IDENTIFICATION AND CHARACTERISATION OF A STEROID RESPONSE ELEMENT-BINDING PROTEIN

by

LOUISE CRAWFORD

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I declare that the studies presented in this thesis are the result of my own independent investigation unless otherwise indicated in the text.

This work has not been and is not being concurrently submitted for any other degree.

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ABSTRACT

The steroid hormone receptors (SR) are nuclear transcription factors which, upon activation by hormone binding, bind specifically to short DNA sequences called steroid response elements (SRE) in steroid regulated genes, and alter the transcription rates of those genes.

The consensus oestrogen response element (ERE) and glucocorticoid response element (GRE) can work alone as hormone-dependent transcriptional enhancers *in vivo*, when linked to a heterologous promoter. However, highly specific binding of purified SR to a SRE *in vitro* has not been demonstrated; in many cases, purified oestrogen (ER) and glucocorticoid (GR) receptor discern between their specific SRE and non-specific DNA with less than 10-fold discrimination.

Several studies have implicated the involvement of accessory proteins that increase the affinity of purified SR for its SRE *in vitro*. *In vivo*, such accessory proteins may be involved in high affinity binding of SR to a SRE to confer transcriptional regulation.

This thesis describes the identification and characterisation of a steroid response element-binding protein (SRE-BP) and argues that by modulating the interaction of different SRs with their target SREs, the SRE-BP plays a role in steroid hormone action.

Whole cell extracts (WCE) of HeLa, GH_3 and CV-1 cells contain SRE-BP activity. SRE-BP activity is also present in nuclear extracts of HeLa cells and WCE of liver tissue.

The SRE-BP binds specifically to two classes of functionally distinct SREs. In gel retardation experiments the SRE-BP binds preferentially to oligonucleotides containing a consensus ERE or a symmetrical GRE; it binds less well to a mutant GRE and does not bind to a symmetrical thyroid response element. The SRE-BP does not recognise transcription factor

binding sites present in the Herpes Simplex Virus thymidine kinase gene promoter.

Using gel filtration chromatography, the SRE-BP has been partially purified and shown to have a relative molecular weight under non-denaturing conditions of 205kD (± 20kD), as it exists in solution. The molecular weight of the SRE-BP when bound to an ERE oligonucleotide is 200kD (± 27kD) as determined by pore gradient gel electrophoresis. Thus, the SRE-BP species that exists in solution is the same as the DNA-bound form of the SRE-BP. Crosslinking experiments show that the SRE-BP is not a single ~200kD polypeptide, but is a protein complex made up of different subunits. Preliminary results suggest the DNA-binding subunit of the SRE-BP is between 88kD and 42kD.

Hence, the SRE-BP is a sequence specific DNA binding protein. It is neither ER nor GR, as demonstrated by its cell type distribution, its DNA sequence specificity, and its relative molecular weight.

Preliminary evidence is presented suggesting that HeLa WCE which contain SRE-BP activity can increase the binding of *in vitro* translated ER to a consensus ERE in a gel retardation assay. A role for accessory proteins in SR DNA-binding activity is further substantiated by the finding that *in vitro* translated ER binds to an ERE as part of a 362kD complex and not simply as a 130kD ER homodimer.

CONTENTS

CHAPTER 1 INTRODUCTION

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INTRODUCTION

1.1 INTRODUCTION

Eukaryotic gene activity can be controlled at various stages during gene expression. These include regulation of transcription, translation and post-translational events. Most frequently, though, gene activity is controlled at the level of transcriptional initiation; the decision of whether or not a gene is switched on and the rate at which it will be transcribed. Understanding the molecular mechanism behind control of transcriptional initiation has become one of the major goals of modern biology. Experimental procedures such as *in vivo* cell transfection systems, *in vitro* DNA-binding assays and protein purification techniques have given much of the knowledge concerning transcriptional control to date.

Traditionally, eukaryotic protein encoding genes transcribed by RNA polymerase II (class II genes) are represented diagrammatically as two-dimensional structures with two distinct regions: the transcribed region and the non-transcribed region. Within the transcribed region, information is encoded which is transmitted from DNA to RNA by the enzyme RNA polymerase II and subsequently to protein after translation of the fully processed mRNA species. Within the non-transcribed 5'- region, information is encoded which confers control over the site at which transcription is initiated and the rate of transcription initiation. This non-transcribed regulatory region exists 5'- to the transcribed region as depicted below in a two-dimensional representation of the steroid hormone controlled rat prolactin gene (Fig. 1.1).

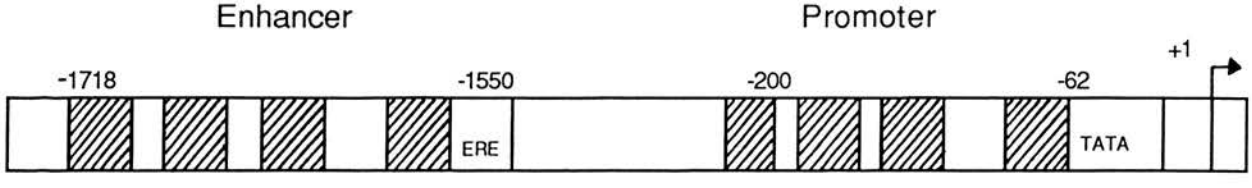


Fig. 1.1 Schematic diagram of the 5' regulatory region of the rat prolactin gene. Hatched boxes represent binding sites for the regulatory factor, Pit-1. The ERE and TATA box are also shown. Adapted from Ingraham et al. (1988).

Functional dissection of regulatory regions in several cloned eukaryotic genes led to the definition of two genetic elements which control gene transcription, called promoter and enhancer elements (see Fig. 1) (for reviews see Serfling et al., 1985; Maniatis et al., 1987; Müller et al., 1988; Dynan, 1989). It is generally accepted that proximal promoter sequences which include the TATA element control the site at which transcription is initiated (called the 'cap' site or +1), and basal transcription level, whereas distal promoter sequences and enhancers control the rate at which transcription is initiated. By definition, an enhancer confers transcriptional control in a manner independent of its orientation and distance relative to the promoter, often existing up to two kilobases from +1 (Müller et al., 1988; Maniatis et al., 1987; Serfling et al., 1985).

It is now clear that promoters and enhancers are modular structures composed of multiple short DNA sequences (see Fig 1.1) that individually confer transcriptional response to different stimuli. Responses are mediated through interaction between particular DNA-sequences with specific DNA-binding proteins, called transcription factors. Transcription factors may be expressed ubiquitously, e.g. TFIID (Buratowski et al., 1989 and 1988 and references therein), or may be cell type specific and/or inducible under certain conditions such as developmental or environmental stimuli. For example, the steroid hormone receptors are cell-type specific but are also only active when exposed to steroid hormones; activated SR then interacts with its DNA sequence, or SRE, in the regulatory region of a particular steroid hormone responsive gene and modulates transcription of that gene (see Yamamoto, 1985 for review). Thus, depending on the array of DNA sequences in the promoter and/or enhancer controlling any one gene, in any particular cell type, distinct complex patterns of transcriptional regulation are seen (for reviews see Dynan, 1987 and 1989; Schaffner, 1989; Berk &

Schmidt, 1990). It appears that different combinations of distinct transcription factors interact with specific genes.

As more and more transcription factors are cloned and their protein structures analysed, it is becoming apparent that there are families of related factors which interact with related DNA sequences. Such families include the steroid hormone receptor superfamily (for reviews see Evans, 1988; O'Malley, 1990 and section 1.6.2) , CTF family (Chodosh et al., 1988a and b; Dorn et al., 1987) and the POU-domain transcription factor family (Herr et al., 1988; He et al., 1989). It is clear that within these families, several structural protein motifs have evolved (reviewed in section 1.2), which are essential to transcription factor function.

A correctly positioned initiation complex within the promoter element allows RNA polymerase II to initiate transcription at +1 (reviewed in section 1.3.2). The present scenario for transcriptional control dictates that DNA-bound transcription factors, directly or indirectly, interact with components of the transcription machinery to promote/stabilise (activation; reviewed in section 1.3.3) or in some way inhibit/disrupt (repression; reviewed in section 1.3.4) the formation of a transcription initiation complex.

In vivo, the native environment of DNA involves its close association with histone and non-histone proteins, and subsequent packaging into higher order chromatin. The ability of transcription factors to modulate transcription from transfected plasmid DNA or to bind naked DNA does not include the effect of chromatin structure on transcriptional control. Moreover, the study of purified transcription factor activity in a DNA-binding assay does not address the possible role of accessory proteins, normally found in the cell, which may modulate DNA-binding activity of the factor. The role of chromatin structure and of accessory proteins in transcription factor activity is reviewed in sections 1.4 and 1.5 respectively, and with specific reference to steroid receptors in sections 1.6.5.2 and 1.6.5.3.

1.2 PROTEIN MOTIFS CHARACTERISED IN TRANSCRIPTION FACTORS

1.2.1 Introduction

DNA-binding transcription factors interact with DNA via their DNA-binding surfaces. Such factors could contact either the negatively charged backbone of B-form DNA or bases , which have innate hydrogen (H) bonding potential, exposed on the major or minor groove (reviewed by Pabo & Sauer, 1984). Characteristically, DNA-binding surfaces of transcription factors contain an excess of basic amino acids and also inherent H-bonding ability (Pabo & Sauer, 1984; Struhl, 1989; Schleif, 1988)). Thus, electrostatic attraction between the DNA phosphate backbone and the basic DNA-binding surface brings protein and DNA into close proximity. Specificity of the protein-DNA interaction arises from hydrogen bonding and van der Waals interactions between nucleotides and amino acid side chains; hydrogen bond formation is highly dependent on the position and orientation of the hydrogen bond donor and acceptor sites. As the minor groove is small and has less hydrogen bonding potential, the major groove is favoured for specific protein-DNA interactions.

The three dimensional configuration of B-form DNA imposes several structural constraints on the conformation of DNA-binding surfaces capable of interaction. Early model building studies predicted that an α-helix or a pair of antiparallel β-strands could fit into the major groove of B-form DNA (reviewed by Pabo &Sauer, 1984).

Analysis of protein structure of several cloned transcription factors reveals a small number of distinct structural motifs implicated in DNA-binding activity. The formation of either an α-helix or β-sheet has been demonstrated for some of these motifs and implicated in others. These motifs; helix-turn-helix, zinc fingers, leucine zipper, helix-loop-helix and β-sheet are reviewed below.

1.2.2 Helix-turn-helix motif

The helix-turn-helix motif was first proposed as a sequence-specific DNA-binding structure in the early 1980s, after X-ray crystallographic analysis of three prokaryotic DNA-binding proteins: Bacteriophage λ repressor and cro proteins and *E.coli* cAMP receptor protein (reviewed by Pabo & Sauer, 1984; Brennan & Mathews, 1989). Despite differences in size, domain organisation and tertiary structure, each of these proteins binds to DNA as a dimer and uses the helix-turn-helix structure to contact adjacent major grooves along one face of B-form DNA.

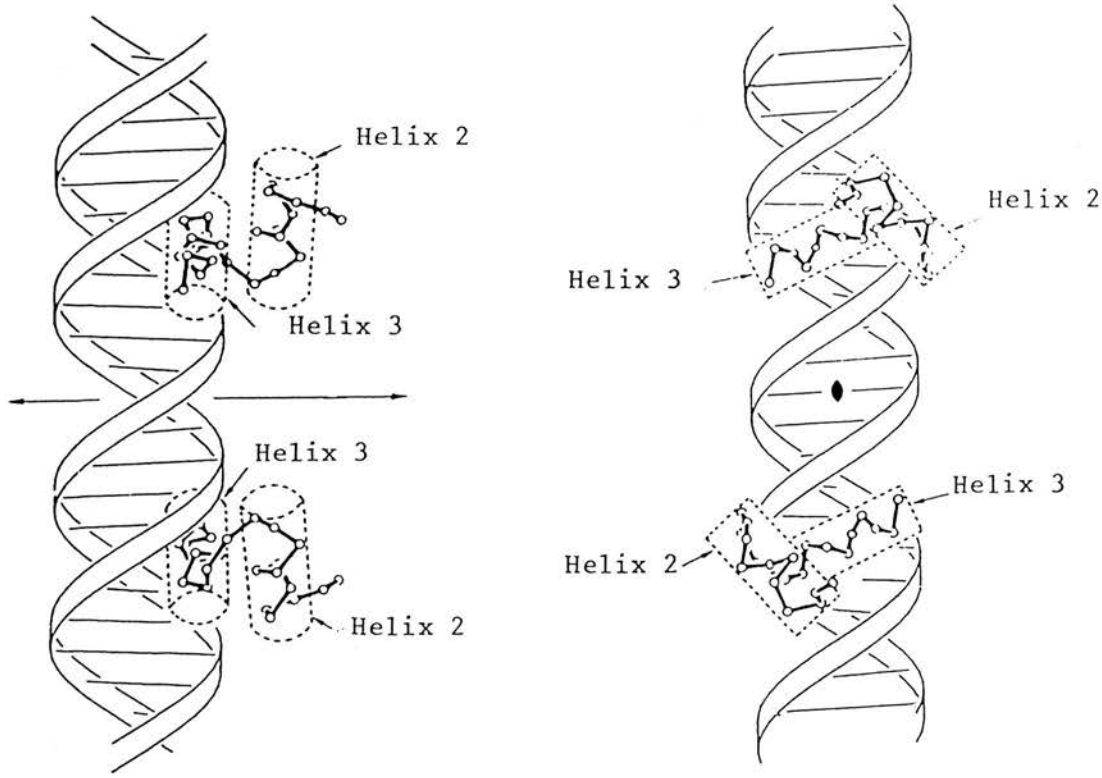


Fig. 1.2 Alpha carbon backbone of helices 2 and 3 of the λ-operator complex, showing the helix-turn-helix region positioned in the major groove of the DNA. Adapted from Pabo & Sauer (1984).

The helix-turn-helix motif consists of two α-helices separated by a tight β-turn (Fig. 1.2). The recognition α-helix (helix 3 in diagram) directly contacts bases exposed in the major groove of the target DNA sequence; the other α-helix (helix 2) lies across, but not in, the major groove. Helix 2 helps to position helix 3 in the major groove and makes non-specific DNA contacts with the phosphate backbone, so securing protein/DNA interaction (Ptashne, 1986a; Pabo & Sauer, 1984). Helix 2 of the λ repressor has also been implicated in protein-protein interaction with RNA polymerase (Hochschild et al., 1983).

Structural predictions based on sequence homologies provoked the proposal that DNA-binding by homeodomain-containing eukaryotic transcription factors involves a helix-turn-helix motif similar to that found in prokaryotic DNA-binding proteins (Laughon & Scott, 1984). The homeodomain is a highly conserved region of approximately 60 amino acids, first described in Drosophila homeotic genes (reviewed by Levine & Hoey, 1988). Mutational analysis (for examples see Treisman et al., 1989; Desplan et al., 1988) and homeodomain "swap" experiments show that the homeodomain is important for sequence-specific DNA binding (reviewed by Levine & Hoey, 1988). In fact, for the Drosophila Antennapedia transcription factor, NMR studies show that the homeodomain does form a helix-turn-helix motif in solution, virtually identical to those observed on prokaryotic DNA-binding proteins (Qian et al., 1989).

Homeodomains have since been characterised in a number of transcription factors from different species including the mammalian POU-domain proteins: Oct-1 (Sturm et al., 1988), Oct-2 (Clerc et al., 1988) and Pit-1/GHF-1 (Ingraham et al., 1988; Bodner et al., 1988; Herr et al., 1988). The POU-domain consists of two subregions: a C-terminal homeodomain and an N-terminal POU-specific domain which are both necessary for effective DNA-binding *in vitro* (Herr et al., 1988; Sturm & Herr,

1988). Indeed, amino acid substitutions within the putative recognition α-helix of Oct-1 homeodomains abolish DNA binding activity, *in vitro* (Sturm & Herr, 1988). The second α-helix (Fig. 1.2, helix 2) of Oct-1 has also been implicated in protein-protein interactions analogous to those proposed for λ repressor (Stern et al., 1989); mutations which abolish Oct-1 interaction with the herpes simplex virus VP16 protein, all map to putative α-helix 2.

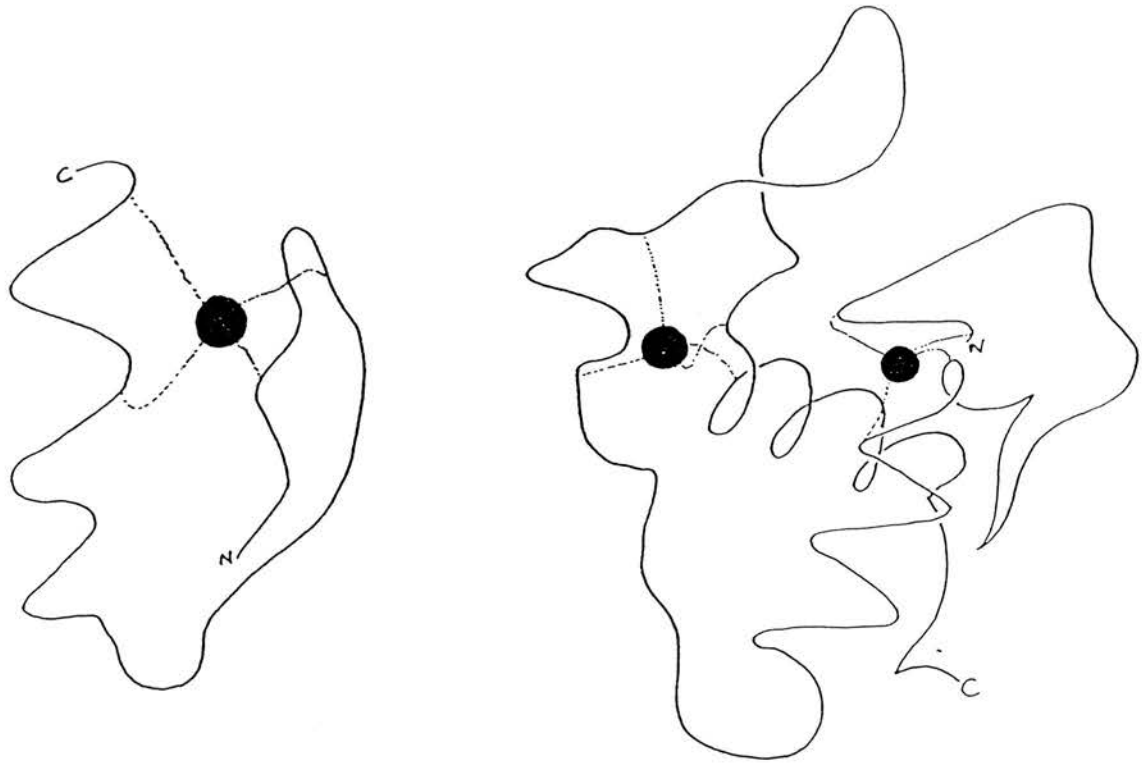
1.2.3 Zinc fingers

Recognition of a second DNA-binding motif, the zinc finger, was first described for the *Xenopus laevis* transcription factor TFIIIA (Miller et al., 1985). TFIIIA contains 7-11 zinc ions per molecule and is composed of nine 30 amino acid repeating units. Each unit incorporates one invarient pair of cysteines and one invarient pair of histidines (C_2H_2 finger protein), and co-ordinates a single atom of zinc (Klug & Rhodes, 1987). Structural analysis of many C_2H_2 finger proteins (Berg, 1986; Vincent, 1986) led to the proposal of a three dimensional model for these domains consisting of an anti-parallel β-sheet followed by an α-helix (Berg, 1988; Gibson et al., 1988). NMR studies of a 25 residue peptide encoding a single C_2H_2 finger of the *Xenopus* protein Xfin have now provided evidence that this ββα structure does indeed form in solution (Fig. 1.3a) (Lee et al., 1989).

The second class of zinc finger is exemplified by the steroid hormone receptor superfamily (Evans & Hollenberg, 1988; Frankel & Pabo, 1988). Receptor family members contain two potential zinc fingers within their DNA-binding domain, each incorporating two invarient pairs of cysteine residues (C_2 C_2 finger proteins) (Evans & Hollenberg, 1988; Berg, 1989). For GR, it has been shown that each finger motif binds one zinc ion which is essential for DNA-binding activity (Freedman et al., 1988).

The deleterious effect on DNA-binding activity observed when one C_2 pair of a C_2 C_2 finger is replaced with an H_2 pair (Green & Chambon, 1987)

indicated that the C_2 C_2 and C_2 H_2 finger structures differed. NMR studies of a peptide containing the DNA-binding domain of GR confirms that the C_2 C_2 finger is structurally different from the C_2 H_2 finger (Fig. 1.3b) (Härd et al., 1990a). In each case a ββα conformation forms, although the cysteine residues co-ordinating a zinc ion are located within the α-helix of the C_2 H_2 finger, whereas only the C-terminal cysteine residue of the C_2 C_2 finger lies within the α-helix (see Figs. 1.3a and 1.3b).



(a) (b)

Fig. 1.3 3D structure of the zinc fingers of Xfin-31 and rat GR

a) Model of the 3D structure of a single zinc finger from Xfin-31 (Adapted from Lee et al., 1989).

b) Model of the 3D structure of the rat GR DNA binding domain. The first finger region is in the top right and the 2nd finger region in the top left of the diagram. (Adapted from Härd et al., 1990a)

The zinc atoms are shown as filled circles, co-ordinated (shown as dotted lines) to protein.

1.2.4 The leucine zipper

The leucine zipper model (Fig. 1.4) for protein dimerisation was first proposed for the CCAAT-box binding factor and enhancer binding protein, C/EBP (Landschulz et al., 1988). When a region of protein sequence of C/EBP is arranged on an idealised α-helix, a periodic repetition of leucine residues, present at every seventh position over a distance of eight helical turns, aligns along one face of the α-helix. A similar periodic array of five leucine residues has been identified in protooncogenes c-Fos and c-Jun in a region which mediates dimerisation between Fos and Jun proteins (Landschulz et al., 1988; Schuermann et al., 1989; reviewed by Curran & Franza, 1988). Homology to the leucine zipper motif has recently been identified in the extensive family of activation transcription factor (ATF) proteins (Hai et al., 1989) and the yeast activator GCN4 (Vogt et al., 1987). Peptides containing the leucine zipper motif of either Fos or Jun proteins independently form α-helices in solution. When mixed, α-helices form heterodimers arranged in a parallel conformation (O'Shea et al., 1989) suggesting that leucine zipper motifs direct protein dimerisation via a short coiled-coil structure (Landschulz et al., 1988; O'Shea et al., 1989).

