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Control of major histocompatibility Class I antigen expression in adenovirus transformed cells.

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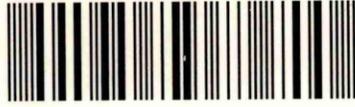
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CONTROL OF MAJOR HISTOCOMPATIBILITY CLASS I
ANTIGEN EXPRESSION IN ADENOVIRUS
TRANSFORMED CELLS

Tina Hollett

A thesis submitted in partial fulfilment of the requirements of
Sheffield Hallam University for the degree of
Doctor of Philosophy

September 1993

Abstract

Two DNA sequences, termed CRE1 and CRE2, located upstream of the Major Histocompatibility Complex (MHC) class I gene have been shown to play an important role in controlling transcription of this gene. MHC class I expression is down-regulated in cells expressing Adenovirus (Ad) 12 E1A compared to cells expressing E1A from Ad2 or Ad5. The possible involvement of these sequences and the factors that bind to them in this down-regulation was investigated using transient expression assays and in the case of CRE1, also by gel retardation and Western blotting. The transient expression assays did not demonstrate a functional role for these sequences in transcriptional down-regulation. The implications of this result in relation to the experimental design are discussed. Gel retardation assays showed that the levels of CRE1-binding factors detected were dependent on the technique employed to prepare the nuclear extracts. Preparation of nuclear extracts by a technique in which the potential for cytoplasmic contamination and proteolytic degradation was minimized showed that the level of CRE1 factors, likely to represent NF- κ B (p50/p65 heterodimers) and H2TF1 (p50 homodimers), was higher in cells expressing Ad2 or Ad5 E1A than those expressing Ad12 E1A. Western blotting using an antibody prepared to the p50 subunit of NF- κ B did not show a difference in levels of this subunit between different Ad-transformed cell lines. These results suggest therefore that the levels of p50 are similar in these cell lines but that the levels of p50 bound to either p65 or another p50 subunit, and capable of binding DNA, are higher in those transformed by Ad2 or Ad5 E1A.

The binding site of CRE2-binding proteins was investigated in Ad-transformed cells by DNase I footprinting. The factors RXR β /H-2RIIBP which bind to this sequence are members of the ER/TR subfamily of steroid/thyroid hormone receptors. Members of this subfamily have the sequence AGGTCA conserved in their binding sites; this sequence formed part of the area of protection found on the DNase I footprint.

The mechanism of nuclear induction of NF- κ B was investigated in Ad-transformed cells by the use of the free radical producer benzoyl peroxide and other inducers such as tumour necrosis factor and cycloheximide. Benzoyl peroxide was shown to activate transcription and scavengers of free radicals prevented the induction. These results were incorporated into a model of NF- κ B induction involving hydrogen abstraction.

Acknowledgements

I would like to thank Dr M. E. Blair and Dr G. E. Blair for their supervision of this work. Also, I would like to thank Mr Stuart MacLaughlin and Dr Ian White for help with the bleeds and injections of the antibody production protocol. I am grateful to Dr Jenny Southgate for her technical advice on Western blotting. The informative discussions with Dr John Colyer and Dr Dan Donnelly proved very useful. The help of Avril Trejdosiewicz in tissue culture and Joan Jarvis in providing clean glassware was very much appreciated. I would like to thank them also for their friendship and support along with that of Dr Aruna Asipu, Matt Hope, Paula Lam, Jan Mahon, Jo Proffitt, Dr Rohana Yusof and the other members of the lab, past and present.

Finally, I would like to thank my family as well as Jon (and his family), Jo, Nim and Linda; they have all provided a good deal of support for which I am grateful.

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Abbreviations

The abbreviations used in this study are in accordance with the recommendations of the Biochemical Journal [Biochem. J. (1992) **281**, 1-19] with the exception of those listed below.

α A-CRYBP1	α A-crystallin binding protein 1
Ad	adenovirus
AGIE-BP1	angiotensinogen gene-inducible enhancer-binding protein 1
APC	antigen presenting cell
APRE	acute phase response element
AT-BP1	α_1 -antitrypsin binding protein 1
AT-BP2	α_1 -antitrypsin binding protein 2
BCG	Bacillus Calmette-Guerin
BCIP	5-bromo-4-chloro-3-indolylphosphate
CAT	chloramphenicol acetyl transferase
CMV	cytomegalovirus
CP1	CCAAT-binding protein 1
CP2	CCAAT-binding protein 2
CR1	conserved region 1
CR2	conserved region 2
CR3	conserved region 3
CRE	class I regulatory element
CRE1	class I regulatory element binding protein 1
CRE2	class I regulatory element binding protein 2
CTL	cytotoxic T-lymphocyte
DCS	donor calf serum
DMEM	Dulbecco modified Eagle's medium
DTT	dithiothreitol
EBP1	enhancer binding protein 1
EC	embryonal carcinoma
ELISA	enzyme-linked immunosorbant assay
ER	estrogen receptor
ERE	estrogen response element
EtBr	ethidium bromide
GR	glucocorticoid receptor

GSH	reduced glutathione
GSSG	oxidized glutathione
HBS	Hepes-buffered saline
H-2RIIBP	H-2 region II binding protein
H2TF1	H-2 transcription factor 1
HIV-1	human immunodeficiency virus 1
HIV-EP1	HIV-1 enhancer binding protein 1
HLA	human leucocyte antigen
HPV	human papilloma virus
HRE	hormone response element
HSV	herpes simplex virus
KBF1	kappa-enhancer binding factor 1
KBF2	kappa-enhancer binding factor 2
ICS	interferon consensus sequence
ICSBP	interferon consensus sequence binding protein
IFN	interferon
I- κ B	inhibitory κ B
I-rel	inhibitory rel
IRF	interferon responsive factor
IRS	interferon response sequence
ISGF	IFN-stimulated gene factor
ISRE	IFN-stimulated response element
LB	Luria broth
MBP-1	MHC enhancer binding protein 1
MDP1	modified Dignam procedure 1
MDP2	modified Dignam procedure 2
MDP3	modified Dignam procedure 3
MHC	major histocompatibility complex
MLP	major late promoter
MuLV	murine leukemia virus
NAC	N-acetyl cysteine
NBT	nitro blue tetrazolium
NK	natural killer cells
NF-1	nuclear factor 1
NF- κ B	nuclear factor of kappa B site

NRE	negative regulatory element
NURP's	nuclear-uptake regulatory proteins
PBS	phosphate buffered saline
PDTC	pyrrolidine derivative of dithiocarbamate
PMA	phorbol 12-myristate 13-acetate
PKC	protein kinase C
PMSF	phenylmethanesulphonyl fluoride
PPD	purified protein derivative of tuberculin
PRDII	positive regulatory domain II
PRDII-BF1	positive regulatory domain II binding factor 1
RA	retinoic acid
RadLV	Radiation leukemia virus
RAR	retinoic acid receptor
RARE	retinoic acid response element
REV-T	reticuloendotheliosis virus strain T
ROI	reactive oxygen intermediates
RT	room temperature
RXR	retinoid X receptor
SDS	sodium dodecyl sulphate
SV40	simian virus 40
TAE	Tris-acetate EDTA
TE	Tris EDTA
TBE	Tris borate EDTA
TcR	T-cell receptor
TEMED	NNN'N'-Tetramethylethylenediamine
TFIIC	transcription factor II C
TFIID	transcription factor II D
TIU	trypsin inhibitory unit
TNF	tumour necrosis factor
TPA	12-O-tetradecanoylphorbol 13-acetate
TR	thyroid receptor
Triton X-100	octyl phenoxy polyethoxyethanol
UV	ultra violet

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1 General Introduction

Major histocompatibility (MHC) class I antigens play a central role in the immune system. They are involved in the presentation of antigens including those of viral and tumour origin to cytotoxic T-cells (Zinkernagel and Doherty, 1979). There are many serotypes of human adenovirus (Ad) and this study was based on work with cells transformed by three serotypes, Ad2, Ad5 and Ad12. The transformation of rat cells with Ad12 is associated with a reduction in the steady state levels of surface MHC class I antigens (Schrier et al., 1983). It has been proposed that this reduction in class I expression may enable these cells to evade immune surveillance by cytotoxic T-cells. The decrease in MHC class I levels is known to involve a reduction in the rate of transcription (Ackrill and Blair, 1988a; Friedman and Ricciardi, 1988; Lassam and Jay, 1989). In the following section human adenoviruses will be described in more detail. This will be followed by an introduction to the MHC, concentrating on class I: its structure, function and regulation by viruses including adenoviruses and other agents. The regions of the MHC class I promoter important to transcriptional control will be described in detail along with the transcription factors which bind to them. Special features of the control of transcription by adenoviruses will be discussed separately.

1.1 Adenoviruses

1.1.1 Introduction

Adenoviruses, which form a family *Adenoviridae*, derive their name from their initial discovery in human adenoid tissue (Rowe et al., 1953). They are widespread in several mammalian and avian species. Human adenoviruses were the first human viruses to be shown to have tumorigenic properties when tumours were induced in newborn hamsters after inoculation with Ad12 (Trentin et al., 1962). Adenoviruses have been intensely studied and several important discoveries in molecular and cell biology were made through the study of these viruses. For example, splicing was first encountered in the formation of adenovirus late mRNA (Chow et al., 1977). One property of adenoviruses is the ability to transform cells *in vitro* and this has been a useful model for tumorigenesis.

1.1.2 Classification of human adenoviruses

More than forty serotypes of human adenovirus have been identified and classified into subgenera A-F based on their oncogenicity in newborn hamsters, their antigenic properties and their DNA homology (Table 1.1). This study used cells transformed by Ad2, Ad5 or Ad12. Ad2 and Ad5 are both members of subgenus C; viruses in this group are non-oncogenic in newborn rodents, as is the case for subgenera E and F. Ad12 is a member of subgenus A; members of this group are highly oncogenic and induce tumours at high frequency and with short latency. Subgroup B viruses induce tumours at low frequency and with long latency. Although group D viruses are not tumorigenic in hamsters, they have been shown to induce mammary tumours in female rats of certain strains (Ankerst et al., 1974). DNA and viruses of all serotypes can transform rodent cells *in vitro* (Flint, 1980; van der Eb, 1980; Jochemsen et al., 1980). Such transformed cells retain the oncogenicity of the original transforming virus, for example, rodent cells transformed by oncogenic Ad12 are tumorigenic when injected into newborn rodents, whereas those transformed by non-oncogenic group C viruses are themselves non-oncogenic (van der Eb and Bernards, 1984).

1.1.3 Diseases associated with human adenoviruses

Adenoviruses have a propensity to generate persistent infections in humans (Jefferies and Burgert, 1990). The different groups of adenovirus are associated with different diseases in humans, common clinical manifestations include conjunctivitis, as well as respiratory, gastrointestinal and urinary tract infections (Table 1.1). There is no evidence that adenoviruses from any of the subgroups form tumours in humans (Green et al., 1980).

1.1.4 The structure of human adenoviruses

Adenoviruses have a linear double stranded DNA genome of approximately 36 kb. They are non-enveloped viruses of 65 - 80 nm in diameter (Horne et al., 1959). The DNA is packaged in a nucleoprotein structure containing at least two basic proteins which are virally encoded (Nermut, 1980). Their DNA/nucleoprotein core is surrounded by a protein coat (capsid). The capsid is comprised of 252 capsomers or protein morphological units arranged to form an icosahedron. There are two types of capsomer; hexons and pentons, the capsid consisting of 240 of the former and 12 of the latter. Hexons are so called because each of these capsomers is surrounded by six neighbouring capsomers. Pentons derive their name by the fact that each is surrounded

Table 1.1 Classification, oncogenicity in newborn rodents and clinical manifestations in humans of human adenoviruses

This table shows the division of different serotypes of adenovirus into six subgroups: A-F, each subgroup being associated with characteristic clinical manifestations in humans and a specific level of oncogenicity in newborn rodents. The table is modified from Waddell et al. (1977) and Paabo et al. (1989).

Subgroup	Serotype	Oncogenicity in newborn rodents	Clinical Manifestations in humans
A	12, 18, 31	high	enteric infections?
B	3, 7, 11, 14, 16, 21, 34, 35	low	respiratory infections, infections of the urinary tract
C	1, 2, 5, 6	nil	respiratory infections
D	8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-47	nil	epidemic kerato- conjunctivitis
E	4	nil	conjunctivitis, respiratory infections
F	40, 41	nil	acute gastroenteritis

by five other capsomers; the peripentonal hexons (Ginsberg et al., 1966). The structure consists of twelve five-sided vertices, a penton is present at the base of each vertice. The capsid, apart from hexons and pentons, contains other hexon associated proteins (Flint, 1980). Knob-like structures are present at the end of the fibres that extend from the twelve five-sided vertices. These structures are considered to be important in viral attachment to cells in order to gain entry (Petersson et al., 1967).

1.1.5 Adenovirus infection of permissive, semi-permissive and non-permissive cells

There are various outcomes of adenovirus infection depending on whether the host cells are permissive, semi-permissive or non-permissive. Permissive cells have the required cellular proteins to allow lytic infection to occur which culminates in the release of virions; human cells are examples of permissive cells. Semi-permissive and non-permissive cells do not support virion production to an appreciable extent (Huebner et al., 1963; Girardi et al., 1964). Rodent cells are examples of semi-permissive hosts. The infection can either be abortive, in which case the virus is eventually lost from the cells, or in a small proportion of cases a process occurs called transformation when viral DNA is incorporated into cellular DNA.

1.1.5.1 Lytic infection

The lytic infection begins with entry of the virion into the cell by attachment of the fiber protein (IV) to specific cell receptor proteins. A part of the virion containing both DNA and proteins (at least II, III, V, VII) gains entry into the cell. The viral DNA reaches the nucleus within 30 - 60 minutes from absorption into the cell. The viral DNA is transcribed by cellular enzymes. Adenovirus genes are denoted 'E' or 'L' indicating 'early' or 'late' genes respectively. The expression of early genes begins before the onset of viral DNA synthesis, while late genes are expressed following viral DNA synthesis. There are three phases during which different genes are transcribed and translated ; the early (1-8 hrs), intermediate (8-12 hrs) and late phase (12-36 hrs). The first viral protein to be made in the early phase is E1A which is required for the transcription of the other early genes (E1B, E2, E3 and E4). The transcription of these genes also begins in the early phase. Viral DNA synthesis commences in the intermediate phase. In the late phase structural proteins are produced and virions are formed. Eventually virions accumulate to such an extent that the cells lyse (reviewed by Darnell et al., 1986; Sharp, 1984). Not all infected permissive

cells undergo lysis; for example, adenoviruses can produce persistent infections in humans (Jefferies and Burgert, 1990).

1.1.5.2 Cell transformation

Transformation is the name given to a process which brings about many changes in cell properties. Before describing the way by which adenoviruses cause transformation, the term itself, along with the term 'cell line', will be defined. When cells are taken from an embryo or an adult animal, most of the adherent cells will proliferate for only a limited number of divisions before senescing. However some cells in some species undergo a change which allows them to grow indefinitely. A culture of cells with an indefinite life span is considered immortal and such a culture is called a cell line. In human cells, only tumour cells grow indefinitely. With rodent cells however, cultures of adherent cells from embryos frequently give rise to cell lines.

Several different treatments, such as exposure to some viruses, certain chemicals or irradiation can dramatically change the growth properties of adherent cells in culture. In addition these treatments can sometimes cause the cells to form tumours when injected into animals. Such changes in growth properties and tumour-forming capacity (when present) are collectively referred to as malignant transformation or transformation. The following is a list of some of the major properties associated with transformed cells (as reviewed by Darnell et al., 1986). However, just as not all transformed cells have tumour-forming capacity, not all transformed cells have all the characteristics listed below:-

1. Increased saturation density ; they continue to grow when normal cells cease growing.
2. Decreased growth factor requirements.
3. Loss of capacity for growth arrest.
4. Transformed cells can generally grow without attachment to substratum.
5. Transformed cells appear more rounded with fewer processes.
6. Loss of contact inhibition of movement.
7. Proteins on the cell surface are more mobile in transformed cells ; possibly due to the loss of cytoskeletal elements.
8. Altered gene transcription.

All transforming agents are thought to cause changes to host DNA. In the case of viruses, segments of the viral DNA become stably integrated into the cellular DNA, as

has been shown for adenoviruses (Bellett, 1975). Ad2-transformed cell lines have been shown to contain viral DNA sequences from the left 14% of the viral genome (Gallimore, 1974; Flint et al., 1976; Johansson et al., 1977, 1978). This part of the genome contains the E1A and E1B genes. These two genes can stably transform primary rodent cells in culture (White and Cipriani, 1990). The various properties of these genes including their ability to cause transformation will be covered in more detail in the following section.

1.1.6 The genome of human adenoviruses

The genome of human adenoviruses has been conventionally divided into 100 map units. The early genes consist of E1A, E1B (together called E1), E2A, E2B (forming region E2), E3 and E4. The late genes are called L1-L5 (Fig 1.1). As E1A and E1B will be discussed in most detail the other gene products will be described first.

1.1.6.1 E2

E2A region encodes a protein called DNA-binding protein which activates several promoters (Chang and Shenk, 1990) and aids viral DNA replication (Stuiver and van der Vliet, 1990). Two other proteins encoded by E2B gene are the terminal protein (Smart and Stillman, 1982) and a DNA polymerase (Stillman et al., 1982).

1.1.6.2 E3

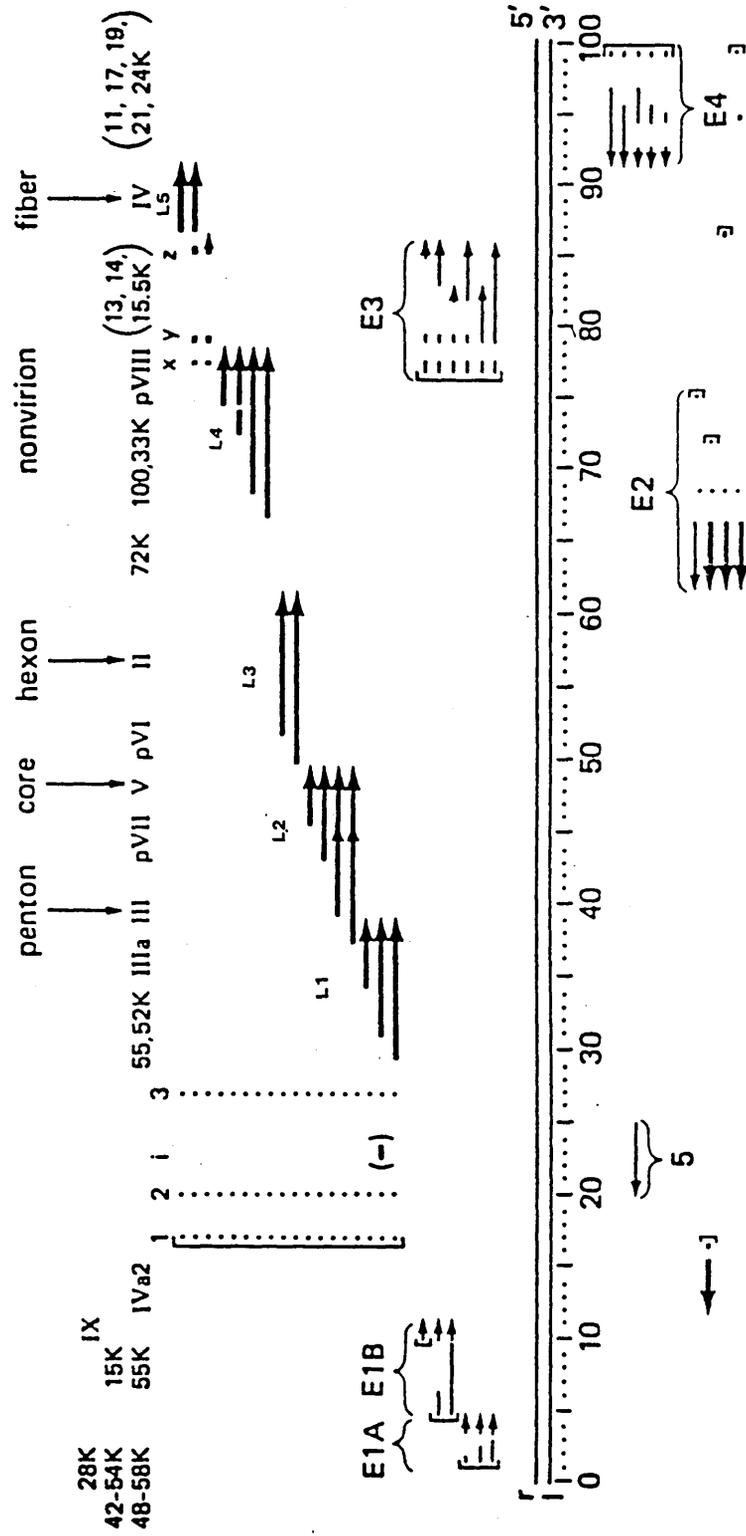
This region contains multiple open reading frames. One protein, of 19 kDa, encoded by this region has been reported to be involved in down-regulation of surface MHC class I antigens during adenovirus infection as will be described in more detail later (Jefferies and Burgert, 1990). Another protein encoded by this region of 14.7 kDa prevents Tumour Necrosis Factor (TNF) α -mediated cytolysis of adenovirus infected cells (Gooding et al., 1990).

1.1.6.3 E4

Two proteins encoded by this region called 294R and 116R (because they consist of 294 and 116 amino acid residues respectively) are involved in permitting the nuclear accumulation of viral RNA (Bridge and Ketner, 1990). 294R is also involved in stimulating transcription from the E2 promoter (Nevins, 1991).

Fig 1.1 Schematic representation of the Ad2 genome showing cytoplasmic RNA transcripts

The 36,500 bp genome of Ad2 is represented by two parallel lines divided into 100 map units. The early transcripts are shown schematically as thin arrows, whereas the late transcripts are shown as thick arrows. The direction of transcription being along either the r or the l strand. The promoters are present at the 5' end of each of the transcripts and are indicated by vertical brackets. The E2 transcription unit is transcribed during the early phase from a promoter located at 75 map units and also during the late phase from a promoter located at 72 map units. Late r-strand mRNAs (from genes L1-L5) are transcribed from a single major late promoter (MLP). Proteins derived from the transcripts are shown at the top of the figure and are designated by K (1000 mol wt) or by Roman numerals. Modified from Fraenkel-Conrat et al. (1988).



1.1.6.4 Late genes

These genes encode structural proteins. They are all under the control of the same promoter, the major late promoter (as reviewed by Paabo et al., 1989).

1.1.6.5 E1A

a. Physical properties of the E1A gene and its products

E1A extends from 1.3-4.5 map units (Berk and Sharp, 1977). E1A proteins are translated from three major mRNAs sedimenting at 13S, 12S and 9S. Two additional mRNAs of 11S and 10S have also been detected (Stephens and Harlow, 1987; Ulfendahl et al., 1987) (Fig 1.2a). All the mRNAs are transcribed throughout infection apart from 9S which is restricted to the late phase. 9S mRNA encodes a structural protein, polypeptide IX, which is part of the viral capsid. The two most abundant mRNAs of 12S and 13S encode proteins of 243 and 289 residues in group C viruses, and these proteins will be referred to as 243R and 289R (as reviewed by Boulanger and Blair, 1991). In the case of group A virus, Ad12, the 12S and 13S mRNAs encode proteins of 235 and 266 residues respectively, and so will be referred to as 235R and 266R (Lamberti and Williams, 1990).

All the E1A proteins except polypeptide IX have the same N- and C- termini and are translated in the same reading frame. Both 243R and 289R appear as a multitude of discrete bands on SDS gels, the different bands representing various phosphorylated forms of these proteins (as reviewed by Boulanger and Blair, 1991).

There are three regions of E1A which have a high degree of homology between different serotypes. These conserved regions are called CR1, CR2 and CR3. In Ad2 CR1 is located between residues 40-80, CR2 between 120-139 and CR3 between 139-186 (Kimelman et al., 1985). Because of differential splicing CR3 is contained within the 46 amino acids unique to the 289R species.

b. Functional properties of the E1A proteins

Transcriptional transactivation

E1A activates transcription from all the other early gene promoters, that is, E1B, E2, E3 and E4 (Osborne et al., 1984; Jones and Shenk, 1979). E1A can promote the transcription of genes transcribed by RNA polymerase II and III (Spangler et al., 1987; Datta et al., 1991). E1A activates transcription from several other viruses including

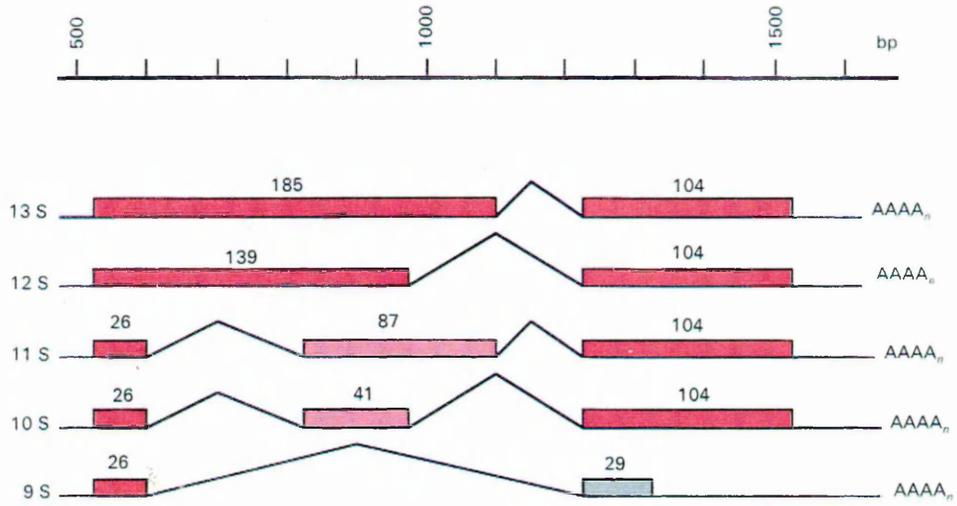
Fig 1.2 Splicing events and gene products of Ad2 E1A and E1B regions

A. The solid line at the top of the figure is the left-hand end of the adenovirus linear genome from 500 to 1600. The Ad2 E1A mRNAs are shown as solid lines and the protein coding sequences as coloured boxes, representing the three alternative reading frames.

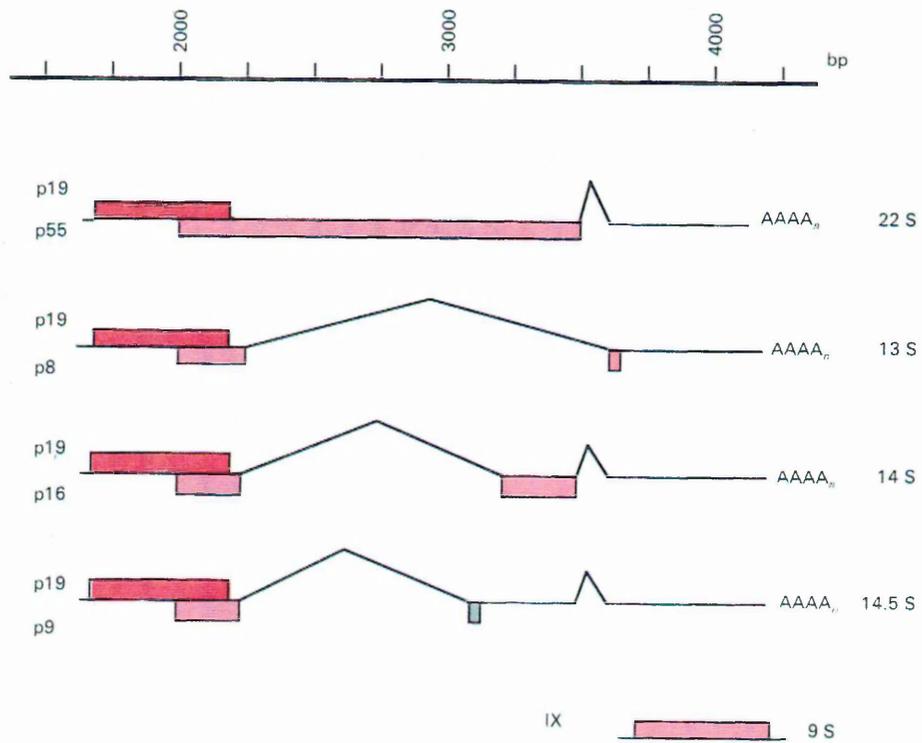
B. The E1B promoter controls a rightward transcript that starts at nucleotide 1699 (+1) in the Ad2 genome. The coloured boxes represent the three alternative reading frames. Synthesis of the virion structural polypeptide IX begins at the intermediate stage of infection, from the E1B 9S mRNA.

Figs A and B from Boulanger and Blair (1991).

A



B



HTLV-1 (Nicholas and Nevin, 1991). E1A can activate cellular genes including p53 (Braithwaite et al., 1990) and c-fos (Simon et al., 1990).

The sequence of 46 amino acids unique to 289R which contains CR3 is able to induce transcription from the E2 promoter and has therefore been described as an autonomous transcriptional activation domain (Lillie et al., 1987). Experiments with a 49 amino acid peptide, suggested that the CR3 region facilitates promoter complex formation (Loewenstein and Green, 1989). However, Braithwaite et al. (1991) have claimed that it is not just CR3 which is involved in transactivation and have proposed that both 289R and 243R are involved. Fahnestock and Lewis (1989) found evidence for sequences outside CR3 being involved in the positive regulation of transcription.

E1A can activate transcription from several unrelated genes that do not share common promoter elements (reviewed by Boulanger and Blair, 1991). It has been proposed therefore that the transcriptional activation of E1A is indirect, mediated by cellular proteins (Nevins, 1991). There is evidence for indirect involvement in several situations, for example, Datta et al. (1991) found in the case of a gene transcribed by RNA polymerase III that E1A mediated its effect through altering the DNA-binding properties of TFIIC. Nevins (1991) suggested that E1A transactivates the E2 promoter in two ways, both involving transcription factor E2F. E1A can dissociate E2F from cellular proteins (Raychaudhuri et al., 1991). E1A also causes E2F to be in an active phosphorylated state. While there is no evidence that E1A acts as a kinase itself, Nevins (1991) has suggested that E1A may either activate a kinase or inhibit a phosphatase. In the case of c-fos, E1A-dependent activation appeared to be mediated through a TATA-specific DNA-binding protein (Simon et al., 1990). Horikoshi et al., (1991) demonstrated that E1A can directly interact with the TATA box transcription factor TFIID.

Transcriptional repression

E1A proteins can repress transcription of certain viral and cellular genes such as the SV40 and polyoma early genes (Borrelli et al., 1984; Lebkowski et al., 1985; Lewis and Manley, 1985), the rat insulin gene (Stein and Ziff, 1987) and rodent muscle specific genes (Webster et al., 1988). Transformation of primary cells by Ad12 is associated with reduced expression of MHC class I, transformation by Ad5 leads to maintained or increased levels of class I (Schrier et al., 1983). Sawada et al. (1985)

used two recombinant viruses containing either E1A of Ad12 and E1B of Ad5 or E1A of Ad5 and E1B of Ad12 in transformation. Oncogenicity of the transformed cells was dependent on the presence of the Ad12 E1A gene. This would be consistent with the Ad12 E1A gene and not the Ad12 E1B gene being responsible for the down-regulation of MHC class I.

The expression of Ad12 E1A proteins reduced steady state levels of MHC class I heavy chain mRNA in transformed cells of rat (Schrier et al., 1983), mouse (Eager et al., 1985), hamster (Ackrill and Blair, 1988b) and human origin (Vaessen et al., 1987). Both 289R and 243R have been reported capable of mediating repression of certain genes. In the case of Ad5 E1A and SV40 early transcription, Jelmsa et al. (1989), using deletion mutants, found that residues 4-25 and 36-60 were important in repression. These residues include part of CR1 (40-80).

Svensson et al (1991) showed that E1A bound to the cellular protein p300 and that CR1 is required for this binding. Using various mutants they found that transcriptional repression correlated with p300 binding. Other groups have found that CR1 and CR2 are both required for repression (Lillie et al., 1987; Schneider et al., 1987). It is possible that the precise amino acid sequences involved in repression depend on the promoter. The repression of class I gene expression mediated by Ad12 E1A could be unusual as it appears to be caused by the 13S and not the 12S product (Bernards et al., 1983), which suggests that CR3, the region unique to 13S, might be involved in causing the repression.

Stimulation of host cell proliferation and cellular DNA synthesis

Cells expressing E1A have been shown to have increased rates of cellular DNA synthesis and cell proliferation (Kaczmarek et al., 1986; Quinlan et al., 1987; Stabel et al., 1985). Smith and Ziff (1988) have localised these properties to the CR1 domain and its flanking N-terminal region. Quinlan et al. (1987) propose that the phenomena of induction of cellular DNA synthesis and cell proliferation by E1A might, at least in part, be due to the secretion of growth factors by infected cells. There is a transcriptional activation of p53 in quiescent normal rat kidney cells when infected with Ad5 (Braithwaite et al., 1990). This effect has been shown to be due to the 289R product of E1A. Since there is evidence that p53 regulates normal diploid cell

proliferation, at least some of the effects on cell proliferation of E1A in Ad5 infected cells could be due to the effect of E1A on p53.

Immortalization and malignant transformation

The E1A region alone is able to immortalize non-permissive rodent primary embryo cells, and in conjunction with E1B or other oncogenes such as activated H-ras, it can induce morphological transformation of these cells (Houweling et al., 1980; White and Cipriani, 1990). In Ad12 the product of the 13S mRNA, 266R, can transform alone, whereas the product of 12S mRNA, 235R, cannot (Lamberti and Williams, 1990). With other serotypes both the 12S and the 13S products are required for stable and complete transformation (Houweling et al., 1980). The transforming properties of E1A have been mapped to CR1 and CR2 (40-80, 120-139) (Velcich and Ziff, 1988). Similar sequences were found to be important by Jelmsa et al. (1989), namely 4-25, 36-60 and 111-138. The 243R product appears to be able to drive G₀ arrested cells into S phase (Montell et al., 1982) and may thus be important in adenovirus-induced cell immortalization (Spindler et al., 1985). It has been suggested that E1A causes transformation through association with cellular proteins. E1A has been shown to be associated with several cellular proteins. These included p300, p105 and p107 (Svensson et al., 1991). Mutations that abolished the ability of E1A to bind p300 or p105 and p107 reduced its capacity to transform primary rodent cells in association with H-ras. Mutations that abolished both p300 and p105/p107 binding abolished the transforming activity of E1A, suggesting that two additive mechanisms were involved (Svensson et al., 1991). p105 is the product of the retinoblastoma tumour suppressor gene (RB gene) (Whyte et al., 1988). It has been suggested that it is the binding of E1A to the products of tumour suppressor genes may somehow lead to their inactivation, which in turn could cause transformation.

Tumour suppressor effect of adenovirus E1A in human tumour cells

Frisch (1991) found that stable expression of Ad5 E1A reduced anchorage-independent growth and tumorigenic potential, caused cytoskeletal reorganization, induced flat morphology and restored contact inhibition in three human tumour cell lines. E1A in this situation seemed to behave like as a tumour suppressor gene. E1A appears to be capable of acting as a transforming agent or a tumour suppressor depending on context.

Degradation of host and viral DNA and cytopathic effect

E1A has been shown to cause cytopathic effect and degradation of host and cellular DNA. These effects have been shown to be mediated by the region of E1A at the amino terminus between amino acids 22 and 86 (White et al., 1991)

Apoptosis

E1A is thought to induce apoptosis in certain circumstances (White et al., 1991). Mapping studies have shown that induction of apoptosis is a function of the N-terminus of E1A that encompasses CR1 (White et al., 1991). Debbas and White (1993) found wildtype p53 was required for the induction of apoptosis by E1A. Lowe and Ruley (1993) found E1A caused the stabilization of nuclear wildtype p53.

1.1.6.6 E1B

a. Physical properties of the gene and its products

The E1B gene is located between 4.6 and 11.2 map units (Berk and Sharp., 1977). The E1B region encodes three major mRNA species of 22S, 13S and 9S as well as two minor species of 14S and 14.5S. The 22S and 13S mRNAs are derived from a common mRNA precursor by alternative RNA splicing (Pettersson et al., 1983). The 22S mRNA encodes two unrelated proteins, p19 and p55 (or 19K and 55K), which are translated in different reading frames. The p19 protein is also translated from a 13S mRNA. Another protein, p8 or 8K, can be encoded from the 13S mRNA (Fig 1.2b). p8 is related to the N-terminal region of p55, although this truncated species has not been detected in infected cells. Both p55 and p19 proteins are post-translationally modified. The p55 protein is phosphorylated on serine and threonine residues (Russell and Blair, 1979) and is also a substrate for poly ADP ribosylation *in vitro* (Goding et al., 1983). A protein kinase activity has also been reported to be associated with p55 (Branton et al., 1985). p19 protein has been found to be covalently linked to lipid (McGlade et al., 1987).

b. Functional properties of the E1B proteins

Viral DNA replication

p55 has been shown to be necessary for viral DNA replication (Mak and Mak 1990; Stillman, 1986). Mak and Mak (1990) found residues between 114 and 155 to be essential for this function.

Accumulation and transport of late mRNAs

p55 is important in the intranuclear maturation of late mRNAs and appears to facilitate the transport of these RNAs to the cytoplasm (Leppard and Shenk, 1989).

Overcoming E1A induced cytotoxic effects

E1A in infection causes the degradation of host and viral DNA and disrupts cytoplasmic architecture. The presence of E1B p19 is sufficient to overcome these effects (White et al., 1991). p19 was found to increase expression from several viral promoters and the cellular promoter hsp70 (Hermann and Mathews, 1989). p19 was found to activate transcription indirectly by stabilizing DNA.

Transformation and tumorigenicity

E1B gene products are required with E1A to produce full morphological transformation of primary rodent cells (Houweling et al., 1980; White et al., 1991). However, high level expression of Ad2 E1A can enable full morphological transformation of established cells to occur in the absence of E1B (Senear and Lewis, 1986). During infection p19 prevents E1A's disruption of cytoplasmic architecture, whereas in the process of transformation p19 itself causes the disruption of the intermediate filaments in the cytoplasm (White and Cipriani, 1990). The morphological differences between primary cells and transformed cells are greater than those between cell lines and transformed cells, and this perhaps explains the need for E1B in the transformation of primary cells and not cell lines. Transformation by p55 and E1A does not cause a gross disruption of intermediate filaments. p19 also confers resistance to TNF α mediated cytotoxic effects, which could be important in allowing tumours to survive in hosts. Deletions of amino acid residues 1-24, 80-96 and 114-155 of p55 in Ad12 greatly reduced the transforming ability of both viruses and plasmids containing any one of these deletions (Mak and Mak, 1990). Cells transformed by plasmids with deletions to residues 80-96 were non-tumorigenic. In Ad5-transformed cells both p19 and p55 appear to contribute to transformation. McLorie et al. (1991) found either p19 or p55 in cooperation with E1A could transform primary baby rat kidney cells, albeit with an efficiency greatly reduced to that observed when both E1B proteins were present. They suggest this indicates that the two processes of transformation by these E1B proteins are additive. However Ad12 mutants where only the p55 and not p19 were produced were found to transform primary BRK cells in association with E1A at efficiencies similar to those of wild-type Ad12 (Edbaeur et al., 1988).

Tumour suppressor properties of p55

Van den Heuwel et al. (1990) found that Ad5 E1-transformed 3Y1 rat cells which express low levels of p55 do not contain a p53/p55 complex. These cells which have nuclearly located p53 are highly oncogenic in nude mice. In 3Y1 cells expressing p55 at higher levels, p53 is complexed to p55 in the cytoplasm. These cells only form tumours after a very long latency period, the tumours appear in cells where selection has occurred to lose this complex. Comparable results were obtained when Ad12-transformed cells which do not normally have the p55/p53 complex were supertransfected with the Ad5 E1B region. Van den Heuwel et al. (1990) suggested that nuclear p53 with a wild-type conformation contributes to the oncogenicity of Ad-transformed cells. Therefore p55, in as much as it retains p53 in the cytoplasm, could be acting as an tumour suppressor gene product.

Suppression of apoptosis

The 19 kDa and the 55 kDa products of E1B are both capable of suppressing E1A induced p53 dependent apoptosis (Lowe and Ruley, 1993; Debbas and White, 1993).