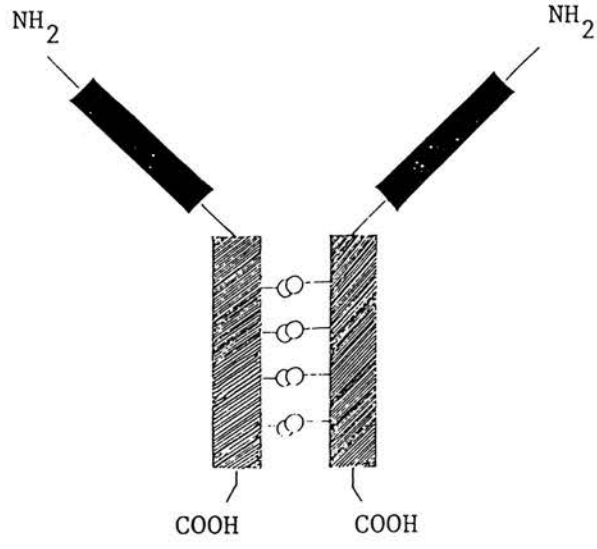


Fig. 1.4 Schematic representation of a leucine zipper protein. Filled rectangles represent the basic DNA binding domain and hatched rectangles represent the leucine repeat region.

Sequence analysis of leucine zipper proteins reveals a short region containing a high proportion of basic amino acids immediately adjacent to the leucine zip (Landschulz et al., 1980; Vinson et al., 1989). Secondary structure predictions of this basic region suggest it could form a stable α-helix (Vinson et al., 1989). Furthermore, this region in C/EBP must remain intact to direct effective DNA-binding (referred to in Landschulz et al., 1988 as unpublished data). This led to the formulation of a 'scissor-grip' model for DNA-binding of leucine zipper proteins (Vinson et al., 1989) in which two polypeptides dimerise to form the 'stem' of a Y-shaped molecule (Fig. 1.4). The arms of this molecule constitute a linked pair of basic regions, arranged in a manner suitable for sequence-specific DNA-binding. The bifurcation point of the Y-shaped molecule closely approaches the centre of the DNA-target site and optimally positions basic regions to allow tracking along each half of the recognition site, hence binding securely and specifically to DNA. Of course, three dimensional protein structural studies are required to confirm or reject this model for DNA binding.

1.2.5 Helix-loop-helix

A second structural motif for dimerisation and DNA-binding has been proposed. This motif is common to a family of proteins capable of forming homo and heterodimers with particular members of the group (Murre et al., 1989a). These proteins include the tissue-specific muscle determination genes (MyoD, Myf-5), two ubiquitous enhancer binding proteins (E12, E47), and several Drosophila genes (*daughterless, achaete-scute, hairy, twist*) (Murre et al., 1989b; Benezra et al., 1990 and references therein). The motif is divided into two conceptual subdomains: the helix-loop-helix (HLH) domain (Murre et al., 1989b) and a short basic region juxtaposed N-terminally to HLH (Tapscott et al., 1988).

Within the HLH are two highly conserved regions, each potentially capable of forming amphipathic α-helices, connected by a less well-conserved β-loop. The HLH has recently been shown to be necessary for MyoD-E12 heterodimer formation (Davis et al., 1990); this heterodimer contains increased specific DNA-binding activity compared to MyoD alone (Murre et al., 1989a). Dimerisation by HLH is probably essential for DNA-binding activity, as deletion of one or both putative α-helices impairs E12 DNA-binding activity *in vitro* (Murre et al., 1989a).

The adjacent basic region is not required for dimerisation but is absolutely necessary for specific DNA-binding *in vitro* (Davis et al., 1990). In fact, heterodimer formation between a ubiquitously expressed HLH protein containing no basic region (called Id), and either MyoD, E12 or E47 inhibits DNA-binding activity (Benezra et al., 1990). Discovery of Id gives new insight into mechanisms by which a family of related transcription factors may be controlled (discussed in Benezra et al., 1990).

1.2.6 Antiparallel β-sheet

The antiparallel β-sheet is the latest addition to an expanding club of structural motifs characterised in DNA-binding proteins. NMR studies of prokaryotic Arc repressor reveal that it binds to DNA via a structural motif involving two anti-parallel β-sheets (Breg et al., 1990). The N-terminally located DNA-binding domain of one Arc monomer dimerises with the analogous region of a second Arc monomer to form an anti-parallel β-sheet (Fig. 1.5). Two such Arc dimers bind to DNA with their β-sheet in successive major grooves on one side of the DNA-helix.

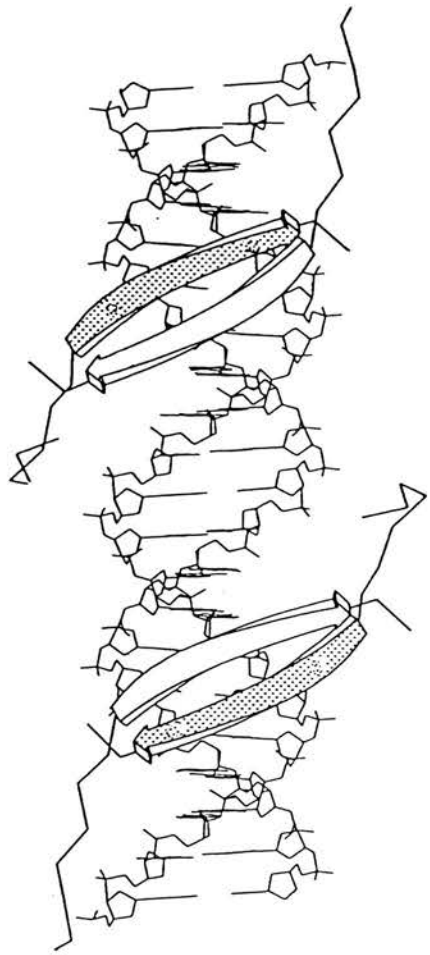


Fig 1.5 Model of the DNA-binding region of bactiophage P22 Arc repressor complexed with DNA. The antiparallel β-sheets (one from each dimer) interact with the major groove of DNA. (Adapted from Breg et al., 1990)

Sequence homologies and structural studies reveal three prokaryotic repressors (Arc, Mnt, Met) which are all members of the same family of β-sheet DNA-binding proteins (Breg et al., 1990; Rafferty et al., 1989). It will be of interest to see if similar β-sheet DNA-binding proteins exist in eukaryotes.

1.3 CONTROL OF TRANSCRIPTION INITIATION

1.3.1 Introduction

The proximal promoter of most eukaryotic class II genes includes an A/T-rich DNA sequence called the TATA-box, which is found 20-30bp upstream of the cap site in mammalian genes (Breathnach & Chambon, 1981). The ubiquitous transcription factor, TFIID, a component of the transcription machinery, binds specifically to the TATA-box. TFIID binding institutes the ordered assembly of other transcription machinery components (TFIIA,-B,-E/F, RNA polymerase II) to form a transcription initiation complex in the vicinity of the TATA box and cap site (Buratowski et al., 1989; Van Dyke et al., 1988 and references therein). The TFIID binding and subsequent initiation complex formation is important in determining the site at which transcription is initiated, and supports basal transcription (Buratowski et al., 1989; Pugh & Tijian, 1990; Hoey et al., 1990). The distal promoter and/or enhancer contain DNA-binding sites for other transcription factors which may act to induce (activate) or inhibit (repress) basal transcription from a particular gene (reviewed by Ptashne & Gann, 1990; Lewin, 1990; Renkawitz, 1990; Levine & Manley, 1989).

The following sections 1.3.2, 1.3.3. and 1.3.4, review aspects of the transcription machinery, and current mechanistic models for transcriptional activation and repression.

1.3.2 The transcription machinery

The finding that purified RNA polymerase II does not accurately initiate transcription *in vitro* led the search for additional cellular factors required for efficient transcription. Fractionation of mammalian cell extracts identified four transcription factors called TFIIA, TFIIB, TFIID and TFIIE/F, which, in addition to purified RNA polymerase II, accurately initiate

transcription from a minimal promoter containing a TATA element and a cap site (Matsui et al., 1980; Samuel et al., 1982; Sawadogo & Roeder, 1985).

TFIID-specific binding to the TATA-box is the first step in transcription initiation (see Fig. 1.6) (Van Dyke et al., 1988; Buratowski et al., 1989). Yeast, Drosophila and mammalian TFIID can be functionally interchanged to mediate basal transcription; for example, yeast TFIID can substitute for mammalian TFIID in a mammalian RNA polymerase II *in vitro* transcription assay (Buratowski et al., 1988; for Drosophila TFIID see Pugh & Tijian, 1990). The recent cloning of TFIID from a number of species, including human, yeast and Drosophila (reviewed by Ptashne & Gann, 1990; Lewin, 1990) reveals that they are all small (~30kDa) and retain a high degree of conservation within the C-terminal 180 amino acids. This conserved region is sufficient both to direct specific binding of a TFIID monomer to DNA and for basal transcription, *in vitro* (Hoey et al., 1990; Horikoshi et al., 1990). It is proposed that the nonconserved N-terminal region determines the differential ability of TFIID from distinct species, to respond to disparate activators (reviewed in section 1.3.3).

TFIIB (also called BFT3) has been purified from HeLa cells as a ~27kD protein (Reinberg & Roeder, 1987a; Zheng et al., 1987). Recent cloning of its cDNA reveals that as well as the full length TFIIB, an N-terminally truncated 22kD TFIIB is also expressed; both TFIIB forms are expressed in various cell lines from different species (Zheng et al., 1990). Although the specific function of TFIIB is not yet defined, it does not independently bind to promoter DNA (Zheng et al., 1987), but purified TFIIB does form a stable complex with purified RNA polymerase II in solution (Zheng et al., 1987; Reinberg & Roeder, 1987; Zheng et al., 1990).

TFIIF is a component of the less purified fraction TFIIE (BF2) and TFIIF is similar or identical to the factor called RAP30/74 (Flores et al., 1988). RAP30/74 has recently been cloned and may be associated with a DNA

helicase activity (Sopta et al., 1989). In addition, TFIIE nuclear fractions also contain a DNA-dependent ATPase activity (Reinberg & Roeder, 1987). TFIIF (RAP30/74) may function to hydrolyse ATP; the energy released would permit the associated helicase to melt DNA, a pre-requisite for RNA polymerase II mediated transcription.

The final characterised component of the transcription machinery is TFIIA. Unlike TFIIB,-D, and -E/F, TFIIA is not absolutely required for most *in vitro* transcription systems but acts as an accessory protein (see section 1.5) which increases the affinity of TFIID for the TATA-box (Buratowski et al., 1988; Buratowski et al., 1989 and references therein).

Efforts to determine the order of events in initiation complex assembly have exploited the major late promoter (MLP) of adenovirus-2 for its simplicity. Recently, gel retardation assays (Buratowski et al., 1989) in conjunction with DNAse 1 footprinting (Van Dyke et al., 1988; Buratowski et al., 1989) resolved a series of preinitiation complexes which suggest the ordered assembly of purified transcription machinery components over the MLP promoter to form a functional transcription initiation complex (Fig. 1.6). Specific binding of TFIID to the TATA-box represents formation of the first preinitiation complex; TFIID binding may be modulated by adjacent, upstream binding of TFIIA (Fig. 1.6). TFIIB binds downstream of the TATA-box in a TFIID-bound dependent manner. RNA polymerase II then binds, probably by direct interaction with TFIIB; TFIIE/F binds downstream of RNA polymerase II probably through its interaction with RNA polymerase II. Reportedly, TFIIE/F binding represents the final step in initiation complex formation (Buratowski et al., 1989).

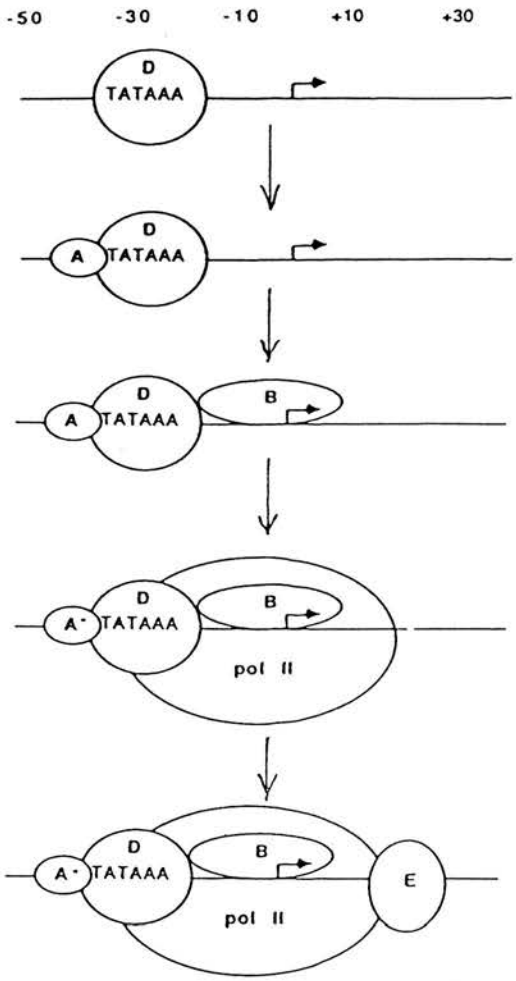


Fig 1.6 Schematic model of the assembly of the transcription initiation complex. The stepwise addition of TFIID, TFIIA, TFIIB, pol II and TFIIE/F are described in the text. (Adapted from Buratowski et al., 1989).

One puzzling observation concerning class II genes is the sequence divergence of the TATA-box (Singer et al., 1990) and its apparent absence from some class II promoters (Smale & Baltimore, 1989 and references therein). It has recently been shown that approximately 1% of random 16bp oligonucleotide sequences can function as basal promoters *in vivo* (Singer et al., 1990). In general, yeast TFIID binds to A/T-rich oligonucleotides *in vitro* even if no sequence homology to the consensus TATA-box (TATAAA) is

observed. The remaining non-TFIID binding oligonucleotides may function through their interaction with an alternative basal promoter binding factor. The lymphocyte-specific terminal deoxynucleotidyl-transferase gene has no TATA-box despite efficient transcription initiation at a single nucleotide. Smale & Baltimore (1989) have identified a 17bp initiator sequence containing within itself a cap site, and capable of mediating efficient basal transcription *in vivo*. The inclusion of an initiator sequence in TATA-less promoters provides one explanation for how eukaryotic promoters direct accurate transcription initiation.

1.3.3 Activation of Transcription

1.3.3.1 Activation

There are two classes of activators: members of one work universally, whereas members of the other work only in certain cells. The yeast transcriptional activator, GAL4 works universally; when introduced into a wide variety of eukaryotic cells, GAL4 activates transcription of a gene encoding a GAL4 binding site (Ma et al., 1988; Kakidani & Ptashne, 1988; Webster et al., 1988). It is proposed that universal activators encode both DNA-binding and activation functions and interact directly with a component of the transcription machinery.

The second class of activators encode either a DNA-binding function or an activation function and can, therefore, only work in cells which provide the missing function. For example, the Herpes Simplex Virus protein VP16 and the mammalian protein Oct-1 are both transcriptional activators although the former has no DNA-binding domain and the latter has no activation domain. VP16 and Oct-1 specifically interact with each other (Stern et al., 1989) to give high level activation from genes containing a binding site for Oct-1. Thus, Oct-1 will activate genes in cells.

Functional analysis of several activators has identified activating regions which can be transferred to heterologous DNA-binding domains and still confer transcriptional activation (Brent & Ptashne, 1984; Webster et al., 1988; Godowski et al., 1988; reviewed by Ptashne, 1988). Sequence analysis of activating regions has grouped activation domains into three loose categories based on their amino acid content (reviewed by Mitchell & Tijan, 1989): acidic activation domain (AAD), so called due to its high content of acidic amino acids (e.g. GAL4, GCN4, VP16, GR) (Ma & Ptashne, 1987 reviewed by Ptashne, 1988); the glutamine-rich activation domain (Sp1) (Courey & Tijan, 1988) and the proline-rich activation domain (CTF/NF-1 family) (Mermod et al., 1989). Additionally, activators such as Ela (Martin et al., 1990) and ER (Webster et al., 1989) do not fall into these categories. For AAD (and possibly others), the potency of activation is directly reflected in the overall negative charge (Ma & Ptashne, 1987). Although AADs appear less structurally defined than DNA-binding domains (section 1.2), an amphipathic α-helix has been implicated in acidic activation (Giniger & Ptashne, 1987).

The mechanism(s) by which activation domains increase the rate of transcription initiation has been addressed in several recent reports (reviewed by Lewin, 1990; Ptashne & Gann, 1990). However, these reports reach different conclusions and so several different models for transcriptional activation are proposed.

Stringer et al. (1990) propose that acidic activators interact directly with TFIID to promote formation of an initiation complex. They find that an affinity column bearing VP16 retains a protein (or proteins) present in HeLa nuclear extracts that restores activity to TFIID-depleted mammalian extract. The affinity column also retains yeast TFIID expressed from a cloned gene in bacteria so demonstrating direct TFIID-VP16 interaction (Stringer et al., 1990). This proposition is supported by the earlier demonstration that GAL4

and the mammalian activator ATF alter the extent of TFIID interaction with the TATA-box (Horikoshi et al., 1988a and 1988b). However, purified cloned mammalian TFIID expressed in *E.coli* can restore basal level transcription, but not VP16-mediated activation to TFIID-depleted mammalian extracts, although a crude fraction containing TFIID activity can restore both basal and activated transcription (Peterson et al., 1990). It therefore appears that at least one other factor is required, in addition to TFIID, to respond to VP16, but not for the VP16-TFIID interaction (reviewed by Ptashne & Gann, 1990). However, Berger et al. (1990) propose the existence of an "adaptor" molecule which arbitrates VP16-TFIID interaction and mediates activation. In their study, VP16-GAL4 fusion protein, specifically bound to a separate oligonucleotide in an *in vitro* transcription system, can inhibit activated (but not basal) transcription from a heterologous promoter (Berger et al., 1990). They proposed that oligonucleotide-bound GAL4-VP16 sequesters the "adaptor" required for interaction with TFIID, but not TFIID itself, and thus prevents activation but leaves basal transcription unaffected. Thus, more work is required to define whether VP16-TFIID interaction is direct or involves adaptors and whether this interaction typifies activation by all acidic activators.

The mechanism by which Ela activates transcription differs from VP16. Ela activates transcription in mammalian, but not yeast, cells (reviewed in Ptashne & Gann, 1990), unlike VP16, which activates in both yeast and mammalian extracts (Kellecher et al., 1990; Berger et al., 1990; Stringer et al., 1990). Martin et al. (1990) show that high level expression of VP16 in *in vitro* transcription assays squelches Ela activity, although Ela does not squelch VP16-mediated activation. They propose that both Ela and VP16 function through interaction with the same target of the transcription machinery (TFIID) although Ela-TFIID interaction is indirect and requires an intermediary adaptor molecule.

Ptashne & Gann (1990) have argued that all activators exert their effects by interacting with TFIID: acidic activators interact directly, and hence universally (although results of Berger et al. (1990) contradict this) and others (Sp1, Ela, Oct 1) do so via intermediary adaptor molecules (e.g. VP16) bearing acidic activating regions. The possibility that activators interact with some other component of the transcription machinery, however, cannot be ignored.

1.3.3.2 Synergy between transcription activators

A striking characteristic of many different eukaryotic transcription activators is their ability to activate gene expression synergistically; transcriptional activation by two activators is greater than the sum of the effects of each working alone. Analysis of a combination of several transcription factor binding sites (NF-1, Sp-1, CP-1) with a SR receptor binding site (GRE/PRE) demonstrates strong synergistic effects on steroid hormone induction of transcription *in vivo* (Schule et al., 1988). The rat glucocorticoid receptor and the yeast activator GAL4 cooperatively activate transcription of a mammalian gene bearing binding sites for each protein (Kakidani & Ptashne, 1988)

Synergy between activators may be achieved by cooperative DNA-binding of transcription factors; binding of an activator to its site may increase binding of a second activator via protein-protein interactions. Interaction between PR complexes bound at distinct target DNA sites has been demonstrated (Theveny et al., 1987); it is possible that this accounts for the observed synergistic transcriptional activation. Alternatively, activators may synergise by simultaneously contacting some part of the transcription machinery so cooperatively promoting/stabilising an initiation complex. In support of this, yeast GAL4 can synergise with itself or mammalian ATF *in vitro*, when either is present at concentrations sufficiently

high to saturate their target DNA sites (Carey et al., 1990; Lin et al., 1990). However, if transcriptional activators directly or indirectly interact with TFIID as proposed, it is difficult to imagine how multiple activating domains can simultaneously touch a small (~ 30kD) TFIID molecule.

1.3.3.3 Looping and scanning

Transcription factors which bind to target DNA sequences and increase the rate of transcription initiation from a linked promoter are termed activators. Two models have been proposed to explain how activators bound to distal promoter and enhancer elements can stimulate transcription initiation from the proximal promoter (reviewed by Ptashne, 1986b). In the first model, the scanning model, a constituent of the transcription machinery initially binds to distal elements, facilitated by already bound activators, then slides in either direction along the DNA until it reaches the proximal promoter; the transcription initiation complex is thus able to assemble. Support for the scanning model was provided by transfection studies in yeast where insertion of a transcription terminator sequence between a TATA-box and an upstream activation site strongly reduced transcription (Brent & Ptashne, 1984). Also, in mammalian transfection studies, preferential enhancer-mediated transcriptional activation from the closer promoter elements of tandemly repeated promoters, can be explained by the scanning model (reviewed by Müller et al., 1988). However, no direct evidence for scanning is documented and neither example above is incompatible with the looping model, described below.

In the second model, it is proposed that distally bound activators directly, or indirectly, interact with the proximally bound transcription factor, looping out the intervening DNA to ultimately facilitate and/or stabilise formation of a transcription initiation complex at the proximal promoter; this is known as the looping model.

The recent demonstration that enhancer bound activators can stimulate transcription *in trans* from a promoter attached non-covalently by a protein bridge favours the looping model; the protein bridge between the enhancer and promoter, which is not required for transcriptional activation, would conceivably be looped out (Müller et al., 1989).