1.2 Major Histocompatibility Complex (MHC)

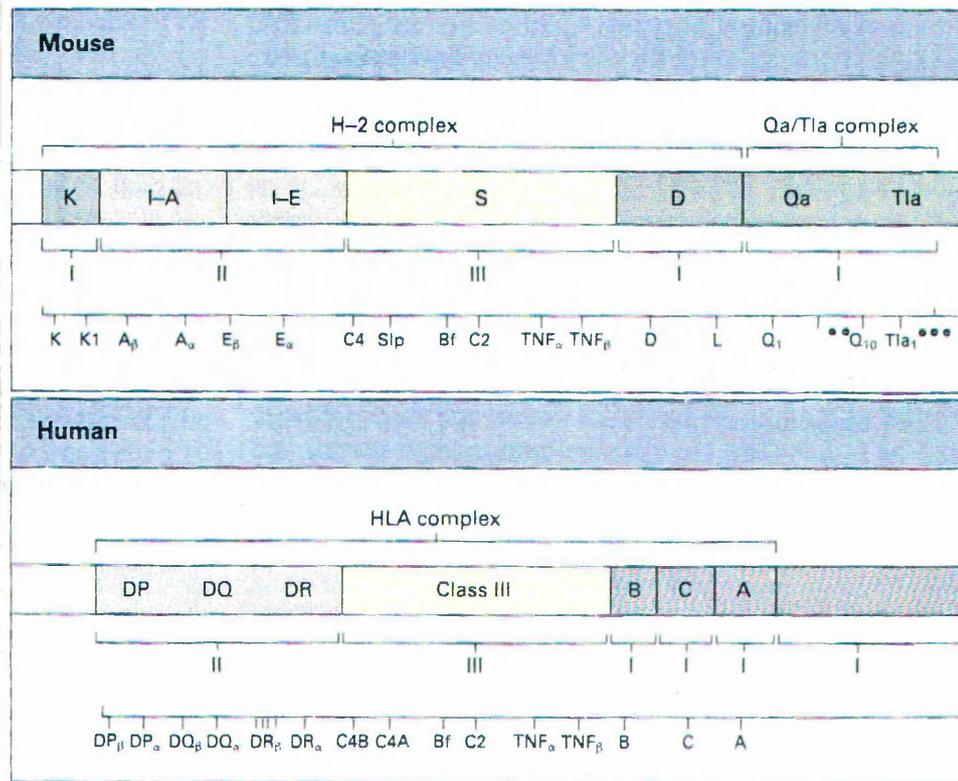
1.2.1 The major subdivisions of the MHC region

The genes responsible for the rejection of transplanted tissues and thus the ability to distinguish 'self' from 'non-self' have been mapped to a region which has become known as the Major Histocompatibility Complex (MHC). All vertebrate species studied so far have been found to have a MHC region (Male et al., 1991). The mouse and human systems are by far the most intensely studied.

MHC is a multigene family approximately 3000 kb in size, located on chromosome 17 in the mouse and chromosome 6 in humans (Male et al., 1991). Both mouse and human MHC regions have been divided into three major classes I, II and III. The class I region can be divided into the classical genes, responsible for transplantation rejection, and the non-classical genes. In the mouse the classical genes map to two regions called K and L; the genes themselves include H-2K, H-2D and H-2L (not all strains carry the L gene) (Fig. 1.3). The non-classical class I genes in the mouse map to a region known as Qa/Tla. In mice the number of class I genes in the haploid genome is around thirty but this number varies amongst different inbred strains (Singer and Maguire., 1990). Most of the class I genes map to the Qa/Tla region and are of

Fig 1.3 Organization of murine and human MHC

The location and major loci of class I, class II and class III regions are shown for the murine and human MHC. Reproduced from Male et al. (1991).



unknown significance. Extensive polymorphism in regions K and D has created a large number of possible combinations of alleles. Each combination of alleles is termed a haplotype, examples of mice haplotypes include b, k and bml. The gene products for a particular haplotype are denoted by the name of the gene followed by the haplotype in superscript, for example H-2K^b. The number of genes in a particular region varies between haplotypes. For example, five genes map to the D region for H-2^d mice, whereas for H-2^b mice only one gene maps to region D (Male et al., 1991).

The number of genes in the human class I region has been estimated to be between 15 and 25 (Singer and Maguire, 1990). The class I region includes A, B and C, these regions contain the major transplantation antigens, 'Human Leucocyte Antigens' HLA-A, -B, and -C which are analogous to H-2K, D and L in the mouse (Fig 1.3). These regions also contain several other genes including HLA-E, -F and -G whose functional significance is unknown. A cluster of class I -like genes is located telomeric to HLA-A; however it is uncertain whether any of these genes represent the human equivalent of the murine Qa/Tla genes (Male et al., 1991).

The class II genes are encoded by regions I-A and I-E in the mouse and by region D in humans. Most class I and class II molecules (with the exception of Qa/Tla antigens) are highly polymorphic structures.

The class III region, although originally defined as encoding four of the components of the complement system, is now known to contain several diverse genes including TNF α and TNF β (Male et al., 1991).

The structural similarities between class I and class II gene products show that they may have evolved from a common origin. There is no evidence for functional or structural similarities between the gene products of class I and class II with those of class III (Male et al., 1991).

1.2.2 The structure of MHC class I

MHC class I molecules are present on the cell surface. These molecules consist of a glycosylated polypeptide chain, the heavy chain, of 42-45 kDa, associated non-covalently with β_2 microglobulin, a 12 kDa polypeptide (Singer and Maguire, 1990).

The heavy chain consists of three extracellular regions α_1 , α_2 and α_3 , a transmembrane region and a cytoplasmic domain.

β_2 microglobulin is a non-polymorphic protein in humans, dimorphic in mice. It has a high degree of sequence homology between species, implying evolutionary conservation. β_2 microglobulin is thought to stabilize the heavy chain (van Bleek and Nathenson, 1992).

There is crystallographic evidence that a large groove between α_1 and α_2 constitutes the binding site for antigen in the form of peptides (Bjorkman et al., 1987; Garrett et al., 1989).

H-2K/H-2D and HLA-A/HLA-B are highly polymorphic, whereas H-2L and HLA-C molecules appear less so. There are three main hypervariable regions in α_1 and α_2 , whereas α_3 appears to be much more conserved. Most of the hypervariable regions are in positions in which the side chains point towards the antigen-binding groove (van Bleek and Nathenson, 1992).

1.2.3 The function of MHC class I gene products

MHC class I is involved in presenting antigenic peptides to cytotoxic T-cells. These T-cells have the cell surface marker CD8+ (Monaco, 1992). They cannot recognise the antigenic peptides alone, their T-cell receptors (TcR) recognise the conformation of antigenic peptide in association with a MHC class I molecule (Zinkernagel and Doherty, 1979). This phenomenon is called MHC class I restriction. The MHC genes are co-dominantly expressed, so any one individual has between three and six different types of class I molecules available to present antigenic peptide to cytotoxic T-cells (Male et al., 1991). Since the variety of class I molecules is so limited, it is not surprising that each class I molecule is capable of binding to several different types of peptide (Jorgensen et al., 1992).

The antigenic peptides presented by class I molecules are generally proteolytically processed from endogenously synthesized proteins, whereas class II molecules bind peptides derived from membrane glycoproteins and serum proteins that have entered the cell by endocytosis (Germain, 1991). Peptides which bind to class I have an optimal length of eight or nine amino acids depending on the class I molecule (van Bleek and

Nathenson, 1992). Little is known about the proteases involved in generating these peptides, however several genes recently found in the MHC region encode proteins which appear to be involved in the supply of peptides to class I molecules (De Mars and Spies, 1992). The peptides include those of viral or tumour origin. Since different class I molecules vary in their ability to present a particular antigenic peptide to T-cells, depending on the haplotype of an individual, particular peptides will be presented to T-cells with varying degrees of success (Jorgensen et al., 1992).

Once the TcR has bound to a particular peptide/MHC class I configuration, the cytotoxic T-cell is stimulated to proliferate and also to release chemicals such as perforins which cause the killing of cells presenting that particular antigen. By this means MHC class I is central to the destruction of tumour cells and virally infected cells by cytotoxic T-cells (Male et al., 1991).

1.2.4 Patterns of classical MHC class I gene expression

Nearly all tissues express class I antigens, however their levels of expression vary. Cells can be divided into those which show high, weak or no detectable expression of classical class I antigens. The highest level of class I has been found on splenocytes. Keratinocytes also display very high levels of class I. Intermediate levels of class I expression occur in the thymus, lymph node, kidney and lung with lower levels in the heart and adrenal glands. Low levels of class I expression are found on skeletal muscle, in the pancreas, thyroid and the small intestine. Class I antigens expression is low on erythrocytes, reticulocytes and endocrine cells (apart from adrenal cells). Class I antigens are not detectable at all in the brain, on corneal endothelium, acinar cells of the parotid gland, the exocrine region of the pancreas, on ova and on mature sperm (Singer and Maguire, 1990; Male et al., 1991).

1.2.5. Patterns of expression of non-classical MHC class I genes

Since this study is concerned with classical MHC class I, little will be said about the expression of non-classical genes. However it is interesting to note that certain non-classical gene products are expressed in just one organ, for example the liver. Although the brain has generally been considered a class I deficient organ, expression of non-classical genes may occur in the brain (Singer and Maguire, 1990).

1.2.6. Regulation of MHC class I expression

1.2.6.1 Regulation of MHC class I expression during development

The expression of MHC class I antigens is developmentally regulated. No cell surface expression of class I is detectable on either ova or mature sperm (Ostrand-Rosenberg, 1980). Class I mRNA has been detected in 8.5 day embryos. Even at the earliest stages at which expression is detected, both classical and non-classical genes are found, and their tissue distribution varies already (Fahrner et al, 1987). Cell surface expression of class I is detectable only after the mid-somite stage of development (Ozato et al., 1985). Since there is a limited availability of embryonic tissue, most research on developmental regulation of class I genes has been done on embryonic carcinoma cells (EC). EC cells such as F9 can be maintained in culture as undifferentiated pluripotent cells. In this state, such cells express no detectable cell-surface class I antigen and barely detectable levels of mRNA (Rosenthal et al 1984; Morello et al., 1982). EC cells can be induced to differentiate and express both class I mRNA and cell surface antigen by treatment with retinoic acid, alone or in combination with cAMP (Rosenthal et al., 1984; Croce et al., 1981; Morello et al., 1982; Daniel-Vedele et al., 1985). Treatment of F9 cells with either α/β or γ interferon similarly induces high levels of class I expression (Wan et al., 1987) but does not cause differentiation. This shows the mechanisms controlling class I expression are separable from those controlling differentiation.

1.2.6.2. Regulation of MHC class I expression by interferons

Interferons (IFNs) were originally identified by their ability to prevent viral replication (Isaacs and Lindenmann, 1957). α , β and γ interferons are all capable of inducing increased cell surface MHC class I (Burrone and Milstein, 1982; Branca and Baglioni, 1981). IFN treatment results in increased rates of transcription of MHC class I (Friedman and Stark, 1985; Blonar et al., 1988), but post-translational control is also important (Revel and Chebath, 1986). IFNs have to react with a specific cell surface receptor to induce an immune response. IFNs α , β share a common receptor; type I, while IFN γ has a separate receptor; type II. Type I IFNs (IFN α and IFN β) are produced following viral infection of leucocytes and fibroblasts respectively. Type II or IFN γ is produced by antigen or mitogen activated T-cells (Male et al., 1991). *In vitro* treatment of cells with IFNs induces MHC class I expression in a wide variety of cells of haemopoietic, lymphoid, epithelial, fibroblastic and neuronal origin (Halloran et al., 1986; Wong et al., 1984). The relative effects of type I and type II IFNs

depend on the target cell type, for example, in mouse fibroblast lines they are equally effective (Korber et al., 1988), whereas in mouse thymocytes type II IFN elicits a greater induction of class I (Sonnenfield et al., 1981). When IFNs are used to treat tumour cells which express low levels of class I, there is a concomitant decrease in tumorigenicity (Tanaka et al., 1985).

1.2.6.3. Regulation of MHC class I expression by TNF

TNF, also known as TNF α , is a protein of 17-18 kDa in size depending upon the species. O'Malley et al. (1962) first reported a tumour necrotizing factor in serum. Although it was first discovered because of its ability to kill tumour cells, TNF is now known to have pleotropic effects. TNF selectively lyses certain transformed cells (Bonavida, 1991) and kills some virally infected cells (Rook et al., 1991). It mediates severe inflammatory reactions, stimulates collagenase and prostaglandin, stimulates fibroblast and endothelial cells, regulates T- and B-cells causing the killing of virally infected cells and stimulates several cytokines from different cell lines. TNF is itself induced by the gram-negative lipopolysaccharide and by lipoteichoic acid of gram-positive cocci. It is possible that all micro-organisms trigger the release of TNF. TNF is produced by macrophages and CD4+ lymphocytes (reviewed by Gifford and Duckworth, 1991).

TNF causes an increase in the cell surface expression of MHC class I (Collins et al., 1986). TNF also causes translocation of the transcription factor NF- κ B from the cytoplasm to the nucleus (Duh et al., 1989). It is possible that many of the actions of TNF including its effect on class I are due to its action on NF- κ B, as will be described in more detail later.

1.2.6.4 Regulation of MHC class I expression in human adenovirus infected cells

The E3 19 kDa protein from adenovirus subgroups B-E but not subgroup A has been shown to bind to MHC class I antigens during adenovirus infection (Wold and Gooding, 1989; Flomenberg et al., 1987; Paabo et al., 1986). The association of E3 19 kDa protein with nascent class I heavy chains within the endoplasmic reticulum prevents terminal glycosylation of the class I molecules, inhibiting their transport beyond the cis Golgi to the cell surface (Severinsson et al., 1986; Kampe et al., 1983). In contrast to the above results, Rosenthal et al. (1985) found that in infected mouse cells, either Ad12 or Ad5 increased the level of class I mRNA.

1.2.6.5 Regulation of MHC class I expression in human adenovirus-transformed cells

Primary cells transformed by oncogenic Ad12 show decreased steady state levels of class I mRNA, whereas non-oncogenic Ad2 and Ad5 either stimulate or do not alter the steady-state levels of cytoplasmic class I transcripts (Schrier et al., 1983; Eager et al., 1985; Vasavada et al., 1986). This has led to a view by several workers that it is the lower level of class I expression in Ad12-transformed cells which allows such cells to escape T-cell-mediated immune surveillance and so cause the increased oncogenicity of Ad12 (Schrier et al., 1983; Eager et al., 1985; Tanaka et al., 1985).

The suppression of class I appears to be due to a reduced level of transcription initiation (Friedman and Ricciardi, 1988; Ackrill and Blair, 1988a; Lassam and Jay, 1989). Shemesh et al. (1991) investigated several Ad12-transformed cell lines, in some the reduced class I appeared to be due to lowered levels of transcription, whereas in other cell lines it seemed to be due to interference with processing of the post-transcriptional product.

Introduction, by transfection, of functional class I genes under the control of a SV40 promoter into Ad12-transformed cells has been shown to abolish their tumorigenicity (Tanaka et al., 1985). This provides evidence for the proposition that it is the lowered levels of class I in Ad12-transformed cells which is in some way responsible for their tumorigenicity. However differences in levels of class I and corresponding variations in susceptibility to cytotoxic T-cells may not be the only factors relevant to the range of tumorigenicity found among the different adenovirus serotypes. Natural killer (NK) cells do not have receptors which bind to MHC class I. Ad12-transformed cells have been shown to be resistant to lysis by NK cells (Sawada et al., 1985). Haddada et al. (1986) have shown that transformation with Ad2 and Ad5 E1A genes can increase susceptibility to natural killer cells and activated macrophages with concomitant reduced tumorigenicity.

1.2.6.6 Regulation of MHC class I expression by infection with other tumour viruses

Viral infections in general cause the release of cytokines such as TNF and interferons which instigate an increase in the transcription of MHC class I antigens. Despite this the overall effect of infection by certain viruses on the levels of MHC class I can be a

reduction. For example, infection by human cytomegalovirus can down-regulate class I expression. A CMV encoded homologue of the class I heavy chain, the UL18 gene product (Berk and Barell, 1988), has been shown to sequester host β_2 microglobulin and so disrupt the assembly of the class I heterodimer (Browne et al., 1990, 1992). Infection with HSV-1 and HSV-2 has been shown to reduce the expression of class I antigens although the mechanism by which this process occurs has not been elucidated. HIV-1 infection has also been associated with the down-regulation of class I in certain cells. (Scheppeler et al., 1989).

1.2.6.7 Regulation of MHC class I expression by transformation with other tumour viruses.

Several viruses which are capable of transforming cells *in vitro* have a concomitant effect on the levels of MHC class I; for example, Rous sarcoma virus transformed human fibroblasts have down-regulated class I expression (Gogusev et al., 1988) due, at least in part, to a reduction in steady state levels of class I mRNA. Levels of class I mRNA were also reduced in cells transformed by Radiation leukaemia virus (RadLV), this reduction was associated with methylation and rearrangement of MHC DNA (Meruelo et al., 1986). SV40 transformation of cells is associated with an up-regulation of class I (Brickell et al., 1985).

1.2.6.8 Regulation of MHC class I expression by virally-induced tumours

Tumours can be induced in animals by treating them with certain viruses. Many of the virally induced tumours show a lack of both H-2K and H-2D antigens. For example, there is a lack of class I antigen expression with a SV40-induced fibrosarcoma, a RadLV-induced Lymphoma, a Gross MuLV-induced lymphoma and several Moloney virus-induced lymphomas (reviewed by Elliot et al., 1989). Human papillomaviruses (HPV)16 and 18 are strongly associated with cervical carcinoma and greater than 30% of such tumours show a complete or partial loss of MHC class I expression (Conner and Stern, 1990).

1.2.6.9 Regulation of MHC class I expression in non-virally induced tumours

Tumours can occur spontaneously and be of unknown aetiology or they can be induced by viruses or by several non-viral inducing agents which include chemical carcinogens, ultraviolet light and γ irradiation.

a. Regulation of MHC class I expression in human tumours

Certain human tumours seem to be strongly associated with a down-regulation of MHC class I; for instance, McDougall et al. (1990) found dramatically reduced levels of MHC class I in colon cancer as compared to normal colonic mucosa samples taken 5-10 cm away from each tumour. HLA class I expression was undetectable in 28% of the specimens, diminished in 68% (as compared to normal control tissue) and normal in only 4% of the samples. Some other tumours are associated with little or no class I, for example, neuroblastomas (Lampson et al., 1983), however since the normal neural tissue from which these tumours originate is also class I negative, the levels of MHC class I probably is not of relevance to the development of tumours in these tissues. Other tumours seem to be associated with down-regulation of MHC class I, but the relationship is less strong, for example, Garrido and Ruiz-Cabello (1991) found 10% of melanomas and 27% of breast carcinomas to be HLA class I negative. Further evidence that reduced MHC class I can be important for immune evasion comes from results with human tumours, where it has been shown over a wide range of tumours including melanomas (Ruiter et al., 1984) and breast cancer (Natali et al., 1983) that tumours with lower class I expression have greater metastatic potential. Additional support for a role of class I in the development of at least some tumours comes from studies of the human malignant leukemia line K562; introduction of specific MHC genes into this cell line, with resulting cell surface class I expression, restores the susceptibility of these cells to antigen-specific cytolysis by cytotoxic T-cells (Maziarz et al., 1990).

Some tumours do not appear to be associated with changes in HLA expression, for example, colorectal adenomas and nonmucinous colorectal carcinomas (Elliot et al., 1989). Certain tumours are even associated with increased class I expression (Smith, 1991). It has been demonstrated that some human tumours selectively lose HLA-A or HLA-B locus products (Lopez Nevot et al., 1989).

Overall, the results with human tumours suggest reduction in the levels of MHC class I does seem to be of importance in the development of certain tumours. It is not known what causes MHC class I levels to be suppressed in some tumours and not in others.

b. Regulation of MHC class I expression in non-virally induced tumours in animals

Some carcinogen-induced and spontaneous tumours in animals still express all class I antigens, for example, the methylcholanthrene-induced mastocytoma P815, whereas in others there is noncoordinate loss of H-2K, -D antigens as in the spontaneous mammary carcinoma TA3 which is K^k, D^{k+}. In other tumours the situation is that commonly found in virally induced tumours, in that there is loss of expression of H-2K and H-2D antigens, as for instance in the spontaneous mammary carcinoma SPI (reviewed by Elliot et al., 1989).

Transfections of class I genes into animal tumour cells have provided further support for the view that expression of MHC class I can, at least in certain circumstances, make tumours susceptible to attack by the immune system. Such experiments have been performed by several workers with a variety of tumours. Wallich et al.(1985) found a highly metastatic methylcholanthrene-induced sarcoma lost its metastatic properties after expression of new H-2 gene products on its surface. A spontaneous murine melanoma line was rendered non-tumorigenic in syngeneic mice by the expression of the transfected class I gene (Tanaka et al., 1988).

1.2.6.10 Regulation of MHC class I expression by oncogenes

Certain oncogenes have been shown to have an effect on MHC class I expression, for instance, induction of class I by 12-O-tetradecanoyl phorbol-13-acetate or dimethyl sulfoxide was preceded by a transient burst of c-fos (Barzilay et al., 1987). A positive correlation between MHC class I and c-fos expression has been found in other tumours such as PCI2 pheochromocytoma and Lewis Lung carcinoma (Kushtai et al., 1988). In the latter system low-metastatic clones express c-fos and high levels of class I antigens, whereas highly metastatic clones are deficient in class I and do not express c-fos. Transfection of c-fos or v-fos into class I negative cells resulted in increased levels of class I proteins and gave such clones a low-metastatic phenotype. Bernards et al. (1986) have shown that class I expression in neuroblastoma cells correlates inversely with N-myc expression. Transfection of rat neuroblastoma cells with N-myc resulted in down-regulation of class I expression. Versteeg et al. (1988) demonstrated that transfection of c-myc into two human melanoma cell lines resulted in a reduction of class I expression, whilst IFN γ treatment both restored class I expression and suppressed c-myc mRNA levels. Alon et al. (1987) have shown that k-ras mRNA

levels were elevated in low MHC-expressing variants of T10 sarcoma which have high metastatic^{at} ability.

In summary, there is strong evidence that certain oncogenes can influence levels of MHC class I in certain tumour systems. However, because these oncogenes have pleiotropic effects it is difficult to know whether the tumorigenic/metastatic^{at} properties of these proteins are directly related to their effects on expression of class I antigens.

1.2.6.11 Regulatory sequences within the class I promoter and the transcription factors that bind to them

As described in the previous sections, the MHC class I genes are of great importance to the immune system; because of this the control of transcription of these genes has been the subject of intensive investigation.

Several sequences within the promoter region have been shown to control the transcription of class I genes, some sequences appear to play different roles in different circumstances such as at certain developmental stages. The interspecies similarity of regulatory sequences within the promoters of MHC class I genes suggests that similar mechanisms of regulation of class I exist in different species.

The following section will describe these sequences and what is known about the factors which bind to them (Fig 1.4), starting with sequences actually in introns, then considering sequences progressively further upstream from the transcription start site.

a. Intron enhancer-like sequences

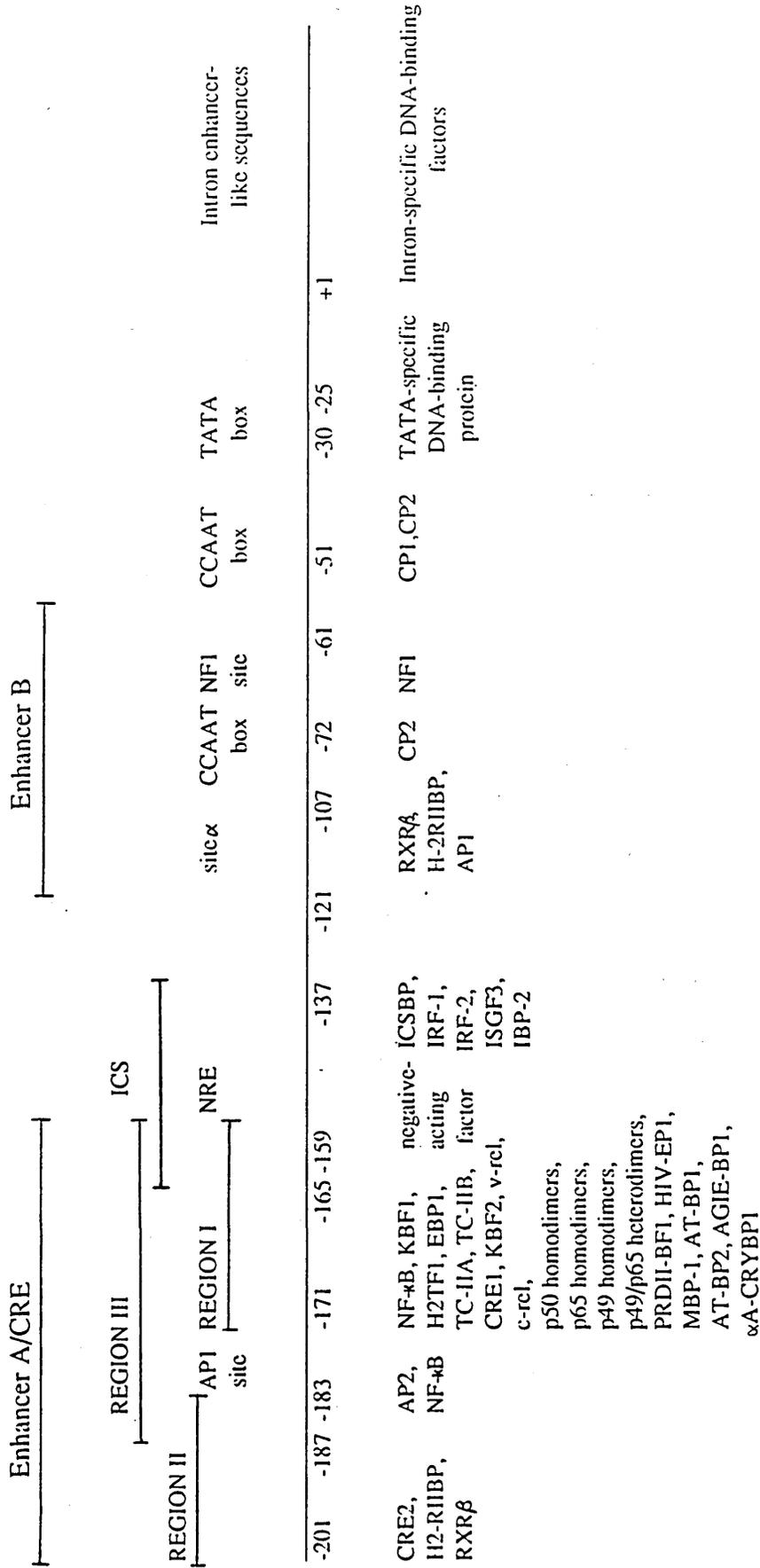
In the human MHC class I gene HLA-B7, enhancer-like sequences have been identified in introns 3 and 5 (Ganguly et al., 1989).

b. TATA box

The first important regulatory sequence upstream from the transcription start site is the TATA box, a TA-rich region. This highly conserved sequence is required for the correct positioning of RNA polymerase II. Its exact position varies between different class I genes in different species, but it occurs around -30 to -25, where +1 represents the transcription initiation site (David-Watine et al., 1990).

Fig 1.4 Regulatory sequences within the MHC class I promoter and the transcription factors which bind to these sites

The major sites that have been shown to be important in the control of transcription of MHC class I genes are represented schematically in this figure. The transcription factors that bind to these sequences are also shown.



c. CCAAT boxes

Many eukaryotic genes possess one or more transcriptional regulatory element(s) that contain the pentanucleotide sequence CCAAT between -80 and -60, MHC class I are examples of genes with such sequences. In the mouse H-2K^b gene, one CCAAT element is present at -51 and a second at -72, the two CCAAT elements motifs being AACCAAT and ACCCAAT respectively. The CCAAT element at -51 is on the coding strand, whereas the one at -72 is on the non-coding strand (David-Watine et al., 1990).

A DNA-binding protein called CP1 binds with high affinity to the CCAAT element at -51. CP1 binds with high affinity to several CCAAT elements including the adenovirus major late promoter and the human α globin gene. A second DNA-binding protein called CP2 binds to both CCAAT elements in the H-2K^b upstream region with high affinity. CP2 also binds to the rat γ fibrinogen promoter with high affinity (Chodosh et al., 1988). CCAAT sequences are thought to be involved in regulating the frequency of transcription initiation (David-Watine et al., 1990)

d. Enhancer B

This enhancer region, like enhancer A, is conserved within the upstream region of several genes coding for classical transplantation antigens but not in the promoter of Qa-TIa region genes. Enhancer B was first described by Kimura et al. (1986) and spans from -121 to -61 in the H-2K^b promoter. The -72 CP2 site lies within enhancer B. Between this site, and the -51 CP1/CP2 binding site, has been found the binding site for another member of the CCAAT family of DNA-binding proteins called Nuclear factor 1 (NF-1). The NF-1 site also lies within enhancer B (Driggers et al., 1992). This is a cellular DNA-binding protein that binds to the adenovirus origin of replication and is required for the initiation of adenoviral replication (Chodosh et al., 1988).

The region between -121 and -107 of enhancer B has been called site α (Dey et al., 1992). In this study two MHC class I genes, the transgene HLA-B7 and the endogenous H-2K^b gene, are occupied in a tissue-specific fashion that correlates with the expression of these genes. *In vivo* protection was not present in the brain where class I expression was virtually absent, whereas protection of certain sequences occurred in spleen tissue which expresses class I. The strongest *in vivo* protection was

seen at site α . Dey et al. (1992) performed chloramphenicol acetyl transferase (CAT) assays to investigate the functional role of this sequence. They used a CAT construct linked to -260 to +1 of the H-2K^b promoter and one with a deletion in site α . The latter gave 50% lower CAT activity in Namalwa B lymphocytes, but the deletion had no effect on CAT activity in LTK⁻ DAP fibroblasts. This demonstrates that site α enhances class I transcription in a cell type-specific fashion. Using a DNA-immunoprecipitation assay, it was shown in the same paper that RXR β , expressed in baculovirus infected insect cells, bound to site α . RXR β is the human homologue of the mouse protein H-2RIIBP. The same group originally isolated a cDNA clone for this factor (Hamada et al., 1989). The latter binds to a GGTC A motif present in the MHC class I promoter in a sequence called region II, which will be described in more detail later. The same GGTC A motif is present in site α . This site has also been shown to bind purified AP-1 (Israel et al., 1989; Korber et al., 1988).

e. Interferon responsive sequences

MHC class I is one of several interferon-responsive genes. Friedman and Stark (1985) identified a 28bp consensus sequence located 5' of the transcription start site in several IFN inducible genes, including two class I genes. Further investigation by several groups has led to the identification of a consensus sequence present in several IFN inducible genes:



Where N = A, C, T or G and x is a positive integer.

This sequence has been termed the IFN-stimulated response element (ISRE). The mouse H-2K^b gene between -153 and -141 has all but the first two bases of this sequence. It has been proposed that IFNs mediate their effects through their binding to their cell surface receptors. This, after several intermediate steps, is thought to lead to ISRE binding factor(s) binding to particular ISREs and so activating transcription. Various ISREs have been found to bind at least three distinct factors called IFN stimulated gene factors, ISGFs (Levy et al., 1988). ISGF1 binds constitutively to ISRE and its function is unknown. The other two factors are inducible. ISGF2 appears to be identical at the DNA level to independently identified factors IRF-1 (Pine et al., 1990) and IBP-1 (Blanar et al., 1989). IRF-1 was identified as an IFN α/β responsive factor and IBP-1 as a IFN γ responsive factor. However this factor might

be post-translationally modified in various forms. Ali Imam et al. (1990) suggested that different forms may be involved in IFN α/β and IFN γ induction of IFN-responsive genes. IRF-1 has been cloned (Harada et al., 1989, 1990). Expression of IRF-1 cDNA constructs in IRF-1 negative EC cells resulted in the activation of endogenous IFN α genes and co-transfected promoters of IFN α , IFN β and MHC class I; ISGF2/IRF-1 could therefore be important in mediating the increase in MHC class I induced by IFNs α/β . A second cDNA clone was isolated for a factor which also binds ISRE referred to as IRF-2. Activation of all the above genes was strongly repressed by co-transfection with IRF-2 cDNA constructs (Harada et al., 1990). These workers suggested IRF-2 acts to switch off IRF-1 induced responses.

The third ISGF, ISGF3 has been shown to be a major factor mediating IFN α induction of several IFN responsive genes. However, Yusof (1993) found that ISGF3 did not bind to the class I promoter ISRE, therefore ISGF3 is unlikely to be important in IFN-mediated class I induction.

In different genes containing ISRE sequences, the flanking sequences also seem to play a role in mediating the IFN response. In the case of class I a sequence from -165 to -137 called the IFN consensus sequence (ICS) or IFN response sequence (IRS) has been shown to play a role (Israel et al., 1986). The IRS was found to be active with both types of IFNs only in conjunction with the adjacent sequence, enhancer A. However, others have observed that the IRS alone could confer responsiveness to IFNs α/β or γ in certain cell types (Korber et al., 1987; Sugita et al., 1987). Nielsch et al. (1991) found that IFN β caused the nuclear translocation of NF- κ B. It is possible that NF- κ B is involved in causing the increase in class I transcription in response to IFN, maybe in cooperation with other IFN-inducible factors.

Another cDNA clone has been isolated which encodes a protein called ICSBP which binds to the ICS of MHC class I genes (Driggers et al., 1990). A negative regulatory element (NRE) has been identified within the ICS and is located 5' of the ISRE. It binds a factor present in undifferentiated but not differentiated F9 EC cells and it has therefore been proposed that this binding activity could play a role in negative regulation during development (Flanagan et al., 1991).

f. Enhancer A

Results from CAT assays have shown a sequence between approximately -200 and -160 which acts as an enhancer in many cells (Kimura et al., 1986; Katoh et al., 1990; Israel et al., 1989; Baldwin and Sharp, 1987). This region has been called enhancer A (Kimura et al., 1986) or a very similar region has been described as CRE (class I regulatory element) (Miyazaki et al., 1986). Enhancer A/CRE can act as a negative transcription element in undifferentiated F9 EC cells (Miyazaki et al., 1986).

Shirayoshi et al. (1987) used a variety of probes from segments of enhancer A in gel retardation analysis to distinguish regions bound by separate DNA-binding proteins. These regions were called I, II and III and are positioned between -173 to -161, -203 to -185 and -189 to -161, respectively, in the H-2K^d promoter and found between -171 to -159, -201 to -183 and -187 to -159 in the H-2K^b promoter.

Region I

This region has been shown to act as an enhancer region in CAT assays with certain cell lines, for example, Mauxion and Sen (1989) cloned a dimer of -175 to -156 into a CAT plasmid and found CAT activity was double that of the plasmid alone in a murine plasma cell line. However the dimer was ineffective in a murine T-cell line and in Hela cells. Baldwin and Sharp (1987) used a CAT plasmid containing the sequence from -190 to +1 and in vivo competition with a trimer of region I or a mutated version of region I (mutated at G residues found to be important for protein binding). The trimer of region I but not the mutated version reduced CAT activity. Burke et al. (1989) used mouse cells and a plasmid containing 1.4 kb of the 5' flanking region of H-2L^d gene and an identical plasmid apart from mutations to the first two G's of region I, they found the latter had 50% lower CAT activity.

Binding to region I correlates with the cell surface expression of MHC class I in a variety of adult and developing tissues (Burke et al., 1989). Dey et al. (1992) showed that there was *in vivo* protection of region I in cells expressing class I genes but not in cells where class I expression was absent.

Region I has been found to bind a wide variety of DNA-binding factors, some of which have been well characterised, others less so. The precise role played by all of these proteins in the enhancer activity displayed by region I is not known, however, in cases

where the protein has been cloned and expressed, its transcriptional regulatory properties are better understood. The following section will describe DNA-binding proteins that have been found to bind to region I and what precise role such proteins have on transcription in general and in particular that of class I genes, if known. The proteins which bind to region I can be divided into two major groups: one group, which includes NF- κ B, is called the rel-related protein group and the other which includes larger proteins such as PRD-BF1 have zinc fingers and the latter shall be described as the PRD-BF1-related protein family.

The family of rel-related proteins

NF- κ B is a transcription factor found to be very important in the control of MHC class I genes and several other important genes, as will be described in more detail in the following section. NF- κ B has been shown to consist of two subunits, a p50 and a p65 (Kawakami et al., 1988; Nolan et al., 1991). Sequence analysis of human and murine cDNAs encoding p50 (Kieran et al., 1990; Ghosh et al., 1990) and p65 (Nolan et al., 1991; Ruben et al., 1991) have shown that in these two proteins there is a sequence at the N-terminal which shows extensive homology. This region of homology has been found to be present in several other proteins, including one very similar to p50 called p49, for which the cDNA has been isolated from a human cDNA library (Schmid et al., 1991). The region of homology is also present in p100, the precursor of p49. p50 has its own precursor, p105, which not surprisingly contains the region of homology. This region is also present in v-rel and c-rel (Gilmore, 1991; Hannink and Temin 1991) and in the maternal effect gene dorsal in *Drosophila* (Govind and Steward 1991) and RelB (Blank et al., 1992).

The region of homology has been described by Blank et al. (1992) as the rel/dorsal homology region. p50 has been shown to be able to dimerize *in vitro* with p65 (Nolan et al., 1991), v-rel (Kieran et al., 1990), c-rel (Logeat et al., 1991) or RelB (Blank et al., 1992) and *in vivo* with p65 (Schmitz et al., 1991) or c-rel (Ballard et al., 1992). p65 can probably form heterodimers with c-rel *in vivo* (Hansen et al., 1992). All these interactions demonstrate the control of transcription by rel-related DNA-binding proteins is very complicated .

The family of rel-related proteins will be described in more detail in the following section.

NF- κ B (p50/p65)

The term NF- κ B was originally given by Sen and Baltimore (1986) to a DNA-binding protein that binds specifically to the sequence GGGGACTTCC present in the immunoglobulin κ light chain enhancer. This sequence is similar to the sequence TGGGGATTCCCCA known as region I. It was found that NF- κ B also binds to region I (Israel et al., 1989). Several other genes have been found to have sequences similar to those found in region I and the κ light chain enhancer. These sequences can all bind NF- κ B and are known as NF- κ B-like DNA-binding motifs. These motifs are found in the promoter regions of several genes important to the immune system such as IFN β , TNF α , lymphotoxin and T-cell receptor β . Such motifs are also present in several viruses such as HIV-1, cytomegalovirus and SV40. As detailed above NF- κ B has been cloned and found to consist of a p50 and a p65 subunit. This heterodimer is normally only constitutively present in the nucleus of a small subset of cells which includes T-cells, B-cells and monocytes (Blank et al., 1992) and some virally infected cells such as those transformed by certain serotypes of adenovirus (Nielsch et al., 1991). In all other cells NF- κ B is retained in the cytoplasm by its attachment through its p65 subunit to one of a family of proteins called NURP's (nuclear-uptake regulatory proteins) (Schmitz et al., 1991). NURPS which include I- κ B α and I- κ B β are proteins which inhibit the DNA-binding ability and nuclear uptake of NF- κ B and related proteins. I- κ B α and I- κ B β have been isolated from the cytosol of human placenta (Zabel and Baeuerle, 1990) and are acidic proteins of 37 and 43 kDa respectively. I- κ B β inhibited the stimulation of transcription mediated by NF- κ B in an *in vitro* transcription system (Kretzschmar et al., 1992). It is yet to be clarified whether I- κ B β enters the nucleus and has a similar function *in vivo*. Another inhibitory protein of NF- κ B has recently been cloned called I-rel (Ruben et al., 1992). It differs from I- κ B α and I- κ B β in that it binds to p50 not p65. It is a 66 kDa rel-related protein that attenuates the DNA-binding activity of p50 and the transcriptional induction of NF- κ B. I- κ B γ (the C-terminal half of the p105 NF- κ B precursor) appears to be another new member of the I- κ B family (Inoue et al., 1992). Inhibitory proteins of NF- κ B can play three roles: retaining NF- κ B in the cytosol so it is unable to act as a transcription factor, allowing induction to occur through NF- κ B release and entry to the nucleus where it acts as a transcription factor, or binding to NF- κ B once again and so attenuating its action. Although evidence for the latter role has still to be established, the first two steps do seem to occur, at least in the case of I- κ B α and I- κ B β (Baeuerle 1991).

NF- κ B is not constitutively present in the nucleus in most cell types, therefore basal levels of expression of MHC class I are probably not due to this factor. A variety of agents including TNF α (Duh et al., 1989) and lipopolysaccharide (Sen and Baltimore, 1986) induce the release of the inhibitory proteins from NF- κ B. Dissociating agents such as formamide or sodium deoxycholate can have the same effect (Baeuerle and Baltimore 1988).

The induction of NF- κ B by several agents such as TNF α , phorbol ester and interleukin-1 can be inhibited by free radical scavengers such as pyrrolidine derivative of dithiocarbamate (PDTC) and N-acetyl cysteine (NAC) (Schreck et al., 1991, 1992).

These results imply that induction by all these agents leads to the release of reactive oxygen intermediates (ROI), which by some as yet unknown mechanism, cause NF- κ B to dissociate from I- κ B, and enter the nucleus. Other workers have suggested phosphorylation of I- κ B causes it to be released from NF- κ B (reviewed by Baeuerle, 1991). The importance of NF- κ B to class I transcription is indicated by the positive correlation between levels of class I expression and the amount of NF- κ B binding activity detected in various cell lines and tissues (Burke et al., 1989; Blanchet et al., 1992). Although transient expression assays have shown region I to act as an enhancer sequence, in certain circumstances, such experiments do not reveal which transcription factor is responsible for the enhanced transcription. Region I has been shown to act as a particularly strong enhancer when cells are treated with TPA or TNF α , two agents which both cause the nuclear localization of NF- κ B (Israel et al., 1989). This suggests that NF- κ B could be responsible for this enhancer activity. Cotransfection of p65 and p50 with a CAT plasmid linked either to the κ enhancer or HIV NF- κ B-motif caused an induction of CAT activity (Ballard et al., 1992). Further evidence for the involvement of NF- κ B in transcriptional activation comes from the use of *in vitro* transcription systems, where a single protein can be investigated without contaminating proteins giving misleading results. Under these conditions NF- κ B purified from HeLa cytosol stimulated transcription from the HIV-1 promoter (Kretzschmar et al., 1992). NF- κ B purified from Jurkat cells stimulated transcription from a PRDII NF- κ B-like DNA-binding motif using an *in vitro* transcription assay (Cohen and Hiscott, 1992). Overall, the available evidence suggests that NF- κ B plays an important role in enhancing transcription from MHC class I genes especially in situations where transcription has been induced by factors such as TNF α , LPS etc.

p50 homodimers

A protein was purified by Kawakami et al. (1988) from human Burkitt Lymphoma cells by affinity chromatography using κ enhancer DNA-binding motif. The protein was estimated to be 51 kDa, and was considered to be NF- κ B. KBF1 (kappa-enhancer binding protein 1) was the name given by Yano et al. (1987) to a protein isolated from a mouse T-cell line by its affinity to region I. It became clear when both subunits of NF- κ B were cloned and sequenced (Ghosh et al., 1990; Ruben et al., 1991) that Kawakami et al. (1988) had in fact only purified the p50 subunit of NF- κ B and not the whole protein. When KBF1 was cloned and sequenced it was found to be identical to the p50 subunit of NF- κ B (Kieran et al., 1990).