1.3.4 Repression of transcription

In recent years, many reports describing negative transcriptional effects have accumulated (reviewed by Levine & Manley, 1989; Renkawitz 1990), and in several cases the components involved in transcriptional repression have been characterised. It appears that there are loosely grouped mechanisms by which transcriptional repression is mediated *in cis* which are 1) inhibition of a transcriptional activator and 2) silencing (reviewed below in 1.3.4.1 and 1.3.4.2). The former mechanism arises when a repressor interferes with either the DNA-binding or activation activity of an activator. The latter mechanism of silencing can be considered as "direct" repression whereby a repressor binds to a defined DNA sequence and somehow inhibits transcription without interfering with the function of an activator. Both can be regarded as cis-repression mechanisms as each affects transcriptional activity from a linked promoter. Squelching (reviewed in 1.3.4.3) has been proposed to explain trans-repression of transcription observed when an activator is over-expressed in either cell transfection systems or *in vitro* transcription assays. It will become apparent in the following sections that a single transcription factor can behave as an activator in one particular context and a repressor in another. For the purpose of this review, I will use the term "repressor" for any factor which negatively regulates gene transcription, independent of whether the factor positively regulates another gene in a different context.

1.3.4.1 Inhibition of a transcriptional activator

As reviewed in 1.3.3, activators must have or must acquire two surfaces; a DNA-binding domain and an activation domain, in order to fulfil their function. Accordingly, repressors can elicit their effect by interfering with the function of either domain.

Inhibition of DNA-binding can be mediated by either a cytoplasmic or a nuclear inhibitor which specifically interacts with an activator to prevent DNA-binding. Alternatively, competition between two transcription factors, one which mediates transcriptional activation and the other, repression, for binding to the same or overlapping DNA site can also result in negative transcriptional control.

Untransformed steroid hormone receptors are retained in a form incapable of binding to DNA, by specific interaction with the inhibitor, hsp90 (Denis et al., 1988b; Renoir et al., 1990). Only after stimulation with steroid hormone does SR dissociate from hsp90 and bind to DNA (reviewed in section 1.6.3). The more recently identified nuclear inhibitor, Id, which contains a helix-loop-helix (HLH) domain but no adjacent DNA-binding domain (reviewed in 1.2.5) can associate specifically with other DNA-binding HLH proteins and inhibit their DNA-binding activity *in vitro* (Benezra et al., 1990). Id is expressed at varying levels in all cell lines tested and it is proposed that it negatively regulates at least three HLH proteins (MyoD, E12, E47) in a dose-dependent manner, by formation of a non-functional heterodimeric complex (Benezra et al., 1990).

A recently characterised DNA-binding repressor, GCF, binds specifically to GC-rich sequences (Kageyama & Pastan, 1989) similar to sequences recognised by the activator Sp1 (Kadonaga et al., 1986). Kageyama & Pastan (1989) propose that GCF repressor and Sp1 activator compete for binding to the same GC-rich DNA site, thus eliciting negative or positive transcriptional effects, respectively.

Unlike GCF which so far appears only to mediate negative transcriptional effects, GR, which activates transcription of some genes (see Beato 1989 for review), also represses transcription of others (for examples see Camper et al., 1985; Charron & Drouin 1986; Frisch & Ruley, 1987; see also section 1.6.4.2). Akerblom et al. (1988) suggest that GR-mediates repression of gonadotrophin α-subunit gene by steric hindrance; in the presence of glucocorticoids and increased cAMP levels, GR and CRE-binding protein, which confer repression and activation respectively, compete for binding to their respective DNA sites which overlap with each other. Why GR behaves as an activator in the context of some genes and a repressor of others is unknown. A suggestion has been made that some GREs which differ only slightly from the consensus GRE (see section 1.6.4.2 for more complete review) behave as negative GREs (nGRE) by altering the structure of bound GR and preventing it functioning as an activator (Sakai et al., 1989). The nGRE hypothesis would explain GR-mediated repression, whether it be by steric hindrance (Akerblom et al., 1988) or by silencing (see below, 1.3.4.2).

Inhibition of transactivation happens when a repressor blocks or masks the activation domain of a DNA-bound activator, so preventing it promoting/stabilising (see 1.3.2) assembly of an initiation complex. Such repressors may, or may not, be DNA-binding proteins. For example, transcription activation by DNA-bound c-Myc is repressed by adjacent binding of the negative factor Myc-PRF (Kakkis et al., 1989). Presumably, protein-protein interactions between c-Myc and Myc-PRF interfere with activation domain function. The yeast repressor GAL80, which does not bind to DNA, interacts directly with DNA-bound GAL4 and somehow blocks the function of both GAL4 activating domains (Ma & Ptashne, 1988 and references therein).

1.3.4.2 Silencing

Silencing can be regarded as "direct" repression, in which one or more repressors bind to a DNA element (silencer) and negatively regulate transcription, independent of interfering with activator function. Repression conferred by a silencer often displays characteristics analogous to activation by an enhancer in that both elicit their effects in a manner independent of orientation and distance from the controlled promoter (Brand et al., 1985). The negative response element (NRE) of human immunodeficiency virus behaves as a silencer (Smith & Greene, 1989 and references therein). Several viral proteins have been implicated in mediating repression of HIV transcription through the NRE although for only one of these, SP50, has DNA-binding and repression been shown (Smith & Green, 1989). However, NRE probably mediates repression by interaction with more than one repressor, as a single SP50 binding site is incapable of negatively regulating a heterologous promotor *in vitro* (Smith & Greene, 1989 and references therein).

Several enhancer and silencer elements have been described upstream of the chicken lysozyme gene which together control macrophage-specific expression of this gene (Baniahmad et al., 1987 and 1990). One of these silencers (S-2.4kb) is comprised of multiple short DNA sequences that independently repress gene activity (Baniahmad et al., 1990), comparable to the molecular structure of enhancers (reviewed by Dynan, 1989). Furthermore, the individual modules of S-2.4kb silencer interact synergistically to repress transcription from a linked promoter (Baniahmad et al., 1990; compare with synergistic activator effects reviewed in 1.3.3.2). V-erbA, unliganded TR or unliganded RAR are directly implicated in repressor function by binding to one module of the S-2.4kb silencer (Baniahmad et al., 1990).

An interesting mechanism for silencer function has been deduced from the finding that a silencer-binding protein, RAP-1, is involved in chromatin loop formation at the silent mating type locus of yeast (Hofmann et al., 1989). Such a configuration may lock activator binding sites critical to transcription activation within the chromatin loop, and inhibit activator-target interaction (see 1.3.3.1).

1.3.4.3 Squelching

Squelching is a form of repression where neither direct modulation of activator activity (see 1.3.4.2) nor a silencing mechanism applies. Squelching constitutes trans-repression and requires over-expression of a non-DNA-bound activator which apparently titrates the target protein (1.3.3.1) of a DNA-bound activator, essential for transcriptional activation (Gill & Ptashne, 1988). Over-expression of GR, PR or ER interferes with transcriptional induction mediated by each of the other receptors (Meyer et al., 1989). Since neither heterodimer formation nor any other kind of interaction between distinct SRs has been demonstrated, the favoured explanation for repression is that the interfering SR sequesters (in solution) the target factor required for SR activation. Whether squelching is a physiologically important form of repression remains to be seen; it has, however, proved indispensable in the characterisation of the target factors of activators (reviewed by Lewin, 1990; Ptashne & Gann, 1990; reviewed in section 1.3.3).

1.4 THE INFLUENCE OF CHROMATIN ON TRANSCRIPTION FACTOR ACTIVITY

1.4.1 Introduction

As reviewed in section 1.3, processes by which basal transcription, transcriptional activation and transcriptional repression of eukaryotic genes

occur, depend on specific interactions between nuclear proteins and DNA sequences. Therefore, recognition and binding of target DNA sites requires that specific DNA sequences are accessible to transcription factors.

Much of the work towards characterising eukaryotic gene transcription has relied on the use of DNA templates which are essentially devoid of chromatin structure and, therefore, do not reflect the natural environment of nuclear genomic DNA. For example, *in vitro* DNA-binding assays and *in vitro* transcription systems allow characterisation of transcription factors using naked DNA. Transient transfection systems depend on introduced supercoiled plasmid DNA which is not packaged into chromatin.

1.4.2 DNA in the nucleosome

Unlike these experimental situations, the DNA in eukaryotic cells is highly packaged in a chromatin hierarchy that stretches from the initial wrapping of DNA around histone octamer cores to the fully condensed structure of chromosomes (reviewed by Pederson et al., 1986). The basic repeating unit of chromatin, the nucleosome, constitutes two copies each of the histones H2A, H2B, H3 and H4 around which some 146bp of DNA is wrapped 1.8 times. This introduces torsional stress into the DNA wrapped around the histone octamer, causing it to bend and kink (reviewed by Morse & Simpson, 1988). The influence of these structural alterations, and the close DNA-DNA, DNA-histone proximities, on the accessibility of specific DNA sites and interaction with transcription factors, is poorly understood (for reviews, see Gross & Garrard, 1988; Morse & Simpson, 1988; Elgin, 1988).

There is convincing evidence that nucleosome presence can repress transcription activation. PH05 is usually repressed by high, and activated by low, levels of inorganic phosphate, although, enforced nucleosome depletion of genetically engineered yeast derivatives results in constitutive activation of PH05 independent of inorganic phosphate levels (Han &

Grunstein, 1988 and references therein). Furthermore, Workman & Roeder (1987) demonstrate that preassembly of nucleosomes inhibits basal transcription from the adenovirus major late promoter *in vitro*, and Bresnick et al. (1990) argue that inhibiting nucleosome dissociation from the MMTV promoter in stably-transfected cells by chemical modification represses steroid hormone induction. Thus, the role of the nucleosome as a transcriptional repressor is strongly implicated; the nucleosome must therefore be removed or modified to allow transcriptional activation of particular genes.

Nucleosomes are phased in some genes with respect to the underlying DNA sequence (reviewed by Gross & Garrard, 1988). The yeast PH05 locus (Almer et al., 1986), the long terminal repeat of MMTV (Richard-Foy & Hager, 1987) and the mouse major β-globin gene promoter (Benezra et al., 1986) are examples of inducible promoters in which a pattern of nucleosome phasing is well characterised. Phasing could dictate the rotational orientation of a particular DNA sequence on the nucleosome relative to the histone octamer, or may place a DNA sequence within the linker DNA region between adjacent nucleosomes. Therefore, some DNA sites will be accessible to their specific transcription factor, whereas others, facing into the nucleosome, will be inaccessible. The proposal that certain transcription factors, able to bind to DNA since their specific site is exposed on the nucleosome, can act as specific nucleosome displacement factors, is reviewed in section 1.6.5.2.

Other mechanisms for nucleosome displacement have been proposed which include DNA replication and demethylation of cytosines (reviewed by Gross & Garrard, 1988). However, for the latter, it is not clear whether demethylation is the cause or consequence of nucleosome displacement.

1.4.3 Nuclease hypersensitive sites

In chromatin, nucleosome-free regions, also called nuclease hypersensitive sites, are believed to represent the "open windows" that allow enhanced access of transcription factors to crucial DNA sequences; hypersensitive sites generally represent a minor (< 1%) but highly selective fraction of the genome which encompasses functional elements including enhancers, silencers, promoters, transcription terminators and replication origins (for examples see Cordingley et al., 1987; Burch & Weintraub, 1983; Weintraub, 1985; reviewed by Gross & Garrard, 1988). Analysis of the fine structure of hypersensitive sites reveals footprints that indicate the presence of bound *trans*-acting factors (see, for example, Becker et al., 1986; Cordingley et al., 1987; Lohr et al., 1987; Liberator & Lingrel, 1987). However, as promoters and enhancers often possess multiple and sometimes overlapping protein binding sites (reviewed by Dynan, 1989), the question of which *trans*-acting factors are present within hypersensitive sites *in vivo* is ambiguous.

The link between hypersensitive sites and transcriptional activation is well established. The promoter regions of genes on the active X-chromosome in mammals possess hypersensitive sites while their allelic partners residing in the inactive X-chromosome lack such sites (Wolf & Migeone, 1985; Yang & Caskey, 1987). The mouse β-globin gene, which is expressed in erythroid cells, has a 700bp hypersensitive region encompassing its promoter from which 4 nucleosomes have been removed; this hypersensitive site is absent in non-erythroid cells which do not express β-globin (Benezra et al., 1986). When the chicken vitellogenin gene is transcriptionally activated in the liver by oestrogen treatment, three hypersensitive sites are induced in the 5' region of the gene (Burch & Weintraub, 1983). Two of these sites are stable and are propagated to daughter cells after hormone withdrawal. The third site is

oestrogen-dependent and is proposed to coincide with activated ER binding to its response element upstream of the vitellogenin gene (Burch & Weintraub, 1983).

Thus, in addition to current models of transcription regulation in which DNA-bound factors mediate regulation of transcription via protein-protein interactions (see section 1.3), additional control at the level of chromatin organisation must not be forgotten. Control over nucleosome displacement and maintenance of "open" chromatin structure are, undoubtedly, important in transcriptional control.

1.5 A ROLE FOR ACCESSORY PROTEINS IN TRANSCRIPTION FACTOR DNA-BINDING ACTIVITY

1.5.1 Introduction

An accessory protein is the term given to a particular protein which modulates DNA-binding activity of a specific transcription factor for its target DNA site. The accessory protein may elicit its effect by increasing (or decreasing) transcription factor affinity for its 'usual' DNA sequence (1.5.2) or by altering factor specificity so that it preferentially binds to a different, related DNA sequence (1.5.3). In addition, one accessory protein may alter both DNA-binding affinity and sequence specificity of a particular transcription factor (see below).

The role of accessory proteins in transcription factor DNA-binding activity is a developing concept with respect to transcription regulation research. This is probably due to the experimental techniques used to study aspects of transcriptional regulation to date. For example, *in vivo* transient transfection systems ensure high levels of the transcription factor of interest (over-expressed from a co-transfected expression plasmid) and/or the specific DNA site (encoded on a reporter plasmid). Also, high concentrations of transcription factor (present in cell extracts enriched for

factor or partially/totally purified factor) and/or DNA site (encoded on naked, linear DNA fragment or oligonucleotide) are also used for *in vitro* DNA-binding systems. However, transcriptional regulation occurring in the nucleus is dependent on a limiting concentration of transcription factors specifically interacting with a small number of target DNA sites, present within a large mass of chromosomal DNA. Therefore, any specific transcription factor must encode or acquire extremely high, sequence-specific DNA binding affinity in order to elicit a significant transcriptional response. To achieve this, accessory proteins may be enlisted to modulate transcription factor DNA-binding activity; the importance of such accessory proteins is, therefore, overlooked under the artificial *in vivo* and *in vitro* experimental conditions described above. Furthermore, the observation that many purified transcription factors discriminate weakly between their specific DNA-binding site and non-specific DNA (see for example Compton et al., 1983; von der Ahe et al., 1985; Maurer & Notides, 1987; Peale et al., 1988) implies a need for additional factors (accessory proteins) *in vivo* which assist high affinity DNA-binding.

1.5.2 Altering the DNA-binding affinity of a transcription factor

The prokaryotic histone-like protein HU (Varshavsky et al., 1977) alters the structure of DNA when it binds non-specifically as a multimeric complex (Broyles & Pettijohn, 1986). Flashner & Gralla (1988) show that HU differentially regulates the interaction of three diverse prokaryotic transcription factors with their DNA sites. In the presence of HU, specific DNA recognition by Lac repressor and cAMP receptor protein is increased more than 10-fold, whereas Trp repressor binding is slightly decreased. This differential modulation of DNA-binding is mimicked by the chemical agent $BaCl_2$, known to promote DNA bending (Lauden & Griffith, 1987).

Frashner & Gralla (1988) propose that HU binding to DNA may promote a variety of alternative DNA structures that either facilitate or inhibit specific interaction of transcription factors with their target DNA sites.

Cleat & Hay (1989) propose that the non-specific adenovirus DNA-binding protein, DBP, acts in an analogous fashion to HU, in modulating eukaryotic NF-1 binding to the adenovirus origin of replication. The presence of DBP in a footprinting experiment has no effect on NF-1 binding when NF-1 is present at saturating concentrations. In contrast, DBP significantly increases the DNA binding activity of limiting quantities of NF-1 to either its viral or human DNA site, *in vitro* (Cleat & Hay, 1989). Furthermore, DNaseI footprinting results suggest that the NF-1 site may be distorted upon NF-1 binding. Thus, DBP may distort and bend DNA in its vicinity into a conformation which resembles DNA when it is incorporated in an NF-1/DNA complex, and so facilitating NF-1/DNA interaction (Cleat & Hay, 1989). If DBP selectively binds to adenovirus DNA *in vivo*, then limiting quantities of NF-1 in the cell nucleus may be attracted to the adenovirus replication origin in preference to host NF-1 sites. Several different CCAAT-binding proteins have been identified and although CCAAT elements seem grossly similar (but probably have different flanking sequences), recent evidence suggests that different proteins are capable of distinguishing between different elements (Dorn et al., 1987; Chodosh et al., 1988a, 1988b; and references therein). It is tempting to speculate the existence of DBP-like accessory proteins in higher eukaryotes, which act to increase the affinity of limiting CCAAT-binding proteins for their preferential sequences.

Studies on the role of TFIIA in formation of a transcription initiation complex (reviewed in 1.3.2) are confounded by its variable requirement in *in vitro* transcription reactions; reports have ranged from TFIIA being totally dispensable (Sawadogo & Roeder, 1985), strongly stimulatory (Samuels &

Sharp, 1986; Buratowski, et al., 1988; Buratowski et al., 1989) to being absolutely necessary (Reinberg et al., 1987). These variable results probably reflect 1) the relative TFIIA concentration (see below) and/or 2) the TATA-box sequence (see 1.5.3). Buratowski et al. (1988) show that TFIIA positively modulates TFIID DNA-binding activity to the adenovirus major late TATA-box, only when TFIID is present at non-saturating concentrations. Furthermore, approximately two-fold less TFIID is required to saturate TATA elements when TFIIA is present *in vitro* (Buratowski et al., 1989). Although TFIIA binding alone to DNA has not been demonstrated, TFIID binding to DNA creates a larger footprint when TFIIA is present (Buratowski et al., 1989). Whether this reflects TFIIA binding to DNA adjacent to the TATA-element, or conformational change in TFIID caused by a protein-protein interaction between TFIIA and TFIID, remains to be clarified.

1.5.3 Altering the sequence specificity of transcription factors

In the yeast *Saccharomyces cerevisiae*, cell type is determined by three key regulatory DNA-binding homeodomain proteins, α1 and α2 (encoded within *Mat α* locus) and *a*l (encoded within *Mat a* locus) (reviewed by Herskowitz, 1989). In their haploid state, two yeast cell types exist called a and α which express *a*1 and α1+α2 respectively; diploid yeast express all three regulatory proteins. Early genetic analysis of *Mat* locus mutants (Strathern et al., 1981) led to the proposal that in haploid α-cells, α1 switches on α-specific gene expression and α2 represses *a*-specific gene expression, whereas in diploids α2 and *a*1 work together to repress expression of haploid-specific genes (in diploids, α2 alone still represses *a*-specific genes). Goutte & Johnson (1988) demonstrate specific binding of α2 to a 32bp DNA site found upstream of *a*-specific genes *in vitro*. However, in combination with *a*1, α2 DNA-binding sequence specificity is

modulated so that it no longer recognises the 32bp *a*-specific DNA site, but instead acquires the ability to recognise a 29bp related sequence found upstream of haploid-specific genes.

The DNA-binding specificity of another homeodomain protein, Oct-1, may be modulated by its interaction with the viral activator VP16. Oct-1 binds to the conserved octamer motif ATGCAAAT and is a ubiquitous mammalian transcriptional activator of small nuclear RNA and histone H2B genes (Baumruker et al., 1988, and references therein); Oct-1 is a member of the POU-domain family (Herr et al., 1988). VP16 is a non-DNA binding viral transactivator which acquires DNA-binding activity by interacting with Oct-1. This VP16-Oct-1 complex activates Herpes Simplex Virus immediate early gene expression through binding to the degenerate viral octamer motif, TAATGARAT (Preston et al., 1988; O'Hare et al., 1988; Stern et al., 1989), although on its own, Oct-1 preferentially binds to the cellular conserved octamer motif (Baumruker et al., 1988). Stern et al. (1989) demonstrate that VP16-Oct-1 interaction is dependent on an intact Oct-1 DNA-binding domain. They speculate that VP16 interaction with the Oct-1 DNA-binding domain could alter the sequence preference of Oct-1 so it no longer binds with high affinity to cellular octamer motifs but directs preferential binding of VP16-Oct-1 complex to the degenerate viral sequence. Thus, preferential expression of viral genes compared to cellular genes, would occur.

In vitro DNA-binding studies reveal that a given homeodomain protein can interact with a range of related sequences (Baumruker et al., 1988; reviewed by Levine & Hoey, 1988) and that a given DNA sequence can be recognised by more than one homeodomain protein. For example, different homeodomain proteins, from Drosophila to man, containing a broad range of homeobox sequences, can recognise the same Drosophila DNA site (Hoey & Levine, 1988; Desplan et al., 1988; Ko et al., 1988). It is, however, unclear as to what extent this lack of selectivity reflects *in vivo* DNA-binding

affinities. In light of the discovery that DNA-binding specificity of yeast α2 homeodomain protein is modulated by *a*1, perhaps there are a number of (related) accessory proteins which mediate high affinity, sequence-specific binding of homeodomain proteins *in vivo*.

As mentioned in section 1.5.2, TFIIA appears to positively modulate TFIID affinity for the adenovirus major late promoter when TFIID is present at limiting concentrations. TFIIA also mediates TFIID binding to a different TATA sequence found upstream of the adenovirus proximal promoter; TFIID binding to this sequence is absolutely dependent on the presence of TFIIA (Buratowski et al., 1989). The upstream site differs from the promoter box sequence by only one nucleotide, although both elements have diverse flanking sequences. Possibly, TFIIA, or TFIIA-like factors, interact with TFIID and permit TFIID binding to the wide variety of TATA-elements occurring in different genes (for review of TATA-element diversity, see Singer et al., 1990). If TFIIA or TFIIA-like factors are differentially distributed in distinct cell types, this could lend some degree of specificity to certain genes encoding a particular TATA-element.

1.6 STEROID HORMONE REGULATION OF GENE TRANSCRIPTION

1.6.1 Introduction

In vertebrates, there are five major classes of steroid hormone which are subdivided into the adrenal steroids (glucocorticoids and mineralocorticoids) and the sex steroids (oestrogens, progestins, androgens) (O'Riordan et al., 1982). Together they play a central role in triggering and governing developmental and physiological processes by coordinating regulation of tissue-specific gene networks (see Yamamoto, 1985 and Evans, 1988 for reviews). Steroid hormones elicit their effects on gene expression after traversing the cell membrane and specifically activating endogenous steroid hormone receptors.

The steroid hormone receptors are nuclear transcription factors which, when activated (transformed) by steroid hormone binding, acquire the ability to regulate transcription of steroid hormone responsive genes. The transformed SR binds specifically to short DNA sequences called steroid response elements, found usually in the 5'- regulatory region of target genes. This SR-SRE interaction either activates or represses basal transcription, presumably through SR interaction with a component(s) of the transcription machinery. One class of steroid hormone specifically transforms a distinct SR which subsequently interacts with its specific SRE; for example, oestradiol binds to and transforms ER which then binds specifically to an ERE.

Within the past five years, the human cDNAs of the major SRs have been cloned: ER (Green et al., 1986), GR (Hollenberg et al., 1985), PR (Misrahi et al., 1987), AR (Trapman et al., 1988), and MR (Arriza et al., 1987). Various cDNA receptor sequences from other organisms including rat, rabbit and mouse have also been cloned (reviewed by Evans, 1988). Comparative analysis of SR sequences with each other and with other nuclear receptors led to the identification of a steroid hormone receptor superfamily (reviewed in 1.6.2).

Many steroid hormone responsive genes have also been cloned (reviewed Beato, 1989; Beato et al., 1989). By using the *trans*-acting receptor and *cis*-acting SRE in *in vitro* DNA-binding studies and *in vivo* cell transfection systems, different functional domains of the SR have been defined, as well as its interaction with the SRE. So far, there appear to be five main reactions involving different functional domains of the SR, which culminate in steroid hormone mediated transcriptional regulation (reviewed in 1.6.3 and 1.6.4). They are 1) Steroid hormone (ligand) binds

specifically to its SR and transforms it from an inactive to active state (reviewed in 1.6.3.1). This involves SR dissociation from the inhibitory protein Hsp90 and possibly other inhibitory proteins; 2) SR dimerisation which is essential for efficient DNA-binding activity of the SR (reviewed in 1.6.3.2); 3) Nuclear localisation of cytoplasmically located SR (reviewed in 1.6.3.3). Whether this reaction occurs before, after or coincides with receptor dimerisation, is not known; 4) Specific interaction between SR and SRE (reviewed in 1.6.3.4), and 5) SR-mediated activation or repression of transcriptional initiation (reviewed in 1.6.3.5).

However, the precise mechanism of steroid hormone regulation of gene transcription is still not fully characterised. Enzymes which metabolise particular hormones have been identified which play a critical role in the fate of a specific hormone (reviewed in 1.6.5.1). There is also increasing evidence that nucleosome phasing plays an integral role in steroid receptor mediated control of transcription and that SRs may function as nucleosome displacement factors (reviewed in 1.6.4.3). Finally, a number of reports indicate that accessory proteins exist which modulate SR-SRE interaction (reviewed in 1.6.4.4).

1.6.2 The steroid hormone receptor superfamily

Analysis of SR structure is essential for understanding how SRs mediate transcriptional control. Before SRs were cloned, biochemical and immunochemical analysis of purified rat liver GR provided the first suggestion of a domain structure for SRs. GR is composed of three domains which can be separated by limited proteolytic digestion (Wrange & Gustafsson, 1978; Carlstedt-Duke et al., 1982). Two domains contain information for DNA-binding and steroid hormone binding, which function

independently of each other (Wrange & Gustafsson, 1978), and the third domain contains a major epitope for immunoreactivity (Carlstedt-Duke et al., 1982).

After the molecular cloning of GR and subsequently other receptors (see 1.6.1 for cloning references), it became possible to carry out functional, structural and comparative analysis of SR molecules. Perhaps not surprisingly, the SRs are all structurally related. It is now clear that the steroid hormone binding domain is C-terminally located and the DNA-binding domain is found in the middle of the receptor molecule (see Fig. 1.7) (reviewed by Evans, 1988; O'Malley, 1990; Green & Chambon, 1988; Beato, 1989). Throughout all the SRs these two domains, in particular the DNA-binding domain, share maximal sequence homology (see above reviews). The N-terminal domain of SRs is hypervariable with respect to both sequence and length; this non-conserved region corresponds to the major immunoreactivity epitope of GR, discussed above. Chambon's group have subdivided the three regions into six sub-regions (A-F) based on varying levels of homology (Krust et al., 1985) (Fig. 1.7). Thus, regions C and E are commonly used to denote the DNA-binding and steroid binding domains, respectively.

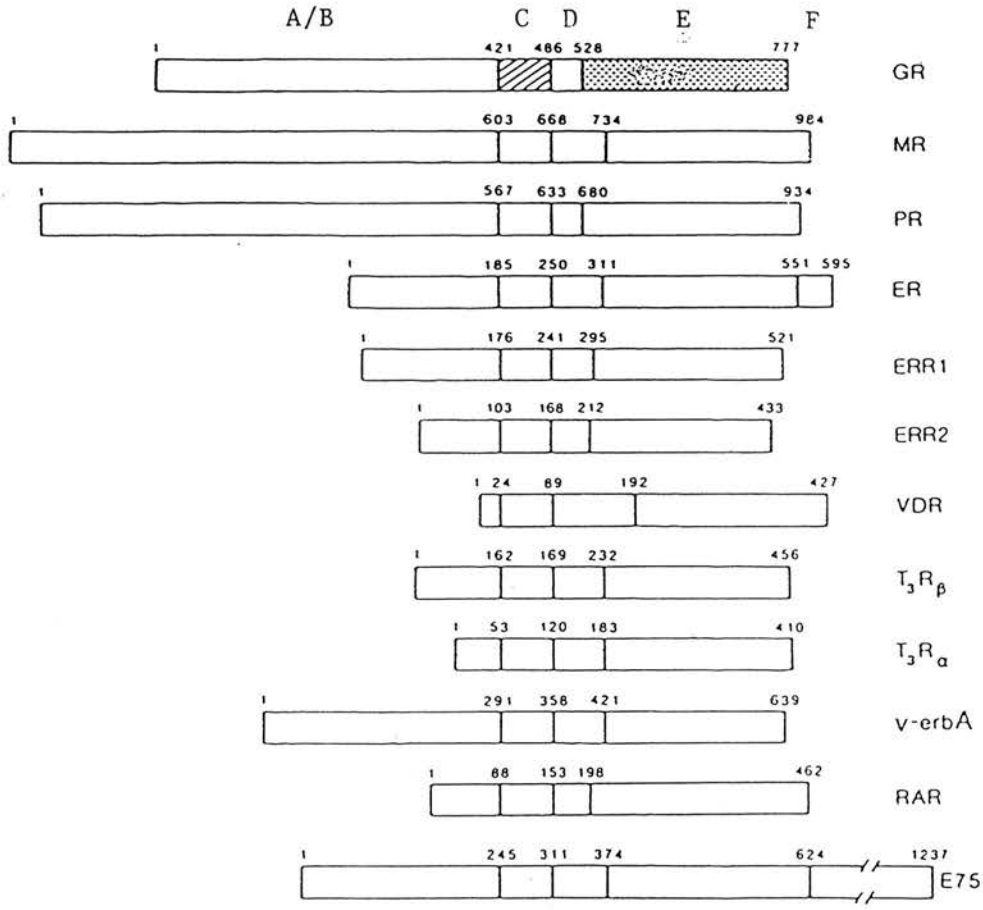


Fig 1.7 Schematic representation of members of the steroid hormone receptor superfamily. Receptors have been aligned by the DNA binding domain (shown as a hatched box for GR). Numbers correspond to amino acid residues, and the amino acid positions of the boundaries of the DNA binding domain (hatched box for GR) and the ligand binding domain (stippled box for GR) are shown. (Adapted from Evans, 1988).

The existence of a superfamily of nuclear receptors that respond to ligands other than steroid hormones, was suggested by the finding that the GR DNA-binding domain bore remarkable similarlity to the viral oncogene

v-erbA (Weinberger et al., 1985) and by the subsequent identification of *c-erbA* as the receptor for thyroid hormone (Weinberger et al., 1986; Sap et al., 1986). Molecular cloning and sequence analysis of two more TRs (Miyajima et al., 1989), at least three receptors for retinoic acid (RAR) (Zelent et al., 1989), and the vitamin D receptor (VDR) (McDonnell et al., 1987) show that they are all superfamily members (reviewed by Evans, 1988 and O'Malley, 1990).

Other superfamily members have been identified, although their activating ligand is unknown. For this reason, these superfamily members are referred to as 'orphans' (O'Malley, 1990). Orphans have been identified either by low stringency hybridisation between receptor cDNA and either cDNA or genomic libraries, or by cloning other transcription factors to find that they too bear structural similarity to the SRs. Orphans include two ER-related proteins called ERR1 and ERR2 (Giguere et al., 1988), COUP transcription factor (Wang et al., 1989), the NGF-induced NGFI-B protein (Milbrandt, 1988), the drosophila gene encoded by E75 locus (Seagraves & Hogness, 1990) and the drosophila gene tailess (Pignoni et al., 1990). O'Malley (1990) predicts that these orphan receptors are activated by ligands indigenous to the cells in which particular orphans are found.

It appears then that the steroid hormone receptor superfamily is ever expanding and it will be of particular interest to find out exactly what ligands activate different superfamily members.

1.6.3 Functional Domains of Steroid Receptors

1.6.3.1 The ligand binding domain

Steroid hormone binding to receptor is absolutely essential for the *in vivo* activity of the wild type SR (Denis et al., 1988a; Becker et al., 1986).

Upon hormone binding, the receptor is transformed from a non DNA-binding 8-9S state containing the inhibitory protein Hsp90, to an active 4-5S state, from which Hsp90 is dissociated (Denis et al., 1988a and 1989 and references therein). SR transformation affects all aspects of characterised receptor activity which includes receptor dimerisation (section 1.6.3.2), nuclear localisation (section 1.6.3.3), DNA-binding (section 1.6.3.4) and transcriptional activation (section 1.6.3.5).

The steroid binding domain (region E, Fig. 1.7) alone confers steroid dependence on SR activity. Transferring the hormone binding domain of GR to the DNA binding domain of prokaryotic factor LexA (Godowski et al., 1988) or yeast GAL4 (Webster et al., 1988), or fusing it to the adenovirus E1A gene product (Picard et al., 1988), confers strict glucocorticoid regulation on each factor. Similarly, fusion of the ER steroid binding domain to c-Myc (Eilers et al., 1989) or GAL4 (Webster et al., 1988) imposes oestrogen dependence on these factors. In fact, deletion of this domain creates a mutant receptor which constitutively binds DNA, although it retains only 5% of wild-type transcriptional activity (Kumar et al., 1987).

The steroid binding domain contains all the information necessary for high affinity hormone binding (Kumar et al., 1986; Rusconi & Yamamoto, 1978; Gronemeyer et al., 1987). Mutational analysis of the hormone binding domain of GR (Giguere et al., 1986; Rusconi & Yamamoto, 1987), ER (Kumar et al., 1986) and PR (Dobson et al., 1989) reveal that integrity of the entire domain is necessary for efficient steroid binding. Photoaffinity labelling of GR with radioactive hormone, identifies three hydrophobic amino acids within the steroid binding domain, which directly interact with hormone (Carlstedt-Duke et al. (1988). The reacting amino acids reside in distinct hydrophobic segments of the steroid binding domain; this hydrophobicity is

conserved throughout GR, ER, PR and c-erbA (Carlstedt-Duke et al., 1988). Fawell et al. (1990) demonstrate that amino acids involved in steroid binding overlap with a conserved hydrophobic region which is involved in oestrogen receptor dimerisation (reviewed in 1.6.3.2). Fawell et al. (1990) propose that by analogy to the structure defined for dimeric uteroglobulin which binds progesterone (Bally & Delettre, 1989), the steroid binding domain of SRs forms a hydrophobic pocket at the dimerisation interface, within which the steroid hormone binds. This proposal assumes that conserved hydrophobic residues are important for pocket structure and non-conserved residues determine specific ligand binding.

1.6.3.1.1 The non-transformed SR

When SRs are not induced by hormone, they reside mainly in the cytosol in a non-transformed, multimeric 8-9S state capable of binding steroid hormone but unable to bind to DNA (Dalman et al., 1989; Denis et al., 1988a). These 8-9S non-transformed SRs contain the 90kD heat shock protein, Hsp90 (Jaob et al., 1984; Renoir et al., 1990 and references therein) and probably other non-SR proteins (Kost et al., 1989; Bagchi et al., 1990 and references therein).

Hsp90 interaction with the GR steroid binding domain has been demonstrated. Limited trypsin digestion of crude GR preparations yields a 27kD fragment which does not bind DNA, but does bind steroid (Wrange & Gustafsson, 1978). In the presence of molybdate, a metal ion which stabilises the labile Hsp90/SR interaction, the 27kD steroid binding fragment is immunoprecipitated by anti-Hsp90 antibodies (Dennis et al., 1988b). As SRs are highly conserved, it is therefore likely that Hsp90 interacts with each SR through their steroid binding domains. However, the number of Hsp90

molecules associated with each SR may differ from one receptor to another. Non-transformed cytosolic GR probably contains one molecule of GR and two molecules of Hsp90 (Mendel & Orti, 1988; Bresnick et al., 1990), whereas immunological data suggest that two ER molecules are associated with two Hsp90 molecules in the non-transformed ER complex (Redeuilh et al., 1987).

Several lines of evidence suggest that Hsp90 association with SR generates or stabilises a SR conformation capable of binding steroid. High salt conditions which disrupt Hsp90/ER complexes also destroy steroid binding capacity in cytosols (Bresnick et al., 1988), and glucocorticoid binding capacity of immunopurified GR correlates with the relative concentration of Hsp90 (Bresnick et al., 1988). Further, GR *in vitro* translated in reticulocyte lysate is associated with endogenous Hsp90, during or immediately at termination of translation, and binds glucocorticoid with high affinity; GR expressed in wheat germ lysates, where there is no identifiable Hsp90, does not bind steroid (Dalman et al., 1989).

1.6.3.1.2. SR transformation

Hsp90 association with SR inhibits DNA-binding activity (Groyer et al 1987; Denis et al., 1988a) and it is, therefore, generally accepted that Hsp90 dissociation is required to transform SR from an inactive to active DNA-binding state (see Groyer et al., 1987; Kost et al., 1989; Dalman et al., 1989; Denis et al., 1988a for examples). Several lines of evidence indicate that steroid binding promotes Hsp90 dissociation. *In vitro,* molybdate-stabilised Hsp90/GR complexes cannot bind DNA unless treated with dexamethasone (Dalman et al., 1989; Denis et al., 1988a). *In vivo,* protein/DNA interactions within GREs of the tyrosine aminotransferase gene can be detected only

after hormone activation of endogenous GR (Becker et al., 1986). In contrast, TR, which does not associate with Hsp90, does bind DNA *in vitro,* in the absence of hormone (Dalman et al., 1990).

Recent evidence, however, demonstrates that Hsp90 dissociation alone is not sufficient to attain a transcriptionally active SR (Bagchi et al., 1990). Thermal or high salt treatment can mediate Hsp90 dissociation (see Bagchi et al., 1990; Dalman et al., 1989 for examples). However, the 4S form of PR devoid of Hsp90, recovered in nuclear salt extracts prepared from hormone-untreated cells, cannot activate transcription *in vitro* unless stimulated by hormone (Bagchi et al., 1990). Thus, steroid hormone may be required to induce further allosteric alterations to generate an active receptor or remove other inhibitory proteins which may mask receptor domains essential for activation. There are, in fact, several reports of SR association with other proteins whose functions are not yet defined (Kost et al., 1989; Tai et al., 1986; Sanchez et al., 1990 and references therein); one or more of these proteins may mask essential SR functional domains which are also exposed upon steroid binding.

1.6.3.1.3 Steroid independent DNA-binding *in vitro*

In vivo, steroid hormone is absolutely required for SR DNA-binding and transactivation. However, several reports demonstrate DNA-binding and *in vitro* transcription by purified SRs in the absence of hormone treatment (Bailly et al., 1986; Tsai et al., 1988; Klein-Hitpass et al., 1990). It is conceivable that during purification, SR is purified from inhibitory proteins normally associated with non-transformed receptor. Alternatively, *in vitro* manipulations may accomplish an irreversible conformational change in the SR, converting it to a fully transformed state. *In vivo,* such a conformational

change and/or inhibitory protein dissociation from SR would be absolutely dependent upon steroid binding.

There are also conflicting reports concerning steroid dependence on DNA-binding of SRs in crude protein extracts. For example, *in vitro* DNA-binding of ER expressed in HeLa WCE (Kumar & Chambon, 1988), or of liver cytosol GR (Willmann & Beato, 1986), is dependent on hormone treatment, whereas ER translated in reticulocyte lysate can bind DNA in a hormone independent manner (Fawell et al., 1990). This disparity probably reflects the conditions in which cells are grown prior to extract preparation, the method by which protein extracts are prepared, the labile nature of Hsp90/SR complex or a combination of all three.

1.6.3.2 Steroid receptor dimerisation

As the SRE is a palindromic sequence (see section 1.6.3.4.2), it has been speculated for some time that SRs bind to their target DNA sites as protein dimers (Scheidereit et al., 1986; Chalepakis et al., 1988b. There is now direct evidence showing that human ER (Kumar & Chambon, 1988), rat GR (Wrange et al., 1989) and rabbit PR (Guichon-Mantel et al., 1989) homodimerise in solution, in a hormone-dependent manner, and bind to their respective SRE as dimers.

Analysis of dimer formation reveals that the information for dimerisation is predominantly contained within the steroid binding domain of the receptor. Proteolytically generated fragments of ER which contain only the hormone binding domain can independently form homodimers in solution (Sabbah et al., 1989). Most recently, mutagenesis of a small N-terminal region in the hormone binding domain of mouse ER identified a heptad repeat of hydrophobic residues, the integrity of which is essential to

both receptor dimerisation and steroid hormone binding (Fawell et al., 1990). The dimerisation and steroid binding activities within this region overlap, but are discrete; dimerisation amino acids are located N-terminally and residues important for steroid binding are located towards the C-terminus of the heptad repeat (Fawell et al., 1990).

Comparison of amino acid sequences shows that the heptad repeat is conserved throughout members of the nuclear receptor superfamily (Fawell et al., 1990). Scrutiny of the amino acid sequence encompassing the ER dimerisation motif reveals certain features in common with the leucine zipper (see section 1.2.4), the helix-loop helix (see section 1.2.5) and the uteroglobulin (Bally & Delettre, 1989) dimerisation motifs (Fawell et al., 1990). The three-dimensional structure of uteroglobulin has been characterised by X-ray diffraction analysis (Bally & Delettre, 1989). Two uteroglobulin monomers interact to form a globular protein with two-fold symmetry centred around the dimer interface, and forming a hydrophobic pocket within which one molecule of steroid hormone is proposed to bind (Bally & Delettre, 1989). Fawell et al. (1990) propose that SR dimerisation occurs via a novel structure that resembles the dimerisation structure of uteroglobulin. This is consistent with the finding that ER dimerisation and steroid binding activities overlap.

The demonstration of *in vitro* receptor dimerisation occurring when steroid binding ability is abolished (Fawell et al., 1990) contrasts with the strict hormone dependence of receptor dimerisation reported by other workers (Kumar & Chambon, 1988; Guichon-Mantel et al., 1989; Wrange et al., 1989). However, Fawell et al. (1990) do report that, although observed receptor dimerisation is hormone-independent, mutation of amino acids important for steroid binding reduces the level of ER dimerisation. Steroid

binding (within the proposed hydrophobic pocket) is probably necessary to stabilise receptor dimers.

Deletion of the dimerisation domain within the steroid binding domain of ER, vastly reduces DNA-binding activity, but does not completely abolish it (Fawell et al., 1990; Kumar & Chambon, 1988). Kumar & Chambon (1988) have proposed that there is a second, weak dimerisation function in the DNA-binding domain of ER, since dimers between wild type and steroid binding domain-truncated ERs still form and bind to target DNA. Härd et al. (1990a) have constructed a model of the interaction between a GR DNA-binding domain dimer and DNA. In this model, amino acids in the second zinc finger are involved in protein-protein interactions between GR DNA-binding domain monomers. Consistent with this model is the observation that purified GR DNA-binding domain binds to DNA *in vitro* as a homodimer (Tsai et al., 1988). This GR dimerisation is dependent on the initial binding of one GR DNA-binding domain monomer to DNA followed by cooperative binding of a second monomer.

1.6.3.3 Nuclear localisation

Proteins enter the nucleus by one of two mechanisms; 1) the protein harbours a nuclear localisation signal which directs it to the nucleus, probably by interaction with constituents of the nuclear pore, or 2) the protein diffuses through the nuclear membrane and is then trapped by binding to an intranuclear component (reviewed by Hunt, 1989; Dingwall & Laskey, 1986).

The subcellular localisation of the steroid hormone receptors has been the subject of many studies over the years, yielding conflicting results. Initially, it was observed that, when cell homogenates were prepared from

animals lacking hormone, the corresponding receptor was recovered in the soluble fraction. After hormone administration, the activated SR complexes were found attached to chromatin. The hypothesis was then put forward that receptor is cytoplasmic in location and migrates into the nucleus when activated by hormone (Gorski et al., 1968).

The advent of monoclonal antibodies for various SRs and their use in immunocytochemical procedures allowed the cellular localisation of receptors to be defined. Ligand-free GR is localised in the cytoplasm (Wikstrom et al., 1987; Picard & Yamamoto, 1987). However, receptors for progesterone (Perrot-Applanot et al., 1985), oestrogen (King & Greene, 1984) and probably androgens (Trapman et al., 1988) appear to be localised also in the nucleus, even in the absence of hormone. Other superfamily members, VD3 and TR, are located in the nucleus bound to chromatin in the absence of activating ligand (Walters et al., 1981). Thus, nuclear translocation of GR would appear to differ mechanistically from nuclear localisation of other receptors.