Purified p50 and p65 have been denatured and renatured as p50 homodimers and as p50/p65 heterodimers (Cohen and Hiscott, 1992). The two proteins can be distinguished as the homodimers have faster gel mobility. NF- κ B binds with twice the affinity of p50 dimers for the κ enhancer DNA-binding motif, whereas region I is recognised equally well by both (Baeuerle, 1991). p50 dimers appear to be constitutively present in most cell types (Baeuerle, 1991), however Blanchet et al. (1992) found some human tumour cell lines which did not appear to have p50 dimer/KBF1 binding activity. They found that cell lines which retained NF- κ B binding but had lost KBF1 binding still retained normal levels of MHC class I expression, suggesting KBF1 is not essential for class I gene transcription. *In vitro* transcription assays have given different results as to the role of p50 dimers as activators of transcription. Kretzschmar et al. (1992) found p50 dimers to be strong transcriptional enhancers when using the HIV NF- κ B DNA-binding motif, whilst Cohen and Hiscott (1992) found p50 to be ineffective in stimulating transcription from a PRDII NF- κ B DNA-binding motif. Kretzschmar et al. (1992) suggest the specific NF- κ B motif used could be critical as to whether p50 dimers act as transcriptional enhancers or not. The importance of the particular NF- κ B motif is further shown by the cotransfection experiments of Schmitz and Baeuerle, 1991. In these experiments p50 and p65 expression plasmids were transfected separately or together with a CAT plasmid controlled by the κ enhancer. Here p50 alone did not induce transcription and in fact reduced the transcriptional activation by p65. Since the role of p50 dimers appears to vary between different NF- κ B motifs, their role in the transcriptional regulation of class I genes requires experiments using region I before it can be determined.

p65 homodimers

p65 dimers have not been detected when probes of NF- κ B-like DNA-binding motifs were used with cellular extracts in gel retardations, and it is not known if such dimers occur *in vivo* (Schmitz and Baeuerle, 1991). However gel purified and renatured p65, although binding with a lower affinity than p50 dimers or NF- κ B, has been shown to produce dimers in gel retardations (Baeuerle, 1991; Urban et al., 1991; Cohen and Hiscott, 1992). When eukaryotic expression vectors of p65 were cotransfected with a CAT reporter gene linked to multiple copies of the κ enhancer, CAT activity was significantly enhanced (Schmid et al., 1991; Perkins et al., 1992; Schmitz and Baeuerle, 1991). However similar experiments using region I linked to CAT did not show induction by p65 (Perkins et al., 1992) suggesting p65 may not be an important activator of class I transcription.

p49

p49 was isolated from a λ gt11 library prepared from a human B-lymphoid leukemia (Schmid et al., 1991). The library was screened at low stringency for hybridization to a human c-rel probe. p49 is derived from a large protein p100 which was also cloned by the same workers (Schmid et al., 1991). p49/p100 are members of the rel-related family most closely related to p105 (60% identity). Schmid et al. (1991) investigated the functional role of this protein, alone and dimerized to p65, by use of expression plasmids cotransfected into Jurkat cells with CAT reporter genes linked either to the HIV or κ enhancer NF- κ B DNA-binding motifs. p49 alone slightly increased CAT activity, however, when cotransfected with p65, it significantly enhanced CAT activity (more so than p50/p65). In fact p50/p65 were only effective in stimulating the κ enhancer, not the HIV enhancer. p49 did not stimulate the region I enhancer either alone or with p65, despite the ability of p49 to bind to this sequence (Perkins et al., 1992).

H2TF1

Baldwin and Sharp (1987) described a factor, termed H2TF1 (H-2 transcription factor 1) which binds to region I. They found this binding activity to be present in many different cell types. Baldwin et al. (1990) estimated H2TF1 to have a molecular weight of 110 kDa. Baeuerle (1991) has suggested that H2TF1 represents a p50 dimer.

KBF2

Another factor which binds specifically to region I, KBF2 (kappa-enhancer binding factor 2) was purified from differentiated mouse cells. This factor was estimated to have a molecular weight of 58 kDa. Whether KBF2 is a post-translationally modified form of p50 remains to be determined.

CRE1

This is a binding activity described by Ackrill and Blair (1989) which binds specifically to a probe which includes region I. It is possible that this binding activity represents p50 homodimers.

EBP1

A protein called EBP1 (enhancer-binding protein 1) of molecular weight 57 kDa, has been isolated from Hela cells (Clark et al., 1989). EBP1 binds to the core region of the SV40 enhancer (an NF- κ B-like motif) and to region I. Biochemical analysis of EBP1 suggests EBP1 is a p50 dimer (Clark et al., 1990).

TC-IIA and TC-IIB

Macchi et al. (1989) describe two DNA-binding proteins, TC-IIA and TC-IIB, which bind to the SV40 NF- κ B-like motif (present in the TC-II enhancer) and to region I. TC-IIB has a higher affinity for region I. Macchi et al. (1989) suggest TC-IIB is a H2TF1/KBF1-like protein and TC-IIA is a NF- κ B-like protein.

v-rel

The avian reticuloendotheliosis virus, REV-T, was isolated from a turkey tumour by Theilen et al. in 1958. The virus transforms immature lymphoid cells *in vitro* and produces fatal lymphomas (Theilen et al., 1966; Beug et al., 1981; Hoelzer et al., 1980). The REV-T genome includes the gene *rel* which is known as v-rel, for viral *rel*. This gene is expressed as a phosphoprotein of 59 kDa. Twelve in-frame residues at the N-terminus and 19 amino acids at the C-terminus are derived from the viral *env* gene. v-rel differs from cellular *rel* (c-rel) in terms of size and composition. v-rel is smaller than c-rel, it also has 21 base changes and three small in-frame deletions (Stephens et al., 1983; Wilhemsen et al., 1984; Sylla and Temin, 1986; Rice et al., 1986). v-rel is highly transforming for avian spleen cells (Kamens et al., 1990).

v-rel has been shown to bind to NF- κ B-like DNA-binding motifs (Kochel and Rice, 1992; Kabrun et al; 1991). v-rel has been found to suppress transcription from NF- κ B sites (Inoue et al., 1991; Ballard et al., 1990). However, since v-rel is not generally present in cells, it is not relevant to the normal transcriptional control of MHC class I genes.

c-rel

The protein c-rel is of cellular origin. The v-rel oncogene is a truncated and mutated form of the avian c-rel protein. c-rel has two N-terminal and 118 C-terminal amino acids that are not present in v-rel. There are also several internal differences between c-rel and v-rel. Chicken c-rel, a 68 kDa protein, is weakly transforming at best (Kamens et al., (1990). The protein c-rel has been shown to bind to NF- κ B motifs including region I (Inoue et al., 1991; Kochel and Rice, 1992). There is evidence that c-rel can associate with p50 (Logeat et al., 1991) and p65 (Hansen et al., 1992). In cotransfection experiments c-rel has activated transcription from CAT genes linked to NF- κ B sites including region I (Inoue et al., 1991).

Richardson and Gilmore (1991) found that full length chicken c-rel and mouse c-rel only weakly activated transcription. They found that c-rel proteins consist of an N-terminal inhibitory domain and a C-terminal activation domain. The activation domain is absent in v-rel. The transient expression results obtained by Inoue et al. (1991) were with plasmids only containing the activation domain. In conclusion c-rel may be a transcriptional activator *in vivo*, including in the regulation of class I genes, but only if mechanisms exist to overcome the inhibitory effect of the N-terminal domain. c-rel also contains a cytoplasmic retention sequence (Hannink and Temin, 1989) which may interfere with its ability to transactivate in the nucleus.

Other rel-related proteins

When proteins have been purified through their affinity for NF- κ B-like DNA-binding motifs, several proteins have been isolated apart from p50 and p65. For example Cohen and Hiscott (1992) also isolated a p72 protein which could heterodimerize with p50, a p47 and a p56. Moliter et al.(1990) have also isolated several rel-related NF- κ B specific proteins. They isolated a p75 and cloned p75 cDNAs based on peptide sequence information derived from affinity-purified protein and have demonstrated the complete identity of p75 and NF- κ B p65 at the protein level (Ballard et al., 1992).

This suggests that post-translational modifications could be responsible for the difference in molecular weight. This group also found a p55 which was identical to p50 at the protein level, again suggesting post-translational modification (Moliter et al., 1990). A p42 proteolytic break-down product of p50 has been described which retains DNA-binding capabilities (Baeuerle, 1991). The functional role of the proteins described in this section is yet to be determined.

PRDII-BF1-related DNA-binding proteins

Several cDNA clones have recently been isolated from mouse, rat and human cDNA libraries. The proteins encoded by these cDNAs all have in common the property that they bind to region I and the proteins are large.

PRDII-BF1 (Fan and Maniatis, 1990), HIV-EP1 (Maekawa et al., 1989) and MBP-1 (Baldwin et al., 1990) are all clones from human cDNA libraries. All three clones are thought to cover different amounts of sequence from the same protein (Baeuerle, 1991; Michelmore et al., 1990). A full length cDNA was isolated from screening a λ gt11 cDNA expression library prepared from MG63 cell mRNA (Fan and Maniatis, 1990). The library was screened with a probe containing multiple copies of PRDII which is one of the two positive regulatory domains of the IFN β gene promoter. The cDNA has a single open reading frame of 8151 nucleotides and is estimated to encode a protein of 298 kDa called PRDII-BF1 (positive regulatory domain II binding factor 1). The protein contains two sets of two zinc fingers. Either set of zinc fingers can bind both PRDII or region I NF- κ B-like DNA-binding motifs with approximately fivefold higher affinity for the latter. Little is known about the functional role of this protein. Fan and Maniatis (1990) found that the steady state level of HLA class I mRNA increased upon serum induction. Treatment with serum also caused an increase in PRDII-BF1, which occurred more rapidly than the increase in HLA class I mRNA.

Several overlapping cDNA clones were isolated from a B-cell library screened with probes to region I. 6.5 kb of sequence was obtained (Baldwin et al., 1990). The cDNAs were found to encode a protein they called MBP-1 (MHC enhancer binding protein 1).

Maekawa et al. (1989) used a Southwestern method to isolate a cDNA encoding a protein that binds to the HIV NF- κ B-like DNA-binding motif. The cDNA was obtained

from a human B-cell λ gt11 library. The protein was called HIV-1 enhancer binding protein 1 (HIV-EP1). The insert consisted of 1035 bp and included two zinc finger domains.

Nakamura et al. (1990) screened a λ gt11 expression library of mouse transformed lens cells for proteins that bind to a section of the α A-crystallin gene promoter which contains a NF- κ B-like DNA-binding motif. They isolated a 2.5 kb cDNA which encoded a protein, called α A-CRYBP1 (α A-crystallin binding protein 1) which also binds to region I. This cDNA hybridized to a 10 kb mRNA. They suggest α A-CRYBP1 is either homologous or extremely similar to human MBP-1/PRDII-BF1 and HIV-EP1.

Michelmore et al. (1990) screened a rat liver cDNA library in λ gt11 for proteins that bind to the B domain of the α_1 -antitrypsin promoter, an element which is important for the liver-specific expression of α_1 antitrypsin. Two partial cDNAs coding for DNA-binding proteins (AT-BP1 and AT-BP2; α_1 -antitrypsin binding proteins) have been isolated. Both proteins contain two zinc fingers and both bind to the NF- κ B-like DNA-binding motif in region I with higher affinity than to the one in the immunoglobulin κ light chain gene enhancer or to the B domain of α_1 -antitrypsin gene promoter.

Northern blot analyses with probes derived from AT-BP1 and AT-BP2 identified mRNA species of 9.5 and 9.0 kb. Fan and Maniatis (1990) detected a 9.5 kb mRNA for 298 kDa PRDII-BF1 protein. Although the predicted amino acid sequence of the complete AT-BP1 and AT-BP2 proteins are not yet known, the sizes of their RNAs suggests that they too are unusually large for transcription factors.

Ron et al. (1991) screened a rat liver cDNA expression library for proteins that bind to the acute-phase response element (APRE) in the promoter of the angiotensinogen gene. APRE binds a factor indistinguishable from NF- κ B. They isolated a single clone that encodes a protein which they called angiotensinogen gene-inducible enhancer-binding protein 1 (AGIE-BP1). It contains a zinc finger motif virtually indistinguishable from those of MBP-1/PRDII-BF1 or α A-CRYBP1. Outside this domain the sequences diverge considerably. On Western blotting, antiserum to AGIE-BP1 detects a protein larger than 250 kDa and Northern blotting detected a mRNA

greater than 10 kb in a variety of tissues. Because the mRNA is larger than those of AT-BP1 and AT-BP2 it may encode a different protein.

In summary, PRDII-BF1, MBP-1 and HIV-EP1 may correspond to the same human protein. However, several related proteins might be present in human cell lines; Fan and Maniatis (1990) detected two to three high molecular mass proteins (250-290 kDa) by Western blotting using anti-PRDII-BF1 antiserum. AGIE-BP1, AT-BP1 and AT-BP2 probably represent the rat and α A-CRYBP1 the mouse form(s) of these high molecular weight proteins that bind to NF- κ B-like DNA-binding motifs. Little is known about their function but their zinc fingers suggest that they may act as transcription factors. Their mRNAs have been found in many different tissues (Maekawa et al., 1989; Baldwin et al., 1990; Fan and Maniatis, 1990; Michelmore et al., 1990; Nakamura et al., 1990; Ron et al., 1991). Since they bind to region I, it is possible that they play a role in the control of MHC class I transcription and their widespread distribution suggests that they may be active in many different cell types.

Region II

Shirayoshi et al. (1987) found a factor in mouse L cells and LH8 T lymphocytes which bound to a H-2L^d sequence; using synthetic oligonucleotides, they found that the sequence required for binding was GGTGAGGTCAGGGGTGGGG which extended from -203 to -185 and they called this region II. Hamada et al. (1989) screened a mouse liver cDNA expression library with a radiolabelled region II binding site and isolated a cDNA clone which encoded a protein (termed H-2RIIBP) which bound to region II. This protein belongs to the steroid/thyroid hormone receptor superfamily and it recognises the estrogen response element as well as region II (Hamada et al., 1989).

Following binding of the appropriate ligand, members of the steroid/thyroid receptor superfamily translocate to the nucleus where they act as transcription factors and bind to specific sequences called hormone response elements (HREs). The DNA-binding domain of the receptors mediates specific recognition of the HREs and includes two zinc finger structures (Severne et al., 1988). Three amino acids in the stem of the first finger, the P box, are critical to specify the half-site sequence (Danielsen et al., 1989; Mader et al., 1989; Umesono and Evans, 1989). The receptors in this superfamily have been divided into two subfamilies based on their P box sequence (Danielsen et al.,

1989; Mader et al., 1989; Umesono and Evans, 1989). The subfamilies have been called the GR (glucocorticoid receptor) and the ER/TR (estrogen receptor/thyroid hormone receptor) subfamilies. The GR subfamily includes glucocorticoid, mineralocorticoid, androgen and progesterone receptors. These recognise common HREs consisting of an inverted repeat of TGTTCT half-sites. All the other receptors, including ER, two isoforms of TRs, retinoic-acid receptors (RARs) α , β , and γ , retinoid X receptors (RXRs) α , β , and γ and human vitamin D₃ receptors belong to the ER/TR subfamily. Members of the ER/TR class display cross-recognition of the palindromic HREs consisting of a half-site AGGTCA or a closely related sequence (reviewed by Umesono et al., 1991; Leid et al., 1992). RARs bind retinoic acid then bind to retinoic acid response elements (RAREs) in retinoic acid-responsive promoters in response to nanomolar concentrations of retinoic acid. RXRs activate different target genes to RARs and bind to different response elements in response to higher concentrations of retinoic acid (reviewed by Yu et al., 1991) some receptors such as ER are known to bind as homodimers (Gordon and Notides, 1986), others such as RAR, TR and vitamin D₃ receptors have been thought to bind as heterodimers with a coregulator which enhances binding and transcriptional activation. The identity of any coregulator was unknown until recently when a cDNA was isolated that encodes a protein that binds to RARE and to RAR. The protein was found to be a member of the RXR subgroup of receptors and was called RXR β (Yu et al., 1992). RXR β forms heterodimers with RAR, TR and vitamin D₃ receptors, and enhances binding and activates transcription from each receptor's specific response element (Hallenbeck et al., 1992; Yu et al., 1992). RXR β isolated from a human cDNA library was found to be equivalent to H-2RIIBP, a clone isolated from a mouse cDNA library. In fact the term RXR β has been used interchangeably with H-2RIIBP (Dey et al., 1992).

Ackrill and Blair (1989) described a binding activity termed CRE2 in Ad12-transformed cells which interacted with a probe which includes region II. It is possible that CRE2 can be distinguished from H-2RIIBP (Griffiths, 1992).

The functional role of region II and RXR β / H-2RIIBP in class I is not well understood. Burke et al. (1989) performed CAT assays with plasmids containing the 1.4 kb sequence 5' of the H-2L^d gene and with identical constructs apart from mutations to G residues within region II which had been shown to interfere with binding when methylated (Shirayoshi et al., 1987). The mutated form had 30% lower CAT activity,

indicating weak enhancer activity of this sequence. The distribution of region II binding activity, if anything, suggests a negative effect on transcription; Burke et al. (1989) found region II binding in the brain and early in gestation, when both region I binding and MHC class I antigens are undetectable. Region II has been shown to act as a repressor of the transcriptional activation caused by region I in Ad12 but not in Ad5 cells (Kralli et al., 1992; Ge et al., 1992). Retinoic acid is known to play a critical role in vertebrate development (Sporn and Roberts, 1983). However there is evidence that the role of RXR β /H-2RIIBP is not in fact negative and that it plays a critical role in the initial activation of MHC class I expression in development. During the early stages of embryonic development cells are class I negative (Ozato et al., 1985). Cell lines have been created from such tissue and are called EC cells. As mentioned earlier such cells can be made to differentiate and express class I under the influence of retinoic acid (Morello et al., 1982). It seems likely that the effects of retinoic acid are mediated by RXR β /H-2RIIBP. Nagata et al.(1992) found that a RXR β /H-2RIIBP expression plasmid enhanced the CAT activity of a plasmid containing the MHC class I promoter linked to a CAT gene when using N-tera, a human EC cell line, which is class I negative. The enhancement was retinoic acid-dependent and mediated through region II. Both the DNA-binding domain and the ligand-binding domain were required for transactivation of the MHC class I promoter. No significant transactivation by RXR β /H-2RIIBP was observed in differentiated class I positive N-tera 2 cells or in murine L fibroblasts or monkey COS-7 cells. Kralli et al. (1992) found retinoic acid treatment caused region II to no longer act as a repressor. *In vivo* footprinting studies performed by Dey et al (1992) failed to identify any protection of region II in the transgene HLA-B7 and the endogenous H-2K^b gene from adult spleen or brain tissue. However, if RXR β /H-2RIIBP plays a role in development one might only expect to see region II binding in developmental tissue at stages which respond to retinoic acid. Although Dey et al. (1992) presented evidence for the presence of RXR β /H-2RIIBP in brain tissue, the lack of *in vivo* protection suggests that for some reason, possibly unfavourable chromatin structure, RXR β /H-2RIIBP is prevented from binding to region II. The inability of RXR β /H-2RIIBP to bind to region II could contribute to the lack of expression of class I antigens in this tissue.

Region III

Shirayoshi et al (1987) describe a region between -189 and -161 which overlaps region I (-173 to -161) but binds distinct factors. Israel et al. (1989) propose that in untreated

Hela cells KBF1 binds to region I and AP2 binds to the adjoining region which is part of region III. In contrast TNF α stimulated cells NF- κ B dislodges KBF1 from region I and binds itself to this region while also dislodging AP2 from the adjoining region. AP2 then binds slightly further upstream but still within region III. NF- κ B binds to the two imperfect direct palindromic repeats, one within region I and one outside it. Israel et al. (1989) found that region III activated a heterologous promoter after TNF α or TPA induction. Three copies of region III were particularly active following TNF α induction. A multimer of either repeat was also responsive to TNF α and TPA (particularly the former), whereas a single copy of either repeat was not. These results suggest region III is important in enhancing class I transcription under the influence of TPA and especially TNF α .

1.2.6.12 Transcription factors and promoter sequences involved in the regulation of transcription by adenovirus E1A

As described previously E1A can effect MHC class I transcription in different ways depending on the serotype of E1A and cells investigated. In primary cells transformed by E1, Ad12 E1A appears to be involved in a process of down-regulation of MHC class I expression, however down-regulation does not always take place when Ad12 E1A is introduced into immortalized rodent cell lines (Vaessen et al., 1986; Nielsch et al., 1991). However Katoh et al. (1991) found that class I expression was down-regulated in the rat fibroblast cell line 3Y1 following introduction of the Ad12 E1A gene. Ad5 E1A can cause an up-regulation of class I expression in primary cells. This means that in different circumstances, differences in the properties ascribed to Ad12 and Ad5 E1A could reflect down-regulation by the former or up-regulation by the latter, or both processes could account for the different properties of cells transformed or transfected with these genes. Both Ad5 E1 and Ad12 E1 transformed or transfected cells would be expected to have sequences within their class I promoters which act as enhancers or repressors. However, for an overall difference in promoter efficiency to exist one would expect enhancer sequences to be more efficient under the influence of Ad5 E1 and/or repressor sequences to be more active under the influence of Ad12 E1. Kimura et al. (1986) found the H-2K^b and H-2K^d promoter drove approx. ten times lower CAT activity in primary mouse cells transformed by the Ad12 E1 region than in those transformed by Ad5 E1. Using a construct containing the region -254 to -61, which acts as an enhancer in untransformed fibroblasts, CAT activity in cells transformed by Ad12 E1 was between half and two-thirds of that in cells transformed

by Ad5 E1. This suggests that although some of the differences in CAT activity between cells transformed by Ad5 or Ad12 E1 is mediated by the sequence -254 to -61, sequences outside this region are more important in contributing to these effects.

Katoh et al (1990) used 3Y1 cells, a diploid Fisher rat embryo fibroblast cell line in transformation experiments with Ad12 13S or Ad5 13S expression plasmids. In cells expressing Ad12 13S there was a greater than twenty fold reduction in CAT activity driven by the promoter from -2014 to +12, while the promoter activity was only slightly inhibited by expression of Ad5 13S. This difference in the properties of Ad5 13S and Ad12 13S could not be ascribed to an enhancer activity being stronger in Ad5, since a plasmid which contained sequences from -194 to -136 and -61 to +12 linked to CAT, enhanced CAT activity more in cells expressing the Ad12 13S product than the Ad5 13S product. This result is in conflict with those obtained by Kimura et al. (1986) using Ad-transformed primary mouse embryo cells. The reason for the variability in results obtained could be related to the different cells used and the different enhancer regions used, although both included region I. Also these results were with transfected rat cells, whereas those described of Kimura et al. (1986) were with transformed mouse cells.

Work by other groups have identified factors which bind to sequences within enhancer A could be important in mediating down-regulation by Ad12 E1A. Meijer et al. (1992) cotransfected primary BRK with a construct linking region I to CAT and either an Ad12 12S or an Ad12 13S expression plasmid. Region I displayed enhancer activity in the presence of Ad12 12S but not in the presence of Ad12 13S. They also found reduced binding of NF- κ B and KBF1 in whole cell extracts from BRK cells transformed by Ad12E1 compared to cells transformed by Ad5E1. Nielsch et al. (1991) found that nuclear levels of NF- κ B, but not KBF1, were lower in Ad12- compared to Ad5-transformed cells but their results were obtained by transformation of an embryo fibroblast cell line and not BRK cells.

Meijer et al. (1992) also found increased binding of a region II factor in Ad12 E1-transformed cells as compared to Ad5 E1-transformed cells, which had been found previously by Ackrill and Blair (1989), who suggested this might be relevant to the down-regulation of class I in these cells. However Meijer et al. (1992) found no evidence that binding to region II mediated a repressor function. The work of Ge et

al. (1992) and Kralli et al. (1992) did demonstrate a repressor function for region II, however, in order to demonstrate this function multiple copies of region II were necessary and each copy of region II needed to be linked to a copy of region I. In fact it was the repression of the transcriptional activation by region I in Ad12-transformed cells but not Ad5-transformed cells which was demonstrated. The differences in experimental design could explain the different results obtained by Meijer et al. (1992) as compared to Ge et al. (1992) and Kralli et al. (1992).

It has been suggested that the CRE is not the only region of the class I promoter which might be of importance to the down-regulation found in Ad12 E1A transformed cells (J. Proffitt, E. Sharma & G. E. Blair, unpublished results). BRK cells transfected with constructs lacking the CRE were still susceptible to down-regulation by E1A. When the region -1490 to -1180 was removed from the 2 kb class I promoter this relieved transcriptional repression in Ad12-transformed cells but did not alter promoter activity in Ad5-transformed cells. This suggests that the region -1490 to -1180 acts in down-regulation in Ad12-transformed cells. Consistent with these results, a construct from -1440 to -1070 caused down-regulation of a heterologous (thymidine kinase) promoter in Ad12-transformed cells and not in Ad5-transformed cells. Gel retardation experiments using approx. 100 bp probes from this region revealed three probes: -1350 to -1230 as well as 1260 to -1150 and -1180 to -1070 which all formed specific complexes using nuclear proteins from Ad12-transformed cells whereas no complexes were formed using extracts of Ad5-transformed cells. The probes -1350 to -1230 and -1180 to -1070 formed faint complexes with proteins from BRK cells suggesting the involvement of cellular factors whose binding activity may be modulated by Ad-transformation.

In the study of Katoh et al. (1990), as well as showing in rat embryo fibroblast cells that the region -254 to -61 is positively regulated by E1A, they also identified a region of negative regulation. They identified a sequence between -1837 and -1521 in the H-2K^{bml} promoter that caused down-regulation of MHC class I transcription in Ad12-transformed cells. This sequence was specifically bound by nuclear factors derived from Ad12-transformed cells but not from untransformed cells or Ad5-transformed cells. However this sequence was only twice as active in cells cotransfected by Ad5 13S as compared to those cotransfected by Ad12 13S, which shows that it may not be the only sequence involved in causing the twenty fold lower

CAT activity of the 2 kb promoter when cotransfected with Ad12 13S compared to Ad5 13S.

The aim of this study was to investigate the possible involvement of the sequences CRE1 and CRE2, and the transcription factors that bind to them, in the down-regulation of MHC class I transcription in Ad12-transformed cells compared to those transformed by Ad2 or Ad5. The transient expression assay, CAT, was used to investigate the functional role of these sequences. The nuclear levels of CRE1-specific DNA-binding factors was investigated in Ad-transformed cells by gel retardation and Western blotting. An antibody was raised to the p50 subunit of the CRE1-specific factor, NF- κ B; this antibody was used in Western blotting. Other aspects of CRE1- and CRE2-binding factors were also investigated in Ad-transformed cells: the binding site for CRE2-specific factors was studied by DNase I footprinting along with the mechanism of nuclear induction of CRE1-binding factors.

Materials

2.1 Reagents

The following reagents were obtained from Sigma, Dorset, UK: DTT, HEPES, 2-mercaptoethanol, piperidine, PDTC, NAC, Freund's Incomplete Adjuvant, Rabbit ExtrAvidin[™] Biotin Staining Kit, goat anti-rabbit alkaline phosphatase, aprotinin, leupeptin, BCIP, NBT, Ponceau S, O-phenyldiamine, cycloheximide.

Glycerol, magnesium chloride, chloroform, ammonium persulphate, TEMED and urea were purchased from FSA Laboratories, Loughborough, UK. Protein Assay kit and broad range molecular weight markers were obtained from Biorad, Munchen, Germany. Benzoyl peroxide, triethylsilane and DMS were obtained from Aldrich Chemical Company, Dorset, UK.

Brewers yeast tRNA and PMSF were purchased from Boehringer Mannheim Biochemica, East Sussex, UK. Bactotryptone, yeast extract and 'Lab M' agar were obtained from Amersham International plc, Buckinghamshire, UK. Deoxyribonucleoside triphosphates were obtained from BCL, East Sussex, UK. Sephadex G-10, G-25 and G-50 (fine), poly(dI-dC) and other polydeoxyribonucleotides were purchased from Pharmacia Ltd., Milton Keynes, UK. Glass distilled phenol was obtained from Rathburn Chemicals Ltd., Scotland. Ethanol was bought from James Burroughs Ltd, Essex, UK. Donor calf serum was obtained from Sera Labs, Sussex, UK and cell culture media and constituents were purchased from Gibco, Paisley, Scotland. Ellmans reagent and sulpho-SMCC were obtained from Pierce, Illinois, USA. Insta-gel liquid scintillation cocktail was purchased from Packard, Groningen, Netherlands. Purified protein derivative of tuberculin (PPD) and Bacillus Calmette-Guerin (BCG) vaccine were purchased from Statens Seruminstitut, Tuberculin Dept., DK2300, Copenhagen, Denmark. Water used was distilled and filtered through a Millipore 'Milli Q' system. All other reagents were obtained from BDH. All reagents used were Analar grade or equivalent.

2.2 Enzymes

All enzymes were obtained from Boehringer Mannheim Biochemica, East Sussex, UK.

2.3 Radiochemicals

Deoxyadenosine triphosphate α -thiol [^{35}S] 500 Ci mmol $^{-1}$ 10 mCi ml $^{-1}$ was purchased from NEN (Dupont), Hertfordshire, UK.

D-threo (dichloroacetyl -I ^{14}C) chloramphenicol with a specific activity of 57 mCi mmol $^{-1}$ (25 mCi ml $^{-1}$) and [^{14}C] methylated proteins (5 mCi mmol $^{-1}$) were obtained from Amersham International plc, Buckinghamshire, UK. The markers comprised the following components:

myosin 200 kDa

phosphorylase b 92.5 kDa (sometimes an extra band at 100 kDa was also present)

bovine serum albumin 69 kDa

ovalbumin 46 kDa (sometimes an extra band at 50 kDa was also present)

carbonic anhydrase 30 kDa

lysozyme 14.3 kDa

Adenosine 5' triphosphate (ATP), tetra (triethylammonium) salt [γ - ^{32}P] > 4000 Ci mmol $^{-1}$ 10 mCi ml $^{-1}$ in water and deoxyguanosine 5'-triphosphate (dGTP), tetra(triethylammonium) salt [α - ^{32}P] > 3000 Ci mmol $^{-1}$ 10 mCi ml $^{-1}$ in water were supplied by ICN Biomedicals Ltd, Buckinghamshire, UK.

2.4 Plasmids

The pBLCAT2 expression vector was used for subcloning, in which the CAT gene is under the control of the herpes simplex virus thymidine kinase basal promoter (Luckow and Schutz, 1987).

2.5 Antibodies

The rel antibody SB66 was kindly donated by Dr P. Enrietto, New York State University, Stony Brook. Three antibodies to p50 of NF- κ B were kindly donated by Dr R. Hay, St Andrews, Scotland. One of these antibodies was raised to the DNA-binding domain, amino acids 35-381, from the sequence of Kieran et al. (1990). The other two antibodies were raised to the N- and C-terminal peptides, their sequences are, C-terminal: CDYGQEGPLEGKI, N-terminal: MAEDDPYLGRPEC

All the antisera were polyclonal and were raised in rabbits.

2.6 Oligodeoxynucleotides

The oligodeoxynucleotides used in gel retardation and/or subcloning are shown in table 2.1. kappa, CRE1, mCRE1 and CRE2 were synthesized in this Division, on a

Table 2.1 Sequences of the oligonucleotides used in cloning and gel retardation

The sequences are shown of the oligonucleotides used in gel retardations and cloning: CRE1, mCRE1 and CRE2 along with the oligonucleotides used solely in gel retardation: kappa and Sp1. The residues in bold print differ between CRE1 and mCRE1. Each oligonucleotide was double stranded, however, for clarity only a single strand is shown.

OLIGONUCLEOTIDE	SEQUENCE
CRE1	GATCCGGCTGGGATTCCCCATCT
mCRE1	GATCCGGCTGCGGATTCCCAATCT
Kappa	GATCCAGAGGGGACTTCCGAGAG
CRE2	GATCCAGTGAGGTCAGGGTGGGAAA
Sp1	ACTTGATTAACTGGGCGGAGTTATGATTGA

Gene Assembler (Pharmacia) using phosphoramidite chemistry. The kappa probe corresponds to the NF- κ B-like DNA-binding motif present in the immunoglobulin kappa light chain enhancer. CRE1 includes region I, the NF- κ B-like DNA-binding motif present in the H-2K^b gene. The Sp1 oligonucleotide was a gift from Dr S. P. Jackson, Wellcome CRC Institute, Cambridge.

2.7 Cells and cell lines

Ad2-transformed cells

F19: Ad2-transformed rat embryo brain cells (Harwood and Gallimore, 1975).

Ad5-transformed cells

BMK 2.4: Ad5 virus-transformed baby mouse kidney cells (Blair-Zajdel and Blair, 1988).

Ad5Xho: Ad5-transformed baby rat kidney cells. Kindly supplied by K.Raska.

Ad12-transformed cells

C57AT1: Ad12-transformed mouse embryo kidney cells (Maeta and Hamada, 1979)

Ad12#1: baby rat kidney cells transformed by Ad12 virus. Kindly supplied by K.Raska

RFC-1: Ad12-transformed Hooded Lister (LIS) rat kidney cells, transformed by the Eco RI-C fragment (containing the E1A and E1B genes) of Ad12 (Raska et al., 1980).

Cells transformed by recombinant virus

5A12: Baby rat kidney cells transformed with recombinant Ad5 virus containing the Ad12 E1A gene (Sawada et al., 1985).

5B12: Baby rat kidney cells transformed by recombinant Ad5 virus containing the Ad12 E1B gene (Sawada et al., 1985).

Human cell lines

Hela: Human cell line derived from a cervical epitheloid carcinoma (Scherer et al., 1953).

2.8 Other materials and reagents

Recombinant TNF α was obtained from Genzyme Corporation. IFN γ was purchased from Biotechnology BV, Holland. Dialysis tubing was obtained from Medicell International Ltd. Nitrocellulose was obtained from Schleicher and Schuell, Dassel,

Germany. Microcollodion bags were purchased from Sartorius, Gottingen, Germany. Glass beads (425-600 microns) were obtained from the Sigma Chemical Company Ltd. Silica gel TLC plates were purchased from Merck, Darmstadt, Germany. The 96 well ELISA plates used were obtained from Corning, U.K.

2.9 Buffers and Solutions

2.9.1 Electrophoresis buffers

10 x Tris-acetate EDTA (10 x TAE)

Tris base 48.4 g l⁻¹

glacial acetic acid 11.42 ml l⁻¹

0.5 M EDTA (pH 8.0) 20.0 ml l⁻¹

10 x Tris/glycine buffer

glycine 139.6 g l⁻¹

Tris base 30.3 g l⁻¹

SDS 10 g l⁻¹

5 x Tris borate EDTA (5 x TBE)

Tris base 54 g l⁻¹

boric acid 27.5 g l⁻¹

0.5 M EDTA (pH 8.0) 20.0 ml l⁻¹

Wet blot transfer buffer

Tris base 9.69 g

glycine 45.04 g

methanol 0.8 l

made up to four litres with water

2.9.2 Polyacrylamide gel mixtures

6% retardation gel

57 g acrylamide

3 g bisacrylamide

made up to 1 l in 0.5 x TBE. To 30 mls of gel mixture 225 μ l of 10% w/v ammonium persulphate was added, the mixture was degassed and 30 μ l of TEMED was added.

15% SDS/polyacrylamide gel

Separating gel

30% acrylamide 0.8% bisacrylamide, 10.0 mls (30 g acrylamide and 0.8 g bisacrylamide made to 100 mls with water)

1.5M Tris HCl, pH 8.8, 6.0 ml

10% ammonium persulphate 67 μ l

10% SDS 200 μ l

TEMED 3.8 μ l

Stacking gel

30% acrylamide 0.8% bisacrylamide 2.5 ml

0.5 M Tris HCl, pH 6.8, 3.75 ml

water 8.5 ml

10% ammonium persulphate 0.15 ml

10% SDS 0.15 ml

TEMED 20 μ l

For both the stacking and the separating gel the mixture was degassed after the addition of ammonium persulphate and TEMED was then added.

6% sequencing gel

40% acrylamide:bis (19:1)

114 g of acrylamide and 6 g of bisacrylamide was made up to 300 mls with water. It was stirred gently with 6 g of mixed bed resin, Amberlite MB1. The mixture was filtered through sintered glass to remove the resin.

Gel mixture

40% acrylamide:bis (19:1) 150 ml

10 x TBE 100 ml

urea 480 g

made up to 1 l with deionized water.

12% footprinting gel mixture

40% acrylamide:bis (19:1) 300 ml

10 x TBE 100 ml

urea 480 g

made up to 1 l with deionized water

For both the sequencing and footprinting gels, per gel, 60 ml of the gel mix was used with 120 μ l of 25% ammonium persulphate and 70 μ l of TEMED.

2.9.3 Mammalian cell culture media

Dulbecco-modified Eagle medium (DMEM) 1 L

- 50 ml 10 x DMEM
- 5 ml penicillin/streptomycin (10,000 U ml⁻¹)
- 5 ml 0.1 M sodium pyruvate
- 5 ml 0.2 M L-glutamine
- 20 ml 7.5% w/v sodium bicarbonate
- 415 ml water

RPMI-1640 1 L

- 50 ml 10 x RPMI-1640
- 5 ml penicillin/streptomycin (10,000 U ml⁻¹)
- 5 ml 0.2 M L- glutamine
- 13 ml 7.5% w/v sodium bicarbonate
- 427 ml water

RPMI-1640 medium was made to pH 7.3 before use with sterile HCl

2.9.4 Bacterial growth media

Luria broth (LB)

- Bactotryptone 10 g
- NaCl 10 g
- yeast extract 5 g

made up to 1 l with water and autoclaved.

LB agar

- Bactotryptone 10 g l⁻¹
- NaCl 10 g l⁻¹
- yeast extract 5 g l⁻¹
- agar 15 g l⁻¹

made up as LB then agar was added just before autoclaving. Plates were poured when the LB agar had cooled to approximately 50°C. LB/ampicillin plates were made exactly like LB plates except ampicillin was added at a concentration of 100 µg/ml just before pouring.

2.9.5 Plasmid DNA miniprep solutions

Solution A

0.05 M glucose
0.025 M Tris HCl pH 8.0
8 mM EDTA

Lysozyme solution was prepared at a concentration of 12 mg/ml in 50 mM Tris HCl pH 8.0

Solution B

0.2 M NaOH
1% SDS

2.9.6 Nuclear extract preparation buffers

Buffer A

10 mM Hepes
1.5 mM MgCl₂
10 mM KCl
0.5 mM PMSF
0.5 mM DTT

Buffer C

20 mM Hepes
0.42 M NaCl
1.5 mM MgCl₂
0.2 mM EDTA
0.5 mM DTT
25% v/v glycerol
0.5 mM PMSF

Buffer D

20 mM Hepes
100 mM KCl
0.2 mM EDTA
0.5 mM PMSF
0.5 mM DTT
20% v/v glycerol

DTT and PMSF were added fresh at time of use. These buffers were sometimes prepared with 10 mM NaF as specified in the text. Buffers AM, CM and DM (M for modified) were the same as A, C and D except in AM and CM there was 50mM sodium fluoride, 0.5 mg/ml leupeptin and 0.24 TIU (trypsin inhibitory units) of aprotinin, buffer DM had 50 mM sodium fluoride and 0.5 mg/ml leupeptin.

2.9.7 Western blotting solutions and reagents

AP buffer

Tris HCl pH 9.5 100 mM
NaCl 100 mM
MgCl₂ 5 mM

BCIP

0.5 g of BCIP was dissolved in 10 ml of 100% dimethyl formamide.

NBT

0.5 g of NBT was dissolved in 10 ml of 70% dimethyl formamide.

2.9.8 Reagent for ELISA

o-phenylenediamine was freshly prepared at 10 mg/ml in methanol and stored in the dark until use.

2.9.9 Colony hybridization buffers

Prehybridization buffer

50% formamide
6 x SSC
5 x Denhardt's
0.5% SDS

100 $\mu\text{g/ml}$ denatured sonicated salmon sperm

Hybridization Buffer

Prepared as prehybridization buffer with the addition of 5% w/v dextran sulphate

Washing Solution

0.5 x SSC

0.5% SDS

50 x Denhardt's

Ficoll 5 g

Polyvinylpyrrolidone 5 g

BSA (pentax fraction V) 5 g

water to 500 ml

Filtered through disposable Nalgene filter, stored in aliquots at -20°C .

20 x SSC

Dissolved 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mls of water, pH was adjusted to 7.0 with concentrated NaOH and the volume was adjusted to 1 L. The solution was sterilized by autoclaving.

2.9.10 Other buffers and solutions

Triton X-100 lysis buffer

Triton X-100 20 ml

1 M Tris pH 8.0 5 ml

0.5 M EDTA pH 8.0 2 ml

made up to 100 ml in distilled water

10 x Kinase buffer

0.5 M Tris HCl pH 7.6

0.1 M MgCl_2

50 mM DTT

1 mM spermidine

1 mM EDTA pH 8.0

Tris EDTA (TE)

10 mM Tris HCl pH 8.0

1 mM EDTA pH 8.0

PBS

NaCl 8 g

KCl 0.2 g

Na₂HPO₄

KH₂PO₄

Made up to 1 L with distilled water

10 x Hepes-buffered saline (HBS)

Hepes 5 g

NaCl 8 g

KCl 0.37 g

Na₂HPO₄

glucose 1.0 g

made up to 100 ml with distilled water then filter sterilized. 0.25 M CaCl₂ was prepared and filter-sterilized.

2.9.11 Sample Buffers

For gel retardations and sequencing gels

sucrose 2.5 g

0.25 M EDTA 2 ml

bromophenol blue 10 mg

made up to 5 ml with distilled water

For SDS/polyacrylamide gels

10% SDS

urea 4.8 g

1 M Tris HCl pH 6.8

bromophenol blue 10 mg

made up to 10 mls with distilled water

For footprinting

formamide 9.8 ml
0.5 M EDTA 0.2 ml
xylene cyanol 10 mg
bromophenol blue 10 mg

5 x DNA-binding buffer for CRE1 and Kappa probes

100 mM Hepes pH7.9
250 mM KCl
12.5% glycerol
1 mM DTT

DNA-binding buffer for the SP1 probe

20 mM Hepes pH 7.9
100 mM KCl
12 mM MgCl₂
0.1 mM EDTA pH 8.0
17% glycerol

both buffers were made up with distilled water

2.10 Preparation of materials

All plastics and glassware used for nucleic acid analysis was sterilized by autoclaving. Dialysis tubing was prepared by boiling for 15 minutes in 500 ml of 10 mM EDTA containing 1 g NaHCO₃. The tubing was then washed extensively in sterile water and stored in 0.05% sodium azide at 4°C.

Methods

2.11 Production of antibody to the C-terminal peptide of NF-κB

The sequence DYGQEGPLEGKI is present at the C-terminus of p50 of NF-κB (Kieran et al., 1990). It was decided to synthesize this peptide and its derivative CDYGQEGPLEGKI, in which an N-terminal cysteine was added in order to link the peptide to a crosslinker and carrier. The peptides were synthesized, purified and sequenced by Dr J. Keen and J. Johnson (University of Leeds).

2.11.1 Peptide synthesis

The peptides were made on a MilliGen/Biosearch 9050 automated solid-phase peptide synthesizer, 9-fluoroenylmethoxycarbonyl (Fmoc)-based chemistry with side-chain-protected amino acids was used.

2.11.2 Peptide purification

Each peptide was purified by reverse-phase h.p.l.c. on a Pharmacia PepRPC™ HR 5/5 C₂/C₁₈ column. The major peptide peak was collected for subsequent analysis and use. An aliquot of peptide was subjected to amino acid sequence analysis to assess quantity and purity.

2.11.3 Amino acid sequence analysis

An aliquot of each peptide (1 μg approximately) was dissolved in water and dried onto a glass-fibre disc for sequence analysis on an Applied Biosystems 477A liquid-pulse machine. Analysis was performed using standard manufacturers instructions.

2.11.4 Conjugation of the peptide to the carrier protein

a. Reduction of the peptide

12 mg of peptide (of which approximately 50% contained an N-terminal cysteine) was resuspended in 500 μl of 0.1 M sodium phosphate buffer pH 6.0 containing 5 mM EDTA and 50 mg of DTT and left overnight at 37°C.

b. Desalting of the peptide on a G-10 column

DTT and EDTA were removed from the peptide on a G-10 column. A slurry of G-10 Sepharose was prepared; 15 g of G-10 Sepharose were suspended in 100 ml of 0.1 M sodium phosphate buffer, pH 6.0, containing 5 mM EDTA. The suspension was shaken overnight at room temperature. A column was prepared in a 10 ml sterile pipette, washed with 3 column volumes of 0.1 M sodium phosphate buffer, pH 6.0, and stored in the cold room.

The reduced peptide/DTT mix was added to the column and was eluted with 0.1 M sodium phosphate buffer, pH 6.0, at 4°C. Fractions (1 ml) were assayed with the Ellman's reagent. The three fractions containing most of the reduced peptide were pooled.

c. The Ellman's Assay

Reduced Ellman's reagent gives a yellow colour, the absorbance of which can be read at 412 nm. The free sulphhydryl groups of the peptide or DTT reduce the Ellman's reagent. The Ellman's reagent was prepared by dissolving 4 mg in 1 ml of 0.1 M disodium hydrogen orthophosphate, pH 8.0. 20 μ l of the reagent was mixed with 20 μ l of each fraction and 1 ml of 0.1 M disodium hydrogen orthophosphate, pH 8.0. The absorbance was read at 412 nm.

d. Preparation of activated PPD

7 mg of lyophilized PPD was mixed with 1.4 ml of water and 1.75 mg of sulpho-SMCC and placed in an ultrasonic bath for 10 minutes. The pH of the solution was adjusted to 7.5 with NaOH. The solution was left at 25°C for 30 minutes with occasional gentle mixing.

Activation of PPD results from the n-hydroxysulphosuccinimidyl active ester moiety of sulpho-SMCC reacting with the free amino groups of PPD. This reaction occurs at pH > 7.

e. Separation of activated PPD from unreacted sulpho-SMCC on a G-25 column

11 g of G-25 Sepharose was shaken overnight at room temperature in 100 ml of 50 mM sodium phosphate buffer, pH 6.0. A 45 ml column was prepared, washed with three column volumes of 50 mM sodium phosphate buffer, pH 6.0, and stored in the cold room.

The activated PPD was loaded on the column and eluted with 50 mM sodium phosphate buffer, pH 6.0, at 4°C. 1 ml fractions were collected and the absorbance of each fraction was read at 280 nm. Activated PPD was eluted in the void volume of the column and unreacted sulpho-SMCC was present in later fractions. Three 1 ml fractions containing most of the activated PPD were pooled.

f. Crosslinking of the reduced peptide to the activated PPD

Reduced peptide was coupled to PPD cross-linked to sulpho-SMCC via the maleimide moiety which reacts with the free thiol group of the peptide's terminal cysteine between pH 6.5-7.5.

The G-25 and the G-10 columns were run simultaneously so that the 3 x 1 ml fractions of reduced peptide could be reacted immediately with the 3 x 1 ml fractions of the activated PPD. The pH was adjusted to pH 7.0 with NaOH and the solution was gassed with nitrogen and stored at room temperature overnight. The next day distilled water was added to give a final volume of 10 mls. The PPD conjugated peptide was stored in 1 ml aliquots at -70°C until use.

2.11.5 Preparation of the peptide-PPD conjugate for injection

An emulsion was prepared of the peptide-PPD conjugate (1.0 ml) and Freund's incomplete adjuvant (1.0 ml). The mixture was sonicated until it was of a consistency to produce spherical droplets on water.

2.11.6 Antibody Production Protocol

Two New Zealand white rabbits between 2-3 kg were allowed to settle in new accommodation for two weeks, then a sample of pre-immune blood was collected from each animal. After the collection of blood, the rabbits were injected with reconstituted freeze dried BCG vaccine for intradermal use. Each rabbit was given 2 x 0.1 ml BCG injections in the scruff of the neck. The first injection of the peptide-PPD conjugate took place one month later. PPD only elicits its delayed-type hypersensitivity reaction in animals that have encountered tubercle bacillus either through infection or immunization with BCG. The initial encounter with the PPD-peptide conjugate was in the form of 2 x 0.5 ml intramuscular injections in the hind legs and 2 x 0.2 ml subcutaneous injections in the scruff of the neck. This was followed by three boost injections at three, six and nine weeks after the initial injection. The boost consisted of 4 x 0.25 ml injections into the scruff of the neck. The rabbits were bled ten days after each of these four injections. Thirteen days after the final boost the rabbits were bled out by cardiac puncture.

2.12 ELISA

The peptide was dissolved in 0.06 M NaHCO₃ buffer (pH 9.6) at a concentration of 5 ng/ μ l. 200 μ l of this solution, the equivalent of 1 μ g of peptide, was added to each well of the ELISA plates and left overnight at 4°C.

The following day the peptide solution was removed and the wells were blocked with 0.1% BSA in PBS-Tween (0.05%) for 30 minutes at 37°C. Then the wells were

washed four times with PBS-Tween (0.05%) using a Wellwash 4 plate-washer (Denley). The test sera were diluted in PBS-Tween. Three fold dilutions were made from 1:100 to 1:218700 for each serum. 200 μ l of each dilution was added to two wells (the sera were tested in duplicate) and incubated for 30 minutes at 37°C. The wells were then washed four times with PBS-Tween (0.05%). When the specificity of binding was tested, the serum was preincubated with peptide before use in the ELISA. 1 μ l of a 1:100 dilution of serum was mixed with 100 μ g of peptide in an Eppendorf tube on a rotary mixer in a 30°C warm room for 1 hour. Either the peptide to which the antibody was raised or an unrelated peptide of the 5/6 loop of the human neurokinin 2 receptor was used.

To detect bound antibody the Sigma ExtrAvidin™ Biotin Staining kit was used. The biotinylated goat anti-rabbit IgG was diluted 1:1000 in PBS-Tween, 200 μ l of this dilution was added to each well and incubated for 30 minutes at 37°C. The solution was removed from the wells and the plates were washed four times with PBS-Tween. The plates were incubated with ExtrAvidin Peroxidase at 1:500 dilution in PBS-Tween for 30 minutes at 37°C, then were washed four times in PBS-Tween. After all liquid was carefully removed from the wells, 200 μ l of O-phenylenediamine substrate (prepared as described in section 2.9.8) was added to each well and incubated for 30 minutes in the dark. The reaction was stopped with 25 μ l of 4 M H₂SO₄. The absorbance was read at 492 nm on an Anthos™ plate reader 2001 (Anthos labtec instruments).

2.13 Western blotting

30 μ g of MBP3 nuclear extract was boiled in sample buffer and loaded onto a 15% SDS/polyacrylamide gel along with molecular weight markers. The gel was run in 1 x Tris/glycine SDS buffer at 250 V for four hours.

The proteins were transferred to nitrocellulose by a standard wet blotting technique (Sambrook et al., 1989) for three hours at 0.3 A using a TE-42 Transphor unit blotting apparatus (Hoeffer scientific instruments). When transfer was complete the nitrocellulose was stained for protein using 0.1% Ponceau S in 3% TCA. Strips corresponding to the sample tracks were cut out and the positions of the molecular weight markers indicated. After destaining in water the blots were incubated in blocking buffer (PBS containing 2% marvel, 0.1% NP40 and 0.1% sodium azide) for

1 hour. Antibodies raised in rabbits A and B against the C-terminal peptide of the p50 subunit of NF- κ B were used in Western blotting. Controls with pre-immune sera were also included. Antibodies to c-rel (donated by Dr P. Enrietto) and to the DNA-binding domain as well as the N- and C-terminal peptides of p50 (donated by Dr R. Hay) were also used on Western blots. The specificity of the antibodies to p50 raised in rabbits A and B was tested by blocking with either the peptide used for immunization or with an unrelated peptide (the 5/6 loop of the human neurokinin 2 receptor). 50 μ g of peptide was added to 1 μ l of serum in 500 μ l of PBS containing 0.1% NP40, 0.1% NaN₃ and left for 1 hour at 30°C on a rotary mixer, then 500 μ l of PBS containing 4% marvel, 0.1% NP40 and 0.1% NaN₃ was added. The nitrocellulose strips were sealed in bags with the appropriate primary antibody diluted in the blocking buffer, and incubated overnight at 4°C on a rotary mixer. The following day the blots were washed thoroughly in several changes of PBS containing 0.1% NP40. The blots were incubated for two hours in goat anti-rabbit alkaline phosphatase immunoconjugates diluted 1:1000 in blocking buffer, washed several times in PBS containing 0.1% NP40 and rinsed in alkaline phosphatase buffer. The substrate was made up fresh before use; 33 μ l of BCIP and 66 μ l of NBT in 10 ml of alkaline phosphatase buffer (prepared as described in section 2.9.7). Blots were incubated in substrate until blue coloured band(s) appeared, then washed in distilled water and dried between filter paper.

2.14 Cloning

The vector used was pBLCAT2. Table 2.1 shows the oligonucleotides that were cloned into pBLCAT2; these were termed CRE2, CRE1 and mCRE1. Each of these oligonucleotides was synthesized as complementary strands which were annealed together. The annealing conditions used were 3 minutes at 100°C, 15 minutes at 68°C, 30 minutes at 37°C and 2 hours at RT. The oligonucleotides were constructed with Bam HI/Bgl II compatible ends and were cloned into the Bam HI site of pBLCAT2. 10 μ g of the vector was digested with Bam HI. The digest was treated with phenol/chloroform and precipitated with 0.1 volume of 3M sodium acetate and 2.5 volumes of cold ethanol at -70°C, then spun for 15 minutes at 10,000 rpm. The cut vector was treated with alkaline phosphatase. For each of the three ligations, with CRE2, CRE1 and mCRE1, 0.1 μ g of Bam HI digested vector was used with 6 ng of the oligonucleotide to give the latter approximately a 10-fold molar excess. The ligations were performed at 15°C overnight, using 1U of T4 DNA ligase.

2.14.1 Preparation of competent cells

The bacteria used in these experiments was the DH5 α strain of *E.coli*. A single colony was taken from an agar plate and grown overnight in 5 ml of LB in a shaking incubator at 37°C. 1 ml of this culture was taken and grown in 100 ml of LB, under the above conditions, until it reached an OD₆₅₀ of 0.6. The culture was chilled on ice, then centrifuged. The cell pellet was resuspended in 50 ml of 0.1 M MgCl₂ and spun. The pellet was resuspended in 50 ml of 0.1 M CaCl₂ left on ice for 30 minutes then spun. Each centrifugation was at 3000 rpm for 5 minutes at 4°C in a bench-top centrifuge. The final pellet was resuspended in 5 ml of 0.1 M CaCl₂ and either stored on ice or kept frozen in aliquots at -70°C (with 50% glycerol) until use.

2.14.2 Transformation

Each of the three ligation mixtures was transformed into competent DH5 α . pBLCAT2 cut with Bam HI and treated with alkaline phosphatase then ligase was also transformed as a control for religation of the vector. Uncut vector was transformed as a control of transformation efficiency. 50 ng of DNA (in 10 μ l) was mixed with 200 μ l of bacteria for each transformation and left for 30 minutes on ice. The bacteria were heat shocked for 2 minutes at 42°, then 1 ml of Luria broth was added to each tube and incubated for 2 hours at 37°C. 50 μ l was plated out onto an agar plate containing ampicillin (100 μ g/ml).

2.14.3 Colony hybridization

Over one hundred colonies were present on the plate for each ligation. In the case of the CRE2 subcloning experiment, a nitrocellulose filter was placed onto the plate in order to obtain a mirror image of the colonies on the plate. The filter was treated with 0.5 M NaOH for 7-8 minutes, followed by 2 minutes treatment in 1.0 M Tris-HCl, pH 7.4, and 4 minutes in 1.5 M NaCl, 0.5 M Tris-HCl, pH7.4. The filter was dried on filter paper, air dried for 2 hours and dried in an oven at 80°C for 2 hours. The filter was prehybridized for 2 hours at 42°C. Then hybridized overnight with a CRE2 probe end-labelled with [γ -³²P] ATP (>4000 Ci mmol⁻¹ 10 mCi ml⁻¹) by T₄ polynucleotide kinase (prehybridization and hybridization solutions as described in section 2.9.8). The filter was given two 2 hour washes at 55°C in 0.5 x SSC/0.5% SDS. The filter was washed with gentle agitation. The filter was exposed wet to X-ray film. Many colonies gave a strong signal, twenty colonies which gave the strongest signal were spotted onto a fresh agar agar plate (containing ampicillin, 100 μ g/ml) and a second

nitrocellulose filter. The six colonies which gave the strongest signal were grown up in overnight cultures which were used to make minipreparations of DNA. Because a high number of positive clones was found for CRE2, it was decided in the case of CRE1 and mCRE1 to omit the colony hybridization step, instead several colonies of each transformant were grown overnight and used to make minipreparations of DNA for sequencing.

2.15 Mini-preparation of plasmid DNA

A colony was grown overnight in 5 ml of Luria broth containing ampicillin (100 $\mu\text{g/ml}$) in a shaking incubator at 37°C. Three ml of culture was spun in a microfuge for 1 minute. The cell pellet was resuspended in 200 μl of solution A and 6 μl of freshly prepared lysozyme solution (12 mg/ml) was added. After 5 minutes at RT, 400 μl of solution B was added, mixed by inversion and left on ice for 5 minutes. Then 300 μl of 7.5 M ammonium acetate pH 7.8 was added, mixed gently and left on ice for 10 minutes. The precipitate was removed by centrifugation for 15 minutes. 540 μl of isopropanol was added to the supernatant, incubated for 10 minutes at room temperature, and spun for 10 minutes. The pellet was dried and resuspended in TE, pH 8.0, then treated with phenol/chloroform and ethanol precipitated as described previously. After the final spin (all centrifugations were performed at 10,000 rpm) each miniprep was taken up in a final volume of 20 μl of TE, pH 8.0.

2.16 DNA Sequencing

Sequencing was performed on minipreparations of DNA (8 μl) using a Pharmacia T7 kit. The reactions were carried out according to the manufacturer's instructions, except that the template DNA was denatured in 0.4 M NaOH for 30 minutes. The universal primer provided with the kit was used. The annealing buffer was however replaced by the manganese buffer which was included in the Sequenase sequencing kit (USB). 1 μl (10 μCi) [α -³⁵S] dATP (10 mCi/ml) was used in each reaction.

The reaction products were separated by electrophoresis on 6% polyacrylamide gels. The upper tank of the sequencing gel apparatus contained 300-500 ml of 0.5 x TBE and the lower tank contained 150 ml of 1 x TBE. The gel was pre-run for 1 hour at 1500 V. After the pre-run, 75 ml of 3 M sodium acetate was added to the lower chamber. The gel was run for 2-3 hours at 1500 V, then the gel was fixed in 10%

acetic acid for 40 minutes, washed for 5 minutes in water, dried for approximately 90 minutes at 60° C and exposed to film for at least 24 hours.

2.17 Large scale plasmid preparation

Plasmid DNAs were prepared on a large scale by lysozyme-Triton X-100 lysis followed by caesium chloride/ethidium bromide equilibrium gradient centrifugation (Clewell and Helinski, 1972). Clones which were found by sequencing to contain the desired insert in the required orientation were grown up to make large scale plasmid preparations. CRE2, CRE1 and mCRE1 were used in the forward orientation in CAT assays, CRE2 was also cloned in the reverse orientation for footprinting. The bacterial culture used for the minipreparations was streaked onto agar plates (containing ampicillin, 100 µg/ml), a colony was picked from these plates and grown overnight in 10 ml of Luria broth (containing ampicillin, 100 µg/ml) at 37°C in a shaking incubator. 4 ml of this culture was grown in 400 ml of Luria broth (containing ampicillin, 100 µg/ml) until an OD₆₅₀ of 0.7 was reached. 6 ml of chloramphenicol (10mg/ml) was added to the culture, which was maintained overnight in a shaking incubator at 37°C. The cultures were centrifuged in a Sorvall GSA rotor at 5000 rpm for 10 minutes at 4°C, the pellet was then resuspended in 3 ml of 25% sucrose in 50 mM Tris HCl pH 8.0. Lysozyme solution was prepared (10 mg/ml) in 50 mM Tris HCl pH 8.0 and 0.5 ml was added to the bacterial suspension which was left on ice for 5 minutes. 1 ml of 0.25M EDTA was added and, after a further 5 minutes on ice, 4 ml of Triton X-100 lysis solution was added. After a one hour spin at 18,000 rpm at 0°C (Sorvall SS34 rotor), caesium chloride was added to the supernatant (clear lysate) at a concentration of 1 g/ml. Once the caesium chloride was dissolved, ethidium bromide (10 mg/ml) was added at 23.5 µl/ml of lysate. The lysate was then divided between two Beckman polyallomer quick seal tubes and centrifuged at 65,000 rpm for 3.5 hours at 21°C in a Beckman VTi65 rotor. Centrifugation resulted in two UV-fluorescent bands. The lower band was removed using a hypodermic needle and the ethidium bromide was extracted six times with isopropanol saturated with caesium chloride, then dialysed overnight with TE pH 8.0 (three changes of buffer). Then plasmid DNA was ethanol precipitated and spun at 0°C in a SS34 rotor at 10,000 rpm for one hour. The pellet was washed in 70% ethanol, spun for 15 minutes at 10,000rpm, dried and resuspended in 1 ml of TE pH 8.0. This was extracted with phenol/chloroform, ethanol precipitated and resuspended in 1 ml of TE pH 8.0.

2.18 Cell culture

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) donor calf serum (DCS) and incubated in a humidified atmosphere containing 5% CO₂. In experiments involving the induction of NF- κ B by hydrogen peroxide and benzoyl peroxide, the medium was removed one hour before the addition of these reagents, the cells were washed in PBS and the iron-free medium, RPMI-1640, was added without serum.

2.19 CAT reporter gene transfection assay

2.19.1 Transfections by the calcium phosphate method

Cells were seeded out onto 90 mm plates such that the cells were between 50-75% confluent one day after plating. The medium was changed four hours before transfection. 15 μ g of DNA (1 mg/ml) was used in the transfections for each condition. 2 x HBS was prepared from 10 x HBS stock and made to pH 7.05 before use. 2 x HBS was added to the DNA to give a total volume of 250 μ l, to which was added dropwise 250 μ l of 0.25 M CaCl₂ (stored as 2.5 M). The solution was gently mixed and left at room temperature for 20 minutes during which time a fine precipitate formed. Three ml of fresh medium was added to each plate and the DNA/calcium phosphate precipitate was added dropwise to the plates and left at room temperature for twenty minutes. Then 7 ml of medium was pipetted onto each plate. Eighteen hours later the medium was removed, the cells were washed with PBS and fresh medium added.

2.19.2 Preparation of cell lysates

After a further 24 hours the medium was removed and the cells washed with cold PBS, fresh PBS was poured on the plates and the cells were scraped with a 'rubber policeman'. The cells were spun at 2000 rpm for 5 minutes at 4°C in a MSE coolspin. The pellets were resuspended in 1 ml of cold PBS and spun at 10,000 rpm for three minutes at 4°C. The PBS was removed and the pellets were resuspended in 100 μ l of 0.25 M Tris HCl (pH 7.8). Each sample was sonicated for 30 seconds in a bath-type sonicator followed by three cycles of freezing in dry ice and ethanol and thawing in a water bath at 37°C. After a fifteen second period of sonication the tubes were spun at 10,000 rpm for three minutes at 4°C, and the supernatants (cell lysates) were collected.

2.19.3 Protein estimation of cell lysates

2 μ l of cell lysate was added to 798 μ l of 0.25 M Tris HCl pH 8.0. Standards ranging from 1-25 mg/ml were prepared with bovine serum albumin. 200 μ l of reagent from the Biorad Protein Assay Kit was added and left for five minutes at RT. The OD₅₉₅ were read against a blank. A standard curve was plotted and used to calculate the protein concentration of the cell lysates.

2.19.4 Analysis of CAT activity

Cell lysates were diluted with 0.25 M Tris HCl pH 8.0 so that the CAT assay for a particular transfection experiment was performed for each condition with an equal amount of protein (150- 250 μ g depending on the cell line) in an equal volume (100 μ l). The cell lysates were heated at 65°C for 10 minutes. A master mix was prepared to ensure that the same batch of frozen acetyl CoA (5 mg/ml) and frozen [¹⁴C] chloramphenicol (0.025 mCi/ml) would be used for all the samples in any one transfection experiment. 8 μ l of [¹⁴C] chloramphenicol (25 μ Ci ml⁻¹) was used with 40 μ l of acetyl CoA per assay. The components were mixed and incubated at 37°C for three hours or overnight depending on the cell line used. At the end of the incubation period 0.5 ml of cold ethyl acetate was added to each tube and after mixing, the tubes were spun at 10,000 rpm for three minutes at 4°C. The upper, organic, phase was transferred to a new tube and the tubes were spun in a centrifugal evaporator (Univap) for 30 minutes at 40°C until all the ethyl acetate was removed. The residue was resuspended in 20 μ l of ethyl acetate and spotted on a silica gel TLC plate. The TLC plates were subjected to ascending chromatography in chloroform: methanol (95:5), air dried and exposed to X-ray film. Spots containing separated chloramphenicol species were scraped into scintillation vials, mixed with 2 ml of Instagel and counted in a scintillation counter. The counts for the converted and the unconverted forms of chloramphenicol were used to calculate the percentage conversion of each sample.

2.20 Footprinting

The DNase I footprinting was performed in collaboration with Mark Griffiths.

2.20.1 Preparation of DNA for labelling

The plasmid pCRE2CAT, containing CRE2 cloned in the reverse orientation was used in footprinting. 200 μ g of the plasmid was digested with Eco RI and Hind III in a volume of 50 μ l for 90 minutes at 37°C. These enzymes were chosen because they

gave an 85 bp fragment (a suitable size for footprinting) with different 5' overhanging ends. The coding strand of the vector, containing the non-coding strand of CRE2, had a 5' overhanging end 5' AGCT- 3'. The non-coding strand of the vector, containing the coding strand of CRE2, had a 5' overhanging 3'-TTAA 5'. Because of the different ends, end-labelling with [α -³²P] dGTP or dCTP would result in only the non-coding strand being labelled at the 3' end. The plasmid pCRE2CATR was used in preference to pCRE2CAT, because only the former contains the coding strand of CRE2 on the non-coding strand of the vector, and it is the non-coding strand of the vector which is labelled by the labelling procedure as described above.

The restriction fragments were resolved by electrophoresis on a 1% (w/v) agarose gel in 1 x TAE, the gel was run in 1 x TAE. The fragment of the correct size to represent the 85 bp fragment was excised from the gel and diced finely with a razor blade. A 0.5 ml eppendorf was pierced at the base of the tube and glass wool was placed in the bottom of the tube and the agarose pieces were placed on top. The DNA was eluted by centrifugation at 10,000 rpm for 10 minutes at RT. The eluate was then extracted twice with phenol/chloroform and the DNA was ethanol precipitated as described previously. The DNA was resuspended in 50 μ l of TE buffer pH 8.0. The 85 bp fragment was approximately 2% of the plasmid DNA. The maximum concentration of the fragment expected would be 80 ng/ μ l.

2.20.2 Labelling DNA for footprinting

2 μ l (approx. 160 ng) of the Eco RI/Hind III fragment of pCRE2CAT was labelled with 5 μ l (50 mCi) [α -³²P] dGTP (>3000 mCi/mmol). The reaction mixture (10 μ l) also contained 0.5 μ l of 1 mM dATP, dCTP, dTTP and 1 μ l (4U) of Klenow fragment in 50 mM Tris HCl (pH 7.8), 5 mM MgCl₂ and 10 mM 2-mercaptoethanol. It was left for 30 minutes at room temperature then passed through a G-50 spin column. The G-50 spin column was prepared in a 1 ml syringe; a small amount of glass wool was pushed to the bottom of the syringe and a suspension of G-50 in water was added up to the top of the syringe using a pasteur pipette. The G-50 was compacted in the column by centrifugation at 1000 rpm for two minutes in a MSE coolspin. The process was repeated until the G-50 column reached the 1 ml mark on the syringe. 100 μ l of water was added to the top of the syringe and the column was spun again at 1000 rpm for two minutes. The column was then ready for use. 1 μ l of the probe after

it had passed through the G-50 column was counted and found to have an activity of 20,000 cpm/ μ l.

2.20.3 DNase I footprinting

2 μ l of the above probe was digested with various concentrations of DNase I between 0.8 and 32 μ g/ml. The reaction mixture (100 μ l) also contained 20 μ l of 5 x DNA-binding buffer, 4 μ l of dIC (1 mg/ml), 1 μ l of 50 mM CaCl₂ and 1 μ l of 100 mM MgCl₂. The DNase I was the final component to be added to reaction mixture. The reaction was allowed to proceed for one minute at 25°C and was stopped with 60 μ l of 0.6 M sodium acetate (pH 5.2), 20 mM EDTA. 100 μ l of water was added and the solution was extracted once with phenol/chloroform and once with chloroform. DNA was precipitated with ethanol at -70°C, centrifuged for 10 minutes at 10000 rpm in the cold room, washed in 70% ethanol and resuspended in 5 μ l of loading buffer. The samples were run on a 12% polyacrylamide gel at 1500 V for 2-3 hours. The gel was fixed in 10% acetic acid for 30 minutes, dried in an oven at 60°C for 2 hours and exposed to X-ray film.

From the DNase I titration it was decided that the best cleavage for footprinting was obtained using a final concentration of 6 μ g/ml DNase I. The reactions were prepared for footprinting in the same way as in the titration experiment except that nuclear extract from 5A12 cells (9 mg/ml) was added to all but one of the reactions. The nuclear extract was prepared by MDP1 (see following section).

2.20.4 Maxam and Gilbert sequencing reactions

The G-specific Maxam and Gilbert sequencing reactions were performed as described by Sambrook et al. (1989). These reactions were run alongside the footprinting reactions so that the nucleotides in the footprint could be identified.

2.21 Nuclear extract preparation

Nuclear extracts were prepared by three methods, all of which were modifications of that described by Dignam et al. (1983). The methods are termed MDP1, MDP2 and MDP3 for modified Dignam procedures 1,2 and 3.

2.21.1 MDP1

Cells were grown on twenty 90 mm plates. The medium was removed and the cells were washed with cold PBS. The cells were harvested in PBS by scraping with a 'rubber policeman'. The cells were spun down in 50 ml falcon tubes at 2000 rpm in an MSE coolspin for five minutes at 4°C, resuspended in 4 ml of cold buffer A (see Section 2.9.6) and held on ice for 10 minutes. Finally the cells were disrupted with 10 strokes with a tight-fitting Dounce homogenizer and nuclei pelleted by centrifugation at 16,000 rpm for 20 minutes at 4°C using a Beckman SW50.1 rotor. The pellet was resuspended in 4 ml of cold buffer C and nuclei disrupted by 10 strokes of a tight-fitting Dounce homogeniser. The nuclear extract was stirred gently on ice for 45 minutes and chromatin and nuclear membranes were pelleted by centrifugation for 20 minutes at 25,000 rpm at 4°C using the SW50.1 rotor. Ammonium sulphate was used to precipitate the proteins present in the supernatant. Solid ammonium sulphate (0.33 g per ml of supernatant) was gently stirred into the supernatant which were stirred on ice for 30 minutes. The nuclear proteins were pelleted by centrifugation as in the previous step. The protein pellet was resuspended in 200 μ l of cold buffer D and dialysed against the same buffer overnight at 4°C, unless stated otherwise in the text. The following day the nuclear extract was snap frozen in liquid nitrogen in aliquots and stored at -70°C.

2.21.2 MDP2

This modification of the Dignam et al. (1983) procedure was described by Andrews and Faller (1991), our further modification was to use buffers AM and CM instead of A and C (see Section 2.9.6). Using this method nuclear extracts could be prepared from a single 90 mm plate of cells. Cells were scraped in 1.5 ml of cold PBS and transferred to an Eppendorf tube. Cells were pelleted for 10 seconds and resuspended in 400 μ l of cold buffer AM. The cells were left on ice for 10 minutes, vortexed for 10 seconds and centrifuged. The supernatants were discarded and each pellet was resuspended in 20-50 μ l (depending on the starting number of cells) of cold buffer CM and left on ice for 20 minutes. Nuclear membranes and chromatin were pelleted by a two minute centrifugation at 4°C. The supernatant was snap frozen in liquid nitrogen in aliquots and stored at -70°C. All centrifugation steps took place in a bench-top microfuge, the 10 second spins took place at RT.

2.21.3 MDP3

This method was exactly the same as MPD1 except buffers A, C and D were replaced by buffers AM, CM and DM (see Section 2.9.6).

2.21.4 Estimations of protein in nuclear extracts

This was performed as described in Section 2.23

2.22 Oligonucleotide end-labelling for gel retardation

CRE1 and kappa were 3' end-labelled using the Klenow fragment of *E.coli* DNA polymerase I or 5' end-labelled using T₄ polynucleotide kinase. DNA (40 ng) was incubated with 4 U of Klenow fragment and 50 μ Ci of [α -³²P] dGTP (>3000 mCi/mmol) for 30 minutes at room temperature in 20 μ l final volume, containing 2 μ l of 10 x Klenow labelling buffer, 50 μ M of unlabelled dATP, dCTP and dTTP. Alternatively, the same amount of DNA was incubated with T₄ polynucleotide kinase (20U) and 50 μ Ci of [γ -³²P] ATP (> 3000 mCi/mmol) at 37°C for 30 minutes in 20 μ l final volume containing 2 μ l of 10 x kinase buffer. In both cases after 30 minutes, 10 μ l of 50 mM EDTA was added and the mixture was incubated at 65°C for 10 minutes to inactivate the enzyme. DNA was precipitated by adding 40 μ l of water, 35 μ l of 7.5 M ammonium acetate, 1 μ l of 1% linear polyacrylamide and 350 μ l of ethanol for one hour at -70°C and pelleted at 10,000 rpm for 15 minutes at 4°C. The pellet was resuspended in 300 μ l of 70% ethanol and centrifuged at 10,000 rpm for 15 minutes at 4°C dried *in vacuo* and resuspended in 100 μ l. All centrifugations were performed in a bench-top microfuge.

The Sp1 oligonucleotide was labelled according to the instructions provided by Dr S.P.Jackson. 50 ng of one strand was incubated with 20 U of T₄ polynucleotide kinase, 5 μ l of 10 x kinase buffer, 50 μ Ci of [γ -³²P] ATP (>3000 mCi/mmol), made up to 50 μ l with water. After 45 minutes another 10 U of T₄ polynucleotide kinase was added and the reaction allowed to proceed for a further 45 minutes at 37°C. Then 250 μ l of 1 M ammonium acetate/0.1%SDS and 250 μ l of phenol/chloroform were added. The oligonucleotide was precipitated from the aqueous phase with ethanol for 15 minutes on dry ice and pelleted by centrifugation for 15 minutes. The ammonium acetate/0.1%SDS, phenol/chloroform and ethanol precipitation steps were repeated. After a 70% ethanol wash, the pellet was dried. To this 75 ng of the other

strand in 20 μ l of TE was added and the strands were annealed by warming to 90°C for two minutes and then allowed to cool slowly.

2.23 Gel retardation assay

2.23.1 CRE1 and kappa probes

The method employed was based on the procedure originally described by Fried and Crothers (1981). Nuclear extracts and labelled oligonucleotide probes were prepared as described previously. The binding reactions were carried out in 25 μ l and contained 10 μ g of nuclear extract in 5 μ l of buffer C or CM or buffer D or DM, 0.05-0.2 ng of end-labelled DNA (approximately 20,000 cpm), 5 μ l of 5 x DNA-binding buffer and 0.25 μ g of poly dI-dC made up to 25 μ l with water if the extract was prepared in buffers C or CM. However if the extract was prepared in buffer DM, the NaCl concentration was adjusted to that of C or CM (80 mM). Extracts prepared in buffer D did not have NaCl added to the binding reaction unless otherwise stated on the figure legends. Complexes were allowed to form during a twenty minute incubation at RT. Complexes were resolved from free probe by electrophoresis in 6% native polyacrylamide gels. The gels were run in 0.5 x TBE, for 90 minutes at 4°C. Gels were fixed for 10 minutes in 10% v/v acetic acid, transferred to Whatman 3MM paper, dried under vacuum at 80°C for 30 minutes and exposed to X-ray film.

2.23.2 Sp1 probe

The binding conditions used for this oligonucleotide were as recommended by Dr S.P.Jackson. The binding reaction was carried out in 10 μ l and contained 1 ng of probe, 0.5 μ g of sonicated salmon sperm, 10 μ g of nuclear extract and 6 μ l of Sp1 DNA-binding buffer. When competitor DNA was used it was added before the probe. The reaction mixture was incubated for 15 minutes at 30°C without the probe and incubated for a further 15 minutes at 30°C with the labelled probe. The samples were loaded on a 6% native polyacrylamide gel. The fixing and drying steps were performed as described previously.

2.24 DNA-protein crosslinking

The method employed was modified from that of Dorn et al. (1989). A standard gel retardation assay was run except the reaction volume was increased three-fold, the radio-labelled DNA was increased 40-fold, the poly[dI-dC] and the extract were increased three fold. The wet gel was exposed to X-ray film for several hours and the

developed film was used to locate the DNA-protein complexes which were excised from the gel and incubated in 20 ml of formaldehyde solution at 4°C overnight on a rotary mixer. Crosslinked complexes were extracted from the gel by electroelution in 400 μ l of 1 x TAE for three hours at 100 V. The 400 μ l of TAE was divided equally between 2 eppendorfs, to each was added 1 ml of cold acetone and 1 μ l of linear polyacrylamide (as a carrier). After 1 hour at -70°C, the samples were spun for 15 minutes at 10,000 rpm at 4°C. The pellets were washed in 70% ethanol and spun for a further 15 minutes at 10,000 rpm at 4°C. The pellets were dried and taken up in water, mixed with sample buffer and loaded onto a 15% SDS/polyacrylamide gel. [¹⁴C] methylated molecular weight markers were run alongside the samples.

2.25 Autoradiography

Dried gels were exposed to X-ray film for an appropriate length of time. For the detection of ³²P a phosphotungstate intensifying screen was used and the autoradiograms were left at -70°C. Films were developed using an X-OGRAPH automatic developer.

Chapter 3
Functional and Molecular Characterization of CRE2

3.1 Introduction

Ad12-transformed cells have lower levels of MHC class I expression and higher levels of CRE2-binding factors (Ackrill and Blair, 1989) than cells transformed by Ad2 or Ad5. It has been proposed that CRE2-binding factors repress MHC class I transcription in Ad12-transformed cells (Ge et al., 1992), however the role of CRE2-binding factors in the control of class I transcription is still unclear. It was therefore decided to investigate the role of this sequence in transcriptional control in Ad-transformed cell lines by the use of an assay in which the transient expression of the reporter gene CAT is monitored. One copy of the oligonucleotide CRE2 (see table 2.1) was cloned into the vector pBLCAT2, in which the reporter gene CAT is under the control of the herpes simplex virus thymidine kinase basal promoter (Luckow and Schutz, 1987). Bacterial colonies containing recombinant plasmids were identified by colony hybridization with a CRE2 radio-labelled probe. The orientation of the CRE2 insert was determined by DNA sequencing. The recombinant plasmid in which one copy of the CRE2 insert was present in the forward orientation in the vector pBLCAT2 was termed pCRE2CAT. This plasmid and pBLCAT2 were transfected separately into Ad-transformed cell lines and the influence of the CRE2 sequence on transient CAT expression was evaluated. A plasmid in which CRE2 was inserted in the reverse orientation into pBLCAT2, termed pCRE2CATR, was used in DNase I footprinting in an attempt to elucidate the binding site for CRE2. The possible role of phosphorylation on CRE2 binding was investigated by the use of sodium fluoride in nuclear extract preparation, sodium fluoride being an activator of protein kinase C (PKC) as well as an inhibitor of phosphatases (Seifert & Schultz, 1991; Hewitt & Nicholas, 1963).

3.2 Cloning of CRE2 and the use of colony hybridization to detect colonies containing recombinant plasmid

The vector pBLCAT2 was digested with Bam HI and the CRE2 oligonucleotide was ligated into the vector. The ligation mixture was used to transform competent *E.coli* strain DH5 α . The transformed bacteria were plated onto a LB plate containing ampicillin (100 μ g/ml). A nitrocellulose filter was placed on the transformation plate to make a mirror image of the pattern of colonies present on the plate. The filter was then used in colony hybridization with a CRE2 probe radio-labelled by T₄ polynucleotide kinase (Fig 3.1a) and many of the colonies gave a strong signal. Twenty of the colonies which gave the strongest signal were picked and spotted onto both a second nitrocellulose filter and a master LB agar plate containing ampicillin (100 μ g/ml)

Fig 3.1 Colony hybridization using the a radiolabelled CRE2 probe

The vector pBLCAT2 was digested with Bam HI and the CRE2 oligonucleotide was ligated into the vector. *E.coli* strain DH5 α was transformed with the ligation mixture. Fig 3.1a: the transformed bacteria were plated onto a LB agar plate containing ampicillin (100 μ g/ml). A nitrocellulose filter was placed over the plate, the filter was then used in colony hybridization with the radio-labelled CRE2 probe. Fig 3.1b: twenty of the colonies which gave the strongest signal were picked and spotted onto a second filter and a master LB agar plate containing ampicillin (100 μ g/ml). The second filter was also hybridized to the radio-labelled CRE2 probe.

(Fig 3.1b). The six colonies which gave the strongest signal were grown up in 10 ml of LB containing ampicillin (100 μ l/ml) overnight and mini-preparations were made of the DNA.