In the SV40 large T-antigen, a seven amino acid nuclear localisation sequence is sufficient to direct T-antigen to the nucleus (Kalderon et al., 1984; Landford et al., 1986). A T-antigen-like sequence has been defined in the hinge region, or region D (see Fig. 1.7) of both the rabbit PR (Guiochon-Mantel et al., 1989) and the rat GR (Picard & Yamamoto, 1987), which mediates receptor nuclear localisation.

For PR, the T-antigen-like sequence acts as a constitutive nuclear localisation signal; deletion of 5 amino acids within this sequence renders the PR cytoplasmic. However, such a cytoplasmic mutant PR can be shifted to the nucleus on addition of hormone, indicating the existence of a distinct hormone-dependent nuclear localisation signal (Guiochon-Mantel et al.,

1989).

Similarly, two nuclear localisation signals, NL1 and NL2, have been defined in GR, although both appear to be hormone-dependent (Picard & Yamamoto, 1987). NL1 constitutes the T-antigen-like sequence and NL2 is located within the steroid binding domain of GR. Deletion of the GR steroid hormone binding domain renders this mutant nuclear, revealing that the T-antigen-like sequence NL1 is, in fact, a constitutive nuclear localisation signal, analogous to T-antigen-like activity of PR (Picard & Yamamoto, 1987). Perhaps in wild type GR, NL1 activity is masked by the steroid binding domain or inhibitory proteins associated with non-transformed GR (see section 1.6.3.1.1). In either case, steroid binding would be required to expose NL1 activity, thus NL1 appears hormone-dependent. The second GR nuclear localisation signal, NL2, is strongly hormone-dependent. In fact, fusion of 258 amino acids of GR, which includes NL2, to β-galactosidase creates a fusion protein which exhibits strict hormone-dependent nuclear localisation (Picard & Yamamoto, 1987).

Perhaps for all SRs, there is a hormone-dependent and a hormone-independent nuclear localisation signal. Indeed, the T-antigen-like sequence is conserved in region D of all SRs (Guiochon-Mantel et al., 1989). Guiochon-Mantel et al. (1989) propose an eight amino acid consensus sequence, rich in basic residues, which is sufficient to mediate constitutive nuclear localisation of SRs.

1.6.3.4 The DNA-binding domain

The DNA-binding domain of SRs, region C (diagram 1.7), is a small (66 to 68 amino acid) domain located in the middle of the SR molecule. This domain alone is sufficient for DNA-binding *in vitro* (Rusconi &

Yamamoto, 1987; Kumar & Chambon, 1988), and deletion of region C abolishes DNA-binding activity (Kumar et al., 1986). However, additional information encoded within N- and C-terminal sequences are required for high affinity DNA-binding as demonstrated by deletion analysis (Danielsen et al., 1987).

Region C, which is highly basic and rich in cysteine residues, contains two copies of the zinc finger motif (reviewed in 1.2.3). EXAFS spectroscopy confirmed that region C of GR binds two zinc ions; furthermore, zinc incorporation is essential for DNA-binding activity (Freedman et al., 1988). Recently, Härd et al. (1990a) demonstrated, by NMR, that a peptide encoding the GR DNA-binding domain does form a ββα zinc finger structure in solution, which is similar to, but distinct from, the ββα zinc finger of TFIIIA (see section 1.2.3 for review and finger structures).

The DNA-binding domain encodes information essential for target gene specificity of the SR; substituting ER region C with GR region C creates a hybrid receptor which activates transcription from a GRE but not from an ERE (Green & Chambon, 1987). Mutational analysis within region C of either ER (Mader et al., 1989) or GR (Umesono & Evans, 1989; Danielsen et al., 1989) demonstrates that the ability of these receptors to discriminate between the closely related ERE and GRE (see section 1.6.3.4.2 for review of SREs) resides in two amino acids located between the two C-terminal cysteines of the N-terminal zinc finger (Fig. 1.8).

		GRE	ERE
GR	CLVCSDEASGCHYGVLTCGSC	√	–
	⇊		
	EG	–	√
ER	CAVCNDYASGYHYGVWSCEGC	–	√
	⇊		
	GS	√	–

Fig. 1.8 The amino acid sequence of the first zinc finger of both GR and ER DNA-binding domains are shown. Mutant receptors in which the two 'specificity' amino acids of GR or ER were substituted with 'specificity' residues of ER or GR, respectively, are indicated below each parent receptor; only mutated amino acids are shown. The ability of parent or mutant receptor to transactivate transcription from a GRE or ERE-containing promoter are indicated to the right of amino acid sequences. (Data taken from Mader et al., 1989 and Danielsen et al., 1989).

Additional amino acids within this finger region are also required for maximal DNA-binding activity although they do not appear to alter target gene specificity (Mader et al., 1989).

Härd et al. (1990a) have proposed a model for GR DNA-binding domain interaction with DNA, in which the two amino acids that determine specificity are located at the beginning of the α-helix which directly interacts with DNA. The consensus ERE and GRE are very similar, differing by only two nucleotides within each arm of the response element (see 1.6.3.4.2). It is conceivable that the two 'specificity' amino acids interact directly with the distinguishing nucleotides of either response element, and that other amino acids aligned along the α-helix interact with nucleotides conserved between

the ERE and GRE.

Mutational analysis of the second zinc finger indicates that this region is also required for efficient DNA-binding; deletions extending into this region abolish transcriptional activation (Danielsen et al., 1987; Hollenberg et al., 1987). Umesono & Evans (1989) show that replacing the 'specificity' amino acids of GR with those of TR (which are identical to ER), together with five amino acids located between the N-terminal cysteines of the C-terminal finger, creates a hybrid receptor which activates from a consensus TRE; a hybrid receptor with just the 'specificity' residues replaced activates only from an ERE and not from a TRE. Umesono & Evans (1989) therefore propose that residues in this region of the second zinc finger are involved in discriminating between half site spacing of the SRE; the consensus TRE encodes identical conserved nucleotides to the consensus ERE, but does not retain the 3bp spacer (see section 1.6.3.4.2). In the DNA-GR interaction model (Hard et al., 1990), the C-terminal zinc finger is involved in protein-protein dimer interaction. Perhaps for TR, which can bind both an ERE and a TRE (Glass et al., 1988), the second finger sequence allows formation of a flexible dimer which can 'open' and 'close' to accommodate response elements with different half site spacing. This flexibility would not be allowed by second finger sequences of the ER and GR.

1.6.3.4.1 Promiscuous DNA-binding of SRs

Identification of the two amino acids critical for specificity (see section 1.6.3.4) divides the steroid hormone receptor superfamily (see section 1.6.2) into sub-families (Mader et al., 1989; Danielsen et al., 1989). Receptors for mineralocorticoids, progestins and androgens all contain the identical 'specificity' residues to GR and constitute one subfamily. This reflects the

fact that GR, PR, AR and MR can all activate transcription from a GRE (for examples see Strahle et al., 1987; Cato et al., 1988; Chalepakis et al., 1988a; Arriza et al., 1987; Ham et al., 1988; Otten et al., 1988). Similarly, receptors for TR, RAR and VDR have the same 'specificity' residues as ER, which may indicate that these receptors all recognise similar response elements. Indeed, hormone response elements which can mediate thyroid, retinoic acid or vitamin D_3 responsiveness share the same conserved arms as the consensus ERE, except that each exhibits differential spacing between the arms (see section 1.6.3.4.2). TR can bind to a consensus ERE and mediate transcriptional repression (Glass et al., 1988), and there is evidence that TR and RAR recognise the same response element (Umesono et al., 1988).

This promiscuous DNA-binding behaviour observed amongst certain members of the steroid hormone receptor superfamily contrasts with the hormone-specific activation of genes in specialised cell types. This may be explained, in part, by the differential expression of SRs in particular cell types. For example, introduction of PR by transfection into glucocorticoid responsive hepatoma cells, confers progesterone responsiveness upon several endogenous glucocorticoid regulated genes (Strahle et al., 1989). This strongly suggests that simply the lack of PR in liver cells prevents progesterone from activating glucocorticoid responsive genes. However, this does not explain why endogenous GR does not activate progesterone, androgen or mineralocorticoid responsive genes. Perhaps such genes are packaged into inactive chromatin (see section 1.4); thus, the SREs in question will be inaccessible to active GR. Human ER transfected into oestrogen-nonresponsive kidney cells can induce transcription from an exogenous ERE-containing promoter, but is unable to activate endogenous

vitellogenin genes in the presence of oestrogen (Seiler-Tuyns et al., 1988). This is probably because the endogenous vitellogenin genes are located within inactive chromatin.

Promiscuous DNA-binding of SRs to response elements of genes activated by different steroid hormones may also be minimised by the differential ability of SRs to recognise distinct features of a common response element. For example, although PR and GR binding to a GRE is very similar (Chalepakis et al., 1988a; Slater et al., 1988), there are also distinct differences (von der Ahe et al., 1986; Cato et al., 1988; Chalapakis et al., 1988a). These differences are probably functionally relevant to hormone-specific gene activation *in vivo,* as mutations in the MMTV GRE differentially affect induction by progesterone or glucocorticoid (Chalepakis et al., 1988a). Also, it is becoming clear that SRs contain promoter-specific and cell-type specific transcriptional activation domains (reviewed in section 1.6.4.1) that restrict the transcriptional activity of certain SRs to particular promoters and cell-types. Thus, differential DNA-binding of SRs to a common SRE, together with their promoter and cell-type specific transcriptional activation activity, may explain, in part, why a particular SR only induces transcription of specific genes.

1.6.3.4.2 The steroid response element

The steroid hormone response elements defined for the various steroid hormones are short DNA sequences, consisting of 5 or 6bp inverted repeats (arms) separated by a 3bp spacer. DNA-binding studies show that the SRE is recognised specifically by purified SR *in vitro* (Compton et al., 1983; Payvar et al., 1981; Maurer & Notides, 1987; Klein-Hitpass et al., 1989), and gene-transfer experiments demonstrate that the SRE is required

for hormone-dependent transcriptional modulation of transfected genes *in vivo* (for examples see Klock et al., 1987; Strahle et al., 1987; Klein-Hitpass et al., 1988).

Comparative analysis of DNA sequences responsible for oestrogen inducibility of the vitellogenin genes, the chicken apo VLDLII gene (Walker et al., 1984; Klein-Hitpass et al., 1986) and the rat prolactin gene (Maurer & Notides, 1987) have allowed the identification of a 13bp ERE consensus sequence. The ERE consensus sequence comprises a 5bp inverted repeat of conserved nucleotides GGTCA separated by a non-conserved 3bp spacer (see Fig. 1.9). Similarly, a consensus sequence responsible for glucocorticoid and progesterone inducibility has been deduced by comparative analysis of a number of responsive genes (Compton et al., 1983; Sheidereit et al., 1986; Strahle et al., 1987). The 15bp consensus GRE/PRE consists of a 6bp inverted repeat of conserved nucleotides AGAACA separated by a non-conserved 3bp spacer (see Fig. 1.9). As can be seen, the consensus ERE and GRE are similar in both structure and, in fact, the consensus ERE can be converted to a functional GRE by only two base pair changes in either arm of the SRE (see Fig. 1.9) (Klock et al., 1987; Martinez et al., 1987).

		Oestradiol	Dexamethasone
ERE consensus	AGGTCACAGTGACCT TCCAGTGTCACTGGA	√	X
	↓ ↓ AGGACACAGTGTCCT TCCTGTGTCACAGGA	X	X*
	↓ ↓		
GRE consensus	AGAACACAGTGTTCT TCTTGTGTCACAAGA	X	√

*minimal responsiveness to dexamethasone was detected

Fig. 1.9 The consensus ERE and GRE; nucleotide substitutions convert an ERE into a GRE. Plasmids containing the 15bp oligonucleotide inserted upstream of the HSV tk promoter/CAT gene fusion were transfected ± oestradiol or dexamethasone. Responsiveness to hormone (√); non-responsiveness to hormone (X). (Data from Klock et al., 1987).

The consensus ERE or GRE alone is able to confer specific hormone inducibility on a linked promoter *in vivo* (Klein-Hitpass et al., 1988; Klock et al., 1987; Martinez et al., 1987; Strahle et al., 1987). However, although consensus EREs are found in the *Xenopus laevis* vitellogenin A1 and A2 genes and in the chicken vitellogenin II gene (Klein-Hitpass et al., 1986; Walker et al., 1984), the EREs found in *Xenopus laevis* vitellogenin B1 and B2 genes (Walker et al., 1984; Martinez et al., 1987; Seiller-Tuyns et al., 1986), human pS2 gene (Berry et al., 1989) and the rat prolactin gene (Maurer & Notides, 1987) all deviate from the consensus sequence. This deviation reflects the decreased ability of such response elements to behave as independent transcriptional enhancers *in vivo*: Alone, neither the vitellogenin B1 EREs (Martinez et al., 1987) nor the rat prolactin ERE (Maurer & Notides, 1987) can confer oestrogen inducibility on a linked

promoter, the human pS2 ERE functions as a less efficient transcriptional enhancer than does the consensus ERE (Berry et al., 1989). It would appear that imperfect EREs require additional flanking DNA to elicit their full transcriptional effects (for example see Maurer & Notides, 1987; Waterman et al., 1988), although a combination of two imperfect palindromes can act co-operatively as an efficient oestrogen-dependent enhancer *in vivo* (Martinez et al., 1987). Similarly, imperfect GRE/PREs resulting from deviations in the GRE consensus sequence behave as less efficient hormone dependent transcriptional enhancers *in vivo* (see for example Kock et al., 1987; Strahle et al., 1987; Cato et al., 1988).

Although consensus response elements for other classes of steroid hormone have not been identified, cell transfection studies have shown that a GRE/PRE is also capable of functioning as a response element for androgens and mineralocorticoids (Ham et al., 1988; Otten et al., 1988; Cato et al., 1988; Arizza et al., 1987).

A consensus thyroid hormone-responsive element has been postulated which contains a 5bp inverted repeat identical to that of the consensus ERE, but no 3bp spacer (Glass et al., 1988). The consensus TRE alone can confer both thyroid hormone and retinoic acid-dependent transcriptional enhancement on a linked promoter (Umesono et al., 1988). However, the consensus TRE is not found endogenously in either thyroid hormone or retinoic acid-responsive genes studied so far and, therefore, the exact DNA sequence requirements for responsiveness of genes to thyroid hormone or retinoic acid are unknown. Indeed, the laminin B1 retinoic acid response element , which deviates from the consensus TRE and exhibits differential spacing between the conserved arms, can only confer retinoic acid-dependent transcriptional enhancement and is not influenced by

thyroid hormone (Vasios et al., 1989). A vitamin D_3 response element has also been identified in the 5'-flanking region of the osteocalcin gene that resembles the consensus ERE, although this response element contains three conserved arms, each separated by a 1 bp spacer (Morrison et al., 1989).

Thus, the hormone response elements can be divided into two groups: elements that mediate response to glucocorticoids, progesterone, androgens and mineralocorticoids, and elements that mediate response to oestrogens, thyroid hormones, retinoic acid and vitamin D_3. These two groups reflect the two subfamilies of the steroid hormone receptor superfamily reviewed in 1.6.3.4.1: GR, PR, AR and MR all encode the 'GS' pair of 'specificity' amino acids and interact with the GRE, whereas ER, TR, RAR and VDR encode the 'EG' pair of 'specificity' amino acids which are distinct from the first subfamily, and mediate transcriptional effects through ERE-like sequences.

1.6.4 SR Mediate Transcriptional Activation and Repression

1.6.4.1 SR mediated activation of transcription

As reviewed in section 1.3.3, DNA-bound transcriptional activators enhance basal transcription by interacting with a component of the transcription machinery. This interaction, mediated via the activation domain of the transcriptional activator, is proposed to initiate or stabilise formation of an active transcription initiation complex (see section 1.3).

Identification of transcriptional activation domains in SRs has been the subject of controversy and it now appears that this is because SRs contain multiple activation functions located in different regions of the SR molecule. Two experimental approaches have been used to determine the

location of SR activation domains: 1) examining the transcriptional activity of SR deletion mutants in *in vivo* transfection studies and 2) transferring putative activation domains to heterologous 'anchor' DNA-binding domains and examining the transcriptional activity of these chimeric proteins *in vivo*. As a result, three areas of the SR have been described which contain transcriptional activation domains; these are the central DNA-binding domain, the N-terminal A/B domain, and the C-terminal steroid binding domain.

The DNA-binding domain alone of either GR (Hollenberg et al., 1987; Schena et al., 1989) or PR (Klein-Hitpass et al., 1990) can transactivate transcription of a reporter plasmid in a cell transfection study, or from a synthetic PRE-containing template in an *in vitro* transcription assay, respectively. This demonstrates the existence of a transcriptional activation function within region C (see Fig. 1.7) of both GR and PR, although additional activation functions are required as wild-type GR or PR exhibits a higher level of transcriptional enhancement. Within the DNA-binding domain of GR, DNA-binding and transcriptional activation activities can be separated by mutation; two single amino acid substitutions in the tip of the C-terminal zinc finger abolish transcriptional activation but do not affect DNA-binding (Schena et al., 1989). In contrast, no such transcriptional activation function within region C of ER exists. The DNA-binding domain alone of ER cannot mediate transcriptional activation in cell transfection studies (Kumar et al., 1987; Kumar & Chambon, 1988), nor can a chimeric protein containing region C of ER fused to the DNA-binding domain of GAL4 (Webster et al., 1989).

Deletions within the N-terminal region or C-terminal region of GR (Danielsen et al., 1987; Hollenberg et al., 1987), ER (Kumar et al., 1987;

Kumar & Chambon, 1988) and PR (Gronemeyer et al., 1987) indicate that these regions also contain transcriptional activation domains. Fusion proteins containing the A/B region of GR or ER fused to a heterologous DNA-binding unit demonstrate that the activation domain in this region behaves constitutively (Godowski et al., 1988; Hollenberg & Evans, 1988; Tora et al., 1989). In contrast, fusion of the steroid binding domain of GR or ER to a heterologous DNA-binding unit reveals that this region encodes a hormone-dependent transcriptional activation function; such chimeric proteins only activate transcription after hormone treatment (Hollenberg & Evans, 1988; Picard et al., 1988; Eilers et al., 1989; Webster et al., 1988; Godowski et al., 1988).

The activation functions located in regions A/B and E of the GR are both acidic in character and are proposed to behave in a similar manner to that proposed for other acidic activators (Hollenberg & Evans, 1988; see section 1.3.3). However, both the N- and C-terminal activation regions of ER are distinct from acidic activation domains (Tora et al., 1989) and it is likely that the mechanism by which ER activates transcription differs from acidic activator mediated transcriptional regulation.

Nevertheless, despite the different mechanisms proposed for transcriptional activation mediated by GR or ER, all SRs probably interact with a component of the transcription machinery and initiate or stabilise the formation of a transcription initiation complex (see section 1.3). Klein-Hitpass et al. (1990) demonstrate that PR acts as a hormone-dependent transcriptional activator by facilitating the formation of a stable initiation complex. Pre-incubation of a minimal template containing a PRE and a TATA element with HeLa whole cell extract results in formation of a stable and functional initiation complex only when purified PR is added

(Klein-Hitpass et al., 1990).

1.6.4.1.1 Promoter- and cell-type specific transcriptional activation domains

The N-terminal activation domain of ER has been implicated in promoter-specific transcriptional activation. N-terminally deleted ER, transfected into HeLa cells, can activate transcription from a co-transfected reporter plasmid containing the vitellogenin A2 gene ERE linked to the HSV tk promoter, but not from either the human pS2 gene promoter (Kumar et al., 1986) or from an ERE linked to the adenovirus 2 major late promoter (Ad2MLP) (Tora et al., 1989). This indicates that the A/B transcriptional activation domain of ER is differentially required for transcriptional activation of distinct promoters.

Cell-type specific transcriptional activation by the N-terminal and C-terminal activation domains of different SRs has also been observed. C-terminally deleted ER mutants can activate transcription from an ERE linked to the Ad2MLP in chicken embryo fibroblast cells but not in HeLa cells (Tora et al., 1989). Thus, some factor which acts through the N-terminal activation domain of ER, and is required for ER-mediated transcriptional induction of the ERE-Ad2MLP promoter construct, must be missing from HeLa cells but present in chicken embryo fibroblast cells.

In nature, PR receptor exists in two forms, PRa and PRb, the former corresponding to an N-terminally truncated PRb (Gronemeyer et al., 1987). Interestingly, PRa can mediate transcriptional enhancement from a reporter plasmid containing the chicken ovalbumen gene whereas PRb cannot (Tora et al., 1988). It therefore appears that the presence of the N-terminal domain in PRb blocks transcriptional activation of this promoter. The mechanism by which PRa does, and PRb does not, activate transcription

from the chicken ovalbumen promoter is not clear, as both receptors can activate this same promoter in HeLa cells (Tora et al., 1988).

There is probably a differential distribution of 'adaptor' molecules with which different activation domains of the SRs interact and ultimately contact a component of the transcription machinery. Defining the mechanism by which SRs interact with the transcription machinery will undoubtedly help explain the mechanism behind cell-type and promoter-specific gene expression.

1.6.4.2 SR-mediated repression of gene transcription

In addition to the myriad of genes which are positively regulated by steroid hormones (cited throughout section 1.6; see also Beato et al., 1989 for review), several genes are down-regulated by steroids. In most documented cases, SR-mediated transcriptional repression of certain genes is conferred after glucocorticoid treatment. Genes repressed by glucocorticoids include the bovine prolactin gene (Camper et al., 1985), the proopiomelanocortin gene (Charron & Drouin, 1986), the human glycoprotein hormone α-subunit gene (Akerblom et al., 1988) and the stromelysin gene (Firsch & Ruley, 1987). Although the precise mechanism(s) by which glucocorticoids repress gene transcription are not fully understood, both silencing (reviewed in 1.3.4.2) and steric hindrance (reviewed in 1.3.4.1) are involved in steroid-dependent transcriptional repression of certain genes.