3.3 Sequencing of the CRE2 insert in the vector pBLCAT2

DNA from the six mini-preparations chosen from the results of the colony hybridization were sequenced according to the method of Sanger et al. (1977) using a Pharmacia sequencing kit. One clone was obtained in which the CRE2 oligonucleotide was inserted in the forward orientation into the Bam HI site of the vector pBLCAT2. The plasmid was referred to as pCRE2CAT (Fig 3.2). This plasmid was used in CAT assays. Another of the six clones appeared to have the CRE2 sequence present in the reverse orientation (data not shown). The orientation of CRE2 in the vector was confirmed by sequencing reactions as described by Maxam and Gilbert (1980) The plasmid was referred to as pCRE2CATR; this plasmid was used for DNase I footprinting and Maxam and Gilbert G-specific sequencing reactions which were run alongside the DNase I footprint (Fig 3.3, lane 8).

3.4 Investigation of the functional role of the CRE2 region by CAT assays

The CAT assay is a transient expression assay used to investigate the functional role of specific DNA sequences. The CAT gene is a bacterial gene and there is no endogenous CAT activity present in eukaryotic cells. The CAT enzyme can be monitored by a sensitive assay which can be used to demonstrate the properties of sequences which act as enhancers, repressors or promoters. The latter require different vectors from those required for enhancers or repressors. Vectors to test promoters have no promoter region placed upstream of the CAT reporter gene, whereas in vectors designed to test enhancers or repressors a weak promoter is present. This was the case for the vector used in this study, pBLCAT2, which has the basal promoter of the herpes simplex virus thymidine kinase gene present (Luckow and Schutz, 1987).

In this study the plasmids pBLCAT2 and pCRE2CAT were transfected separately into Ad-transformed cell lines by calcium phosphate-mediated transfection. Cellular extracts were prepared approximately 40 hours after transfection. Depending on the rate of transcription from the CAT gene, these extracts would contain varying amounts of the bacterial enzyme CAT. This enzyme can convert [14 C] chloramphenicol to its acetylated forms. In order to determine the amount of CAT enzyme activity present in

Fig 3.2 Sequencing of the CRE2 insert in the plasmid pCRE2CAT

The CRE2 oligonucleotide is shown to be inserted in the forward orientation in the Bam HI site of pBLCAT2, creating the vector pCRE2CAT. The sequence in bold is that of the CRE2 insert, the pBLCAT2 sequence surrounding the Bam HI site is in italics.

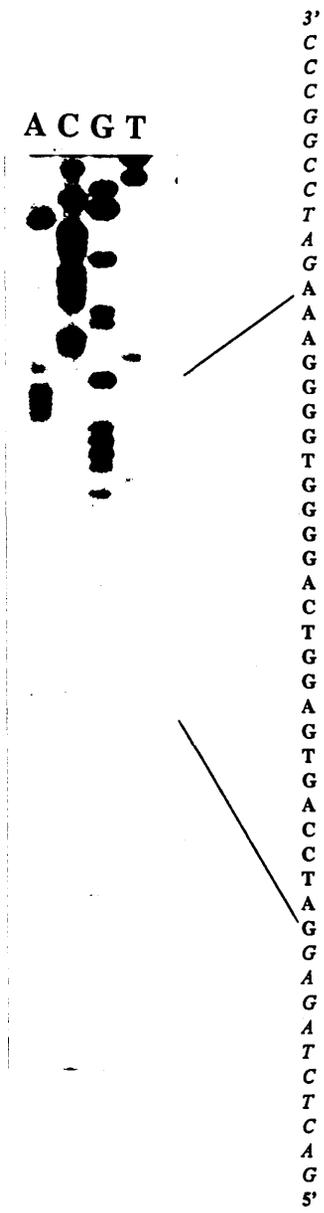
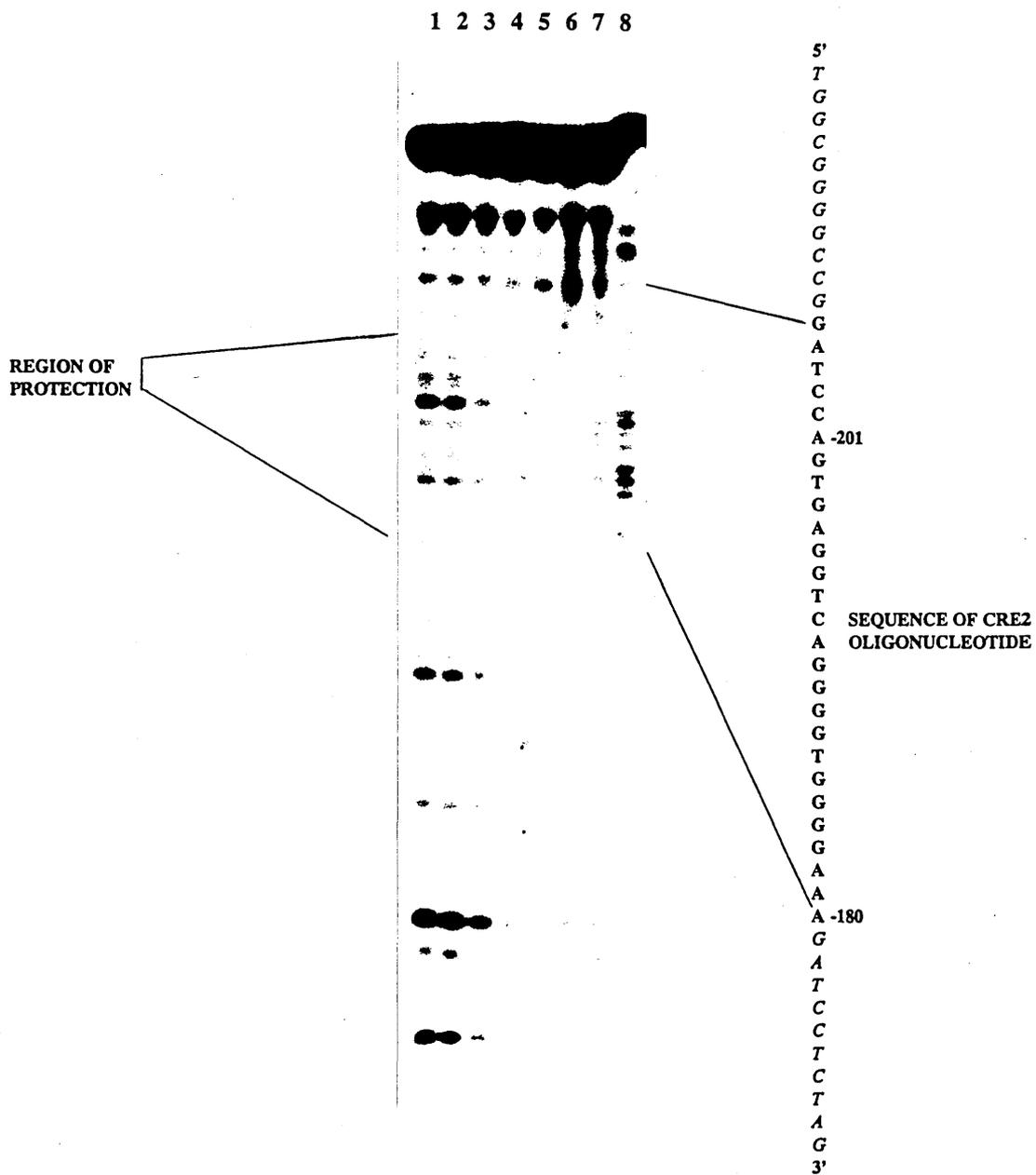


Fig 3.3 DNase I footprint of the CRE2 binding in 5A12 nuclear extracts

Lane 1: no protein, lane 2-6: 9, 18, 45, 90, 180 μg of nuclear extract, lane 7: 180 μg of nuclear extract and a 100-fold excess of unlabelled CRE2, lane 8: G-specific sequencing reactions. The CRE2 oligonucleotide sequence is shown to the right of lane 8 in bold, the pBLCAT2 sequence surrounding the Bam HI insert is shown in italics. The region of protection is shown to the left of lane 1.



individual cellular extracts from the transfected cells, a fixed amount of cellular extract was incubated with a fixed amount of [¹⁴C] chloramphenicol and acetyl CoA (as a source of acetyl groups). After the incubation period the acetylated and unacetylated forms of [¹⁴C] chloramphenicol were extracted and spotted onto thin layer chromatography (TLC) plates. The acetylated forms were separated from the unacetylated form by thin layer chromatography as the acetylated forms migrate further (compare the positions of c and d with that of b in Fig 3.4). The radioactivity associated with the acetylated and unacetylated forms was counted and used to calculate the % conversion as a measure of CAT activity. Although % conversion gives a more accurate measure of CAT activity, an indication of this activity can be obtained from observing the intensity of the spots corresponding to the acetylated forms. The more CAT enzyme present, the more [¹⁴C] chloramphenicol would be converted and so the spots corresponding to the acetylated forms would be more intense. Conversely, the presence of less CAT enzyme would be associated with less intense spots corresponding to the acetylated forms. Since enhancers increase CAT gene transcription which in turn has been shown to correlate with CAT enzyme activity (Latchman, 1993), the CAT assay gives a measure of enhancer activity. Repressors reduce CAT gene expression and, because of the direct correlation to CAT enzyme activity, CAT assays can also be used to detect repressor function.

Fig 3.4 shows a typical CAT assay result comparing the CAT activity in extracts from 5A12 cells transfected either with pBLCAT2 or pCRE2CAT. Extracts from cells transfected by pCRE2CAT did not show significantly altered CAT activity compared to those from cells transfected by the vector pBLCAT2. Fig 3.5 summarizes the CAT assay results obtained in four cell lines: 5A12, RFC-1, BMK and Ad5Xho. The presence of the CRE2 sequence was not associated with strong enhancer or repressor activity in any of the cell lines tested. However in RFC-1 and Ad5Xho, it did appear to repress CAT activity to some degree.

3.5 DNase I footprinting of the binding site for CRE2-specific DNA-binding factor(s)

DNase I footprinting is based on the cleavage of DNA molecules by this enzyme. The DNA of interest is radioactively labelled at one end of one of the two strands. It is necessary to establish conditions which produce limited cleavage for fragments to be present over the full range of sizes from full-length to a few bps. If too much cleavage

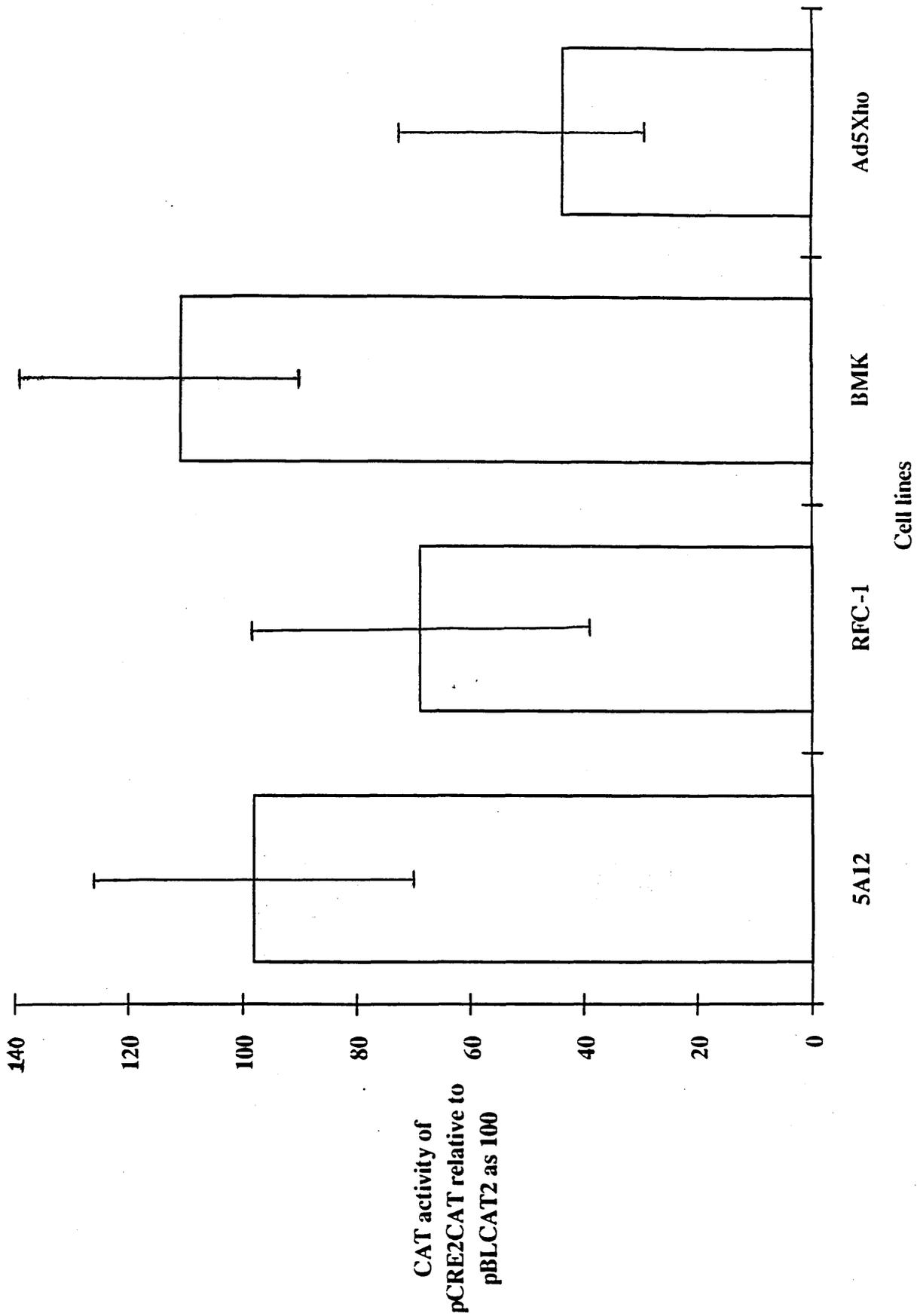
Fig 3.4 The CAT activity present in extracts from 5A12 cells transfected with either pCRE2CAT or pBLCAT2

Extracts were prepared from 5A12 cells transfected with either 15 μg of pBLCAT2 or 15 μg of pCRE2CAT. An aliquot of cell extract corresponding to 150 μg was mixed with 8 μl (200 μCi) of [^{14}C] chloramphenicol and 40 μl of acetyl CoA (5 mg/ml) and incubated overnight. The acetylated and unacetylated forms of [^{14}C] chloramphenicol were extracted and spotted onto a TLC plate at position a, b represents the position of the unacetylated and c/d represent the positions of the acetylated forms.

Lane 1: extract from cells transfected with pBLCAT2, lane 2: extract from cells transfected with pCRE2CAT.

Fig 3.5 Expression of the pCRE2CAT and pBLCAT2 plasmids in Ad-transformed cells

The CAT activity of extracts from cells transfected by the vector pBLCAT2 is presented as 100 and that of extracts from cells transfected by pCRE2CAT is presented relative to it. Results are shown for four cell lines: 5A12, RFC-1, BMK and Ad5Xho. Each column represents the average of at least 3 separate experiments. The range of values are as shown.



occurs all the fragments will be small and if too little, the smaller fragments will not be properly represented. These fragments can be separated by gel electrophoresis to form a ladder of bands with each band in the ladder differing from the next by one nucleotide. Maxam and Gilbert sequencing reactions can be performed on the same radio-labelled DNA fragment. These reactions cut the DNA into a series of fragments each terminating in a specific nucleotide or nucleotides, for example, G or G+A. When Maxam and Gilbert sequencing reactions are run alongside DNase I cleavage products, the identity of the terminal nucleotide of the cleavage product can be ascertained. If the DNA is complexed to protein(s), for example from a nuclear extract, prior to being treated with the cleavage agent, DNase I, the nucleotides to which the protein is bound will be protected from cleavage. Fragments of a particular length will not be formed, resulting in a gap in the DNA ladder; this region is called a footprint and represents the region of DNA protected from cleavage by bound protein. If the protein does not completely hinder DNase I cleavage of a particular region, a site of partial protection will be created in which the radioactive bands have not completely disappeared but are of reduced intensity.

The non-coding strand of the Eco RI-Hind III fragment of pCRE2CATR was radio-labelled using the Klenow fragment for DNase I footprinting and Maxam and Gilbert sequencing reactions. A titration was performed to ascertain the correct conditions to obtain a full range of cleavage product sizes. DNase I was used at a final concentration of 0.8-32 $\mu\text{g/ml}$; 6 $\mu\text{g/ml}$ was found to give the best cleavage pattern (data not shown). The non-coding vector sequence in this fragment contains the coding strand of CRE2 as shown by the G-specific sequencing reactions shown in lane 8 of Fig 3.3. Lanes 1 to 7 show DNase I footprinting of this fragment in the absence of nuclear extracts and in the presence of increasing amounts of 5A12 nuclear extracts (from 9 to 180 μg). A region of protection between -180 and -201 was evident with 180 μg of nuclear extract (lane 6). Some degree of protection began with 18 μg of nuclear extract (lane 3) and the size of the footprint enlarged with increasing amounts of nuclear extract up to 180 μg (lane 6). The protection seen with 180 μg was not complete throughout region II. The section of best protection covered the sequence AGGTCA between -197 and -192, this is the conserved sequence present in the ER/TR subfamily of hormone receptors that includes RXR β /H-2RIIBP. The protection was lost in the presence of excess unlabelled CRE2 (lane 7) which indicates that the protection observed was due to specific binding to CRE2.

3.6 The effect of sodium fluoride in the preparation of nuclear extracts on CRE2-binding activity

Sodium fluoride is an inhibitor of phosphatases and an activator of PKC (Seifert and Schultz, 1991; Hewitt and Nicholas, 1963). Nuclear extracts prepared in the presence of sodium fluoride therefore contain proteins in a more phosphorylated state, so the effect of phosphorylation on CRE2-binding can be investigated. 5A12 nuclear extracts were prepared by MDPI with and without 10 mM sodium fluoride in buffers A, C and D. The use of sodium fluoride in the preparation of extracts caused intensification of one binding activity and the appearance of the second of slower mobility (compare lanes 1 and 3 in Fig 3.6).

3.7 Discussion

The functional properties of CRE2-binding proteins were studied using CAT assays in which a single copy of the CRE2 sequence was placed upstream of the CAT reporter gene in the plasmid pBLCAT2. The binding site of CRE2-specific binding factors was analysed by DNase I footprinting. The influence of phosphorylation on binding was investigated by preparation of extracts in the presence and absence of sodium fluoride.

It has been proposed that a region of the class I promoter approximately equivalent to CRE2, termed R2, acts as a repressor of MHC class I transcription in Ad12-transformed cells but not in Ad5-transformed cells and this difference in repressor activity accounts, at least in part, for the higher levels of MHC class I expression found in Ad5-transformed cells (Kralli et al., 1992; Ge et al., 1992). The tissue distribution of CRE2-binding also suggests a negative role for factors binding to this sequence; CRE2-binding factors is higher in Ad12- than Ad5-transformed cell lines (Ackrill and Blair, 1989; Ge et al., 1992). Burke et al. (1989) found high levels of CRE2-binding activity in the brain and in the early stages of gestation when MHC class I antigens are undetectable. In this study the role of CRE2-binding factors was investigated by the transient CAT expression assay in cells transformed by Ad2, Ad5, Ad12 or recombinant virus. In two cell lines termed 5A12 and 5B12, the transforming agent was Ad12 E1A/Ad5 E1B and Ad5 E1A/Ad12 E1B respectively. Because of this, the cell lines used in this study will be described as Ad2, Ad5 or Ad12 E1A expressing cells. Four cell lines were chosen which expressed Ad12 E1A and four were chosen which expressed either Ad2 or Ad5 E1A. A single copy of CRE2 was placed upstream of the

Fig 3.6 The effect of sodium fluoride in the preparation of nuclear extracts on CRE2-binding activity

5A12 nuclear extracts were prepared by method MDP1 with (lane 1 and 2) or without (lane 3 and 4) 10 mM sodium fluoride. Lane 2 and 4 contained 100-fold excess of unlabelled CRE2. A CRE2 radiolabelled probe was used.

CAT reporter gene in pBLCAT2 creating the construct pCRE2CAT. The CAT assays did not show CRE2 to greatly influence CAT activity when pCRE2CAT was compared to pBLCAT2. In two of the four cell lines tested, RFC-1 and Ad5Xho, it was associated with a small degree of repression. The results of Ge et al. (1992) and Kralli et al. (1992) could explain why CRE2 was not found to have a strong repressor function in this study. They could only demonstrate a weak repressor function for R2 (the sequence of which is virtually identical to CRE2) in Ad12-transformed cells, when a tetramer of this sequence was cloned in front of a H-2K^b minimal promoter or the thymidine kinase (TK) promoter taken from the vector pBLCAT2. However, the R2 sequence did appear to repress R1 activation (a sequence virtually identical to CRE1) in Ad12- but not Ad5-transformed cells. To demonstrate this effect R1 and MR1 were used, the latter being a mutated version of R1 which did not bind transcription factors. The cassettes R2₂R1₂ and MR2₂R1₂ were used, cloned as one, two or four copies upstream of the H-2K^b minimal promoter. MR2₂R1₂ and R2₂R1₂ showed increased transcriptional activity as the number of copies increased from one to four in Ad5-transformed cells, whereas only the former showed an increase in transcriptional activity with increased copy number in Ad12-transformed cells suggesting the sequence R2 represses R1 activation in Ad12- but not in Ad5-transformed cells. There was no difference in CAT activity associated with a single copy of MR2₂R1₂ or R2₂R1₂ in either Ad12 or Ad5 cells. If these results are generally valid, the repressor function of R2/CRE2 would not be expected to be demonstrated using the experimental design adopted in this study, firstly because R1 is required to be present and secondly because both R1 and R2 need to be present in multiple copies. R2 appears to need to be adjacent to R1 to repress R1 induced activation; Kralli et al. (1992) suggest that factors binding to R2 could prevent proteins binding to R1. In the MHC class I promoter R1 and R2 do exist as adjacent sequences so transcription factors binding to one sequence would have the opportunity to interact with factors binding to the adjacent sequence. It is unclear why multiple copies of R1 and R2 should be required as this situation does not exist in the MHC class I promoter. R2/CRE2 sequence has been reported to act as an enhancer; Kralli et al. (1992) report that retinoic acid treatment causes this sequence to no longer act as a repressor. It seems likely that R2/CRE2 can have different roles in transcriptional control depending upon the presence or absence of agents such as retinoic acid.

The coding strand of the CRE2 oligonucleotide was footprinted using 5A12 nuclear extracts. The region of protection which was visible between -201 and -180, increased with increasing amounts of nuclear extract from 9 to 180 μ g. The protection was not complete within this region and was best between -197 and -192 which covers the sequence AGGTCA, the conserved sequence present at the binding site for RXR β /H-2RIIBP and other members of the ER/TR subfamily of steroid/thyroid hormone receptors. Since this is a conserved sequence one would expect binding to occur to this sequence. The protection shown is unlikely to be due to non-specific binding because in the presence of an excess of the CRE2 oligonucleotide the protection disappears. Methylation interference has been performed on the coding strand of CRE2 (Griffiths, 1992) using 5A12, HeLa and rat brain extracts, and on a similar sequence referred to as region II (Shirayoshi et al., 1987). Griffiths (1992) found the eight G residues between -200 and -188 to be protected. Shirayoshi et al. (1987) used the H-2L^d region II sequence and LH8 lymphocyte line and found protection at G residues between -200, and -190 (equivalent to between residues -198 and -188 in the H-2K^b sequence). The precise sequence protected was different in the various cell types; this could reflect the presence of different binding factors found in these cells. Shirayoshi et al. (1987) found mutant oligonucleotides with mutations in the G residues at -198, -197, -192 and -191 were unable to compete for wildtype binding further demonstrating the importance of these G residues. Griffiths (1992) confirmed these results using oligonucleotides with mutations to the equivalent G residues in the H-2K^b sequence.

The result shown here with DNase I footprinting suggest a larger area of binding than that found by methylation interference with the same extract. This is not unexpected as DNase I is a large molecule; because of this it not only is prevented from cutting DNA at the site where protein is bound to DNA, but also it is too large to reach the bases adjacent to the binding site. For this reason, it tends to overestimate the size of the binding site. Another disadvantage of DNase I is that it cuts preferentially at certain sites and at sites of poor cutting it is not possible to tell if protection has occurred.

Overall, more detailed results about the binding site of transcription factors can be obtained by methylation interference, although this method has the disadvantage of only giving information about G residues. DNase I footprinting, methylation interference and competition analysis with mutant oligonucleotides all suggest the conserved

sequence AGGTCA to be an important part of the binding site for CRE2-specific binding factors.

Sodium fluoride was shown to have a marked effect on the binding pattern obtained by gel retardation. It caused intensification of a band already present in the absence of sodium fluoride and the appearance of a second fainter band of slower mobility. Sodium fluoride acts as a phosphatase inhibitor and it is also an activator of protein kinase C (PKC) (Chabre, 1990; Seifert and Schultz, 1991). As a result of these properties, certain proteins of extracts prepared in the presence of sodium fluoride are more likely to be phosphorylated than those from extracts prepared in the absence of sodium fluoride. This suggests that the phosphorylation of certain proteins, possibly CRE2-binding proteins themselves, could lead to their enhanced binding to this DNA-binding motif. Work by Griffiths (1992) supplied further evidence for the potential role of phosphorylation in CRE2-factor binding; it was found that the binding activity disappeared when extracts were treated with potato acid phosphatase and binding was restored when extracts were treated with protein kinase A.

4.1 Introduction

As described previously, Ad12-transformed cells have reduced MHC class I expression compared to cells transformed by Ad2 or Ad5 (Schrier et al., 1983; Eager et al., 1985). Although some researchers have suggested that this difference might be due to differences in the levels of CRE2-binding factors (Ackrill and Blair, 1989; Ge et al., 1992), others have suggested that the lowered level of class I expression is due to reduced NF- κ B binding (Nielsch et al., 1991) or reduced NF- κ B and H2TF1 binding in Ad12-transformed cells (Meijer et al., 1992). Both these factors bind to a region called region I (Shirayoshi et al., 1987) or CRE1 (Ackrill and Blair, 1989). It was decided to investigate the functional role of this sequence in Ad-transformed cells by the use of CAT assays. Two oligonucleotides were used: CRE1 and mCRE1, the latter had mutations (Table 2.1) which have been shown to prevent transcription factor binding (Shirayoshi et al., 1987).

A single copy of the CRE1 or mCRE1 sequence was cloned into the vector pBLCAT2. The orientation of the insert in either case was determined by sequencing. The plasmid in which one copy of CRE1 was present in the forward orientation in the vector pBLCAT2 was termed pCRE1CAT, similarly the plasmid pmCRE1CAT contained a single copy of mCRE1. These plasmids and pBLCAT2 were transfected separately into Ad-transformed cell lines and the influence of CRE1 and mCRE1 sequence on transient CAT expression was investigated.

4.2 Cloning the CRE1 and mCRE1 oligonucleotides into the vector pBLCAT2

These two oligonucleotides were cloned into pBLCAT2 in exactly the same manner as CRE2. In cloning the CRE2 oligonucleotide in pBLCAT2 (Chapter 3), a large number of bacterial colonies was found by colony hybridization to contain recombinant plasmids, therefore it was decided that this step could be omitted. Several colonies of transformants were chosen at random and grown up overnight in 10 ml of LB/ampicillin (100 μ g/ml) and mini-preparations of DNA were made the following day.

4.3 Sequencing of the CRE1 and mCRE1 inserts in plasmids pCRE1CAT and pmCRE1CAT

The minipreparations of DNA from colonies of *E.coli* DH5 α transformed by CRE1/pBLCAT2 ligation mix were sequenced and one was found which had the CRE1 oligonucleotide present in the forward orientation. Similarly the minipreparations of

DNA from colonies of *E.coli* DH5 α transformed by mCRE1/pBLCAT2 ligation mix were sequenced and one was obtained which had the mCRE2 oligonucleotide present in the forward orientation. The sequences of the CRE1 and mCRE1 oligonucleotides are shown in table 2.1, these oligonucleotides have Bam HI/Bgl II compatible ends. Fig 4.1a shows the CRE1 oligonucleotide inserted in the forward orientation in the Bam HI site of the vector pBLCAT2, creating pCRE1CAT. Fig 4.1b shows the mCRE1 oligonucleotide inserted in the forward orientation in the Bam HI site of pBLCAT2, creating pmCRE1CAT.

4.4 Investigation of the functional role of CRE1 using plasmids pCRE1CAT, pmCRE1 and pBLCAT2 in CAT assays

Fig 4.2 shows a typical CAT assay result in which transient expression directed by plasmids pCRE1CAT and pmCRE1CAT was compared to that directed by the vector pBLCAT2 using 5A12 cell extracts. There was little difference in the CAT activity in extracts from cells transfected with pCRE1CAT compared to extracts from cells transfected with pBLCAT2, whereas extracts from cells transfected with pmCRE1CAT had significantly reduced CAT activity. Fig 4.3 summarizes the CAT assay results obtained in four cell lines: 5A12, RFC-1, BMK and Ad5Xho. The transient expression of CAT directed by plasmids pCRE1CAT was approximately equivalent to that directed by pBLCAT2 in 5A12 and RFC-1 (Fig 4.3a) whereas it was slightly less than that directed by pBLCAT2 in BMK and Ad5Xho. The CAT activity driven by pmCRE1CAT was below 40% of that directed by the vector pBLCAT2 in all the cell lines tested (Fig 4.3b).

4.5 Discussion

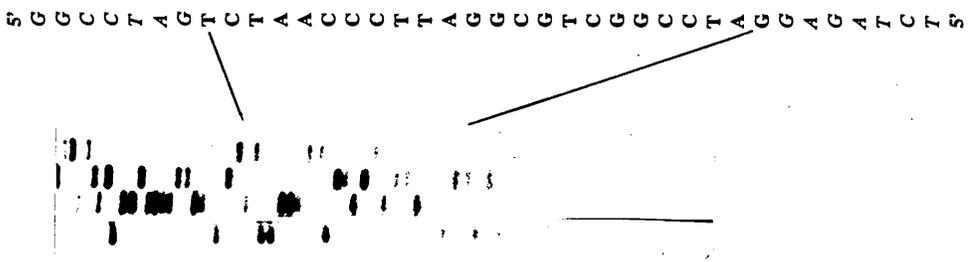
It is possible that the reduced levels of MHC class I in Ad12-transformed cells compared to Ad2- or Ad5-transformed cells could reflect a reduced functioning of an enhancer in the former. CRE1 is a likely candidate for such an enhancer, as multiple copies of this sequence have been shown to enhance CAT expression (Mauxion and Sen, 1989; Kralli et al., 1992). In this study the functional properties of CRE1-binding factors was investigated by the transient CAT expression assay system in cells transformed by Ad2, Ad5, Ad12 or recombinant virus. Four cell lines expressed Ad2 or Ad5 E1A and four expressed Ad12 E1A. A single copy of the CRE1 sequence was placed upstream of the CAT reporter gene in the plasmid pBLCAT2 to create the construct pCRE1CAT. A mutant CRE1, containing mutations that inhibit CRE1-

Fig 4.1 Sequencing of pCRE1CAT and pmCRE1CAT

The sequence of the CRE1 and the mCRE1 inserts are shown in Fig 4.1a. and Fig 4.1b. respectively. In each case the sequence in bold is that of the insert and the surrounding sequence which is that of the vector pBLCAT2 around the Bam HI site is shown in italics.

b. pmCREICAT

ACGT



a. pCREICAT

ACGT

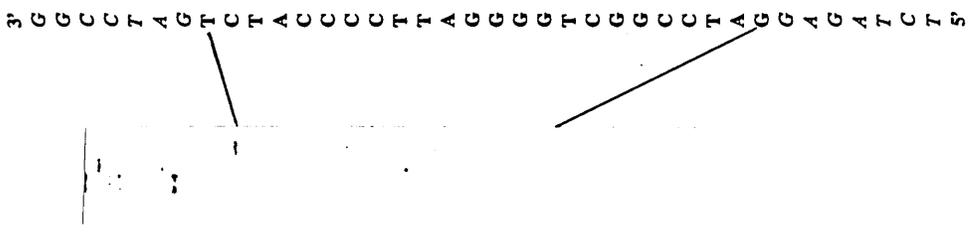


Fig 4.2 The CAT activity in extracts of 5A12 cells transfected with the vector pBLCAT2 or one of the constructs pCRE1CAT or pmCRE1CAT

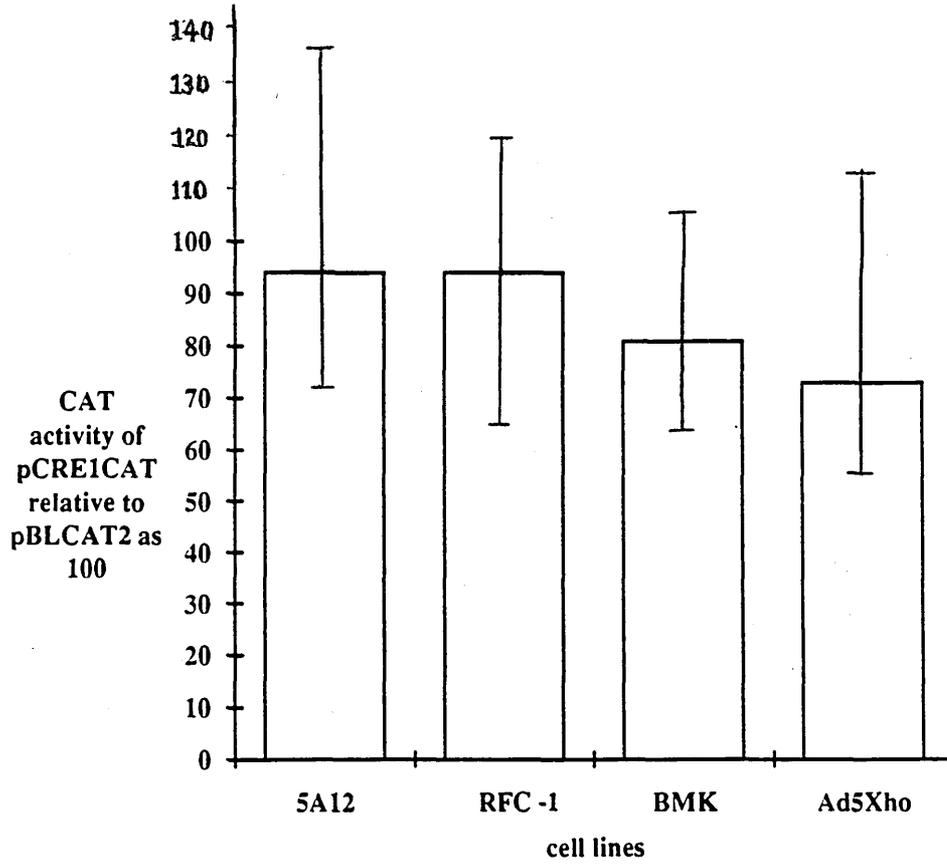
Extracts were prepared from 5A12 cells transfected with one of the following: 15 μg of pBLCAT2, 15 μg of pCRE1CAT or 15 μg of pmCRE1CAT. An aliquot of cell extract corresponding to 150 μg of protein was mixed with 8 μl (200 μCi) of [^{14}C] chloramphenicol and 40 μl of acetyl CoA (5 mg/ml) and incubated overnight. The acetylated and unacetylated forms of [^{14}C] chloramphenicol were separated by TLC. Lane 1: extracts from cells transfected with pBLCAT2, lane 2: extracts from cells transfected with pCRE1CAT and lane 3: extracts from cells transfected with pmCRE1CAT.

Fig 4.3 Expression of plasmids pBLCAT2, pCRE1CAT and pmCRE1CAT in Ad-transformed cells

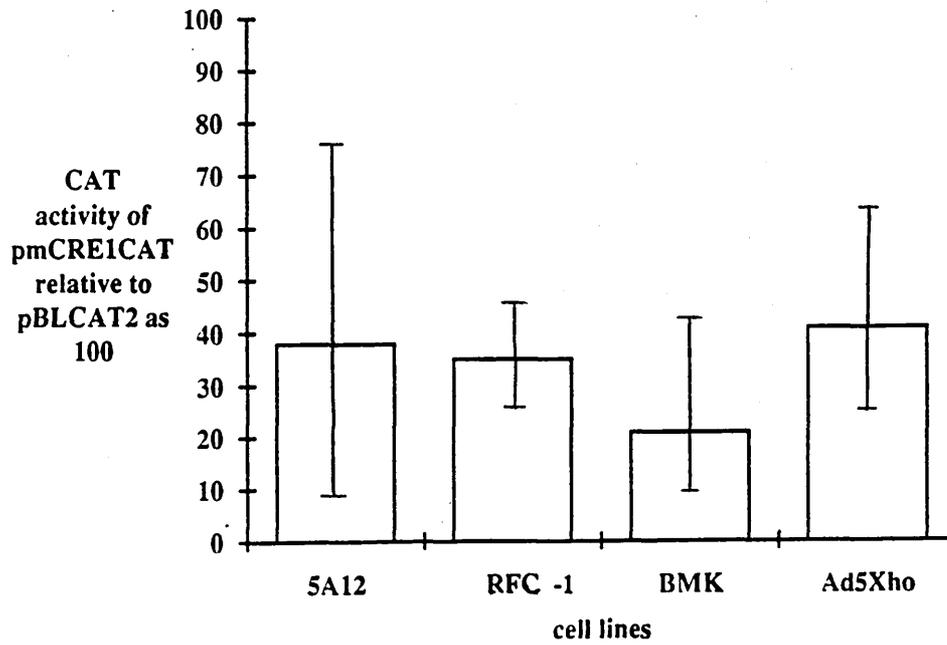
The CAT activity detected in extracts from cells transfected with the vector pBLCAT2 was assigned a value of 100 and those from extracts of cells transfected with pCRE1CAT or pmCRE1CAT are presented relative to it in Figs 4.3a and 4.3b respectively. Results are presented for four cell lines: 5A12, RFC-1, BMK and Ad5Xho. Each column represents the average of at least 3 separate experiments.

The range of values are as shown.

a



b



binding was also cloned into the same vector. The CAT activity of the two constructs was compared with that of the vector alone.

The CRE1 sequence was not shown to have enhancer activity. It is possible that the sequence did not demonstrate enhancer activity because it was present only as a single copy and multiple copies are necessary to demonstrate its activity. However, Meijer et al. (1992) showed enhancer activity in certain circumstances using a single copy of a sequence virtually identical to CRE1 which they called H2TF1. They found a single copy of this sequence was associated with enhancer activity in cells transformed by Ad12 12S E1A but not by Ad12 13S E1A. In this study cell lines expressing both the 12S and 13S products of Ad12 or Ad5 were studied. The different cell systems used make comparison between this study and that of Meijer et al. (1992) difficult.

mCRE1CAT was found to be associated with reduced CAT activity compared to the vector alone. Meijer et al (1992) obtained similar results with a mutated form of H2TF1; reasons for this effect are not known.

Chapter 5

Production and characterization of an antibody to the C-terminal peptide of the NF- κ B p50 subunit

5.1 Introduction

There are two premises to the theory that CRE1-binding proteins are involved in causing the down-regulation of MHC class I in Ad12-transformed cells compared to cells transformed by Ad2 or Ad5. Firstly, that such proteins act to enhance transcription; this was investigated in the previous chapter. Secondly, that such proteins are more abundant in the nucleus in Ad2- or Ad5-transformed cell lines than in Ad12-transformed cell lines (nuclear localization is required for such proteins to be transcriptionally active).

The levels of CRE1-binding factors in the nucleus could be investigated by various approaches, the most commonly used being that of gel retardation using nuclear extracts; this method will be described in a later chapter. Another approach would be to use an antibody to a known CRE1-binding factor and investigate the presence of this factor in nuclear extracts by Western blotting. Dr R. Hay donated three antibodies to the p50 subunit of NF- κ B; to the C-terminus, the N-terminus and the DNA-binding domain. However, because limited amounts were provided, it was decided to produce our own antibody. An anti-peptide antibody was chosen as peptide synthesis facilities were available. The antibody was made to the C-terminal peptide as it binds more specifically to NF- κ B as compared to antibody to the N-terminal peptide. The antibody produced to the C-terminal domain was used to investigate the levels of p50 in Ad-transformed cells. These results will be discussed later in relation to the results obtained by gel retardations with the CRE1 probe and with the kappa probe. In this chapter the production of an antibody to the C-terminal peptide of NF- κ B p50 will be described, followed by a section on its characterization by ELISA and Western blotting alongside the antibodies to NF- κ B provided by Dr R. Hay and the c-rel antibody provided by Dr P. Enrietto.

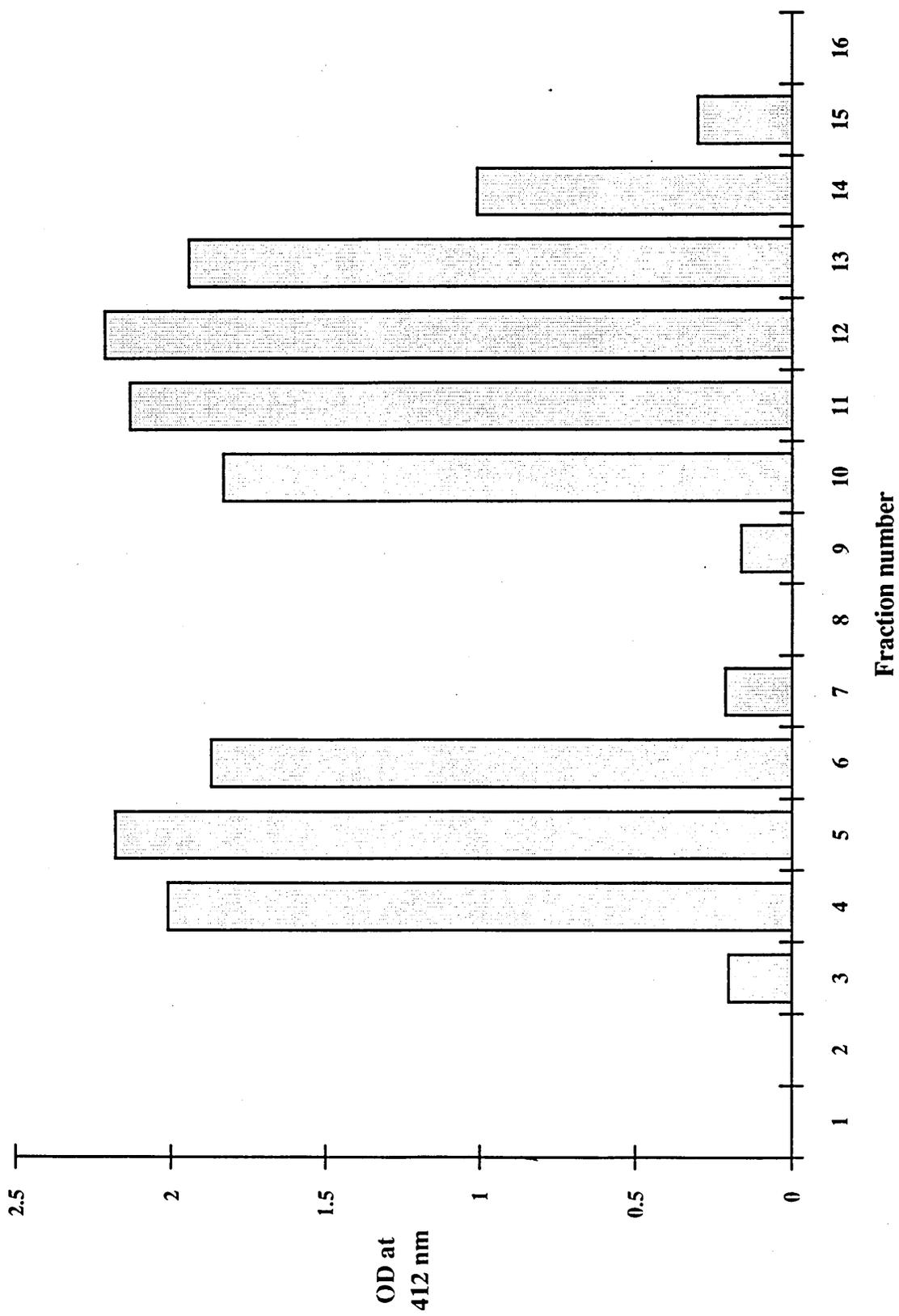
5.2 Coupling of the peptide to a carrier

5.2.1 Removal of DTT from reduced peptide by use of a Sephadex G-10 column

The peptide/DTT mix was added to a Sephadex G-10 column and the column was eluted with 0.1 M sodium phosphate buffer pH 6.0. Fractions (1 ml) were collected and the Ellman's test was performed on 20 μ l of each fraction and the absorbance was read at 412 nm. As shown in Fig 5.1, most of the peptide was eluted in fractions 4 to 6 and the DTT eluted later, mainly in fractions 10 to 14. Fractions 4, 5 and 6 were retained for mixing with the activated PPD.

Fig 5.1 Removal of excess DTT from reduced peptide by use of a Sephadex G-10 column

Fractions (1 ml) were collected from a Sephadex G-10 column, eluted with 0.1 M sodium phosphate, pH 6.0. The Ellman's test was performed on 20 μ l of each fraction and the absorbance was measured at 412 nm. The results are plotted as fraction number versus absorbance reading at 412 nm.



5.2.2 Removal of SMCC from activated PPD using a Sephadex G-25 column

The activated PPD/SMCC mix was added to a Sephadex G-25 column and eluted with 50 mM sodium phosphate buffer pH 6.0. Fractions (1 ml) were collected and absorbance was read at 280 nm. From the absorbance readings, Fig 5.2, it can be seen that the activated PPD (which elutes first) appeared between fractions 13 and 19, mainly in fractions 15, 16 and 17. These three fractions were mixed with the reduced peptide. The SMCC eluted later in fractions 28 to 38.

5.3 ELISA results

The ELISA was used to detect whether the antisera raised in the two rabbits, termed A and B, bound to the peptide to which they were raised when the peptide was itself attached to an ELISA plate. Information about the specificity of the reaction was obtained when conditions were included in which either the antiserum or the peptide were absent. Further information on the specificity of the antibody reaction was obtained by use of an unrelated peptide. The ELISA was also used to calculate the titres of the antibody response in the two rabbits 10 days after the initial injection with the PPD-peptide conjugate and 10 days after subsequent boosts.

Figs 5.3 and 5.4 show the ELISA results obtained with rabbits A and B respectively. Both figures show that negligible absorbance readings were obtained for the pre-immune sera at all the dilutions used. Readings not significantly different from those obtained for the pre-immune sera were found for both rabbits for wells incubated with the p50 peptide but having no contact with serum and for wells which had no contact with either serum or peptide (Table 5.1). Negligible readings were obtained when serum taken 10 days after the second boost from rabbit B was used with wells coated with an unrelated peptide (which forms part of the 5/6 loop of the human neurokinin-2 receptor), or when such serum was pre-incubated with the p50 peptide (Table 5.1). The same serum did produce a strong reaction if not pre-incubated with p50 peptide and used in wells coated with the p50 peptide. In the case of rabbit A the highest titre was obtained after the third boost (15,000). The titre increased from 2,200 after the initial injection to 10,000 following the first boost and to 11,500 following the second boost. For rabbit B the highest titre appeared after the first boost (29,000). The titre rose from 9,000 following the initial injection, peaked with the first boost, reduced to 17,000 after the second boost and dropped to 7,000 following the third boost.

Fig 5.2 Removal of SMCC from activated PPD by use of a Sephadex G-25 column

Fractions (1 ml) were collected from a Sephadex G-25 column, eluted with 50 mM sodium phosphate buffer, pH 6.0. The absorbance at 280 nm of each fraction was read. The results are plotted as fraction number versus absorbance reading at 280 nm.

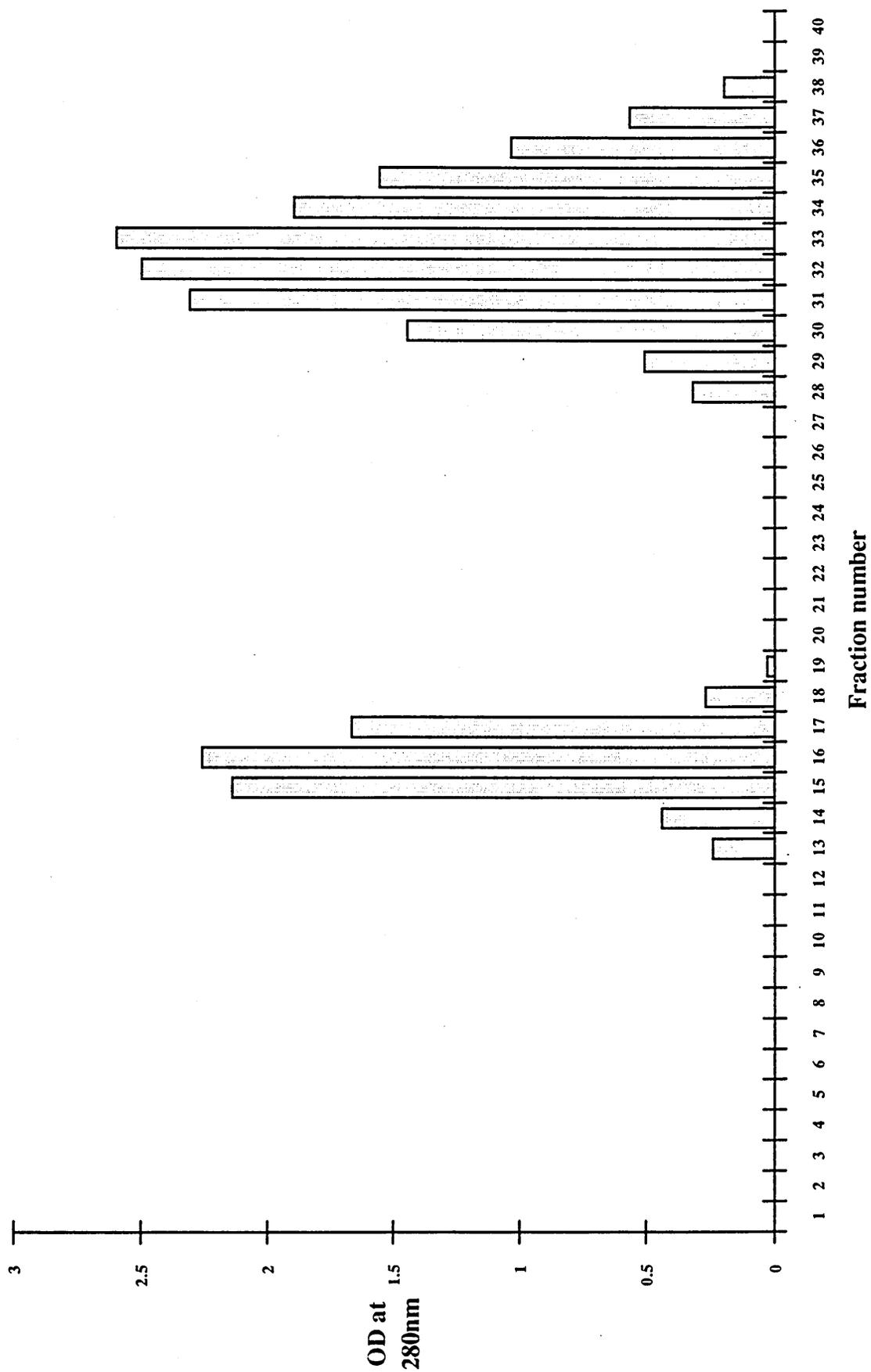


Fig 5.3 Levels of antibody against the C-terminal peptide of p50 in the serum of rabbit A

The results are presented as -log dilutions versus absorbance readings at 490 nm for: pre-immune (\diamond - \diamond), first injection (\square - \square), first boost (\blacktriangle - \blacktriangle), second boost (\triangle - \triangle) and third boost (o-o). The titres are calculated from the graph as the dilution (derived from -log dilution) which corresponds to 50% of the maximal O.D, as shown by the dotted lines.

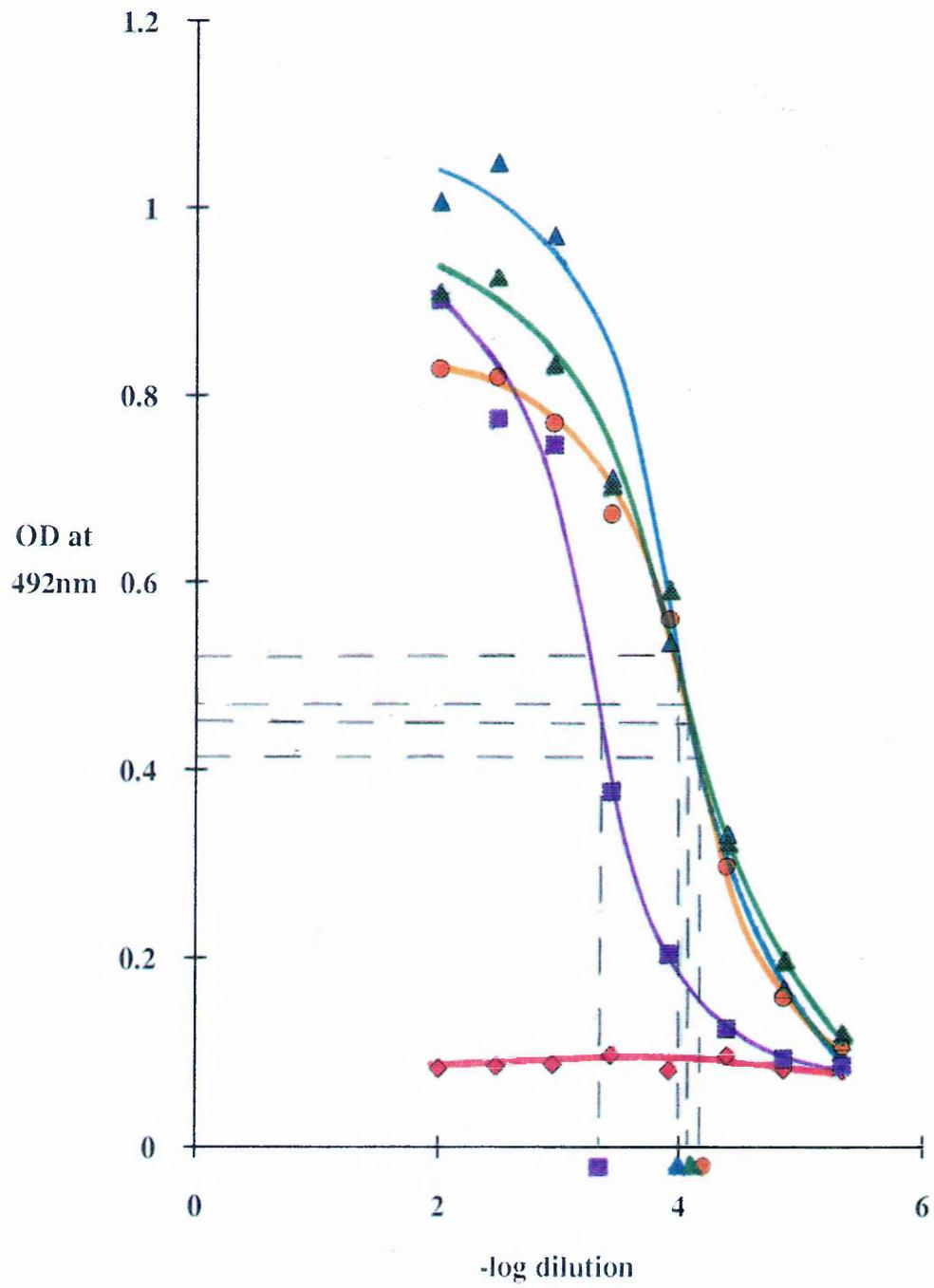


Fig 5.4 Levels of antibody against the C-terminal peptide of p50 in the serum of rabbit B

The results are presented as -log dilutions versus absorbance readings at 490 nm for: pre-immune (\diamond - \diamond), first injection (\square - \square), first boost (\blacktriangle - \blacktriangle), second boost (\triangle - \triangle) and third boost (o-o). The titres are calculated from the graph as the dilution (derived from -log dilution) which corresponds to 50% of the maximal absorbance, as shown by the dotted lines.

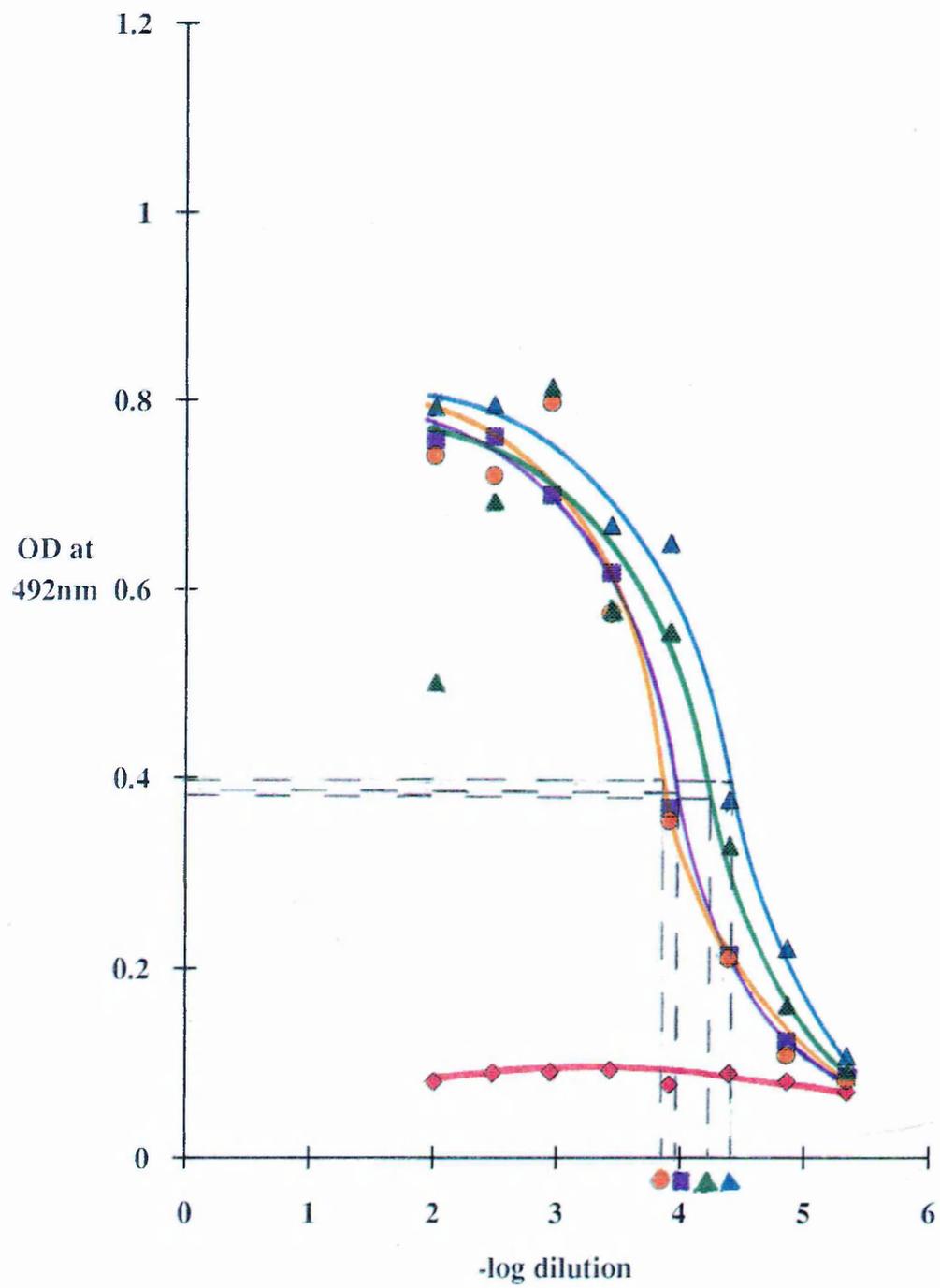


Table 5.1 ELISA results for rabbits A and B

Table A shows the ELISA results for rabbit A for pre-immune serum, and for wells coated with p50 peptide but no serum or for wells which were neither coated with peptide or treated with serum.

Table B shows the ELISA results for rabbit B for all the conditions described in table A for rabbit A, also included in table B are the results for wells treated with p50 peptide and serum taken 10 days after the second boost and a repeat of this condition with the exception that the serum was pre-incubated with p50 peptide. The final condition included in the table consists of wells coated with the neurokinin-2 receptor peptide and serum taken 10 days after the second boost.

A.

RABBIT A			
DILUTION	PRE-IMMUNE	WELLS TREATED WITH p50 PEPTIDE (NO SERUM)	UNTREATED WELLS (NO SERUM)
1/100	.083	.113	.042
1/300	.085	.080	.041
1/900	.087	.080	.042
1/2700	.097	.093	.043
1/8100	.081	.068	.047
1/24300	.096	.084	.044
1/72900	.082	.076	.040
1/218700	.082	.077	.045

B.

RABBIT B

DILUTION	PRE-IMMUNE	WELLS TREATED WITH p50 PEPTIDE (NO SERUM)	UNTREATED WELLS (NO SERUM)	WELLS TREATED WITH p50 PEPTIDE + SERUM TAKEN AFTER SECOND BOOST	WELLS TREATED WITH p50 PEPTIDE + SERUM TAKEN AFTER SECOND BOOST BLOCKED BY PRE-INCUBATION WITH p50 PEPTIDE	WELLS TREATED WITH NEUROKININ-2 RECEPTOR PEPTIDE + SERUM TAKEN AFTER SECOND BOOST
1/100	.082	.084	.042	.764	.085	.042
1/300	.078	.079	.046	1.077	.095	.047
1/900	.065	.083	.047	.855	.087	.052
1/2700	.078	.086	.044	.809	.098	.043
1/8100	.083	.083	.041	.531	.080	.047
1/24300	.079	.078	.042	.490	.092	.045
1/72900	.086	.083	.043	.272	.085	.046
1/218700	.068	.079	.041	.210	.082	.045

5.4 Western Blotting using antisera from rabbits A and B raised to the C-terminal peptide of p50 as compared to other p50 and c-rel antisera

Fig 5.5 shows that for rabbit A a protein of approximately 68 kDa reacted weakly with the immune serum (lane 2) but not with the pre-immune serum (lane 1). The binding of antibody to the 68 kDa protein was not blocked when the immune serum was pre-incubated with 50 μ g of the peptide to which the antiserum was raised (lane 3). The immune serum of rabbit A reacted weakly with a second protein at 55 kDa (lane 2) which did not react with the pre-immune serum (lane 1). The binding of antibody to this 55 kDa protein was partially blocked by pre-incubation of serum with 50 μ g of the C-terminal peptide (lane 3). A protein of approximately 55 kDa also reacted with the immune serum of rabbit B (lane 5) but not with the pre-immune serum (lane 4). The reaction appeared much stronger than for rabbit A serum. The binding of antibody to the 55 kDa protein was almost completely blocked when the immune serum was pre-incubated with 50 μ g of peptide (lane 6). A protein of apparently the same molecular weight also bound to three other antisera raised to p50, these were the antisera donated by Dr R. Hay to the C-terminal peptide (lane 7), the N-terminal peptide (lane 8) and the DNA-binding domain (lane 9). They were all used at 1 in 100 dilution, as recommended. At this dilution they all gave high background, in fact only for the DNA-binding domain antiserum was the strongest binding reaction with the 55 kDa protein. The protein of this size only reacted weakly with the C-terminal peptide antiserum. Four proteins clearly reacted with the N-terminal peptide antibody, one of which was approximately 100 kDa; a protein of the same size also reacted with the c-rel antiserum (lane 10).

5.5 Discussion

It was decided to generate an antibody to the C-terminal peptide of p50. Two peptides of 12 and 13 amino-acids were synthesized (with and without a terminal cysteine used to crosslink the peptide to a carrier). In general, it is uncommon for amino-acids of these lengths to be immunogenic (Lachmann et al., 1986). Normally peptides of these length are considered haptens, incapable of eliciting an immune response alone, they need to be linked to a carrier. The carrier elicits a T-cell response which, by some poorly understood mechanism, activates a B-cell response and subsequent antibody production to the hapten. It was decided to use PPD as the carrier as it is an unusually powerful T-cell antigen, it gives a T-cell mediated delayed-type hypersensitivity (DTH) in animals which have previously encountered the tubercle bacillus, for example, as a

Fig 5.5 Western blotting of rabbit A and rabbit B antisera raised to C-terminal peptide of p50 as compared with antiserum to c-rel and three p50 antisera

Ad12#1 nuclear extracts prepared by method MDP3 were used for Western blotting. Antisera from rabbit A were used in lanes 1-3, antisera from rabbit B were used in lanes 4-6. Lanes 1 and 4 were with pre-immune antisera. Lanes 2, 3, 5 and 6 were with antisera taken 10 days after the second boost. The antisera used in lanes 3 and 6 were pre-incubated for 1 hour with 50 μ g of peptide to the C-terminal of p50. Lanes 7-9 were with p50 antibodies provided by Dr R Hay; lane 7 was with antiserum to the C-terminal peptide of p50, lane 8 was with antiserum to the N-terminal of p50 and lane 9 was with antiserum to the DNA-binding domain of p50. Antiserum to c-rel provided by Dr P. Enrietto was used in lane 10. Antisera from rabbits A and B were used at 1 in 1000 dilution, where as other antisera were used at 1 in 100 dilution.

result of immunization with Bacillus Calmette-Guerin (BCG) (Lachmann et al., 1986). For this reason the rabbits were initially inoculated with BCG before the first injection with PPD-peptide conjugate. PPD is prepared from the culture supernatants of *Mycobacterium tuberculosis* by ultrafiltration, heating to 100°C and precipitation of protein with trichloroacetic acid (Seibert, 1940). Another advantage of PPD is that it does not cause antibody production to itself (Lachmann et al., 1986).

The stages in the preparation of the peptide-PPD conjugate involved the use of two Sephadex columns. The G-10 column was eluted to give two well separated peaks when absorbance at 412 nm was plotted against fraction number from the results of the Ellman's test, showing the DTT was effectively removed from the reduced peptide. Removal of the DTT was necessary to allow formation of a disulphide bond between the reduced peptide and the activated PPD. Elution of the G-25 column also gave two well-defined peaks when absorbance at 280 nm was plotted against fraction number, demonstrating the clear separation of activated PPD from excess SMCC.

The ELISA results showed that both rabbits did not contain antibody in the pre-immune sera which reacted with the p50 C-terminal peptide; readings for the pre-immune serum at all dilutions were as low as for wells treated with p50 peptide but having no contact with serum, and almost as low as for wells which received neither peptide or serum. High titres were obtained with both rabbits with the immune serum and the p50 peptide.

For both, the titres increased from serum taken 10 days after the first injection with the peptide/PPD conjugate to serum taken 10 days after the first boost. One month before the initial injection with the peptide/PPD conjugate, the rabbits were injected with BCG, a potent stimulator of T- and B-cells and macrophages. The BCG enhanced the initial response to the peptide/PPD conjugate. In the initial response there would have been clonal proliferation of the B-cells which had epitopes for the p50 peptide under the influence of T-cells activated by PPD. Memory B-cells specific for the p50 peptide would have been produced as part of the primary response. Some of these memory cells would have been stimulated to proliferate and produce antibody upon each subsequent contact with peptide. Memory B-cells are more sensitive to antigenic triggering, have higher affinity for antigen and respond more effectively to T-cell help (Roitt, 1988). Consequently, the secondary response was greater than the primary response. The time course of the response was quite different in the two different rabbits; in rabbit B the response peaked after the first boost and then declined, whereas

in rabbit A the response was still increasing after the third boost. The highest titre with rabbit B was greater than that obtained with rabbit A. Evidence that the response was specific for the p50 peptide came from the results obtained with peptide to the 5/6 loop of the human neurokinin 2 receptor; very low readings were obtained, as low as for wells untreated with peptide or serum. This shows the antibody did not react with an unrelated peptide. Further evidence for the specificity of the reaction came from the results with serum taken 10 days after the second boost; when this serum was pre-incubated with p50 peptide it no longer reacted on ELISA for wells incubated with the p50 peptide.

The titres obtained with both rabbits were high; certain peptides do not appear to give rise to specific antibody. The reasons for this are not completely understood. It is thought that a hydrophobic part of the peptide is necessary for it to attach to the lipid bilayer of the antigen presenting cell (APC) and a hydrophilic part is required for it to interact with the T cell receptor in an aqueous environment. The C-terminal p50 peptide contains a small hydrophobic region with the majority of the peptide being hydrophilic, it is therefore a likely candidate to give a good antibody response.

A protein of 68 kDa reacted on Western blots with rabbit A immune serum but not with the pre-immune serum. However the reaction was not blocked by pre-incubation of the serum, taken 10 days after the second boost, with 50 μ g of the peptide to which the antiserum was raised. This suggests the protein does not represent p50 and it might represent a protein to which the animal has raised antibodies as a result of infection which occurred between the time the pre-immune and immune blood samples were taken. The size of the protein, at 68 kDa, suggests it is too large to represent p50. The immune serum of rabbit A reacted weakly with a second protein of 55 kDa, this reaction was partially blocked by pre-incubation of serum with 50 μ g of the C-terminal peptide which suggests the protein represents p50. The size is slightly larger than the 50 kDa that would be expected for p50 however, within the accuracy of the markers, the protein band could actually represent a protein of 50 kDa. Stronger evidence that the protein does represent p50 comes from the fact that a protein of the same mobility reacted with Dr Hay's antisera to p50. The reaction of the 55 kDa protein appeared much stronger for rabbit B than for rabbit A. This could be because rabbit B antiserum gave a higher titre on ELISA. Also in Western blotting the anti-peptide antibody has to recognize the peptide to which it was raised in the context of the whole protein; it

is possible that the antibodies raised in rabbit B were better suited to use in Western blotting. The binding of antibody to the 55 kDa protein was almost completely blocked by pre-incubation of the serum with 50 μ g of the peptide to which the antiserum was raised. This again suggests the protein represents p50; it is possible that because of the high titre of the antibody more peptide would be required to produce complete blocking.

The antisera provided by Dr R. Hay did not react as specifically at the recommended dilution of 1 in 100, however, all three antisera reacted with a protein of approximately 55 kDa, with the antibody against the DNA-binding domain showing the highest binding activity. The C-terminal peptide antiserum reacted with so many proteins that, used alone, it would not provide any useful information. The antiserum to the N-terminal peptide would be expected to react with other members of the rel-related family. It reacted with a protein of 100 kDa; the anti c-rel antiserum also reacted with a protein of 100 kDa, suggesting this protein could represent c-rel.

Two proteins, both smaller than 50 kDa reacted weakly with the immune serum. It is possible these proteins represent breakdown products of p50, especially since these reactions were completely blocked by pre-incubation of immune serum with 50 μ g of C-terminal peptide of p50.

Chapter 6
Characterization of CRE1-binding proteins

6.1 Introduction

This study was directed towards characterizing the nuclear levels of CRE1-specific DNA-binding proteins present in Ad-transformed cells. Initially it was necessary to determine which method of nuclear extract preparation would be most suitable to investigate this question as it was found that different methods for the preparation of nuclear extracts produced different patterns of DNA-protein complexes on gel retardation. The methods used have been termed MDP1, MDP2 and MDP3. The complexes of different mobilities will be described in the section below. This will be followed by a section in which various techniques were used to characterize these DNA-protein complexes.

6.2 Comparison of the DNA-protein complexes obtained on gel retardation using different methods for the preparation of nuclear extracts

The DNA-protein complexes obtained with the different methods have been numbered from 1 to 7; complex 1 being that with the slowest mobility and complex 7 being the that with the fastest mobility.

6.2.1 DNA/protein complexes obtained on gel retardations using MDP1 extracts

Nuclear extracts were originally prepared by MDP1. The major differences from the Dignam procedure (Dignam et al., 1983) were that the cells were not spun down in buffer A before homogenization in this buffer and, unlike the Dignam procedure, this modification included an ammonium sulphate precipitation step and overnight dialysis. Four complexes were obtained; 1, 3, 4 and 7 as shown in Fig 6.1a lane 1 and Fig 6.1c lanes 1, 5-7. Not all cell lines used produced all complexes.

Complex 1

This complex had the slowest mobility and was usually present in extracts prepared by this method (Fig 6.1a lane 1, Fig 6.1c lanes 1, 5-7).

Complex 2

This complex was not detected using extracts prepared by the MDP1 method.

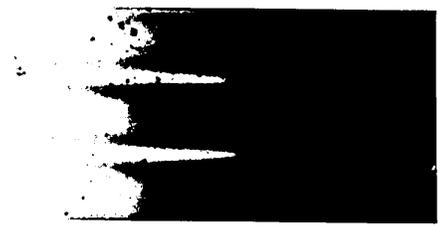
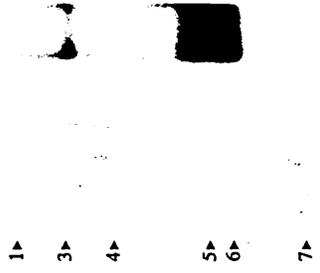
Complex 3

This complex was not always present but when present it usually appeared as a sharp complex, running faster than complex 1 (Fig 6.1a lane 1 and Fig 6.1c lanes 1,5-7).

Fig 6.1 Comparison of the DNA-protein complexes obtained on gel retardations using the CRE1 probe with different methods for the preparation of nuclear extracts

Fig 6.1a: Lane 1: complexes 1, 3, 4 and 7 were obtained using MDP1 extracts from F19 cells. Lane 2: MDP1 extracts from F19 cells containing 100-fold excess of unlabelled CRE1 in the binding reaction. Lane 3: complexes 3, 5 and 6 present with F19 MDP2 extracts. Fig 6.1b : Lane 1: complexes 3, 5 and 6 obtained using MDP3 extracts of Hela cells. Lane 2: MDP3 extracts of Hela cells treated with TNF α (100 U/ml) for 1 hour showed the same complexes as in lane 1 as well as the inducible complex 2. Fig 6.1c: lane 1 to 6 MDP1 F19 extracts, lanes 2, 3 and 4 with 100-fold, 200-fold and 500-fold excess of unlabelled CRE1 respectively and lanes 5 to 7 with 100-fold, 200-fold and 500-fold excess of unlabelled mCRE1 respectively.

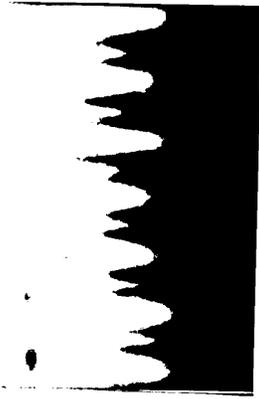
a. 1 2 3
1 2 3 4 5 6 7



b. 1 2



c. 1 2 3 4 5 6 7



Complex 4

This complex migrated slightly faster than complex 3. It usually appeared as a more diffuse complex than complex 3 (Fig 6.1a lane 1 and Fig 6.1c lanes 1, 5-7).

Complexes 5 and 6

These were not detected using nuclear extracts prepared by the MDP1 method.

Complex 7

This complex had the fastest mobility and was quite a diffuse complex. (Fig 6.1a lane 1 and Fig 6.1c lanes 1,5-7).

6.2.2 DNA/protein complexes obtained on gel retardations using MDP2 extracts

This is a modification of the Dignam procedure that is quicker and simpler than MDP1 and requires fewer cells (Andrews and Faller, 1991). Buffers A and C are the same in both procedures, but MDP2 has no ammonium sulphate precipitation step. The complexes obtained on gel retardations using this procedure were 2, 3, 5 and 6 (complex 2 was not always present).

Complexes 2 and 3

Fig 6.1b shows complex 2, running just above complex 3. Complex 1 runs considerably more slowly than complex 3, whereas complex 2 runs much more closely to complex 3; compare Fig 6.1b lane 2 and Fig 6.1c lane 1.

Complexes 5 and 6

These complexes ran as a doublet between complexes 4 and 7 (Fig 6.1a compare lane 3 to lane 1). When the resolution of the gel is good as in Fig 6.1a, they can be clearly visualized as a doublet. When the resolution of the gel is not as good they tend to appear as one complex, as in Fig 6.1b (lanes 1 and 2). These two complexes were not always present on gels. The probe labelled with varying levels of specific activity on different occasions. These complexes were only present when probes of higher specific activity were used.

6.2.3 DNA/protein complexes obtained on gel retardations using MDP3 extracts

This is a modification of MDP1, in which leupeptin (0.5 mg/ml) and aprotinin (0.24 TIU/ml) were used as additional protease inhibitors and sodium fluoride was present at 50 mM. MDP3 produced the same complexes as MDP2 (Fig 6.1b, lanes 1 and 2).

6.2.4 Investigation of factors involved in DNA/protein complex formation

When different complexes were obtained with MDP2, MDP1 was assessed for steps, not present in MDP2, which could potentially cause proteolysis. One such step was the overnight dialysis. When MDP1 was performed with 1 hour as opposed to overnight dialysis, only complex 3 was present (Fig 6.2). In this case, after overnight dialysis complexes 1, 4 and 7 were present.

Binding reactions were set up in 25 μ l, containing 5 μ l of extract. When MDP2 extracts were used 5 μ l of extract in Dig C were used. Dig C contains 0.42M NaCl, therefore the reaction mix (which contained no other source of NaCl) had NaCl present at 84 mM. When NaCl concentration in the reaction mix was increased to 80 mM with MDP1 extracts, complex 5 and 6 were obtained on gel retentions (Fig 6.3).

A TNF α inducible complex was not obtained with MDP1. A TNF α inducible complex (complex 2) was produced by MDP2 (see later). MDP1 was modified by the addition of extra protease inhibitors; the modification was called MDP3. A TNF α inducible complex was obtained with MDP3 as shown with Hela cells in Fig 6.1b lanes 1 and 2.

6.3 Characterization of the DNA-protein complexes obtained by gel retardation

After determining which complexes were present with different methods of nuclear extract preparation and binding reaction conditions, it was of interest to characterize these complexes. Information was obtained as to the nature of these complexes from several different procedures: oligonucleotide competition assays, the use of the kappa oligonucleotide, the effect of non-specific competitors on binding, induction experiments, and a crosslinking procedure.

6.3.1 Competition assays

If the complexes shown on gel retardations represent proteins that bind specifically to NF- κ B and related DNA-binding motifs, then one would expect the binding to be

Fig 6.2 The effect of 1 hour as compared to overnight dialysis on the DNA/protein complexes obtained by gel retardation using the MDP1 method of nuclear extract preparation

Lane 1 and 2: extracts which had been dialysed for one hour. Lanes 3 and 4: extracts which had been dialysed overnight. Lane 2 and 4: binding reactions contained 100-fold excess of unlabelled CRE1. The CRE1 probe was used with F19 extracts.

Fig 6.3 The effect of sodium chloride on the formation of DNA-protein complexes

Nuclear extracts were prepared by MDP1. Lanes 1-8 MDP1 extracts, binding reaction supplemented with 80 mM NaCl. Lane 1: 5B12, lane 2: BMK, lane 3: Ad5Xho and lane 4: F19, lane 5: 5A12, lane 6: C57, lane 7: RFC-1, lane 8: Ad12#1. Lane 9: HeLa MDP2 nuclear extracts from cells treated with TNF α (100 U/ml) for 1 hour. The CRE1 probe was used.

competed out by either CRE1 or the kappa oligonucleotide but not by mCRE1 in which residues shown to be essential for the binding of NF- κ B and related proteins have been mutated (Shirayoshi et al., 1987). The following section will review the information obtained from competition assays for the different complexes.

Complex 1

Fig 6.1c shows that complex 1 was competed out by 100-fold excess of CRE1 (lane 2) but not by 100-fold excess of mCRE1 (lane 5).

Complex 2

Fig 6.4 shows that complex 2 was competed out with the CRE1 (lanes 2-4) or kappa (lanes 8-10) oligonucleotides with as little as a 10-fold excess of the unlabelled oligonucleotide. Complex 2 was not competed with the oligonucleotide mCRE1 (lanes 5-7).

Complex 3

Fig 6.1c shows that complex 3 was not completely competed out at a 100-fold excess (lane 2) but it was competed out at 200-fold excess (lane 3) of unlabelled CRE1. It was not competed out by a 200-fold excess of mCRE1 (lane 6).

Complex 4

Fig 6.1c shows that this binding activity was competed out at a 100-fold excess of CRE1 (lane 2), but not with a 100-fold excess of mCRE1 (lane 5).

Complexes 5 and 6

In Fig 6.4 the probe was not labelled to as high a specific activity as in other experiments, probably because of this, complexes 5 and 6 only appeared faintly. However competition is not complete with a 100-fold excess of CRE1 (lane 4) but did appear to occur with 100-fold excess of the kappa probe (lane 10). In certain experiments, efficient competition was not obtained for these DNA-binding proteins.

Complex 7

Fig 6.1c shows that complex 7 was competed out by a 100-fold excess of CRE1 (lane 2) but not by a 100-fold excess of mCRE1 (lane 5).

Fig 6.4 Specific competition analysis using the CRE1 probe and CRE1, mCRE1 and kappa as competitor oligonucleotides

MBP1 nuclear extracts from 5B12 cells were used. Lane 1: no competitor, Lane 2-4: 10, 50 and 100-fold excess of unlabelled CRE1, lanes 5-7: 10, 50 and 100-fold excess of unlabelled mCRE2 and lanes 8-10: 10, 50 and 100-fold excess of unlabelled kappa oligonucleotide.

6.3.2 Binding to the kappa oligonucleotide

Fig 6.5 shows gel retardations using MDP3 nuclear extracts with labelled kappa (lanes 1-8) and CRE1 (lanes 9-16) probes. Complexes 2 and 3 had the same mobility with either probe. Complex 2 was more intense with the kappa probe. Fig 6.6 shows that both complexes 2 and 3 produced with the kappa probe can be competed with an excess of unlabelled CRE1 (Fig 6.6a) or kappa oligonucleotide (Fig 6.6b).

6.3.3 The effect of non-specific competitors on binding to CRE1

Fig 6.7 and Fig 6.8 show that the proportion of binding activity present as complex 4 or complex 7 could be altered depending on the non-specific competitor. The same batch of frozen aliquots of F19 MDP1 extracts was used in these experiments. Using poly (dG-dC) as a non-specific competitor, complex 4 was more intense than complex 7 (Fig 6.7) whereas the reverse was the case when poly (dI-dC) was used (Fig 6.8).

6.3.4 Induction experiments

One of the most interesting features of NF- κ B is its inducibility. A number of agents can be used to treat cells which are thought to cause the dissociation of NF- κ B from I- κ B in the cytoplasm and allow the nuclear translocation of NF- κ B. On gel retardation, these events are seen as the appearance or increased intensity of a particular complex using nuclear extracts prepared from cells treated with an inducing agent as compared to nuclear extracts made from untreated cells.

If in this study a particular complex could be shown to be inducible by several agents that are known to induce NF- κ B, then it would be indicative that the complex represents NF- κ B; that was indeed found to be the case as complex 2 was shown to be inducible by TNF α , cycloheximide, LPS and H₂O₂. These are all agents that have been shown to induce NF- κ B. Schreck et al. (1991) have proposed that these agents (in common with all others that induce NF- κ B) act through a mechanism that involves ROI; this will be discussed further in chapter 8.