A 500bp upstream regulatory region has been defined in the bovine prolactin gene which behaves as a glucocorticoid-dependent transcriptional silencer (Sakai et al., 1988). This region contains multiple binding sites for purified GR (Sakai et al., 1988). Functional analysis of these GR binding

sites led to the definition of a negative GRE (nGRE) which alone can confer glucocorticoid-dependent repression from a linked promoter, *in vivo* (Sakai et al., 1988). A nGRE has also been defined upstream of the rat proopiomelanocortin (POMC) gene; this region is essential for glucocorticoid-mediated transcriptional repression and also binds purified GR (Drouin et al., 1989). Both defined nGREs, although related to the consensus 'positive' GRE, differ in nucleotide sequence from the consensus GRE. It is proposed that the different nucleotide sequence of the nGRE may result in different receptor-DNA interactions which somehow alter the structure of bound GR, thereby preventing it from functioning as an activator (Sakai et al., 1988).

However, the incapacitation of GR activating ability cannot account entirely for transcriptional repression; GR binding must somehow interfere with the activity of transcriptional activators required for gene expression. Indeed, in the absence of glucocorticoids, the bovine prolactin gene nGRE enhances promoter activity, presumably by the action of activators which bind the same region (Sakai et al., 1988). In rat POMC, the region of DNA encoding the nGRE also contains a putative binding site for a CCAAT-binding protein (Drouin et al., 1987). Thus, in the presence of glucocorticoids the nGRE may compete with overlapping 'positive' DNA elements for binding of distinct transcription factors. This mechanism of steric hindrance is well-documented for glucocorticoid-mediated transcriptional repression of the human gonadotrophin α-subunit gene (Akerblom et al., 1988; reviewed in section 1.3.4.1). The TR has been shown to mediate both ligand-dependent and ligand-independent transcriptional repression, depending on the nucleotide sequence and structure of its binding site. When transfected into CV-1 cells, the

unliganded TR suppresses transcription from a co-transfected TRE-containing reporter construct; upon hormone treatment, transcriptional repression ceases and transcription is stimulated (Damm et al., 1989). Interestingly, the oncogenic derivative of TR, v-*erb*-A, which does not contain a ligand binding domain, and therefore does not bind ligand (Sap et al., 1986; Weinbergeret al., 1986), behaves as a constitutive transcriptional repressor in the presence or absence of ligand (Damm et al., 1989). It therefore appears that ligand binding to TR is required to expose or create a transcriptional activation domain.

In contrast, TR, which can bind to an ERE *in vitro,* represses transcription from an ERE-containing promoter *in vivo* only when stimulated by thyroid hormone (Glass et al., 1988). This repression occurs in the absence of oestrogen stimulation and is, therefore, unlikely to involve competition between TR and ER for binding to the common response element. Thus, the mechanism(s) by which liganded or unliganded TR represses transcription is poorly understood. Purportedly, TR binding somehow interferes with formation of a functional transcriptional initiation complex at the linked promoter.

1.6.5 Additional Factors which Influence Transcription by Steroid Receptors

1.6.5.1 Enzymes that metabolise steroid hormones

As discussed earlier (section 1.6.3.4.1), receptors for glucocorticoids, progestin, androgens and mineralocorticoids can all activate transcription through a common response element.

A second order of promiscuous behaviour has been observed in MR which can bind and be transformed by mineralocorticoid (aldosterone) and

glucocorticoids (cortisol and corticosterone) (Krozowski & Funder, 1983; Arriza et al., 1987). However, MRs present in classical mineralocorticoid target tissues (kidney, colon and parotid) are selective for aldosterone binding and show minimal glucocorticoid binding *in vivo* (Funder et al., 1988 and references therein). It is now clear that glucocorticoid insensitivity, and so mineralocorticoid specificity in these tissues, is achieved by the presence of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD). 11β-HSD specifically metabolises glucocorticoids and the resulting metabolites are unable to bind to either MR or GR (Funder et al., 1988).

Agarwal et al. (1989) claim to have cloned the cDNA of 11β-HSD and show that it hybridises with a single mRNA species in mineralocorticoid target tissues and additional tissues which include liver and testis. However, a recent review (Funder, 1990) casts doubt on this claim and proposes that the cDNA cloned by Agarwal et al. (1989) encodes an 11β-HSD-related enzyme and that 11β-HSD and Agarwal's enzyme constitute members of a multigene family. Indeed, Southern blot analysis using the cloned cDNA suggests that a number of enzyme-related genes exist in rat and human (Agarwal et al., 1988). It is, therefore, tempting to speculate that GR activity is controlled by selective enzyme activity in a number of cell-types.

In conclusion, target tissue specificity is not merely dictated by the presence of specific SR but also by the expression of enzymes which metabolise specific steroids.

1.6.5.2 The SRE in the nucleosome

The influence of chromatin structure on transcription factor activity has been reviewed in section 1.4. In summary, the nucleosome appears to

behave as a transcriptional repressor and nucleosome-free regions, called nuclease hypersensitive sites, are often associated with transcriptionally active DNA. The finding that nucleosomes are phased along certain studied promoters invites speculation that some target DNA sites are always accessible to transcription factor binding, whereas others are always inaccessible.

The MMTV long terminal repeat reproducibly acquires a series of six positioned nucleosomes (A-F) when introduced into mammalian cells, in the absence of hormone (Richard-Foy & Hager, 1987). Upon glucocorticoid treatment, one of these nucleosomes, NucB, is displaced, which coincides with the appearance of a 190bp hypersensitive region encompassing the normally NucB-associated DNA (Richard-Foy & Hager, 1987).

MMTV DNA associated with NucB includes the MMTV GRE and a binding site for NF-1 (Cordingley et al., 1987). NucB can be reassembled *in vitro* and can be bound by purified GR (Pearlmann & Wrange, 1988). In contrast, *in vivo* NF-1 binding can only be detected after hormone stimulation of MMTV-infected murine cells and NucB displacement (Cordingley et al., 1987). However, NF-1 concentration in infected cells is apparently unaffected by hormone treatment, and NF-1 is present in a form capable of binding, with high affinity, to naked MMTV DNA *in vitro* (Cordingley et al., 1987).

Hager's group has developed a cell-free system in which nucleosomes A and B are specifically reassembled on MMTV DNA. NF-1 cannot bind to its recognition site when that site is accurately positioned on NucB, whereas GR can bind and induce displacement of NucB (T. Archer & G. Hager, personal communication). Hager's group propose that GR, through binding to the MMTV GRE exposed on NucB, acts as a nucleosome

displacement factor. NucB displacement thus allows NF-1 access to its DNA site, which was previously concealed within NucB.

Interestingly, inducible hypersensitive sites are frequently associated with steroid hormone regulated genes; the hypersensitive sites often appearing during hormone stimulation (for examples see Burch & Weintraub, 1983; Fritton et al., 1983; Becker et al., 1986; Jantzen et al., 1987; Cordingley et al., 1987; reviewed by Gross & Garrard, 1988). Perhaps one function of the SR is to behave as a nucleosome displacement factor, so allowing transcription factor recognition of previously inaccessible DNA sites.

1.6.5.3 The role of accessory proteins in SR DNA-binding affinity

Accessory proteins can modulate the DNA-binding activity of a particular transcription factor for its target DNA site. The role of accessory proteins in altering either the DNA-binding affinity or sequence specificity of a particular factor has been reviewed in section 1.5.

Purified SRs have been shown to bind directly to their respective SRE *in vitro*; however, the discrimination between a specific binding site and non-specific DNA may be less than 10-fold (see for example Maurer & Notides, 1987; Payvar et al., 1981; Compton et al., 1983; Bially et al., 1986; von der Ahe et al., 1985; Jantzen et al., 1987; Peale et al., 1988; Härd et al., 1990b). This indirectly suggests that other cellular proteins, in addition to the purified receptor itself, are required in order to achieve the high affinity SR-SRE interaction necessary for efficient steroid responsiveness, *in vivo*.

There is mounting evidence which suggests that the affinity of a particular SR for its response element can be modulated by accessory

proteins, *in vitro*. Such accessory proteins have been implicated in increasing the sequence-specific DNA-binding affinity of purified ER (Feavers et al., 1987), GR (Payvar & Wrange, 1983), PR (Edwards et al., 1989) and the SR related TR (Murray & Towle, 1989). Furthermore, crude preparations of PR will bind with higher affinity to DNA already complexed with other nuclear proteins, rather than to naked DNA (Thrall & Spelsberg, 1980), and electron microscopy demonstrates that ER bound to DNA forms a large protein complex (Heggeler-Bordier et al., 1987). The role of accessory proteins in SR DNA-binding activity will be discussed in more detail throughout chapters 3, 4 and 5.

The aim of this thesis was to investigate whether accessory proteins (which are not SRs) can bind directly to a SRE and thus modulate the steroid receptor SR-SRE interaction.

This thesis now reports the identification and characterisation of a steroid response element-binding protein (SRE-BP). The SRE-BP binds preferentially to either an ERE or a GRE *in vitro* and does not recognise a palindromic TRE, nor transcription factor binding sites present in the Herpes Simplex Virus thymidine kinase promoter.

Circumstantial evidence reported in this thesis suggests that the SRE-BP, present in HeLa whole cell extracts, behaves as an accessory protein and increases the affinity of *in vitro* translated ER for its response element. Additionally, the demonstration of *in vitro* translated ER binding to DNA as part of a 362kD complex, and not simply a 130kD receptor dimer (Kumar & Chambon, 1988; Fawell et al., 1990), further substantiates the proposal that accessory proteins are involved in SR binding to DNA.

CHAPTER 2

MATERIALS AND METHODS

2.1 STANDARD SOLUTIONS

2.1.1 Gel electrophoresis buffers

10 x TBE:

108.9g Tris
55.7g Boric acid
4.7g EDTA
Make up to 1000ml with dH_2O

10 x TAE:

8.11g Tris (pH 7.9)
2.7g N-acetate
3.72g EDTA
Make up to 1000ml with dH_2O

5 x SDS Electrode Buffer:

30g Tris
144g Glycine
5.0g SDS
2.7g EDTA
Make up to 1000ml with dH_2O

Gel Juice:

0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol
1mM EDTA
30% glycerol

2.1.2 Bacterial Growth Media

L-Broth:

10g tryptone
5g yeast extract
5g NaCl
Make up to 1000ml with dH_2O and autoclave

L-Agar:

L-broth to which 1% Bacto-Agar (Difco) is added

10 x M9

10g NH_4Cl
100g $Na_2HPO_4.7H_2O$
30g KH_2PO_4
50g NaCl
to 100ml with dH_2O

2.1.3 General solutions

TE:

10mM Tris HCl (pH8), 0.1mM EDTA

10 x PBS:

80g NaCl
2.0g KCl
11.4g Na_2HPO_4
2.0g KH_2PO_4
Make up to 1000ml with dH_2O and autoclave

Tris-saturated phenol

Frozen phenol was thawed by heating to 60°C. Phenol was then extracted 2-3 times with 0.2M Tris HCl (pH 8.0) until buffer pH was > 7.0. Phenol was extracted twice with 0.1M Tris HcL and finally stored at 4°C under a layer of 0.1M Tris HCl (pH 8.0).

2.2 GEL ELECTROPHORESIS

2.2.1 Horizontal agarose gel electrophoresis

Agarose gels were used to visualise linear DNA restriction fragments or supercoiled plasmid DNA. If a DNA restriction fragment was to be recovered from the agarose gel, low melting point agarose (Sea Plaque agarose, FMC) was used.

The percentage of agarose to be used was determined by the size range of linear DNA fragments to be separated. One per cent (w/v) agarose was routinely used to separate DNA fragments of ~7kb to 0.56kb; 1.5% to 2% (w/v) agarose was used to separate smaller DNA fragments of ~1kb to 0.1kb. The correct percentage agarose solution was prepared by dissolving agarose in 100mls $^1/_2$ x TBE buffer in a microwave oven. The agarose solution was then poured into a 11cm x 14cm gel former with a comb in place - the tooth size of the comb depended on sample size - and allowed to set. The gel was placed in a horizontal gel electrophoresis apparatus (Gibco BRL, Model HS) containing sufficient $^1/_2$ x TBE buffer to

just cover the gel. 0.1 volume of Gel Juice was added to DNA samples before loading onto the gel with a Gilson pipette. Gels were usually electrophoresed at 60mA for as long as required to see sufficient separation of DNA fragments. After electrophoresis, gels were stained in Ethidium Bromide ($10mgml^{-1}$) for 5 minutes in order to visualise DNA fragments using a UV transilluminator emission wavelength 312nm. If required, gels were photographed using Polaroid film in a Polaroid Cu-5 camera.

2.2.2 Vertical gel electrophoresis

Glass plates used for vertical gel electrophoresis were soaked in dilute Fairy Liquid solution, scrubbed with a soft nylon brush, rinsed 5 times with dH_2O, and twice with 70% ethanol before use.

2.2.2.1 Denaturing polyacrylamide gel electrophoresis

A 20% denaturing polyacrylamide solution was prepared by dissolving 21g Urea (Aristar, BDH) in 20ml 50% (19:1) bis:acrylamide (Biorad, premixed), 2.5ml 10 x TBE and 300µl 10% ammonium persulphate. Distilled water was added to a final volume of 50ml. The polyacrylamide solution was filtered through Whatman #1 filter paper and degassed using a vacuum pump, before adding 30µl TEMED (BDH). The solution was immediately poured into a 15cm x 17cm x 0.8mm glass sandwich, which was held together with bulldog clips. A 20 tooth comb was inserted and the solution left to polymerise. After polymerisation, the gel was attached to a perspex vertical gel box (Gibco BRL, Model V16) containing $^1/_2$ x TBE. The wells were flushed out with buffer to remove unpolymerised acrylamide/urea solution and the gel was pre-electrophoresed at 35mA for 30 minutes.

One volume of STOP solution (formamide to which 0.03% xylene cyanol, 0.03% bromophenol blue and 20mM EDTA had been added) was added to DNA samples to be electrophoresed, which were then heated to

60°C for 10 minutes. Samples were loaded onto the gel using a Gilson pipette with an elongated pipette tip (multiflex duckbill tips, Bioquote) and electrophoresed for 30 to 40 minutes at 35mA. Radiolabelled samples were visualised by autoradiography (section 2.3).

2.2.2.2 4% Native polyacrylamide gel electrophoresis

A 4% native polyacrylamide gel solution was prepared by mixing 4ml 50% 19:1, bis:acrylamide (Biorad, premixed) with 2.5ml of either 10 x TBE or 10 x TAE, 300µl 10% ammonium persulphate, in a final volume of 50ml. The solution was filtered through Whatman #1 filter paper before adding 30µl TEMED (BDH) and poured immediately into a 15cm x 17cm x 1.5mm glass sandwich which had been previously sealed with 1% (w/v) agarose (dissolved in water); a 20-tooth comb was placed in the top of the gel between the glass plates. After polymerisation, the 4% polyacrylamide gel was attached to vertical gel electrophoresis apparatus (Gibco BRL, Model V16) containing either $^1/_2$ x TBE buffer or $^1/_2$ x TAE buffer respectively and pre-electrophoresed for 30 minutes at 200V. Gels which contained $^1/_2$ x TAE buffer were electrophoresed in circulating buffer. Using a Gilson pipette and an elongated pipette tip (Multiflex round tip, Bioquote), band shift reactions (section 2.8.1) were loaded onto the gel while the gel was electrophoresing, which minimised disruption of any protein/DNA complexes. The gel was run for 45-60 minutes at 250V. Following electrophoresis, the gel was transferred to Whatman #1 paper, covered with Saran wrap and dried using a heated vacuum gel drier (Biorad). Protein/DNA complexed were visualised by exposing the dried gel for 24h to autoradiographic film (section 2.3).

2.2.2.3 Native pore gradient gel electrophoresis

Two different percentage (3% and 25%) polyacrylamide solutions were made as follows: a 3% or 25% solution was prepared by mixing 1.65ml or 13.75ml respectively, of 40% 79:0.8:0.2, acrylamide:bis-acrylamide (BDH):diallyltartardiamide (Sigma) with 1.1ml 10 x TBE, 100µl 10% ammonium persulphate and dH_2O to 22ml. Each solution was separately filtered through Whatman #1 filter paper and degassed under vacuum before adding 7.5µl TEMED (BDH). The solutions were immediately poured into separate chambers of a gradient former, taking care not to trap air between the chambers, which was connected to a peristaltic pump (LKB). As the 25% polyacrylamide solution was pumped from its chamber into an agarose-sealed 15cm x 17cm x 1.5mm glass sandwich at a rate of 2.6mls/minute, the 3% polyacrylamide solution was pulled into this chamber and mixed with a magnetic stirrer to form the pore size gradient. A 20 tooth comb was inserted into the top of the gel, between the glass plates, which was then left to polymerise.

The gradient gel was attached to vertical gel electrophoresis apparatus (Gibco BRL, Model V16) and pre-electrophoresed overnight at 75V in 0.5 X TBE at 4°C. Protein standard markers were loaded onto the gradient gel. The voltage was increased to 300V and band-shift reactions (section 2.8.1) were loaded immediately, in adjacent lanes to markers. Pore gradient gel electrophoresis was carried out at 300V for 5h at 4°C (1500 volt hours). Following electrophoresis, the gel was stained with Coomassie Brilliant Blue R (section 2.6.4.1) to visualise protein standards. The gradient gel was then transferred onto Whatman #1 blotting paper, covered with Saran wrap and dried using a heated vacuum gel drier (Biorad). Protein/DNA complexes were visualised by autoradiography (section 2.3); their molecular weight was calculated by relating the

electrophoretic mobility of radioactive-DNA/protein complexes to the electrophoretic mobility of the protein standard markers.
The precise size of mol. wt. markers used differed depending on their source.

2.2.2.4 SDS polyacrylamide gel electrophoresis

A 10% SDS polyacrylamide separating gel solution was prepared by mixing 8ml of filtered 30% 37.5:1, acrylamide:bis-acrylamide (Biorad, premixed) with 6ml of filtered separating buffer (1.5M Tris HCl (pH 8.8), 8mM EDTA, 0.4% SDS), 200µl 10% ammonium persulphate and dH_2O to 24ml. After adding 30µl TEMED (BDH) the 10% SDS acrylamide solution was poured into a 15cm x 17cm x 0.8mm glass sandwich held together with bulldog clips. The gel was overlaid with water-saturated butan-2-ol. Once polymerised the butan-2-ol was removed and the gel surface was washed several times with dH_2O.

A 5% SDS polyacrylamide stacking gel solution was prepared by mixing 1ml of filtered 37.5:1,acrylamide:bis-acrylamide (Biorad, premixed) with 1.25ml of filtered stacking buffer (0.5M Tris HCl (pH 6.8), 8mM EDTA, 0.4% SDS), 100µl 10% ammonium persulphate and dH_2O to 6ml. After adding 10µl TEMED (BDH), the stacking gel solution was poured on top of the separating gel, a 20 tooth comb was inserted and the stacking gel was left to polymerise. The polymerised gel was attached to vertical gel electrophoresis apparatus (Gibco, Model V16) containing 1 x SDS electrode buffer (section 2.1).

Protein samples were incubated for 10 minutes at 60°C with 0.25 volumes of 4 x sample incubation buffer (0.5M Tris HCl (pH 6.5), 8% SDS, 0.2M EDTA, 40% (v/v) glycerol). 2-3µl of gel juice were added and samples were loaded onto the SDS polyacrylamide gel using a Gilson pipette with an elongated pipette tip (multiflex duckbill tips, Bioquote). Samples were electrophoresed at 70V overnight. The gel was silver stained (section

2.6.4.1) if required, dried using a heated vacuum gel drier and autoradiographed (section 2.3) if necessary.

2.3 AUTORADIOGRAPHY

Detection of radiolabelled material was carried out at -70°C using either Kodak X-omat 5 X-ray film (18cm x 24cm) or Agfa Curix RP-1 X-ray film (24cm x 30cm). X-ray film was placed inside autoradiography cassettes which contained 2 x or 4 x intensifying screens and was in direct contact with either hydrated gels covered with a sheet of Saran wrap or dried gels. Exposed film was developed for 2 minutes in Kodak LX-24 developer (0.25 dilution), rinsed in water, fixed for one minute in Kodak FX-40 fixer (0.25 dilution), rinsed once more in water and left to dry.

2.4 CLONING AND DNA TECHNIQUES

2.4.1 Parent plasmids used in plasmid construction

All plasmids constructed for use in this thesis (listed in Table 2.1) were derived from pBLCAT2 (Luckow & Schutz, 1987).

Plasmids from which fragments were taken and inserted into the pBLCAT2 polylinker were as follows:

1) (a) pKC264-14: pUC18 into which one 21bp ERE_{con} oligonucleotide (oligonucleotide sequence in Chapter 3, Fig. 3.1a) was cloned (Dr. K.E. Chapman).

   (b) pKC264-04: pUC18 into which one 21bp ERE_{con} oligonucleotide was cloned in opposite orientation to pKC264-14 (Dr. K.E. Chapman).

2) pKC264-13: pUC18 into which two 21bp ERE_{con} oligonucleotides had been cloned (Dr. K.E. Chapman).

3) pKC246: M13mp19 derivative containing one ERE_{con} generated by site-specific mutagenesis of *lac* operator sequences (Dr. K.E. Chapman).

Table 2.1: Plasmids constructed and used in this thesis

NAME	DESCRIPTION
pLC4a	pBLCAT2 with 21bp ERE_{PRL}oligonucleotide (Fig. 3.1b) cloned into *BamHI* site
pLC4b	pBLCAT2 with 2 x 21bp ERE_{PRL}oligonucleotide cloned into *BamHI* site
pLC5a	pBLCAT2 with 56bp *BamHI - HindIII* fragment of pKC264-14
pLC5b	pBLCAT2 with 68bp *BamHI - HindIII* fragment of pKC264-13
pGRE32*	pBLCAT2 with one 21bp GRE oligonucleotide (Fig. 3.1c) cloned into *BamHI* (filled) site
pGRE3	pBLCAT2 with two 21bp GRE oligonucleotides cloned into *BamHI* (filled) site
$pGRE_M$ 13	pBLCAT2 with one 20bp GRE_M oligonucleotide (Fig. 3.1d) cloned into *BamHI* (filled) site
$pGRE_M$11	pBLCAT2 with two 20bp GRE_M oligonucleotide cloned into *BamHI* (filled) site
pLC3	pBLCAT2 with 30bp *HindIII - BamHI* fragment replaced with 179bp *PvuII - HindIII* fragment of pKC246

*plasmids were constructed and supplied by Dr. K.E. Chapman, MRC Brain Metabolism Unit, University Department of Pharmacology, Edinburgh University.