TNF α

TNF α has been shown to be a strong inducer of NF- κ B in a number of cell lines (Duh et al., 1989). Duh et al. (1989) produced induction by TNF α present at 100 U/ml. Fig 6.6a shows that complex 2 is demonstrable in Hela cells nuclear extracts after the exposure of the cells to TNF α (100 U/ml) for just 5 minutes (lane 3), although greater

Fig 6.5 Levels of CRE1- and kappa-binding factors in nuclear extracts prepared from Ad-transformed cell lines by the MDP3 method

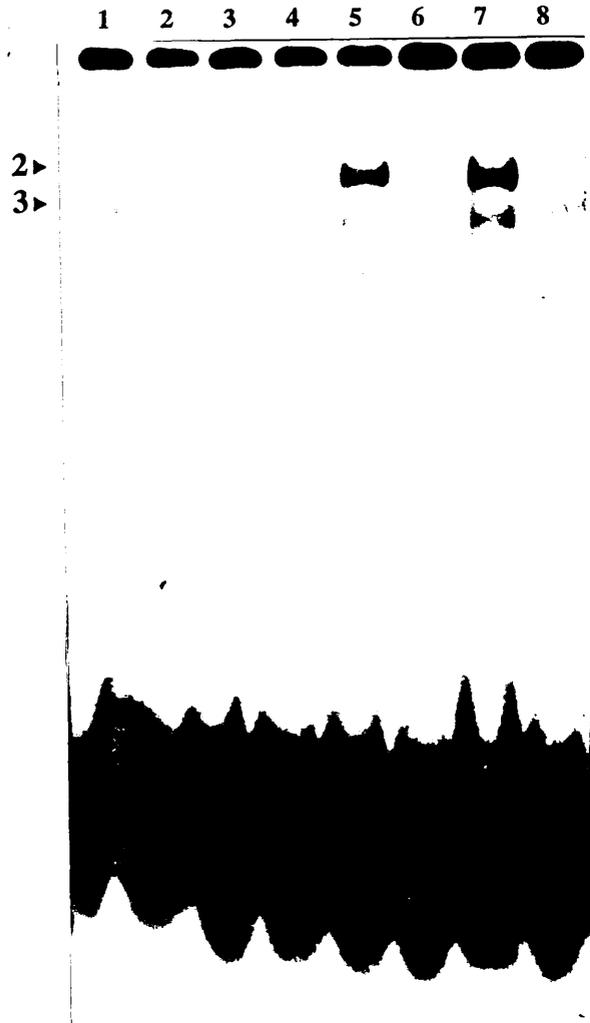
Lane 1-8 were with the kappa probe and lanes 9-16 were with the CRE1 probe. The following Ad2 or Ad5 cells E1A-expressing cells were analyzed: 5B12 (lanes 1 and 9), BMK (lanes 2 and 10), Ad5Xho (lanes 3 and 11), F19 (lanes 4 and 12), along with the following Ad12 E1A-expressing cells: 5A12 (lanes 5 and 13), C57 (lanes 6 and 14), RFC-1 (lanes 7 and 15) and Ad12#1 (lanes 8 and 16).

Fig 6.6 Induction of complex 2 by TNF α detected with the kappa probe and either CRE1 or kappa oligonucleotides as competitors

All extracts were prepared by MDP2. A, In lanes 1-2 extracts were prepared from untreated Hela cells. In lanes 3-8, extracts were from cells treated with TNF α (100 U/ml) for 5 (lanes 3 and 4), 10 (lanes 5 and 6) and 15 minutes (lanes 7 and 8). In lanes 2, 4, 6 and 8 the binding reactions contained a 100-fold excess of unlabelled CRE1 oligonucleotide.

B, In lanes 1 and 2 Hela MDP2 nuclear extracts prepared from cells treated with TNF α (100 U/ml) for 15 minutes. Lane 2: the binding reaction contained a 100-fold excess of the unlabelled kappa oligonucleotide.

a.



b.



Fig 6.7 Competition analysis using the CRE1 probe with CRE1 and kappa as competitor oligonucleotides and dG-dC as non-specific competitor

The competitor present in lanes 2-6 was unlabelled CRE1 and in lanes 8-12 was unlabelled kappa. Competitor was present at 20-fold excess in lanes 2 and 8, 40-fold excess in lanes 3 and 9, 80-fold excess in lanes 4 and 10, 160-fold excess in lanes 5 and 11 and 320-fold excess in lanes 6 and 12.

Fig 6.8 Competition analysis using the CRE1 probe with CRE1 and kappa as competitor oligonucleotides and poly dI-dC as non specific competitor

The competitor present in lanes 2-6 was unlabelled CRE1 and in lanes 8-12 was unlabelled kappa. Competitor was present at 20-fold excess in lanes 2 and 8, 40-fold excess in lanes 3 and 9, 80-fold excess in lanes 4 and 10, 160-fold excess in lanes 5 and 11 and 320-fold excess in lanes 6 and 12.

induction was obtained with 10 or 15 minutes exposure to TNF α (lanes 5 and 7). Complex 2 has been demonstrated to be inducible in several Ad-transformed cell lines for 15 minutes to 18 hours treatment with TNF α (see chapter 8).

Cycloheximide

Cycloheximide interferes with translation by binding to the 60S subunit of ribosomes (Todorov, 1990). Cycloheximide also induces NF- κ B as was first shown by Sen and Baltimore (1986); they treated cells for 4 hours with cycloheximide (10 μ g/ml). A strong induction of complex 2 was obtained by treating cells with cycloheximide (30 μ g/ml) for 9 hours as shown with BMK cells in Fig 6.9 (lane 17).

Lipopolysaccharide

LPS is a constituent of the membrane of gram-negative bacteria (Male et al., 1992). LPS is a B-cell mitogen. Sen and Baltimore (1986) were the first to demonstrate the induction of NF- κ B by LPS. They found induction of NF- κ B binding activity with LPS treatment (10 μ g/ml) of between 30 minutes and 8 hours. Complex 2 was induced following a 1 hour treatment with LPS (20 μ g/ml) of HeLa cells (Fig 6.10, lane 3).

Hydrogen Peroxide

Hydrogen peroxide (H₂O₂) is a powerful oxidizing agent which is broken down within the cell to water, although it can cause the production of some hydroxyl radicals (Halliwell, 1992). Schreck et al. (1991) found that treatment of cells with 100 μ M H₂O₂ for 1 hour resulted in the induction of NF- κ B. Treatment of 5B12 cells with H₂O₂ (100 μ M) for 1 hour was sufficient to induce complex 2 (Fig 6.11, lane 3).

6.3.5 Formaldehyde crosslinking

The DNA and protein of complexes 1, 4, 5, 6 and 7 were crosslinked using formaldehyde. Unfortunately the technique did not work for complexes 2 and 3. In this procedure the complexes are excised from a gel retardation, crosslinked with formaldehyde, eluted and acetone precipitated, then separated by SDS/polyacrylamide gel electrophoresis, with molecular weight markers used to estimate the size of the protein/oligonucleotide complexes. Fig 6.12a shows that complex 1 from a gel retardation did not enter a SDS/polyacrylamide gel; its size can be estimated to be greater than 200 kDa. Fig 6.12b shows the cross-linked products of complex 4, when analysed by SDS/polyacrylamide gel electrophoresis, gave two DNA/protein complexes

Fig 6.9 The effect of TNF α and/or cycloheximide on the levels of CRE1-binding factors in MDP2 nuclear extracts of BMK cells

Lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18 all had a 100-fold excess of unlabelled CRE1 in the reaction mixes. The nuclear extracts used in lanes 1 and 2 were prepared from untreated cells. Lanes 3 to 16 used nuclear extracts from cells treated with TNF α (100 U/ml) for 15 minutes (lanes 3 and 4), 30 minutes (lanes 5 and 6), 1 hour (lanes 7 and 8), 2 hours (lanes 9 and 10), 6 hours (lanes 11 and 12) and 8 hours (lanes 13 to 16). The nuclear extracts used in lanes 15 and 16 were from cells treated with cycloheximide (30 μ g/ml) for 9 hours as well as TNF α . The nuclear extracts in lanes 17 and 18 were prepared from cells treated for 9 hours with cycloheximide (30 μ g/ml) alone.

Fig 6.10 Induction of complex 2 in Hela cells treated with LPS

The CRE1 probe was used with Hela MBP2 nuclear extracts. Lanes 2 and 4 had a 100-fold excess of unlabelled CRE1 in the reaction mixes. The nuclear extracts used in lanes 1 and 2 were prepared from untreated cells. The nuclear extracts in lanes 3 and 4 were prepared from cells treated with LPS (20 $\mu\text{g/ml}$) for 1 hour.

Fig 6.11 Induction of complex 2 in 5B12 cells treated with H₂O₂

The CRE1 probe was used with 5B12 MBP2 nuclear extracts. Lane 2 had 100-fold excess of unlabelled CRE1 in the reaction mix (competition results not shown for cells treated with H₂O₂). The nuclear extracts used in lanes 1 and 2 were prepared from untreated cells. The nuclear extracts in lanes 3 were prepared from cells treated with H₂O₂ (100 μM) for 1 hour

Fig 6.12 Estimation of the size of DNA-protein complexes stabilized by formaldehyde crosslinking

The CRE1 probe was used in all the crosslinking experiments.

Fig 6.12a: Lane 1, complex 1 was excised from a native polyacrylamide gel used to separate retarded complexes in F19 nuclear extracts, the DNA:protein complexes were crosslinked with formaldehyde, eluted, acetone precipitated and separated by SDS/polyacrylamide gel electrophoresis along with ^{14}C molecular weight markers in lane 2.

Fig 6.12b: Lane 1, ^{14}C molecular weight markers. Complexes 4 (lane 2) and 7 (lane 3) from BMK nuclear extracts were excised from a native polyacrylamide gel and treated as described in Fig 6.12a and run on a SDS/polyacrylamide gel.

Fig 6.12c: Lane 1, complexes 5 and 6 from C57 extracts were excised from a native polyacrylamide gel and treated as described in Fig 6.12a and separated by SDS polyacrylamide gel electrophoresis along with ^{14}C molecular weight markers in lane 2.

a.

1 2

>200 kDa▶
<200 kDa▶
<92.5 kDa▶
<69 kDa▶
<46 kDa▶
<30 kDa▶
<14.3 kDa▶

b.

1 2 3

200 kDa▶
92.5 kDa▶
69 kDa▶
46 kDa▶
30 kDa▶
14.3 kDa▶

c.

1 2

65 kDa▶
<200 kDa▶
<92.5 kDa▶
<69 kDa▶
<50 kDa▶
<46 kDa▶
<30 kDa▶

of approximately 65 kDa and 93 kDa, whereas complex 7 gave a single complex of approximately 50 kDa. Fig 6.12c shows that complexes 5 and 6 generated a complex of approximately 65 kDa.

6.4 Discussion

A rapid method for the preparation of nuclear extracts was recently published (Andrews and Faller, 1991) and is referred to as MDP2. When MDP2 was compared to a modification of the Dignam procedure (Dignam et al., 1983), referred to as MDP1, it was found that different complexes were present on gel retardations using the CRE1 probe. MDP1 gave complexes referred to as 1,3,4 and 7 whereas MDP2 gave complexes 2,3,5 and 6. Because MDP2 extracts were prepared in buffer C, the binding reactions for MDP2 extracts had NaCl present unlike MDP1 extracts which were in buffer D which contains no NaCl. When NaCl was added to binding reactions with MDP1 extracts to approximately the same concentrations as that found in binding reactions with MDP2 extracts, complexes 5 and 6, previously only associated with MDP2, were found with extracts prepared by either method. This shows that the proteins that produce complexes 5 and 6 when bound to the CRE1 probe are present in MDP1 extracts but require NaCl to be present at approximately 80 mM in order to bind to the CRE1 probe. Using MDP1, complexes 1, 4 and 7 were present with overnight dialysis, whereas only complex 3 was present with 1 hour dialysis. This suggests complexes 1, 4 and 7 could represent proteolytic breakdown products; this will be discussed further in relation to the results obtained by formaldehyde crosslinking. Since MDP2 is a quicker process there may be less opportunity for proteolysis to occur. Also the proteins might be more stable in the high salt concentration present in Dig C but not Dig D. Either reason could cause the absence of these complexes in MDP2. Since a TNF α -inducible complex was not obtained with MDP1 but was obtained with MDP2, it seemed likely that such a binding activity was being lost due to proteolysis. Therefore MDP1 was modified by the addition of extra protease inhibitors, aprotinin and leupeptin, also sodium fluoride was increased to 50 mM; the modification was called MDP3. As MDP3 extracts gave complex 2 on gel retardation, it seems likely that complex 2 was indeed subject to proteolytic attack in MDP1.

Information was obtained as to the nature of the different complexes from several different procedures including competition assays, the use of the kappa oligonucleotide

as a probe, the effect of different non-specific competitors, induction experiments and crosslinking procedures.

If the complexes shown on gel retardations represent proteins that bind specifically to NF- κ B-like DNA-binding motifs, then one would expect the complexes to be competed out by either CRE1 or the kappa oligonucleotides but not at all or only slightly by mCRE1 in which G residues shown to be essential for binding of NF- κ B-like proteins have been mutated (Shirayoshi et al., 1987). From these criteria complex 1, 2, 3, 4 and 7 were all shown to bind specifically to the CRE1 probe. Complexes 5 and 6 appeared to be competed out with an excess of the kappa oligonucleotide, but not always with an excess of the CRE1 oligonucleotide. The reason for this variation was not known, although it could possibly be due to variations in the specific activity of the probe. The kappa oligonucleotide was made to the same length as the CRE1 oligonucleotide, so that if complexes appeared with the same mobility with either probe it would be indicative that they represent the same protein and that the protein is a NF- κ B-like DNA-binding protein. Complexes 2, 3, 5 and 6 were present with either probe and were competed out by an excess of either of the unlabelled oligonucleotide sequences; this is evidence that they all represent NF- κ B-like DNA-binding proteins (although competition for complexes 5 and 6 by CRE1 was not always obtained).

The same batch of aliquots of MDP1 nuclear extracts gave a more intense complex 4 than complex 7 when poly(dG-dC) was used as a non-specific competitor, whereas the reverse was the case when poly(dI-dC) was used. This suggests poly(dI-dC) and poly(dG-dC) do more than act as non-specific competitors. It appears they can both compete to some extent with the formation of specific binding; complex 4 is partially competed out by poly(dI-dC) whereas complex 7 is to some extent competed out by poly(dG-dC). p50 and p65 homodimers are strongly inhibited by poly(dI-dC) more so than NF- κ B (Baeuerle et al., 1991). This suggests that complex 4 could represent a p50 or a p65 homodimer or their breakdown products. Since p65 homodimers tend to be only represented on gel retardations when special binding conditions are used (Hiscott and Cohen, 1992), complex 4 is more likely to represent p50 homodimers or their breakdown products. Poly(dG-dC) competes for binding of NF- κ B more efficiently than poly(dI-dC) (Baeuerle, 1991). Poly(dG-dC) inhibits the binding of complex 7 more than poly(dI-dC), suggesting it might represent NF- κ B, or one of its breakdown products.

A major feature of NF- κ B is its inducibility. It was considered that if in this study, a particular complex shown by gel retardation was induced by several of the agents known to induce NF- κ B, then this would be strongly indicative that the complex represented NF- κ B. The results obtained indicated that complex 2 could represent NF- κ B as it was shown to be inducible by TNF α , cycloheximide, LPS and H₂O₂; all are agents that have been shown to induce NF- κ B. Furthermore, complex 2 induction occurred after cells were treated with the agents for periods of time and with dosages known to induce NF- κ B.

Formaldehyde crosslinking is a means of obtaining information on the size of the protein component of complexes detected by gel retardation. This information was obtained for complexes 1, 4, 5, 6 and 7. Unfortunately this technique did not work for complexes 2 and 3. Complex 1 was greater than 200 kDa. It could represent one of the large PRDII-BF1-like proteins, alternatively it could represent a tetramer of NF- κ B; Baeuerle (1991) suggests NF- κ B can bind to DNA in this way. When the crosslinked products of complex 4 were analysed on an SDS/polyacrylamide gel, two species were present of approximately 65 and 93 kDa. Because of the relatively small length of the probe and the relatively large size of DNase I it was not considered necessary to DNase I treat the formaldehyde crosslinked products. However, the probe would have caused a certain degree of overestimation of the sizes; it can be said that the complexes on the SDS/polyacrylamide gels should represent proteins no larger than the estimated size. Since the free probe runs faster than the smallest marker, it is not thought that the overestimation problem was too great. Given the potential overestimation, the 65 kDa complex associated with complex 4 could represent p50 and the 93 kDa complex associated with complex 4 could represent c-rel. Similarly because of overestimation, the 65 kDa complex associated with complexes 5 and 6 could also represent p50; perhaps it has a slightly different conformation and so runs differently on gel retardations. Again with overestimation, the 50 kDa protein of complex 7 could represent the p42 proteolytic breakdown product of p50 described by Baeuerle (1991). Complex 7 appeared only after overnight as opposed to 1 hour dialysis of MDP1 extracts, which provides further evidence that complex 7 could represent a proteolytic breakdown product.

Chapter 7
Nuclear levels of CRE1/kappa binding factors
in Ad-transformed cells

7.1 Introduction

It has been claimed that the differences in levels of MHC class I expression found in oncogenic and non-oncogenic Ad-transformed cells is due to differences in the levels of CRE1/kappa binding factors in these cells (Nielsch et al., 1991; Meijer et al., 1992). In this chapter the levels of these binding factors in the nucleus of Ad-transformed cells was investigated by two different approaches; gel retardation using CRE1 and kappa oligonucleotides and Western blotting using the rabbit B antiserum raised to the C-terminal of p50.

7.2 The use of gel retardation to investigate the nuclear levels of CRE1/kappa-binding factors in Ad-transformed cell lines

The levels of CRE1/kappa binding factors were investigated in eight adenovirus cell lines; four expressing Ad2 or Ad5 E1A and four expressing Ad12 E1A. The results obtained appeared to depend upon the method by which the extracts were prepared.

7.2.1 Levels of CRE1-binding factors in nuclear extracts prepared from Ad-transformed cells by the MDP1 method

Fig 7.1, shows the levels of CRE1-binding factors in nuclear extracts prepared by MDP1. Complex 1 appeared to be present in three of the four Ad12 E1A expressing cell lines and not present with any cell line expressing Ad2 or Ad5 E1A. Complex 4 was present in 3 of the 4 Ad2 or Ad5 E1A expressing cell lines and was not present in any cell line expressing Ad12 E1A. Complex 7 appeared as an intense band in BMK, F19 and Ad5Xho cells but it was also present, although fainter, in 5B12 as well as 5A12, C57 and Ad12#1(the three latter cell lines express Ad12 E1A).

7.2.2 Levels of CRE1-binding factors in nuclear extracts prepared from Ad-transformed cells by the MDP1 method with 10 mM sodium fluoride

Fig 7.2 shows the levels of CRE1-binding factors found when nuclear extracts were prepared by MDP1 with the modification that all buffers contained 10 mM sodium fluoride. The intensity of complex 1 varied between different cell lines, but there seemed to be no relation to the serotype of E1A expressed by a particular cell line. Complexes 4 and 7 were present in all cell lines which expressed Ad2 or Ad5 E1A and absent in all Ad12 E1A expressing cell lines.

Fig 7.1 Levels of CRE1-binding factors in nuclear extracts prepared from Ad-transformed cell lines by the MDP1 method

MDP1 nuclear extracts of the following Ad2 or Ad5 E1A-expressing cells were analysed: 5B12 (lane 1), BMK (lane 2), Ad5Xho (lane 3) and F19 (lane 4) along with the following Ad12 E1A-expressing cells: 5A12 (lane 5), C57 (lane 6), RFC-1 (lane 7) and Ad12#1 (lane 8). The CRE1 probe was used.

Fig 7.2 Levels of CRE1-binding factors in nuclear extracts prepared from Ad-transformed cell lines by the MDP1 method in the presence of sodium fluoride

MDP1 nuclear extracts prepared in the presence of 10mM sodium fluoride were analysed from the following Ad2 or Ad5 E1A-expressing cells: 5B12 (lane 1), BMK (lane 2), Ad5Xho (lane 3) and F19 (lane 4) along with the following Ad12 E1A-expressing cells: 5A12 (lane 5), C57 (lane 6), RFC-1 (lane 7) and Ad12#1 (lane 8). The CRE1 probe was used.

7.2.3 Levels of CRE1-binding factors in nuclear extracts prepared from Ad-transformed cells by the MDP2 method

When extracts were prepared by MDP2, there was no difference between cell lines expressing Ad12 E1A compared to those expressing Ad2 or Ad5 E1A, in any of the complexes present; 2, 3, 5 and 6 (Fig 7.3). Complex 2 was clearly present in extracts from BMK and Ad12#1 cells, and faintly present in F19 cells. Complexes 3, 5 and 6 were present in all the cell lines.

7.2.4 Levels of kappa-binding factors in nuclear extracts prepared from Ad-transformed cells by the MDP2 method

When MDP2 nuclear extracts were used with the kappa probe a slightly different binding pattern was found than with the CRE1 probe (compare Fig 7.3 with Fig 7.4). Complex 2, although sometimes faint, was visible in the extracts of more cell lines and complexes 5 and 6 were barely visible. Complex 3 was present in all cell lines except C57. As with the CRE1 probe, there did not appear to be any differences in binding between cell lines which expressed Ad12 E1A and those which expressed Ad2 or Ad5 E1A.

7.2.5 Levels of CRE1- and kappa-binding factors in nuclear extracts prepared from Ad-transformed cells by the MDP3 method

Fig 6.5 shows that complex 2 was found in all the cell lines tested with either probe, but was more intense in cells expressing Ad2 or Ad5 E1A than in those expressing Ad12 E1A. Complex 3 binding more clearly demonstrated with the CRE1 probe and, although it was present with all cell lines, it appeared to be more intense in cell lines which express Ad2 or Ad5 E1A, with the exception of C57 which also showed an intense complex 3. Complex 5 and 6 appeared very faintly with the kappa probe.

7.3 The use of Western blotting to investigate the steady-state levels of p50 in nuclear extracts of Ad-transformed cell lines

The steady-state levels of p50 were investigated using MDP3 nuclear extracts from the eight different Ad-transformed cell lines and rabbit B antiserum taken 10 days after the second boost. Western blotting of these nuclear extracts revealed three polypeptides of approximately 55K, 54K and 50K (Fig 7.5). All the polypeptides appeared to be present in all the cell lines. The intensity of the polypeptides varied between cell lines,

Fig 7.3 Levels of CRE1-binding factors in nuclear extracts prepared from Ad-transformed cell lines by the MDP2

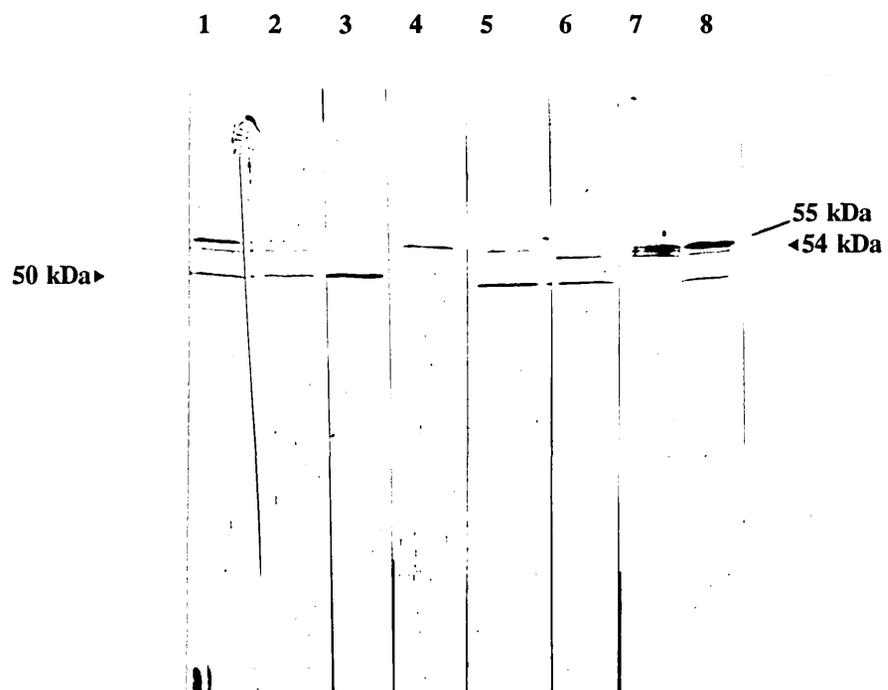
MDP2 nuclear extracts of the following Ad2 or Ad5 E1A-expressing cells were analysed: 5B12 (lane 1), BMK (lane 2), Ad5Xho (lane 3) and F19 (lane 4) along with the following Ad12 E1A-expressing cells: 5A12 (lane 5), C57 (lane 6), RFC-1 (lane 7) and Ad12#1 (lane 8). The CRE1 probe was used.

Fig 7.4 Levels of kappa-binding factors in nuclear extracts prepared from Ad-transformed cell lines by MDP2 method

MDP2 nuclear extracts of the following Ad2 or Ad5 E1A-expressing cells were analysed: 5B12 (lane 1), BMK (lane 2), Ad5Xho (lane 3) and F19 (lane 4) along with the following Ad12 E1A-expressing cells: 5A12 (lane 5), C57 (lane 6), RFC-1 (lane 7) and Ad12#1 (lane 8). The kappa probe was used.

Fig 7.5 The steady-state levels of p50 in nuclear extracts of Ad-transformed cells

The levels of p50 in nuclear extracts prepared by the MDP3 method were investigated by Western Blotting of the following Ad2 or Ad5 E1A-expressing cells: 5B12 (lane 1), BMK (lane 2), Ad5Xho (lane 3), F19 (lane 4) along with the following Ad12 E1A-expressing cells: 5A12 (lane 5), C57 (lane 6), RFC-1 (lane 7) and Ad12#1 (lane 8). Rabbit B antiserum was used, taken 10 days after the second boost. The serum was used at 1 in 1000 dilution.



but there was no general difference in the intensity of the polypeptides between cell lines which expressed Ad12 E1A compared to those which expressed Ad2 or Ad5 E1A.

7.4 Levels of Sp1-binding factors in nuclear extracts prepared from Ad-transformed cell lines by the MDP3 method

It was decided to investigate whether the differences found in CRE1/kappa binding factors between cells expressing Ad2 or Ad5 E1A compared to those expressing Ad12 E1A were restricted to factors which bind to these two probes or were part of a more general phenomenon; for this reason it was decided to investigate the levels of Sp1-binding factors in these cell lines. Fig 7.6a shows that the Sp1-binding activity was specific as it was competed out with 100-fold excess of unlabelled competitor. The level of Sp1-binding activity varied slightly between the eight different cell lines, but there was no overall difference between those expressing Ad12 E1A compared to those expressing Ad2 or Ad5 E1A (Fig 7.6b).

7.5 Discussion

This chapter investigated the levels of CRE1/kappa binding factors in eight different cell lines, four in which Ad12 E1A was expressed and four in which either Ad2 or Ad5 E1A was expressed. Two different approaches were used to investigate these levels: gel retardation and Western blotting. The detection of differences between the two groups of transformed cells by gel retardation depended on the method used for nuclear extract preparation; higher levels of CRE1/kappa-binding factors were present in Ad2 or Ad5 E1A expressing cell lines when extracts were prepared by the MDP1 and MDP3 methods but not when the extracts were prepared by the MDP2 method. Hemar et al. (1992) found a quick method of extract preparation, different to MDP2, gave nuclear NF- κ B binding in cells where such binding was absent when extracts were prepared by the Dignam method (Dignam et al., 1983). The quick method used by Hemar et al. (1992) involved the use of detergent which is not the case for MDP2. They suggest that the quick method leads to erroneous results due to the leakage of cytoplasmic contents into nuclear extracts. Although this is more likely to be the case with methods involving the use of detergent, if this also applied to the MDP2 quick method of nuclear extract preparation, the CRE1-binding found in cells expressing Ad12 E1A with this method could be due to cytoplasmic contamination of the nuclear extract preparation.

Fig 7.6 Levels of Sp1-specific binding factors in nuclear extracts prepared from Ad-transformed cell lines by the MDP3 method

MDP3 nuclear extracts of the following Ad2 or Ad5 E1A-expressing cells were analysed: 5B12 (lane 1), BMK (lane 2), Ad5Xho (lane 3) and F19 (lane 4) along with the following Ad12 E1A-expressing cells: 5A12 (lane 5), C57 (lane 6), RFC-1 (lane 7) and Ad12#1 (lane 8). The Sp1 probe was used.

The binding pattern with MDP1 is likely to reflect the levels of proteolytic breakdown products. Since this method should provide the same level of separation of cytoplasmic and nuclear contents as MDP3, one would expect the difference in CRE1-binding between the cells expressing Ad12 E1A compared to those expressing Ad2 or Ad5 to be present as well with MDP3, as indeed was found to be the case. This study found both the major binding activities detected by this method, complexes 2 and 3, to be more intense for cells expressing Ad2 or Ad5 E1A compared to cells expressing Ad12 E1A. It is likely that complexes 2 and 3 represent NF- κ B and H2TF1/KBF1 respectively. Meijer et al. (1992) also found higher levels of these factors in Ad5- compared to Ad12-transformed cells. However, since they used whole cell extracts, their results do not provide information on the nuclear levels of these factors. Ge et al. (1992) did not find any significant difference between Ad5- and Ad12-transformed cells in levels of CRE1-binding; the lack of agreement with the results of this study could be due to the different method used to prepare nuclear extracts. Nielsch et al. (1991) found only NF- κ B to be present at higher levels in Ad5- compared to Ad12-transformed cells. The cell lines used by Nielsch et al. (1991) were established cell lines before adenovirus transformation, whereas the cell lines used in this study were derived from primary cells transformed by adenovirus; this could account for the differences in the results obtained.

Sp1-binding levels varied slightly between cell lines but there was no overall difference between the cell lines expressing Ad12 compared to Ad2 or Ad5 E1A. Ge et al. (1992) also did not obtain any difference between Ad5- and Ad12-transformed cells in the level of Sp1-binding factors. This demonstrates that the differences found in the levels of CRE1-binding factors between these groups is not just part of a general difference in the level of transcription factors between these two groups.

The Western blotting results did not appear, at first sight, to agree with the results obtained on gel retardations. However, the two systems are capable of investigating different questions. Western blotting was used to detect the levels of p50 in nuclear extracts in which the proteins were denatured and probably broken down into their subunit constituents, therefore this system could not be used to obtain information as to whether the p50 present in the nuclear extract was bound to another p50 or a p65 for example, whereas on gel retardations p50 homodimers and p50/p65 heterodimers have been shown to run with different mobilities as non-denaturing conditions are used

(Cohen and Hiscott, 1992; Baeuerle, 1992). It is reasonable to assume that complexes 2 and 3 are likely to represent NF- κ B (p50/p65 heterodimer) and H2TF1/KBF1 (p50 dimer) respectively. Combining the results of gel retardations with those of Western blotting suggests that the levels of p50 do not differ between the cell lines expressing Ad12 E1A compared to those expressing Ad2 or Ad5 E1A but the conformation of some of the p50 must be different between these groups since it is capable of binding DNA either as a homodimer or a heterodimer with p65 more readily in cell lines expressing Ad2 or Ad5 E1A. Alternatively, the p50 present in Ad12 E1A-expressing cells could be bound to another protein; for example, c-rel and this heterodimer was not detectable on gel retardations under the conditions used.

Chapter 8

Induction of CRE1-binding proteins by $\text{TNF}\alpha$, cycloheximide,
puromycin and benzoyl peroxide in Ad-transformed cells

8.1 Introduction

An understanding of the mechanism of induction of NF- κ B would be of great importance, particularly because the long terminal repeat (LTR) of HIV-1 contains two NF- κ B binding sites and NF- κ B has been implicated in the activation of transcription of HIV genes (Osborn et al., 1989). In this chapter several aspects of NF- κ B induction in Ad-transformed cells were investigated. Induction by TNF α and cycloheximide in different adenovirus cell lines was studied. It was hoped that this information might be relevant to the different properties of cell lines expressing Ad12 E1A compared to those expressing Ad2 or Ad5 E1A. Schreck et al. (1991) proposed that NF- κ B induction involves ROI. The results with puromycin and benzoyl peroxide were hoped to provide further information about the process of induction in general, and in the case of benzoyl peroxide help provide further evidence for the role of ROI. Induction by benzoyl peroxide was investigated in Ad-transformed cell lines as it was hoped this information might be relevant to the different properties of these cell lines.

8.2 The induction of CRE1-binding factor(s) by TNF α and/or cycloheximide

When cells transformed by Ad2 or Ad5 E1A are introduced into syngeneic newborn rodents they are not oncogenic, whereas cells transformed by Ad12 E1A are highly oncogenic. It is known that members of the former group have higher levels of MHC class I antigens than members of the latter group. It could be argued that a more relevant parameter of oncogenicity is the level of surface MHC class I when these cells are introduced into living animals. In such an environment the cells would be under the influence of many cytokines including TNF α which is known to up-regulate the levels of MHC class I and cause the nuclear translocation of NF- κ B (Duh et al., 1989; Osborn et al., 1989). It could be envisaged that members of these two groups respond differently to TNF α in terms of the type of response, degree of response and the kinetics of the response. For this reason the response to TNF α in cells expressing Ad12 E1A compared to Ad2 or Ad5 E1A was investigated over an eight hour time-course.

The nature of the response to TNF α was investigated by use of the protein synthesis inhibitor cycloheximide (Todorov, 1990); if the response to TNF α required protein synthesis it should be inhibited by pretreatment with cycloheximide. The effect of cycloheximide alone was also investigated in these cells as it is another agent known

to induce NF- κ B; as will be described later, free radicals may be involved in its method of activation.

The induction of complex 2 by TNF α occurred in all the cell lines tested except for F19 and 5A12. The induction of complex 2 by cycloheximide was evident in all the cell lines. The four cell lines expressing Ad2 or Ad5 E1A will be discussed first.

In 5B12, TNF α induction appeared by 15 minutes (lane 3) and seemed strongest for the first hour and gradually decreased towards 8 hours (Fig 8.1). Cycloheximide and TNF α appeared to act synergistically, the induction being greater with both agents than with either one alone (compare lane 15 to lanes 13 and 17).

Complex 3, although present before treatment with TNF α or cycloheximide, was stronger after treatment, appearing most intense at 1 hour of TNF α treatment (lane 7) and after 8 hours of TNF α treatment in association with 9 hours of cycloheximide treatment (lane 15).

In BMK cells complex 2 was also induced after only 15 minutes of TNF α treatment (Fig 6.9). Its induction pattern was different from that of 5B12. Induction appeared to increase up to 6 hours and was maintained until 8 hours. The induction of complex 2 by cycloheximide alone was greater than the induction by TNF α alone. Complex 3 did not appear to be induced by cycloheximide or TNF α , in fact there was the disappearance of complex 3 when cells were treated with both TNF α and cycloheximide.

In Ad5Xho cells complex 2 was again shown to be induced by just 15 minutes treatment with TNF α (lane 3, Fig 8.2). The level of induction increased in the first hour of treatment, was reduced at 2 hours (lane 9) and peaked again at 6 hours (lane 11). Relatively strong induction of complex 2 was achieved by cycloheximide (lane 17), while induction with TNF α and cycloheximide (lane 15) did not appear to be greater than that of cycloheximide alone. Complex 3 appeared to show a similar pattern of induction to complex 2.

In F19 cells complex 2 was not induced by TNF α (Fig 8.3). Although TNF α did not induce complex 2, complex 3 was intensified by 15 minutes treatment (lane 3).

Fig 8.1 The effect of TNF α and/or cycloheximide on the levels of CRE1-binding factors in MBP2 nuclear extracts of 5B12 cells

Lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18 all had a 100-fold excess of unlabelled CRE1 in the binding reaction. The nuclear extracts used in lanes 1 and 2 were prepared from untreated cells. Lanes 3 to 16 used nuclear extracts from cells treated with TNF α (100 U/ml) for 15 minutes (lanes 3 and 4), 30 minutes (lanes 5 and 6), 1 hour (lanes 7 and 8), 2 hours (lanes 9 and 10), 6 hours (lanes 11 and 12) and 8 hours (lanes 13 to 16). The nuclear extracts used in lanes 15 and 16 were from cells treated with cycloheximide (30 μ g/ml) for 9 hours as well as TNF α . The nuclear extracts in lanes 17 and 18 were prepared from cells treated for 9 hours with cycloheximide (30 μ g/ml) alone.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

2▶

3▶

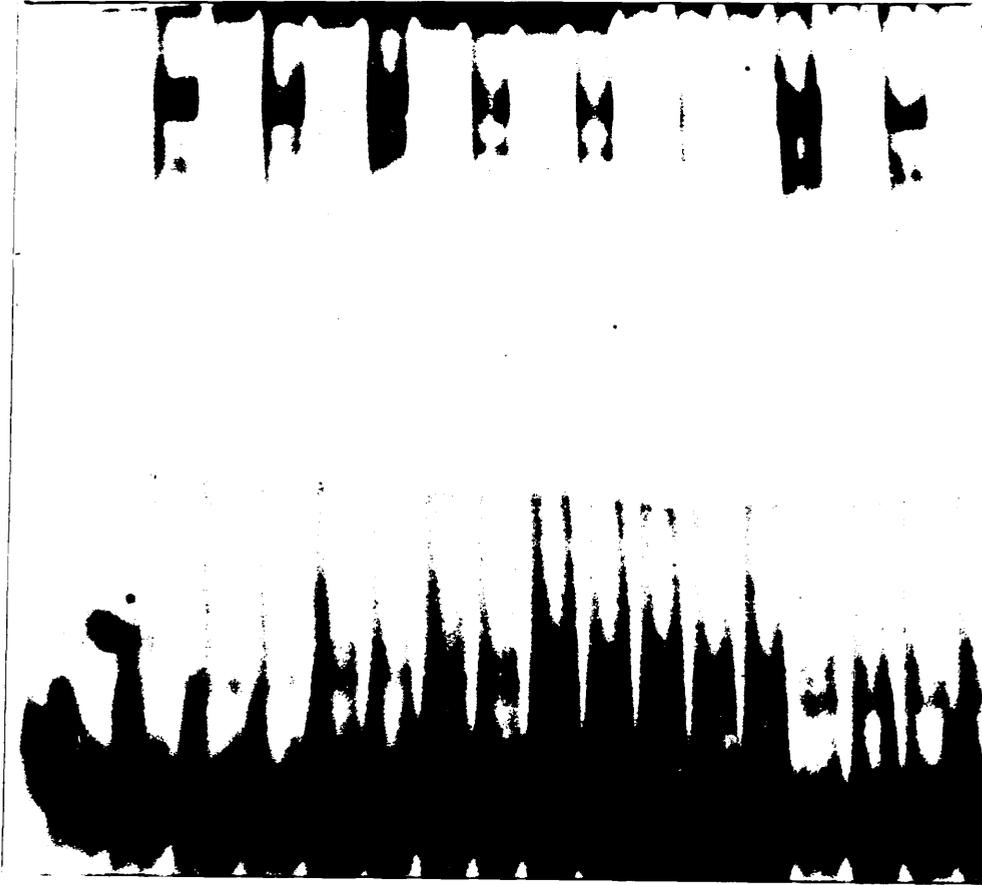


Fig 8.2 The effect of TNF α and/or cycloheximide on the levels of CRE1-binding factors in MDP2 nuclear extracts of Ad5Xho cells

Lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18 all had a 100-fold excess of unlabelled CRE1 in the binding reaction. The nuclear extracts used in lanes 1 and 2 were prepared from untreated cells. Lanes 3 to 16 used nuclear extracts from cells treated with TNF α (100 U/ml) for 15 minutes (lanes 3 and 4), 30 minutes (lanes 5 and 6), 1 hour (lanes 7 and 8), 2 hours (lanes 9 and 10), 6 hours (lanes 11 and 12) and 8 hours (lanes 13 to 16). The nuclear extracts used in lanes 15 and 16 were from cells treated with cycloheximide (30 μ g/ml) for 9 hours as well as TNF α . The nuclear extracts in lanes 17 and 18 were prepared from cells treated for 9 hours with cycloheximide (30 μ g/ml) alone.

Fig 8.3 The effect of TNF α and/or cycloheximide on the levels of CRE1-binding factors in MDP2 nuclear extracts of F19 cells

Lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18 all had a 100-fold excess of unlabelled CRE1 in the binding reaction. The nuclear extracts used in lanes 1 and 2 were prepared from untreated cells. Lanes 3 to 16 used nuclear extracts from cells treated with TNF α (100 U/ml) for 15 minutes (lanes 3 and 4), 30 minutes (lanes 5 and 6), 1 hour (lanes 7 and 8), 2 hours (lanes 9 and 10), 6 hours (lanes 11 and 12) and 8 hours (lanes 13 to 16). The nuclear extracts used in lanes 15 and 16 were from cells treated with cycloheximide (30 μ g/ml) for 9 hours as well as TNF α . The nuclear extracts in lanes 17 and 18 were prepared from cells treated for 9 hours with cycloheximide (30 μ g/ml) alone.

Cycloheximide did induce complex 2 and intensify complex 3 (lane 17). In 5A12 cells complex 2 was not induced by $\text{TNF}\alpha$ (Fig 8.4). However cycloheximide did induce complex 2 (lane 17) and treatment with $\text{TNF}\alpha$ and cycloheximide (lane 15) caused a greater induction of complex 2 than $\text{TNF}\alpha$ alone (lane 13). Complex 3 was intensified by 1 hour of treatment with $\text{TNF}\alpha$ (lane 7).

In C57 cells, complex 2 did not appear to be induced by 15 minutes of treatment with $\text{TNF}\alpha$ (lane 3, Fig 8.5). Complex 2 was induced by 30 minutes of treatment with $\text{TNF}\alpha$ (lane 5) and the level of induction remains approximately constant with up to 8 hours of $\text{TNF}\alpha$ treatment. The induction with cycloheximide and $\text{TNF}\alpha$ (lane 15) appeared to be much greater than that of either agent alone (lanes 13 and 17). Complex 3 did not appear to be induced in this cell line.

In RFC-1 cells complex 2 was induced by 15 minutes of treatment with $\text{TNF}\alpha$ (lane 3). The level of induction decreased after 2 hours of treatment (lane 9) and it was not evident at 8 hours (lane 13, Fig 8.6). The induction produced by cycloheximide alone was barely apparent (lane 17). By far the strongest induction of complex 2 took place by the joint treatment with cycloheximide and $\text{TNF}\alpha$. Complex 3 binding was particularly strong, however, it appeared to be induced by $\text{TNF}\alpha$, particularly after 15 minutes of treatment (lane 3). Cycloheximide also induced complex 3 (lane 17), but the greatest induction was found with the treatment condition of $\text{TNF}\alpha$ (100 U/ml) for eight hours and cycloheximide (30 $\mu\text{g}/\text{ml}$) for nine hours (lane 15). The induction by $\text{TNF}\alpha$ (100 U/ml) for 30 minutes was partially, but not completely, inhibited by treatment of the cells with 0.1 mM PDTC or 30 mM NAC for 1 hour prior to the addition of $\text{TNF}\alpha$ (Fig 8.7). PDTC and NAC are both scavengers of free radicals (Schreck et al., 1991). Fig 8.8 shows that induction of complex 2 by $\text{TNF}\alpha$ (100 U/ml) was still present with 18 hours. Treatment with interferon γ (1000 U/ml) for 12 hours was insufficient to induce complex 2 (lane 3), however, in combination with 18 hours of $\text{TNF}\alpha$ treatment, interferon γ caused greater induction of complex 2 than $\text{TNF}\alpha$ alone (compare lane 7 to lane 9).

In Ad12#1 cells there was no induction of complex 2 by 15 minutes of exposure to $\text{TNF}\alpha$ (lane 3, Fig 8.9). Thirty minutes treatment with $\text{TNF}\alpha$ was sufficient to cause an induction of complex 2. This time period gave the strongest induction by $\text{TNF}\alpha$ alone, thereafter the level of induction remained approximately constant between 1 and

Fig 8.4 The effect of TNF α and/or cycloheximide on the levels of CRE1-binding factors in MDP2 nuclear extracts of 5A12 cells

Lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18 all had a 100-fold excess of unlabelled CRE1 in the binding reaction. The nuclear extracts used in lanes 1 and 2 were prepared from untreated cells. Lanes 3 to 16 used nuclear extracts from cells treated with TNF α (100 U/ml) for 15 minutes (lanes 3 and 4), 30 minutes (lanes 5 and 6), 1 hour (lanes 7 and 8), 2 hours (lanes 9 and 10), 6 hours (lanes 11 and 12) and 8 hours (lanes 13 to 16). The nuclear extracts used in lanes 15 and 16 were from cells treated with cycloheximide (30 μ g/ml) for 9 hours as well as TNF α . The nuclear extracts in lanes 17 and 18 were prepared from cells treated for 9 hours with cycloheximide (30 μ g/ml) alone.

Fig 8.5 The effect of TNF α and/or cycloheximide on the levels of CRE1-binding factors in MDP2 nuclear extracts of C57 cells

Lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18 all had a 100-fold excess of unlabelled CRE1 in the binding reaction. The nuclear extracts used in lanes 1 and 2 were prepared from untreated cells. Lanes 3 to 16 used nuclear extracts from cells treated with TNF α (100 U/ml) for 15 minutes (lanes 3 and 4), 30 minutes (lanes 5 and 6), 1 hour (lanes 7 and 8), 2 hours (lanes 9 and 10), 6 hours (lanes 11 and 12) and 8 hours (lanes 13 to 16). The nuclear extracts used in lanes 15 and 16 were from cells treated with cycloheximide (30 μ g/ml) for 9 hours as well as TNF α . The nuclear extracts in lanes 17 and 18 were prepared from cells treated for 9 hours with cycloheximide (30 μ g/ml) alone.

Fig 8.6 The effect of TNF α and/or cycloheximide on the levels of CRE1-binding factors in MDP2 nuclear extracts of RFC-1 cells

Lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18 all had a 100-fold excess of unlabelled CRE1 in the binding reaction. The nuclear extracts used in lanes 1 and 2 were prepared from untreated cells. Lanes 3 to 16 used nuclear extracts from cells treated with TNF α (100 U/ml) for 15 minutes (lanes 3 and 4), 30 minutes (lanes 5 and 6), 1 hour (lanes 7 and 8), 2 hours (lanes 9 and 10), 6 hours (lanes 11 and 12) and 8 hours (lanes 13 to 16). The nuclear extracts used in lanes 15 and 16 were from cells treated with cycloheximide (30 μ g/ml) for 9 hours as well as TNF α . The nuclear extracts in lanes 17 and 18 were prepared from cells treated for 9 hours with cycloheximide (30 μ g/ml) alone.

Fig 8.7 The partial inhibition of TNF α induction of complex 2 by NAC and PDTC

The CRE1 probe was used with 5 μ g of nuclear MDP2 extracts from RFC-1 cells, the cells were treated as follows: lane 1: untreated cells, lane 2-4 TNF α treated cell for 30 minutes (100 U/ml), lane 3: cells treated with PDTC (0.1 mM) for 1 hour prior to addition of TNF α , lane 4: cells treated with NAC (30 mM) for 1 hour prior to addition of TNF α .

Fig 8.8 The effect of TNF α and TNF α with interferon γ on levels of CRE1-binding factors

RFC-1 MDP2 nuclear extracts were used. Lane 1 and 2: untreated cells, lanes 3 and 4: cells treated for 12 hours with interferon γ (1000 U/ml), lanes 5 and 6: cells treated with interferon γ (1000 U/ml) for 12 hours and cycloheximide (30 μ g/ml) for 13 hours, lanes 7 and 8: TNF α (100 U/ml) for 18 hours, lanes 9 and 10: TNF α (100 U/ml) for 18 hours and interferon γ (1000 U/ml) for 12 hours. Lane 2, 4, 6, 8 and 10 had 100-fold excess of unlabelled CRE1 in the binding reactions.

Fig 8.9 The effect of TNF α and/or cycloheximide on the levels of CRE1-binding factors in MDP2 nuclear extracts of Ad12#1 cells

Lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18 all had a 100-fold excess of unlabelled CRE1 in the binding reactions. The nuclear extracts used in lanes 1 and 2 were prepared from untreated cells. Lanes 3 to 16 used nuclear extracts from cells treated with TNF α (100 U/ml) for 15 minutes (lanes 3 and 4), 30 minutes (lanes 5 and 6), 1 hour (lanes 7 and 8), 2 hours (lanes 9 and 10), 6 hours (lanes 11 and 12) and 8 hours (lanes 13 to 16). The nuclear extracts used in lanes 15 and 16 were from cells treated with cycloheximide (30 μ g/ml) for 9 hours as well as TNF α . The nuclear extracts in lanes 17 and 18 were prepared from cells treated for 9 hours with cycloheximide (30 μ g/ml) alone.

8 hours. The induction found with 8 hours of TNF α and 9 hours of cycloheximide (lane 15) was greater than that achieved by either treatment individually (lanes 13 and 17). There is a slight induction of complex 3 by 30 minutes treatment with TNF α (lane 5) and by a combined TNF α /cycloheximide treatment (lane 15).

8.3 The induction of complexes 2 and 3 by puromycin

Puromycin is a protein synthesis inhibitor which has a unique action; the drug itself reacts with the C-terminus of the growing peptide chain on the ribosome forming a peptidyl-puromycin compound which rapidly dissociates from the ribosome, thus causing premature termination of protein synthesis (Franklin and Snow, 1991). The action of puromycin is equally effectively on 70S and 80S ribosomes. Treatment of C57 cells with puromycin (30 μ g/ml, lane 3) or cycloheximide (30 μ g/ml, lane 5) caused the induction of complex 2, however, puromycin caused a greater induction (Fig 8.10). Cycloheximide did not induce complex 3 in this cell line, whereas puromycin did appear to induce complex 3.

8.4 The induction of complex 2 by benzoyl peroxide

Schreck et al.(1991) have shown that NF- κ B can be induced by H₂O₂. It was proposed that this phenomenon was due to the production of ROI by H₂O₂, since scavengers of ROI prevented the induction of NF- κ B by H₂O₂ and by several other agents. It was decided to investigate whether a chemical, other than H₂O₂, which produces free radicals could also induce NF- κ B.

Benzoyl peroxide is a well known chemical used as a topical treatment for acne and to treat cutaneous ulcers. It is used in several industrial processes because it is a source of free radicals (Hogan, 1991) This chemical is known to act as a tumour promoter in mice (Iverson, 1986).

Benzoyl peroxide is only slightly soluble in water but readily soluble in DMF, DMSO and acetone. Fig 8.11 shows the effect of benzoyl peroxide when dissolved in DMF; 10 μ M (lane 3) but not 1 μ M (lane 2) benzoyl peroxide caused the induction of complex 2 in 5B12 cells. This effect was not due to DMF alone (lanes 4 and 5). 10 μ M benzoyl peroxide in DMF did not induce complex 2 when cells were pretreated for 1 hour with NAC (30 mM) (lane 7).

Fig 8.10 The effect of cycloheximide or puromycin on CRE1-binding factors in C57 cells

The CRE1 probe was used with C57 MBP2 nuclear extracts. Lanes 1 and 2: untreated cells, lane 3 and 4: cycloheximide treated cells (30 $\mu\text{g/ml}$ for 1 hour), lanes 5 and 6 puromycin treated cells (30 $\mu\text{g/ml}$ for 1 hour). Lanes 2, 4 and 6 contained 100-fold excess of unlabelled CRE1 in the binding reactions.

Fig 8.11 The effect of benzoyl peroxide dissolved in DMF on CRE1-binding factors in 5B12 cells

The CRE1 probe was used with 5B12 MBP2 nuclear extracts. Lane 1: untreated cells, lane 2 and 6 cells treated with 1 μM benzoyl peroxide dissolved in DMF. The benzoyl peroxide was added to each plate in a volume of 7 μl (0.1% final concentration). Lanes 3 and 7: cells treated with 10 μM benzoyl peroxide dissolved in DMF. The benzoyl peroxide was added to each plate in a volume of 70 μl (1% final concentration). Lanes 4 and 5: cells treated with 7 μl and 70 μl of DMF respectively (equivalent to 0.1 and 1% final concentration). All treatments with DMF or benzoyl peroxide were for 1 hour. Lanes 6 and 7 were also treated with NAC (30 mM) for 2 hours.

Fig 8.12 shows the effect of benzoyl peroxide on levels of nuclear complex 2 and complex 3 in 5B12 cells when DMSO or acetone is used as the solvent. Both these solvents appeared to cause a slight increase in the level of complex 2 binding (compare lane 1 with lanes 2 and 9). When benzoyl peroxide was dissolved in acetone, low dosages, 1.25 μM and 2.5 μM (lanes 3 and 4), appeared to reduce complex 2 binding levels from those obtained by acetone alone to the levels found in untreated cells. The levels of complex 2 binding increased consistently with increased dosage from 2.5 μM (lane 4) to 20 μM (lane 7). At 10 μM and 20 μM of benzoyl peroxide dissolved in acetone, the intensity of complex 2 was clearly greater than that found with the treatment of acetone alone (lane 2 compared to lanes 6 and 7). When cells were pretreated with 0.1 mM PDTTC 1 hour before treatment with 20 μM benzoyl peroxide in acetone, both complex 2 and complex 3 binding disappeared (lane 8). When benzoyl peroxide was dissolved in DMSO, 1.25 μM (lane 10) appeared to slightly reduce the binding as compared to DMSO alone (lane 9). Levels of complex 2 binding increased with increasing amounts of benzoyl peroxide from 1.25 μM (lane 10) to 5 μM (lane 12), thereafter for 10 μM (lane 13) and 20 μM (lane 14) levels where reduced to that of untreated cells (lane 1). When cells were pretreated with 0.1 mM PDTTC 1 hour before treatment with 20 μM benzoyl peroxide in DMSO (lane 15) complex 2 binding was slightly reduced as compared to the 20 μM benzoyl peroxide in DMSO treatment (lane 14). Complex 3 did not produce as intense a complex as complex 2, despite this, some induction of complex 3 is demonstrable with treatments of 10 μM and 20 μM benzoyl peroxide in acetone (lanes 6 and 7) and 5 μM benzoyl peroxide in DMSO (lane 13)

Fig 8.13 compares the effect of benzoyl peroxide dissolved in acetone with acetone treatment alone on CRE1-binding levels in MDP2 nuclear extracts from cells expressing Ad12 E1A compared to cells expressing Ad2 or Ad5 E1A. Each cell line was treated with either 7 μl (0.1%) of acetone or an equivalent volume of benzoyl peroxide dissolved in acetone which gave a final media concentration of 20 μM . Binding levels were in general quite low in the presence of acetone, with BMK having the highest levels of complex 2 and complex 3 binding. In the presence of benzoyl peroxide dissolved in acetone, complex 2 was induced in 5B12, BMK and F19, most strongly in BMK (lanes 9, 10 and 12). Complex 3 was induced to a lesser extent in BMK and slightly in 5B12 (lanes 9 and 10).

Fig 8.12 The dose-dependence of induction of complex 2 by benzoyl peroxide dissolved in DMSO or acetone

The CRE1 probe was used with 5B12 MDP2 nuclear extracts: lane 1: untreated cells, lane 2: cells treated with 14 μ l (0.2% final concentration) of acetone, lanes 3-8: cells treated with benzoyl peroxide dissolved in 14 μ l of acetone (0.2% final concentration): lane 3: 1.25 μ M, lane 4: 2.5 μ M, lane 5: 5 μ M, lane 6: 10 μ M, lane 7 and 8: 20 μ M. Lane 9 cells treated with 14 μ l of DMSO. Lane 10-15: cells treated with benzoyl peroxide dissolved in 14 μ l of DMSO (0.2% final concentration), lane 10: 1.25 μ M, lane 11: 2.5 μ M, lane 12: 5 μ M, lane 13: 10 μ M, lane 14 and 15: 20 μ M. All treatments with benzoyl peroxide, DMSO or acetone were for 1 hour. Lanes 8 and 15 were also treated with 0.1 mM PDTC.

Fig 8.13 The effect of benzoyl peroxide on CRE1-binding factors in Ad-transformed cells

The CRE1 probe was used to analyse MDP2 extracts from the following Ad2 or Ad5 E1A-expressing cell lines: 5B12 (lanes 1 and 9), BMK (lanes 2 and 10), Ad5Xho (lanes 3 and 11) and F19 (lanes 4 and 12) along with the following Ad12 E1A-expressing cell lines: 5A12 (lanes 5 and 13), C57 (lanes 6 and 14), RFC-1 (lanes 7 and 15) and Ad12#1 (lanes 8 and 16). The nuclear extracts in lanes 1-8 were from cells treated with 7 μ l of acetone (0.1% final concentration) for 1 hour, lanes 9-15 were from cells treated for 1 hour with 20 μ M benzoyl peroxide dissolved in acetone. The benzoyl peroxide was added to each plate in a volume of 7 μ l (0.1% final concentration).

8.5 Discussion

In this chapter the induction of CRE1-binding proteins in Ad-transformed cells was investigated. The agents used were $\text{TNF}\alpha$, cycloheximide, puromycin and benzoyl peroxide. It was hypothesized that cell lines which expressed Ad12 E1A could be less sensitive than those expressing Ad2 or Ad5 E1A to $\text{NF-}\kappa\text{B}$ induction by $\text{TNF}\alpha$, an enhancer of MHC class I transcription, and this could contribute to the lower levels of MHC class I in the former. However, no major difference was found between cell lines expressing Ad12 E1A compared to those expressing Ad2 or Ad5 E1A. Two cell lines 5A12 and F19, expressing Ad12 and Ad2 E1A respectively, failed to respond to $\text{TNF}\alpha$; the reason for this is not known. The 55-60 kDa (TR55) receptor is required for $\text{TNF}\alpha$ induction of $\text{NF-}\kappa\text{B}$ (Kruppa et al., 1992), therefore if this receptor is not present in 5A12 and F19 cell lines, this could explain their failure to respond to $\text{TNF}\alpha$.

Only RFC-1 out of three Ad12 E1A-expressing cells which responded to $\text{TNF}\alpha$ by the induction of complex 2, produced this response after 15 minutes of treatment with $\text{TNF}\alpha$, the other cell lines required 30 minutes of treatment. Whereas all the three cell lines which expressed Ad2 or Ad5 E1A and for which $\text{TNF}\alpha$ treatment caused an induction of complex 2, the induced complex was present after only 15 minutes treatment with $\text{TNF}\alpha$. Overall the induction of complex 2 by $\text{TNF}\alpha$ and the time course does not seem to be related by the serotype of E1A expressed by these cell lines.

All the cell lines showed an induction of complex 2 by cycloheximide, the induction was usually greatest by a combination of treatments with cycloheximide and $\text{TNF}\alpha$. This, as well as the fact that cycloheximide induction was possible when $\text{TNF}\alpha$ induction was absent, strongly suggests that at least the initial part of the induction pathways by these two agents is different. Complex 3 induction was shown to occur in certain cell lines by either cycloheximide, $\text{TNF}\alpha$ or both agents. Other workers who obtain two complexes on gel retardations when using $\text{NF-}\kappa\text{B}$ -like motifs as probes, have not usually shown induction of the lower complex by various agents; this could be a property specific to Ad-transformed cells.

Treatment of C57 cells with puromycin caused an induction of complex 2, since cycloheximide has already been shown to induce complex 2, this result suggests that protein synthesis inhibitors in general might be involved in the induction of this complex.

Benzoyl peroxide, an agent which induces the production of free radicals, was shown to induce complex 2 in certain cell lines. The effect was not due to the solvent used to dissolve this chemical; in the case of DMF, no induction was caused. Acetone and DMSO both produced a slight induction of NF- κ B, but the induction by benzoyl peroxide dissolved in these agents was, at the optimal dosage, greater than that produced by the solvent alone. NAC and PDTC both prevented complex 2 induction. These agents are scavengers of free radicals and so this result suggests that free radicals are involved in the induction of complex 2 by benzoyl peroxide. Optimal induction was obtained by different dosages in DMSO and acetone, in DMSO a lower dosage was found to be optimal. This could be because the benzoyl peroxide enters the cell more efficiently when dissolved in DMSO. Alternatively, acetone could scavenge the free radicals produced by benzoyl peroxide to a greater extent than DMSO, therefore more benzoyl peroxide might be required when dissolved in acetone to give an equivalent number of free radicals available to react with molecules present in the cell.

The level of induction achieved increased with increasing dosage over the whole dose range when acetone was used as the solvent and up to 5 μ M with DMSO as the solvent. This suggests that the effect was a specific effect and not due to over-stimulation of a particular receptor to the point where responses were no longer specific. Induction of NF- κ B by benzoyl peroxide appeared to occur more readily in cells expressing Ad2 or Ad5 E1A. Since TNF induction did not appear to vary between these cell lines, this suggests that part of the induction pathway specific for benzoyl peroxide might be working at lower efficiency in Ad12-E1A expressing cells compared to those expressing Ad2 or Ad5 E1A.

General Discussion

This study focussed on the oncogenic properties of cells transformed by certain serotypes of Adenovirus; Ad12-transformed cells but not Ad2 or Ad5 transformed cells are oncogenic when introduced in to syngeneic rodents (Schrier et al., 1983). It has been proposed that the oncogenic properties of Ad12 transformed cells are due to such cells producing lower levels of the cell surface proteins, MHC class I antigens (Schrier et al., 1983). These proteins are involved in displaying viral and other foreign antigens to cytotoxic T-cells; therefore a reduction in the level of surface MHC class I antigens would be one way to evade immune detection. This reduction in MHC class I has been shown in Ad12-transformed cells to involve down-regulation of transcription (Ackrill and Blair, 1988a; Friedman and Riccardi, 1988; Lassam and Jay, 1989); how this is brought about was a central interest of this study. A region termed the CRE, around 200 bp upstream from the transcription initiation site in MHC class I genes, has been shown to play an important role in the control of transcription (Kimura et al., 1986). This region can be divided into two separate regions called CRE1 and CRE2.

The functional role of these sequences in Ad-transformed cells was investigated by the use of the CAT transient expression assay. The levels of factors which bind to the CRE1 region was investigated by gel retardation and by Western blotting using an antibody which was prepared to the C-terminal peptide of the p50 subunit of NF- κ B, a CRE1-specific binding factor. The binding site for CRE2-specific transcription factors was investigated by the use of DNase I footprinting. NF- κ B plays a central role in controlling the transcription of several important genes in the immune system, it also is involved in the control of transcription from the LTR of HIV. The final section of this study investigated the mechanism of NF- κ B induction in Ad-transformed cells.

The lower level of MHC class I transcription in Ad12- compared to Ad2- or Ad5-transformed cells could be due to an enhancer function being down-regulated or a repressor function being up-regulated in Ad12-transformed cells. In this study cell lines expressing Ad12 E1A were compared with those expressing Ad2 or Ad5 E1A. The sequences CRE1, mCRE1 and CRE2 were each cloned into the vector pBLCAT2. CRE2 has been proposed to act as a repressor (Ackrill and Blair, 1989; Kralli et al., 1992) and CRE1 has been demonstrated to act as an enhancer in certain circumstances (Israel et al, 1989; Mauxion and Sen, 1989); neither sequence was shown to play a role in the control of transcription in this study. It is possible that multiple copies of these control sequences are required to demonstrate their function. How sequence-specific

DNA-binding factors influence transcription is unknown. It has been proposed (Mitchell and Tjian, 1989) that they may function by interacting with proteins of the transcription initiation complex by the looping out of the intervening DNA. The presence of several copies of a DNA-binding motif would enable several transcription factors to interact with the transcription initiation complex, thus stabilizing this interaction. From this one would predict, for example, that the enhancer properties of a sequence would increase as the number of copies of that sequence increased; this has been shown to be the case for CRE1 (Israel et al., 1989) and CRE1/CRE2 (referred to as R1 and R2) (Kralli et al., 1992). However, absence of multiple copies in this study does not appear to be the only reason why CRE2 function was not demonstrated, Kralli et al. (1992) found evidence to suggest the role of CRE2, as a repressor of the enhancer function of CRE1, could only be demonstrated when both sequences were present. The presence of CRE2-specific binding proteins could hinder the interaction of CRE1-specific binding proteins with the transcription initiation complex. In the case of CRE1, Meijer et al. (1992) found that enhancer function can be shown using a single copy of this sequence in cells transformed by Ad12 12S E1A but not by Ad12 13S E1A. The cells used in this study expressed both the 12S and the 13S products; the different cell lines used makes comparison of results found in this study with those of Meijer et al. (1992) difficult.

If CRE1-binding factors enhance transcription and are instrumental in the different levels of MHC class I found in cells expressing Ad12 compared to Ad2 or Ad5 E1A, then the lower levels of transcription in the former could be due to reduced levels of these factors. This was investigated by gel retardation analysis and by Western blotting using an antibody generated to the C-terminal peptide of the p50 subunit of NF- κ B. The specificity and the titre of the antibody was investigated by ELISA. High titres were obtained for both the rabbits used. Specificity of the antibody was suggested by the fact that the immune serum only bound to wells coated with p50 peptide and not an unrelated peptide. Also the immune serum did not bind to the wells coated with the p50 peptide following pre-incubation with this peptide. Western blotting demonstrated that a protein of approximately 55 kDa reacted with serum from both rabbits as well as with the three antisera provided by Dr Hay to the C-, N- and DNA-binding domain of NF- κ B, providing further evidence that the antiserum generated was indeed recognizing NF- κ B. This protein was not found to be present at different levels in cells expressing Ad12 E1A compared to those expressing Ad2 or Ad5 E1A.

The technique used to prepare nuclear extracts was found to be critical to the results obtained by gel retardation using CRE1 and kappa probes. The initial method employed, MDP1, did not appear to be sufficient to protect an inducible CRE1-binding factor (thus likely to represent NF- κ B) from proteolysis. The second method employed, MDP2, could be criticised on the grounds that it involved a greater potential risk of cytoplasmic contamination of nuclear extracts. It was decided that the method most likely to give a true reflection of the nuclear levels of CRE1-binding factors was MDP3; with this method complexes 2 and 3 were found to be present at higher levels in Ad2- or Ad5- as opposed to Ad12-transformed cells. Results with competition experiments, formaldehyde crosslinking and induction experiments suggested that complexes 2 and 3 were likely to represent NF- κ B (p50/p65 heterodimers) and KBF1/H2TF1 (p50 homodimers respectively). These results taken together with the results from Western blotting suggest that cells expressing Ad12 E1A do not differ from those expressing Ad2 or Ad5 E1A in terms of the levels of p50 but in the ability of that p50 to bind DNA either as a heterodimer or as a homodimer. Nielsch et al. (1991) found NF- κ B to be higher in Ad5-transformed cells compared to Ad12-transformed cells with no difference in the levels of H2TF1/KBF1. These results were obtained with established cell lines transformed by adenovirus, unlike the cells used in this study which were derived from primary cells transformed by adenovirus. It is possible that the different cells used accounts for the variation in the results. Meijer et al. (1992) did find differences in the levels of these factors between Ad5- and Ad12-transformed cells. However, these results do not give information on the nuclear level of these factors as whole cell extracts were used. Ge et al. (1992) did not find any differences in the levels of CRE1-binding factors in Ad5- and Ad12-transformed cells. They prepared their nuclear extracts by a method described by Shapiro et al. (1988); this could account for their results differing from those obtained in this study. The levels of Sp1-binding factors were not found to vary between cells expressing Ad12 E1A compared to those expressing Ad2 or Ad5 E1A; this suggests that the differences found on gel retardations for CRE1-binding factors are not just part of a general difference in the levels of transcription factors found between Ad12 E1A-expressing cells and those expressing Ad2 or Ad5 E1A.

mCRE1 was shown in this study to be associated with reduced CAT activity, this confirms the results of Meijer et al. (1992). They found that the mutated sequence did

not bind transcription factors on gel retardations, so the effect is unlikely to be due to the binding of a repressor protein and the reason for this effect is not known.

The functional and the molecular characteristics of CRE2-specific binding factors were investigated. The former were studied by the use of the transient expression assay CAT and the latter through investigation of the effect of sodium fluoride on the binding of CRE2-specific binding factors and DNase I footprinting.

The CRE2 DNase I footprint showed a larger area of protection, suggesting a larger binding site for transcription factors than that obtained using methylation interference (Griffiths, 1992; Shirayoshi et al., 1987). This is a consequence of the large size of the DNase I molecule causing overestimation of the size of the binding site. The DNase I footprint covered the sequence AGGTCA, the conserved sequence present in the binding sites for RXR β /H-2RIIBP and other members of the ER/TR subfamily of steroid/thyroid hormone receptors. Since this sequence is conserved amongst different members of this subfamily, it suggests that it is likely to serve an important function; it is therefore not unexpected that this region should be part of the binding site for transcription factors.

Sodium fluoride has been shown to be an activator of G proteins in association with aluminium (Chabre, 1990) or magnesium (Antonny et al., 1993; Bigay et al., 1987). One such G protein is coupled to phospholipase C and it is involved in its breakdown to products that ultimately lead to the activation of PKC. Sodium fluoride is also a phosphatase inhibitor (Chabre, 1990). Resulting from these properties sodium fluoride treatment can lead to higher levels of phosphorylation of certain proteins. CRE2-binding activity was greater when extracts were prepared in the presence of sodium fluoride; this suggests that the phosphorylation of certain proteins potentiates binding. The work of Griffiths (1992) suggests the proteins which are being phosphorylated are nuclear in origin; it was found that phosphatase treatment of nuclear extracts caused the disappearance of binding which was restored by the use of protein kinase A.

Cell lines expressing Ad12 E1A were not found to differ from those expressing Ad2 or Ad5 E1A in terms of the induction of complex 2 by TNF α or cycloheximide. Two cell lines were found not to be inducible by TNF α , one expressing Ad2 and one expressing Ad12; it is possible that these cell lines did not express the receptor TR55

which has been shown to be required for the induction of NF- κ B by TNF α (Kruppa et al., 1992).

Schreck et al. (1991) were the first to propose the involvement of ROI in the induction of NF- κ B by diverse agents. They found H₂O₂ induced NF- κ B and the induction by several agents including H₂O₂ could be inhibited by reducing agents, metal ion chelators and free-radical scavengers. In this study benzoyl peroxide was shown by gel retardation to induce a complex likely to represent NF- κ B and free-radical scavengers were found to inhibit that induction.

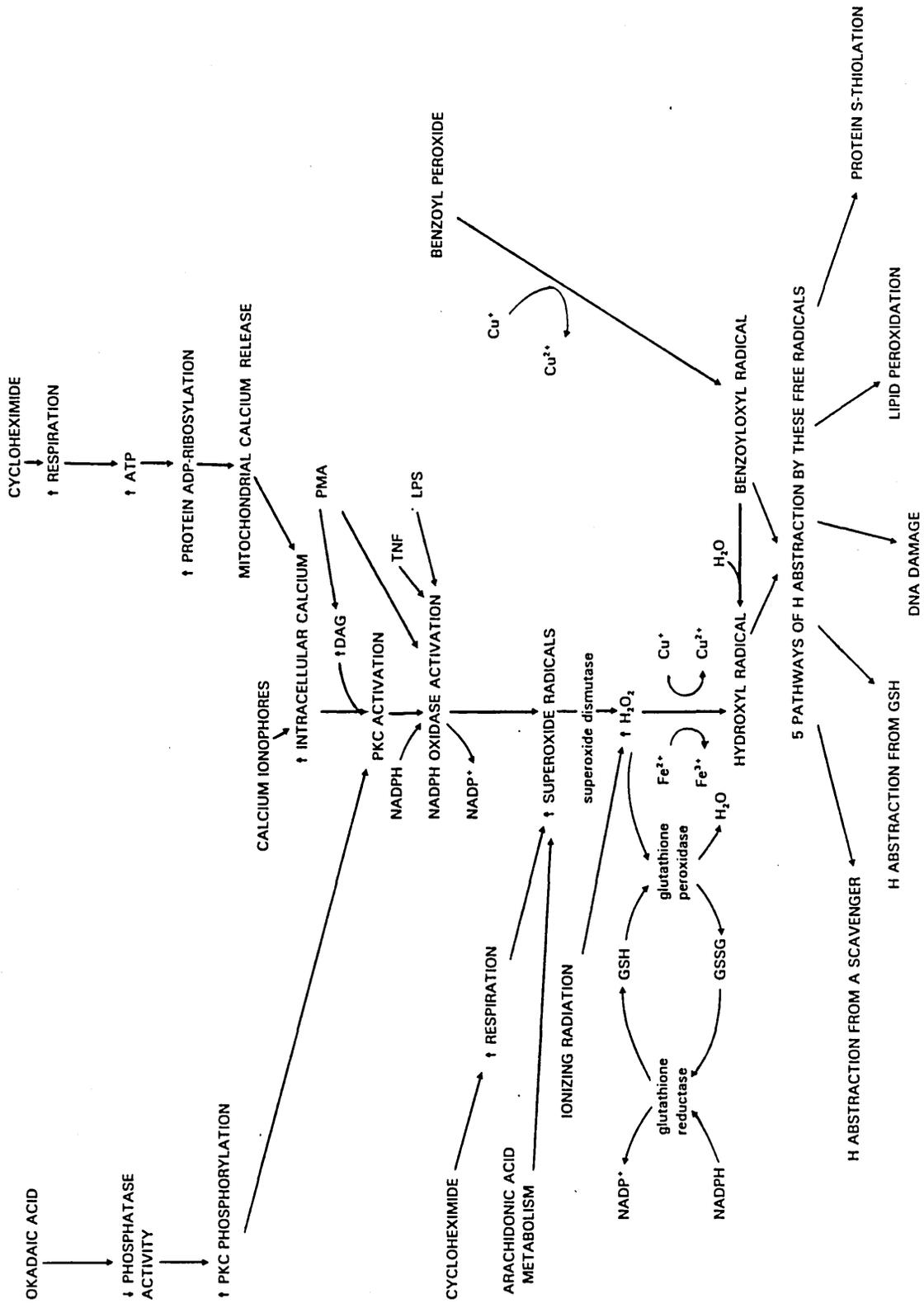
Fig 9.1 depicts a proposed model showing the presumed involvement of free-radicals in the activation of NF- κ B. The model is based on the work of Schreck et al. (1991, 1992) and information on NADPH oxidase and oxidative stress, incorporating the results presented in chapter 8 with benzoyl peroxide. Schreck et al. (1991) proposed that the various agents which induce NF- κ B all cause an increase in ROI. They suggest NADPH oxidase could be involved. NADPH oxidase converts O₂ to the superoxide ion O₂⁻ according to the reaction:



Many of the agents which induce NF- κ B activate NADPH oxidase; for example, TNF α (Tsujiimoto et al., 1986), PMA (Rider et al., 1988; Reibman et al., 1988), LPS (Dahinden et al., 1983) and calcium ionophores (Koenderman et al., 1989). The mechanism by which LPS and TNF α activate NADPH oxidase is incompletely understood. PMA causes an increase in diacylglycerol (DAG) (Rider et al., 1988), which, in association with increased intracellular calcium, activates protein kinase C (PKC) which is thought to activate NADPH oxidase (Seifert and Schultz, 1991). PMA might also activate NADPH oxidase directly (Seifert and Schultz, 1991). Calcium ionophores are thought to activate NADPH oxidase through causing an increase in intracellular calcium which together with DAG activates PKC and thus, indirectly, NADPH oxidase (Smolen, 1984). Cycloheximide, the protein synthesis inhibitor, has an initial effect on cells by causing an increase in respiration (Todorov, 1990). This causes ATP production which increases the level of protein ADP ribosylation which in turn causes a release of mitochondrial calcium and an increase in intracellular calcium (Reed, 1990) and so leads to the same effects as a calcium ionophore. Okadaic acid

Fig 9.1 A model depicting the involvement of free radicals and hydrogen abstraction in NF- κ B induction

This figure shows a model portraying how the different pathways for the induction of NF- κ B by various agents could all involve the generation of free-radicals and hydrogen abstraction.



activates NF- κ B (Thevenin et al., 1990). It is proposed that this activation is caused by its inhibition of certain phosphatases which could enable PKC phosphorylation and thus its activation (Cohen, 1989). Okadaic acid could therefore indirectly activate NADPH oxidase.

NADPH oxidase causes an increase in the level of superoxide radicals. Superoxide radicals are also generated in respiration and by arachidonic acid metabolism (Rice-Evans et al., 1991). Cycloheximide, by increasing respiration, causes increased levels of superoxide radicals by both this NADPH oxidase-independent pathway, as well as the previously described pathway involving NADPH oxidase. The enzyme superoxide dismutase converts superoxide radicals to H_2O_2 .

Ionizing radiation, another inducer of NF- κ B (Brach et al., 1991), is also a producer of H_2O_2 (Rice-Evans et al., 1991). H_2O_2 is detoxified in the cells mainly by the glutathione peroxidase system to water. In peroxisomes catalase plays a minor role and converts H_2O_2 to water and oxygen (Jaescke and Mitchell, 1990).

Whilst H_2O_2 is reduced to water by glutathione peroxidase, reduced glutathione (GSH) is oxidised to its disulphide, GSSG (Reed, 1990). Most of the GSSG formed is immediately reduced back to GSH through glutathione reductase with the cofactor NADPH. When the system is overstretched glutathione reductase cannot convert GSSG back to GSH quickly enough and there is a drop in the GSH/GSSG ratio. NADPH is used by glutathione reductase to reduce oxidised glutathione. Tribble and Jones (1990) suggest the supply of NADPH is the limiting factor for this reaction. NADPH oxidase activity will reduce available NADPH and so reduce the rate of reduction of oxidized glutathione. Several inducers of NF- κ B cause this drop in the GSH/GSSG ratio; for example, such a drop has been shown to be caused by a calcium ionophore (Olafsdottir et al., 1988), H_2O_2 (Perchellet et al., 1986), $TNF\alpha$ and PMA (Staal et al., 1990). It is proposed here that this drop in GSH/GSSG plays an essential role in NF- κ B induction since oxidants such as diamide which reduce this ratio enhance NF- κ B activity (Staal et al., 1990) and reducing agents such as 2-ME which raise the GSH/GSSG ratio inhibit NF- κ B induction (Schreck et al., 1992). The reduction in the GSH/GSSG ratio is considered important in NF- κ B induction, since as a consequence not enough GSH is present to deal with all the remaining H_2O_2 . This allows the opportunity for some H_2O_2 to be converted to hydroxyl radicals by reacting with either

Fe^{2+} or Cu^+ forming Fe^{3+} and Cu^{2+} respectively (Rice-Evans et al., 1991). Schreck et al. (1992) showed evidence that these steps are involved in NF- κ B induction; they found desferal, the iron-chelator and O-phenanthroline, the copper chelator, both reduced NF- κ B induction by H_2O_2 . Neither produced complete inhibition, presumably because hydroxyl radicals could still be created by the other pathway. It is proposed that at least five potential pathways exist for the hydroxyl radical from this point all involving hydrogen atom abstraction. Since a particular radical of benzoyl peroxide is also capable of causing hydrogen abstraction and creating hydroxyl radicals, the generation of this radical will now be discussed. Benzoyl peroxide is thought to react within the cell with Cu^+ to give Cu^{2+} and the benzoyloxyl radical (Swauger et al., 1990). The copper chelator O-phenanthroline can prevent benzoyloxyl radical induced DNA damage (Swauger et al., 1991). This radical can react in one of two ways; firstly degrade to a phenyl radical and carbon dioxide or secondly abstract a hydrogen atom to form benzoic acid. Swauger et al. (1990) present evidence to suggest that it is principally the latter process which occurs. The benzoyloxyl radical is a very reactive species and will abstract a hydrogen atom from molecules in close proximity to its site of generation; in an aqueous environment it is likely to abstract a hydrogen atom from water, creating a hydroxyl radical. It is also capable of abstracting hydrogen atoms from other molecules.

Since TNF α induction involves the production of hydroxyl radicals requiring either Fe^{2+} or Cu^+ , whereas benzoyl peroxide involves benzoyloxyl radical production requiring just Cu^+ , then the situation could arise when Cu^+ but not Fe^{2+} is available in limiting amounts, then TNF α but not benzoyl peroxide induction could occur in a particular cell line. This could explain the results obtained in certain cell lines in which TNF but not benzoyl peroxide induction occurred.

It is proposed that both hydroxyl radicals and benzoyloxyl radicals carry out hydrogen abstraction to form water and benzoic acid respectively. Fig 9.1 shows five potential pathways for hydrogen abstraction. When a free radical scavenger is added to the cells, such as NAC or PDTC, it provides the hydrogen atom for the hydroxyl radical to form water or for the benzoyloxyl radical to form benzoic acid. In the process it becomes a radical. When two such radicals come together a disulphide bridge can form between them. When these free radical scavengers are present the induction of NF- κ B is inhibited; this suggests they divert the hydroxyl radicals from following a

pathway that would ultimately lead to NF- κ B induction. So what other pathways are available when free radical scavengers are not present in cells? GSH is a scavenger of free radicals as well as reacting with H₂O₂. There is evidence that benzoyl peroxide causes a drop in the GSH/GSSG ratio (Perchellet et al., 1986), presumably because GSH participates as a hydrogen atom donor to benzoyloxyl radicals. It is proposed that this drop in GSH/GSSG ratio is essential so that from this point on benzoyloxyl radicals will no longer be efficiently removed by GSH and some will be available to follow other pathways of hydrogen atom abstraction. Swauger et al. (1991) provide evidence for this; they found GSH provided complete protection against the DNA damage which is mediated by the benzoyloxyl radical.

In the case of hydroxyl radicals, the GSH/GSSG ratio would already be reduced by H₂O₂ so little GSH would be available for hydrogen abstraction. For both benzoyloxyl radicals and hydroxyl radicals, once GSH levels are low, other pathways must be used for hydrogen atom abstraction. These alternative pathways include hydrogen abstraction from DNA; it is difficult to see how DNA damage might cause NF- κ B induction which occurs in minutes. Another pathway is hydrogen abstraction from lipids causing lipid peroxidation. This can cause a change in membrane structure, which could enable the more efficient passage of NF- κ B into the nucleus, but it could not be responsible for the dissociation of I- κ B from NF- κ B which is an essential part of NF- κ B induction. Another pathway is that of protein S thiolation, in which proteins donate the hydrogen of their SH groups to form disulphide bridges. Obviously this could change the conformation of these proteins, leading to possible functional change. For example, if S-thiolation were to occur in I- κ B it could lead to conformational change which prevented NF- κ B binding, so enabling NF- κ B to translocate to the nucleus.

It is hoped that future work might help to establish the biochemical pathways involved in NF- κ B induction. Such work might enable the activity of this very important transcription factor to be controlled in situations where it could be having a detrimental effect on health such as in HIV-infected individuals.

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