2.4.2 Plasmid construction

pLC4a and pLC4b were constructed as follows: 10µg pBLCAT2 was digested with 10 units *BamHI* in a 20µl reaction containing 2µl 10 x restriction buffer (recipe, Table 2.2) at 37°C for 1-2h. This reaction was diluted 2-fold to reduce salt concentration; digested plasmid was then dephosphorylated by adding 1µl bacterial alkaline phosphatase (2500 units, $17\mu l^{-1}$; BRL) and incubating for 1h at 65°C. Digested, phosphatased vector was purified from a low melting point agarose gel (section 2.4.4). 500pmol

of each strand of the ERE_{PRL} oligonucleotide (see Fig. 3.1b for sequence) was phosphorylated with 5µl 10mM ATP (Sigma) in a 50µl reaction containing kinase buffer (recipe, section 2.7.1) and 10 units T4 polynucleotide kinase (NBL, 12 units μl^{-1}) at 37°C for 45 mins. Enzyme was then inactivated at 65°C for 10 mins and complementary phosphorylated oligonucleotides were annealed (see section 2.7.1). Phosphatased vector and phosphorylated ERE_{PRL}oligonucleotide were ligated (section 2.4.6) using 100-fold excess of oligonucleotide over vector. Competent cells were transformed with ligation mix (section 2.4.7). Restriction analysis (section 2.4.3) of DNA minipreps (section 2.4.8) confirmed insertion of either one or two ER_{PRL} oligonucleotides inserted in pLC4a, and pLC4b, respectively. Large scale plasmid preparations (section 2.4.9) were then carried out.

pLC5a and pLC5b were constructed as follows: 5µg of pBLCAT2 (vector) and 5µg pKC264-13 (containing 2 x ERE_{con} oligonucleotide, section 2.4.1) or pKC264-14 (containing 1 x ERE_{con} oligonucleotide, see 2.4.1) were digested with *BamHI* and *HindIII* (section 2.4.3). Vector and insert fragments (see 2.4.1) were purified from a low melting point agarose gel (see section 2.4.4). Purified digested pBLCAT2 vector and pKC264-13 or pKC264-14 insert fragments were ligated (section 2.4.6). pLC5a and pLC5b constructions were checked by restriction analysis (section 2.4.3). The insert sequence of pLC5b was determined by sequencing of pKC264-13 by Dr. K.E. Chapman, (insert sequence presented in Chapter 5: Fig 5.1 (DF2)).

pLC3 was constructed as follows: 5µg pBLCAT2 vector was digested with *HindIII* (see section 2.4.3). After inactivating enzyme activity at 65°C for 10 mins, sticky ends were filled in with 2µl dNTPs (mixture of 2mM each of dTTP, dCTP, dATP, dGTP) and 0.5µl Klenow (large fragment of DNA polymerase I) (1000 units $17\mu l^{-1}$; Gibco, BRL or BCL) at room temperature for 15 mins before inactivating enzyme activity (see 2.4.3). Linearised, filled

vector was then digested with *BamHI.* pKC246 was digested with *PvuII* and *BamHI* (section 2.4.3). Insert and vector fragments were purified from a low melting point agarose gel (section 2.4.4) and ligated (section 2.4.6). pLC3 construct was checked by restriction analysis. Sequence of pLC3 insert was as follows:

PvuII
↓
CAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCG

CAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGC

TTTACACTTTATGCTTCCGGCTCGTATAATGTGTGGAATTGG

TCACTGTGACCAATTTCACACAGGAAACAGCTATGACCATGA

TTACGCCAAGCTT
↑
HindIII

Conserved nucleotides of ERE_{con} sequence are indicated by horizontal lines. Insert sequence was determined by sequencing of pKC246 by Dr. K.E. Chapman

2.4.3 Restriction endonuclease digestion

Table 2.2

Restriction Endonucleases and Reaction Buffers

Restriction Endonuclease[b]	Source	Recognition site[a]
EcoRI	NBL, BRL or BCL	G↓AATTC
HindIII	"	A↓AGCTT
BamHI	"	G↓GATCC
AluI	"	AG↓GT
PvuII	"	CAG↓CTG

a. In each case, the sequence is shown 5' to 3' and the point of cleavage is denoted by a downward arrow.

b. Reaction Buffer in which enzymes were used 1: 10 x restriction buffer: 500mMNaCl, 500mM Tris HCl (pH 8.0), 100mM $MgCl_2$, 6mM β-mercaptoethanol, 1μgμl^{-1} Bovine Serum Albumin (DNAse free, BRL).

Plasmids and oligonucleotides used for cloning are described above (section 2.4.1 and Table 2.1) and oligonucleotide sequences are given in Chapter 3, Fig. 3.1. The restriction enzymes and reaction buffers used for plasmid digestion are listed in Table 2.2.

DNA was digested using 1 unit of restriction enzyme per 1μg of DNA, 1 x restriction buffer (Table 2.2) and dH_2O to the required volume. Digestion reactions were incubated at 37°C for 1-2h. Enzyme activity was terminated by heating digests to 60°C for 10 mins and digested DNA was visualised after electrophoresis through an agarose gel (section 2.2.1).

For investigatory purposes, 0.2-0.4μg of DNA was routinely digested in a final volume of 10μl. For cloning purposes, 5-10μg of plasmid DNA

were routinely digested in a final volume of 50µl. The required restriction fragments were recovered after electrophoresis of the entire 50µl digest through a low melting point agarose gel (section 2.2.1 and 2.4.4).

Molecular weight markers routinely used were AluI fragments of pBR322 which give a range of mol. wt. sizes between 910bp and 100bp.

2.4.4 Purifying DNA fragments from low melting point agarose gels

DNA restriction fragments were visualised as described (section 2.2.1). A gel slice containing the required DNA fragment was excised with a scalpel blade, placed into a 1.5ml Eppendorf tube and heated at 70°C for 10-15 minutes or until the agarose was completely melted. 100µl of Tris-saturated phenol was added, vortexed for 10 seconds and then incubated at -70°C for 5-10 minutes. After centrifugation for 10 minutes in an Eppendorf bench top centrifuge, the aqueous phase, which contained the DNA fragment, was carefully removed to a fresh Eppendorf tube. The aqueous phase was extracted three more times to remove residual agarose as follows: once with 0.5 volumes tris-saturated phenol, once with 0.5 volumes tris-saturated phenol and 0.5 volumes chloroform:isoamylalcohol (24:1, v/v), then finally with 0.5 volumes chloroform:isoamylalcohol; each extraction was centrifuged for 2 mins in the Eppendorf bench-top centrifuge. The DNA fragment was then ethanol precipitated and its recovery checked (section 2.4.5).

2.4.5 Ethanol precipitation of DNA

The DNA sample was vortexed for 10 seconds with 0.15 volumes 5M NaCl or 0.05 volumes 8M ammonium acetate, and 2.5 volumes of ethanol, put on dry ice for 5-10 minutes, then centrifuged in an Eppendorf bench top centrifuge for 10 minutes. The DNA pellet was dried briefly at 37°C for

approximately 30 mins then resuspended in the required volume of TE. If the pelleted DNA had been purified from a low melting point agarose gel, it was usually resuspended in 20µl of TE. Recovery of precipitated DNA was then checked by electrophoresing an aliquot (1-2µl or 5-10%) of the total recovered DNA through an agarose gel (section 2.2.1).

2.4.6 DNA ligation

The DNA restriction fragments to be ligated were recovered from a low melting point gel as described in section 2.4.1. Ligation reactions were performed in a total volume of 10µl containing 1µl 10 x ligation buffer (50mM Tris HCl (ph 7.5), 10mM $MgCl_2$,1mM DTT), 1mM ATP pH 7.0, 0.5 units T4 DNA ligase (BCL) and either a 1:3 or 1:6 molar ratio of vector DNA:insert DNA. Ligation reactions were incubated at room temperature overnight. Note: Enzymes used for plasmid digestion all generated DNA fragments with sticky ends which, in some cases, were filled in (section 2.4.2). To ligate sticky ends, 0.5 units of T4 DNA ligase in 10µl total volume was sufficient; to ligate blunt-ended DNA fragments, 2.5 units of T4 DNA ligase was used.

2.4.7 Preparation of competent cells and transformation with recombinant plasmid DNA

Escherichia coli strain HB101 was used for competent cell production and subsequent plasmid transformation.

Genotype: (F^- hsd S20 (r^-_B m^-_B) rec A13, ara I4, pro A2, lac YI, gal K2, rps L20, xyl 5, sup E44)

A single colony of HB101, grown overnight to saturation in L-broth was diluted approximately 50-fold into 40ml of fresh L-broth and grown in an orbital shaker at 37°C to A_{600} of 0.4 to 0.7. The cells were harvested by centrifugation at 6000rpm in a Sorvall SS34 rotor for 5 minutes at 4°C. The

pellet was gently resuspended in 10ml of ice cold 0.1M $CaCl_2$, left on ice for 10 mins and centrifuged as before. The pellet was gently resuspended in 2ml of ice cold 0.1M $CaCl_2$ and stored on ice for > 2h to acquire full competence.

Competent cells were transformed as outlined in Maniatis et al. (1982). Either 1-5µl of ligation mix or 10ng of plasmid DNA in TE, were mixed with 200µl of competent cells and incubated on ice for up to 30 minutes. Cells were heat shocked at 37°C for 5 minutes then returned to ice. Immediately 100µl-200µl aliquots were spread on selective media (L-agar containing 0.1mgml^{-1} sodium ampicillin) using a glass spreader. Plates were allowed to dry for a few minutes with the lids off, before incubating them overnight at 37°C. Colonies that grew on the selective media represented cells which had been successfully transformed by recombinant DNA bearing the ampicillin resistance marker. Single colonies were picked with a tooth pick and streaked out onto fresh selective media and grown overnight at 37°C. Plates with purified transformed colonies were sealed with parafilm and stored at 4°C for up to three months.

2.4.8 Preparation of small quantities of plasmid DNA (Alkaline Lysis Method, Maniatis et al 1982)

1.5ml of selective growth medium (L-broth + 0.1mgml^{-1} sodium ampicillin) was inoculated with a single transformed bacterial colony and grown overnight at 37°C on a rotator. The overnight culture was placed in an Eppendorf tube and cells were pelleted for 1 minute in an Eppendorf bench top centrifuge. The bacterial cell pellet was resuspended in 100µl of ice cold GTE buffer (50mM glucose, 25mM Tris HCl (pH 8.0) 10mM EDTA), containing approximately 4mgml^{-1} lysozyme (Sigma) then gently mixed with 200µl of freshly prepared alkaline-SDS solution (0.2M NaOH, 1% SDS) and stored on ice for 5 minutes. Ice cold potassium acetate, 150µl (3M with

respect to potassium and 5M with respect to acetate, pH 4.8) was mixed in by flicking, and the Eppendorf tube was stored on ice for 5 min. The sample was then centrifuged in an Eppendorf bench top centrifuge for 5 mins to remove denatured chromosomal DNA, cellular proteins and high molecular weight RNA. The supernatant was transferred to a new Eppendorf tube and extracted with 0.5 volumes of tris-saturated phenol/0.5 volumes chloroform:isoamyl alcohol (24:1 v/v). After centrifuging for 2 mins as above, the aqueous phase was ethanol precipitated (section 2.4.5) with 2 volumes of ethanol at room temperature for 5 mins. After centrifuging for 5 mins in an Eppendorf bench top centrifuge, the pellet was dried at 37°C for approximately 30 mins and resuspended in 50µl TE containing 20µgml^{-1} DNase-free pancreatic RNase (Sigma), (prepared by heating at 100°C for 10 mins). 10µl was removed for restriction analysis (section 2.4.3) and the remainder was stored at -20°C.

2.4.9 Preparation of large quantities of plasmid DNA

L-broth, approximately 2ml, containing 0.1mgml^{-1} ampicillin, was inoculated with a single transformed bacterial colony and grown overnight at 37°C on a rotator. The overnight culture was diluted into 40ml of selective minimal medium (0.3% casamino acid, 1XM9, 2ml 1M $MgSO_4$, 1ml 0.01% thiamine, 0.5ml 0.1M $CaCl_2$, 12.5ml 20% glucose, 0.1mgml^{-1} sodiumampicillin, dH_2O to 500ml), grown at 37°C to A_{600} of 0.4-0.7 before adding to 460ml of selective minimal medium. The 500ml transformed *E.coli* culture was grown overnight in an orbital shaker at 37°C then centrifuged at 6000rpm for 5 minutes at 4°C in a Sorvall GSA rotor. The bacterial cell pellet was resuspended in 12ml of ice-cold GTE buffer (50mM glucose, 25mM Tris HCl (pH 8.0), 10mM EDTA) containing approximately 4mgml^{-1} lysozyme (Sigma). Immediately after, 24ml of freshly prepared alkaline-SDS (0.2M NaOH, 1% SDS) was added and the suspension was

stored on ice for 5 minutes. Ice-cold potassium acetate: 16ml, pH 4.8 (3M with respect to potassium and 5M with respect to acetate) was added and the sample was stored on ice for a further 10 mins before centrifuging at 6000rpm for 10 minutes at 4°C in a Sorvall GSA rotor. The resulting yellow supernatant (and as little of the flocculent pellet as possible) was strained through butter muslin into 250ml centrifuge pots. Plasmid DNA was allowed to precipitate out of solution by adding 32ml of isopropanol and leaving at room temperature for 30 mins; plasmid DNA was recovered by centrifuging at 10,000rpm for 2 mins at 4°C in a Sorvall GSA rotor. The DNA pellet was dried at room temperature for 30 mins before resuspending in 7ml of TE. Following resuspension, 8g of CsCl and 0.2ml of Ethidium Bromide (10mgml^{-1}) were then added. The DNA/CsCl solution was transferred into an 11.5ml PK50 Sorvall ultracentrifuge tube and centrifuged at 33,000rpm for 48-72 hours at 20°C in a Sorvall Ti65 rotor. Plasmid DNA bands were clearly visible after centrifugation; they were collected through the tube wall using a syringe and 21G needle and transferred to a new ultracentrifuge tube. Fresh CsCl solution (prepared by adding 100g CsCl to 100ml TE) was added to the recovered plasmid DNA band, enough to fill the tube, and the sample was centrifuged as before for 48-72h. The visible plasmid band was collected through the tube wall as before and transferred to a glass test tube. Ethidium bromide was removed from the plasmid DNA by extracting several times with 0.2 volumes of TE-saturated isopropanol (until aqueous and organic phases were no longer pink). The plasmid preparation was dialysed for 24h against three 2 litre changes of TE, at 4°C.

The concentration of recovered plasmid was calculated by measuring its A_{260} in a Shimadzu UV60 spectrophotometer. Plasmid preparations were stored at -20°C.

2.5 TISSUE CULTURE GROWTH CONDITIONS

HeLa, GH_3 and CV-1 cells were maintained (by myself or Ms. E. Clark) on 75cm^2 tissue culture flasks (J. Bibby Science Products) in DMEM (Dalbecco's Modification of Eagle's Medium; Flow Laboratories) containing 10% foetal calf serum (Globefarm) which had been heat inactivated at 56°C for 30 mins to denature proteases; 2mM L-Glutamine (Gibco, BRL) and 100 units Penicillin/100μg Streptomycin ml^{-1} (Penicillin/Streptomycin solution: 10,000units/10,000μgml^{-1}; Gibco, BRL).

Confluent cells were harvested with trypsin-EDTA (Flow Laboratories) as follows: medium in which cells were grown was discarded into Chloros. Cells were washed twice with 2.5ml trypsin-EDTA to remove residual serum; both washes were discarded into Chloros. Cells were then incubated at 37°C with 2.5ml trypsin-EDTA for 5 mins or until cells detached from flask surface. The trypsin/cell suspension was divided between two or three sterile 75cm^2 tissue culture flasks and 15ml of fresh tissue culture medium (described above) were added to each flask. Cells were then incubated at 37°C in 5% CO_2 until confluent and then cells were harvested and divided between sterile tissue culture flasks again, as described above. If required, tissue culture medium was replaced with fresh medium between cell harvests. Usually 12 flasks of confluent cells were harvested to make cell extracts as described in section 2.6.1. Occasionally, cells were counted using a haemocytometer; approximately 2 x 10^6 cells were recovered from each confluent 75cm^2 flask.

2.6 PROTEIN TECHNIQUES

2.6.1 Preparation of whole cell extracts

GH_3, HeLa and CV-1 cells were cultured as described (section 2.5). WCE were prepared essentially as described by Manley et al. (1980).

Approximately 2.5 x 10^7 cells were harvested with trypsin-EDTA (Flow Laboratories). The cell pellet was washed three times in 1 x PBS, then once in 1 x PBS containing 10mM $MgCl_2$ (occasionally cells were stored in pellet form for up to 3 days at -70°C before the final wash). All further steps were carried out between 0°C & 4°C.

Cells were centrifuged as above, for 5 mins at 4°C in a clinical bench top centrifuge (Chilspin, MSE), resuspended in 4 packed cell volumes of ice-cold TED buffer (10mM tris HCl (pH 7.9), 1mM EDTA, 5mM DTT and allowed to swell on ice for 20 minutes. Cells were then lysed with 8 strokes of a 3ml Uniform glass homogeniser before adding 4 packed cell volumes of TMDSG buffer (50mM Tris HCl (pH 7.9), 10mM $MgCl_2$, 2mM DTT, 25% (w/v) sucrose, 50% (v/v) glycerol) containing 0.15$\mu g ml^{-1}$ PMSF (Sigma) and the mixture was stirred gently for 5 minutes. One packed cell volume of saturated $(NH_4)_2SO_4$ was added slowly, dropwise, and the suspension was stirred gently with a magnetic stirrer for 20 minutes. The resulting cell lysate preparation was placed in either 5ml or 12ml ultracentrifuge tubes (Dupont) and centrifuged at 100,000xg for 3h at 2°C (33,000rpm in a Sorvall AH-650 rotor). The supernatant was decanted taking care not to disturb the pellet, which contained unwanted cellular membranes and nucleic acid material. Protein in the supernatant was precipitated by gradually adding solid $(NH_4)_2SO_4$ (0.33gml^{-1} supernatant). Once $(NH_4)_2SO_4$ had dissolved, 1μl of 1M NaOH was added for every gram of solid $(NH_4)SO_4$ already added (to adjust the pH of the precipitate to approximately pH7) and the suspension was stirred gently for 30 mins with a magnetic stirrer. The suspension was then centrifuged at 15,000xg, for 20 mins at 4°C and the protein pellet was resuspended in $^1/_{20}$ of the high speed supernatant volume in ice cold extract buffer (50mM tris HCl pH 7.9, 6mM $MgCl_2$, 40mM$(NH_4)_2SO_4$, 0.2mM EDTA, 1mM DTT, 15% (v/v) glycerol). The resuspended whole cell protein extract was then dialysed 16-18h against three 1 litre changes of the extract

buffer and centrifuged at 10,000rpm for 10 mins in Eppendorf bench top centrifuge at 4°C to eliminate insoluble matter.

Protein concentration of the WCE was determined using Biorad Protein Assay (section 2.6.4.2). WCE concentration was usually between 8 and 20$\mu g \mu l^{-1}$. Whole cell extract was aliquoted into 20-50µl samples, snap-frozen in liquid nitrogen and stored at -70°C. The protein extracts were stable at -70°C for up to 3 months, after which time they began to lose activity.

WCE was prepared from rat liver tissue by the same method as outlined above, except liver tissue was initially homogenised at 4°C in TED buffer using a chilled 3ml uniform homogeniser. Rat liver tissue was dissected from an adult female Cobb Wistar rat (~ 250g) by Mr. R.C. Dow, MRC Brain Metabolism Unit, University of Edinburgh.

If required, protein extract was denatured either by heating to 60°C for 10 mins or adding 6M urea and incubating on ice for 10 mins. Urea was removed by overnight dialysis at 4°C against two changes of extract buffer (recipe given above).

2.6.2 Preparation of nuclear extracts

The following protocol outlines nuclear extract preparation from 10^6 cells (Schreiber et al., 1989). In general, 6 x 10^6 HeLa cells were used and the volumes given in this protocol were multiplied accordingly.

Approximately 10^6 HeLa cells were harvested by trypsinisation (trypsin 0.02M EDTA (Flow Laboratories)), washed in 5ml of 1 x PBS and pelleted at 1000rpm, for 5 mins at 4°C in a clinical bench top centrifuge (Chilspin, MSE) before resuspending in 1ml 1 X PBS and transferring to an Eppendorf tube. Cells were centrifuged again in an Eppendorf bench top centrifuge. Cells were swollen by resuspending in 400µl of cold buffer A (10mM Hepes (pH 7.9), 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT), containing a protease inhibitor cocktail (200µM PMSF (Sigma), 1µM leupeptin (Sigma), 1µM pepstatin (Sigma)) and allowed to swell on ice for

15 minutes. After adding 25µl of 10% (v/v) Nonidet NP-40, the cells were lysed by vigorously vortexing for 10 seconds; lysed cell homogenate was centrifuged for 30 seconds in an Eppendorf bench top centrifuge. Pelleted nuclei were resuspended in 50µl of ice-cold buffer C (20mM Hepes (pH 7.9), 0.4M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT) containing a protease inhibitor cocktail as described above, vortexed for 10 seconds and rocked vigorously on a shaking platform for 15 minutes at 4°C. The nuclear sample was then centrifuged for 5 mins in an Eppendorf bench top centrifuge to remove nucleic acid material. The nuclear extract supernatant was aliquoted into 20µl samples, snap-frozen in liquid N_2 and stored at -70°C. Nuclear extract concentration was determined using Biorad Protein Assay (section 2.6.3.2); protein recovery was usually about 8-10µgµl^{-1}. Nuclear extracts were fairly unstable, losing activity within two weeks.

2.6.3 Preparation of bacterial lysates

E.coli strain used to prepare bacterial lysate was KC1074 (*cya* Δ *crp* Δ) transformed with pHA5 (Aiba et al., 1982) which constitutively over-expresses cAMP-receptor protein. This strain was engineered by Dr. K.E. Chapman who also prepared bacterial lysates as follows: a 20ml culture in selective L-broth was grown to late log phase (A600 ~ 1.0). Cells were collected by centrifugation at 6000rpm in an SS34 rotor for 5 mins at 4°C, washed in TE and resuspended in 1ml lysate buffer (100µM potassium phosphate pH 7.4), 50mM KCl, 10% glycerol, 1mM EDTA, 1mM DTT, 160µgml^{-1} PMSF). The bacterial preparation was sonicated, generally with two 10 second pulses of a MSE sonicator at 0°C. The sonicated sample was centrifuged in an Eppendorf bench top centrifuge for 10 mins at 4°C. Supernatant was transferred to a new tube and 0.2 volumes glycerol were added. Bacterial lysate was stored at -70°C for several months.

2.6.4 Detection and quantitation of proteins

2.6.4.1 Detection of proteins

Coomassie Blue R Protein Staining: Coomassie blue is a moderately sensitive protein-specific dye which usually allows detection of 0.2-0.5µg of any protein in a sharp band in a gel (Hames & Rickwood, 1987).

Protein gels were fixed for 45 mins in sufficient fix solution (10% glacial acetic acid, 50% Methanol) to completely submerge gel. Fix was removed and replaced with ~300mls Coomassie Blue R staining solution (0.25% Coomassie Blue R (Sigma), 50% methanol (v/v), 7.5% v/v) for 30 mins. The gel was then rinsed twice with tap water and soaked overnight in destaining solution (7% glacial acetic acid, 10% methanol). Small squares of sponge were sunk in the destain solution to soak up excess Coomassie Blue R stain and so ensure even destaining of the gel. The stained gel was then dried using a heated vacuum gel drier (Biorad) and autoradiographed (section 2.3), if required.

Silver Staining: Silver stain is a highly sensitive protein-specific stain which allows detection of as little as 0.4ng of bovine serum albumin per square mm of polyacrylamide gel (Hames & Rickwood, 1987).

The gel was fixed for 45 mins (as described in 2.6.4.1) then washed twice for 1h in 50% methanol and rinsed once after each methanol wash for 15 mins in dH_2O. During the final dH_2O rinse, two solutions were made up for the silver stain: solution A (0.6g $AgNO_3$ (Sigma), dH_2O to 4ml) and solution B (21ml 0.36% NaOH, 1.4ml 14.8M ammonium hydroxide). Solution A was added dropwise to solution B, stirring continuously, and the resulting silver stain solution was adjusted to 100ml with dH_2O. Shaking gently, the gel was stained with stain solution for 20 minutes then washed three times, each for 10 mins in a total volume of 1.5 litres dH_2O. The gel was then rocked gently in freshly made up developer (2.5ml w/v 1% citric

acid, 250µl formaldehyde, dH_2O to 500ml) until protein bands were visibly resolved. The developer was poured off and the gel was rinsed several times in dH_2O. The gel was either stored in fixer (for up to 2 days), or dried using a heated vacuum gel drier (Biorad) and autoradiographed (section 2.3), if required.

2.6.4.2 Quantitation of proteins

Protein concentration was calculated using the Biorad Protein Assay. Biorad Protein Assay was prepared by diluting 1 part in 5 and filtering through Whatman #1 paper; this assay solution was used as follows:

Protein standard solution (bovine serum albumin, usually ~1.46mgml^{-1}) was diluted to 0.05, 0.1, 0.2, 0.3, 0.4 and 0.6 of its original concentration with dH_2O to 20µl final volume. Each dilution, plus a 20µl dH_2O control was vortexed for 2-3 secs with 1ml of prepared Biorad protein assay solution. These seven protein standards were left at room temperature for about 5 mins before transferring to 1ml plastic cuvette tubes. The A_{595} of each protein standard was measured and a calibration curve was determined using a Shimadzu UV/60 spectrophotometer.

Simultaneously, 20µl aliquots of protein extract (prepared as described in sections 2.6.1, 2.6.2 or 2.6.3, and diluted if necessary), were vortexed with 1ml of assay solution and treated similarly. Protein extract concentration was calculated (by spectrophotometer) by relating A_{595} of protein extract to the protein standard curve.

2.7 LABELLING DNA

2.7.1 Preparation of oligonucleotide probe

30pmol of single stranded oligonucleotide was end labelled at 37°C for 45 mins using 50µCi [γ^{32}P]-ATP (~3000Ci/mmol) (Amersham) and approximately 4.5 units T4 polynucleotide kinase (NBL) in a total of 20µl

kinase buffer (100mM tris HCl pH 8.0, 0.01mM $MgCl_2$, 0.005mM DTT). The reaction was stopped by inactivating kinase at 60°C for 10 mins. 1µl of kinased oligonucleotide was diluted 10-fold and stored for gel electrophoresis.

The remaining labelled oligonucleotide was separated from unincorporated label essentially as outlined in Maniatis, 1982. Labelled oligonucleotide was diluted to 100µl with TE and centrifuged (1000rpm, 5 mins, 4°C in clinical bench top centrifuge: MSE Chilspin) through a 1ml Sephadex G-50 (Pharmacia) column which had been pre-equilibrated with three 100µl washes of STE (TE containing 100mM NaCl). Most of the radioactive material, both incorporated and unincorporated label, remained on the column at this stage. Labelled oligonucleotide was removed from the column with three 100µl washes of TE and the effluent from each wash (4 effluents in total) was collected. The recovery of labelled oligonucleotide was estimated, after electrophoresis (section 2.2.2.1) and autoradiography (section 2.3) by comparing the signal intensity of recovered oligonucleotide to that of the known quantity of unseparated oligonucleotide (aliquoted above). As the concentration of recovered oligonucleotide was estimated, the amount of oligonucleotide used in experiments was approximate. Electrophoresis of recovered and unseparated oligonucleotide also allowed the efficiency of Sephadex G-50 column separation to be checked.

Labelled oligonucleotide usually eluted from the Sephadex G-50 column with the first and second 100µl TE washes. The complementary labelled oligonucleotides were mixed and annealed by heating at 60°C for 10 minutes and cooled slowly. Labelled double-stranded oligonucleotide was diluted to 0.1pmolμl^{-1} or 0.05pmolμl^{-1} for use in band shift assays (section 2.8.1).

Periodically, 1µl of labelled oligonucleotide final preparation was removed into 1ml of scintillation fluid and counted in an LKB scintillation

counter to determine specific activity. Oligonucleotide probe was usually labelled to a specific activity of 5-6 x 10^8cpm/pmol

2.7.2 Preparation of DNA restriction fragment probe

One microgram of plasmid (pKC264-13 or pKC264-04, section 2.4.1) was digested with *EcoRI* (section 2.4.1). Sticky ends generated were filled in with 30μCi [α-^{32}P]dATP (~3000Ci/mmol, Amersham) and 1μl of a mixture of 2mM each dTTP, dCTP, dGTP (Sigma) in a final volume of 10μl containing 1 x restriction buffer (see Table 2.2 for recipe) and 2.5 units Klenow (large fragment from DNA polymerase I) (1,000U/17μl, Gibco, BRL or BCL) for 10 minutes at room temperature. Chase was then added: 1μl 2mMdATP, to complete filling in of partially filled ends and incubation continued for 5 mins at room temperature. Enzyme activity was stopped by heating at 60°C for 10 mins. Labelled linear plasmid DNA was then restriction endonuclease digested with *HindIII* (section 2.4.3). The *EcoRI-HindIII* fragment, labelled at the *EcoRI* end was purified from a 1% low melting point agarose gel (section 2.4.2) and finally resuspended in 100μl TE. 1μl of recovered fragment was counted in a LKB scintillation counter to determine specific activity. Specific activity of labelled DNA probes was usually 1-3 x 10^6cpm/μg DNA.

2.8 ANALYSIS OF DNA/PROTEIN COMPLEXES

2.8.1 Gel retardation assay

Gel retardation assays (band shift assays) were carried out as outlined by Singh et al. (1986).

A standard band shift reaction contained approximately 0.1pmol of ^{32}P-labelled double stranded oligonucleotide (section 2.7.1) (or 3fmol of ^{32}P-labelled DNA restriction fragment (section 2.7.2) where stated, 10μl of 2 x binding buffer (2 x = 200mM NaCl, 20mM tris HCl (pH 7.9), 2mM EDTA,

25% (v/v) glycerol), 4μg of the non-specific competitor poly [d(I-C)] (BCL) and 1μg-20μg of protein sample, in a final reaction volume made up to 20μl with dH_2O. All ingredients, except the protein, were mixed together; the DNA-binding reaction was initiated on addition of protein sample which was mixed in by pipetting the reaction up and down a few times using a Gilson pipette. The nature of the protein sample used was either whole cell extract (section 2.6.1), nuclear extract (section 2.6.2), partially purified protein extract from Sephacryl S300 column (section 2.9), bacterial lysate (section 2.6.3) or *in vitro* translated mouse ER (supplied by Dr. S. Fawell, ICRF, London; see Chapter 5). Band shift reactions were incubated at room temperature for 20 mins then loaded immediately and swiftly onto an electrophoresing 4% native polyacrylamide gel (section 2.2.2.2) or gradient gel (section 2.2.2.3). Protein-DNA complexes formed in the band shift reaction were resolved from unbound labelled DNA by electrophoresis at 200V for 45-60 mins. Protein-DNA complexes were detected after drying the gel using a heated vacuum gel drier (Biorad) and exposing to autoradiographic film (section 2.3). Typically, exposure to autoradiographic film was overnight, unless otherwise stated.

In competition band shift assays excess unlabelled competitor oligonucleotide was included into the standard band shift reaction at 3- to 243-fold molar excess over ^{32}P-labelled oligonucleotide, prior to addition of protein extract. Most commonly, 100-fold excess of competitor oligonucleotide was used.

2.8.2 UV Cross-linking of protein-DNA complexes

(Adapted from Kumar & Chambon, 1988)

BdU-containing oligonucleotides were end-labelled with ^{32}P and annealed (section 2.7.1) with unlabelled, unsubstituted complementary oligonucleotide (Fig. 4.7). HeLa WCE was incubated with labelled

oligonucleotide in a series of band shift reactions in which the quantity of each substrate had been increased 10-fold (100μg WCE, 40μg poly [d(I-C)], 1pmol labelled oligonucleotide) and the final reaction volume was increased 5-fold, from 20μl to 100μl. Four reactions were set up in duplicate: 100μg HeLa WCE added to approximately 1pmol each of 1) labelled BdU-ERE1, 2) labelled BdU-ERE2 , 3) labelled ERE_{con} and 4) a control reaction in which no oligonucleotide was added. Reactions were incubated for 20 min at room temperature during which time each 100μl reaction was transferred to four wells (25μl per well) of a microtitre plate. The microtitre plate was covered in Saran wrap and inverted on top of a UV transilluminator (emission wavelength, 312nM; Ultraviolet Products Inc). One set of reactions was UV-irradiated for 40 mins and the duplicate set was irradiated for 20 mins.

To confirm that protein-DNA had formed complexes, a 10μl aliquot from each reaction was subjected to 4% native gel electrophoresis (section 2.2.2.2). To the remaining 90μl of each reaction, 25μl of sample incubation buffer (recipe, 2.2.2.4) was added; reactions were heated as described in section 2.2.2.4 and subjected to 10% SDS polyacrylamide gel electrophoresis (section 2.2.2.4). Protein standard mol. wt. markers (Rainbow Markers, Amersham) were electrophoresed in parallel. The gel was dried using a heated vacuum drier (Biorad) and exposed to autoradiographic film (section 2.3) for up to two weeks.

Rainbow Markers: molecular weights as follows:

Lysozyme	(MW 14,300)
Trypsin inhibitor	(MW 21,500)
Carbonic anhydrase	(MW 30,000)
Ovalbumin	(MW 46,000)
Bovine serum albumin	(MW 69,000)
Phosphorylase b	(MW 97,400)
Myosin	(MW 200,00)

The precise size of mol. wt. markers used differed depending on their source.

2.9 GEL FILTRATION CHROMATOGRAPHY

The Sephacryl S300 column used for gel filtration chromatography was 150cm high and 3cm wide (approximately 1 litre volume). This column had been precalibrated (by Dr. S. Chapman, Chemistry Department, Edinburgh University) with the following molecular weight markers flavocytochrome b_2 (230kD), β-amylase (200kD), alcohol dehydrogenase (150kD), bovine serum albumin (66kD), carbonic anhydrase (29kD), myoglobin (17.5kD) and cytochrome C (12.5kD) and preequilibrated at 4°C with 2 litres of column buffer (recipe below). The protein pellet generated after the final ammonium sulphate precipitation of HeLa WCE preparation (see 2.6.1) was finally resuspended in column buffer (50mM tris HCl (pH 7.9), 6mM $MgCl_2$, 100mM NaCl, 0.2mM EDTA, 1mM DTT, 10% (v/v) glycerol) instead of extract buffer (recipe, 2.6.1). Column buffer differed from extract buffer most importantly in 1) glycerol content: less glycerol (v/v) in column buffer ensured fairly swift progression of protein extract through the column so minimising exposure to proteolytic attack, and retaining glycerol content similar to that of 1 x binding buffer used in the band shift assay (see below) and 2) column buffer did not contain ammonium sulphate but contained 100mM NaCl similar to 1 x binding buffer (see section 2.8.1). This was important since 18µl of each fraction collected from the column was to be assayed for DNA-binding activity in a band shift reaction; ammonium sulphate presence in such an assay prohibits protein/DNA complex resolution by gel electrophoresis, giving rise to extended 'smears' down length of gel (unpublished result)

Approximately 7mg of HeLa WCE was fractionated over the column. 4µg of blue dextran (mol. wt. > 300kD) and 1mg cytochrome c (mol. wt. 12.5kD) were added to HeLa WCE prior to loading to mark the void volume of the column and the lower mol. wt. parameter, respectively within which protein fractions were to be collected (see Chapter 4, Introduction). WCE

was allowed to diffuse into the top of the column before adding a continuous flow of column buffer. A calibration graph was constructed by plotting the mol. wt. of each protein marker against the fraction of its elution volume divided by the void volume on semi-log paper. (Fig 4.1). Fractions of 3.5mls eluted and were collected from the column over 24h-48h at 4°C. In a band shift assay (section 2.8.1), 18µl of each fraction were assayed for SRE-BP DNA-binding to either ^{32}P-labelled ERE_{con} oligonucleotide in the presence of 4µg poly [d(I-C)] in a final reaction volume of 20µl. The reaction was incubated at room temperature for 20 mins before 4% native gel electrophoresis (see 2.2.2.2). The mol. wt of SRE-BP was determined by dividing the buffer volume in which its DNA-binding activity eluted by the void volume and relating this fraction to the calibration graph derived from standard mol. wt. markers (Fig. 4.1). Fractions which contained SRE-BP DNA-binding activity were concentrated using an Amicon concentrator. Approximately 800µl (1µgµl^{-1}, see 2.6.4.2) of concentrated partially purified protein was recovered.

A protein elution profile was attempted by measuring the OD_{595} of each column fraction in a Shimadzu UV/60 spectrophotometer. However, protein concentration in each fraction was too low for detection under these conditions.

CHAPTER 3

IDENTIFICATION OF A SEQUENCE SPECIFIC STEROID RESPONSE ELEMENT-BINDING PROTEIN

3.1 INTRODUCTION

The consensus ERE and GRE are related sequences which show dyad symmetry with 5 or 6-base-pair (bp) arms separated by a 3bp spacer region. Receptors for glucocorticoids, mineralocorticoids, progesterone and androgens can all act specifically through a GRE, whereas the receptor activated by oestrogen acts through the similar, but distinct, ERE (Klock et al., 1987; Martinez et al., 1987; Ham et al., 1988; Cato et al., 1987; Cato et al., 1988; Otten et al., 1988; Strahle et al., 1987; Klein-Hitpass et al., 1988; Arriza et al., 1987). The consensus ERE and GRE are independently able to confer hormone dependent transcriptional enhancement from a linked promoter *in vivo*, as demonstrated by gene transfer experiments (see above references and Fig. 3.2).

Although purified SRs directly bind to the respective SRE *in vitro*, their ability to discriminate between a specific binding site and non-specific DNA may be less than 10-fold (for examples see Maurer & Notides, 1987; Payvar et al., 1981; von de Ahe et al., 1985; Jantzen et al., 1987; Peale et al., 1988; Härd et al., 1990b). This observation provokes speculation that other cellular factors are required to achieve high affinity binding of SR to the SRE *in vivo*.

There are several reported experiments which directly or indirectly suggest the involvement of accessory proteins which increase sequence-specific binding of SRs to DNA (Feavers et al., 1987; Wrange et al., 1986; Payvar & Wrange, 1983; Edwards et al., 1989; Murray & Towle, 1989). Moreover, electron microscopy of the protein-DNA complex at the ERE of the Xenopus vitellogenin gene (Heggeler-Bordier et al., 1987) indicate that this complex is large and may include other proteins in addition to the hormone receptor.

In this study, I wished to determine whether accessory proteins (which are not SRs) could specifically bind to a SRE and thus increase the specificity of SR-SRE interaction. To do this, a DNA-binding assay known as the gel

retardation or band shift assay was used. This assay is based on the altered mobility of protein-DNA complexes relative to unbound DNA, during non-denaturing gel electrophoresis (Fried & Crothers, 1983; Garner & Revzin, 1981). Protein-DNA complexes are formed by mixing protein extract with ^{32}P-labelled DNA which encodes the binding site for a particular protein in a band shift reaction. Distinct protein-DNA complexes are detected when excess heterologous 'nonsense' DNA (poly [d(I-C)]), which titrates out more abundant non-specific DNA-binding proteins present in crude protein extract, is included in the reaction. The sequence requirement for complex formation can be examined by including excess unlabelled competitor DNA encoding a defined sequence in a competition band shift assay; sequences which compete and sequences which do not compete for complex formation can therefore be determined.

DNA sequences examined in this study for their ability to be bound by specific cellular factors are shown in Figure 3.1. Each is a short oligonucleotide which encodes a consensus ERE (ERE_{con}, Fig. 3.1a) (Klock et al., 1987), an ERE derived from 5'-regulatory sequences in the rat prolactin gene (ERE_{PRL}, Fig. 3.1b) (Maurer & Notides, 1987; Waterman et al., 1988), a symmetrical GRE (GRE, Fig. 3.1c) (Klock et al., 1987), a mutant GRE (GRE_M, Fig. 3.1d), and a symmetrical TRE (TRE, Fig. 3.1e) (Glass et al., 1988). These are all related sequences; compared to the conserved nucleotides of the consensus ERE (Fig. 1a), the sequence of and/or the spacing between the conserved nucleotides has been altered.

Each oligonucleotide (Fig. 3.1) was initially tested for its ability to confer either oestrogen or glucocorticoid inducibility on a linked heterologous promoter in a cell transfection system (Fig. 3.2). Both the ERE_{con} oligonucleotide and the GRE oligonucleotide independently enhanced transcription from the Herpes Simplex Virus (HSV) thymidine kinase (tk) promoter when either oestradiol or dexamethasone respectively, were present

Figure 3.1 Sequence of oligonucleotides used in the gel retardation assays

(a) ERE_{CON}

GCAGGTCACAGTGACCTGGAC
CGTCCAGTGTCACTGGACCTG

(b) ERE_{PRL}

GATCTTGTCACTATGTCCT
AACAGTGATACAGGACTAG

(c) GRE

GCAGAACACAGTGTTCTGGAC
CGTCTTGTGTCACAAGACCTG

(d) GRE_M

CAAGAACATGCATGTTCTTG
GTTCTTGTACGTACAAGAAC
Δ

(e) TRE

CATCAGGTCATGACCTGATG
GTAGTCCAGTACTGGACTAC

Horizontal lines indicate the conserved nucleotides within the SRE. A hollow arrow head shows the position of an additional base-pair in the spacer region of oligonucleotide (d). (a) ERE consensus sequence derived from the oestrogen-inducible vitellogenin genes of *Xenopus laevis* and chicken (Klock et al., 1987). (b) ERE sequence from the 5' flanking region of the rat prolactin gene (Maurer & Notides, 1987; Waterman et al., 1988). (c) GRE sequence with perfect 2-fold symmetry (Klock et al., 1987). (d) Mutant GRE sequence. (e) Symmetrical TRE sequence (Glass et al., 1988). Oligonucleotides were synthesised by Oswell DNA Services, Department of Chemistry, Edinburgh University.
ERE_{PRL} oligonucleotide was synthesised with *BamHI* sticky ends for cloning purposes.

(Fig. 3.2). An oligonucleotide encoding the same conserved nucleotides as the symmetrical GRE with a 3bp spacer region has also been shown to independently confer progesterone-dependent transcriptional enhancement from a linked promoter in gene transfer experiments (Strahle et al., 1987). However, the GRE_M oligonucleotide did not mediate dexamethasone-dependent transcriptional enhancement (Fig. 3.2). ERE_{PRL} did not act as a transcriptional enhancer on its own (Fig. 2), but it has been shown to confer oestradiol-dependent transcriptional enhancement on a linked promoter when contained within a 228bp fragment of DNA (Maurer & Notides, 1987). Preliminary results from this laboratory (Clark & Chapman, personal communication) indicated that the palindrommic TRE did mediate oestradiol-dependent transcriptional enhancement although at a much reduced level (2-fold) compared to the ERE_{con} (10-fold induction). Umesono & Evans (1989) have similar findings although Glass et al. (1988) demonstrate non-responsiveness of the rat growth hormone TRE to oestradiol stimulation. The palindromic TRE has been shown to act as an independent enhancer in response to thyroid hormone (Glass et al., 1988). The ERE_{con} and the symmetrical GRE are, therefore, functional SREs *in vivo*, whereas the TRE and GRE_M are non-functional SREs, in the presence of either oestrogen or glucocorticoid hormone, respectively.

This chapter now describes identification of a cellular factor present in whole cell extracts (WCE) of HeLa, GH_3 and CV-1 cells, and in liver tissue WCE. This factor binds preferentially to oligonucleotides which contain either an ERE or symmetrical GRE sequence. The factor has reduced affinity for the GRE_M oligonucleotide and does not bind to the TRE oligonucleotide, nor does it bind to transcription factor binding sites present in the HSV tk promoter. The factor, also present in HeLa cell nuclear extracts, is named the Steroid Response Element-Binding Protein (SRE-BP).

Figure 3.2 **Ability of oligonucleotides (Fig. 3.1) to confer oestradiol or dexamethasone dependent transcriptional enhancement on HSV tk promoter** (unpublished data from E. Clark & K. Chapman)

CAT activity is expressed relative to pBLCAT with hormone treatment, which is given an arbitrary value of 1.0.

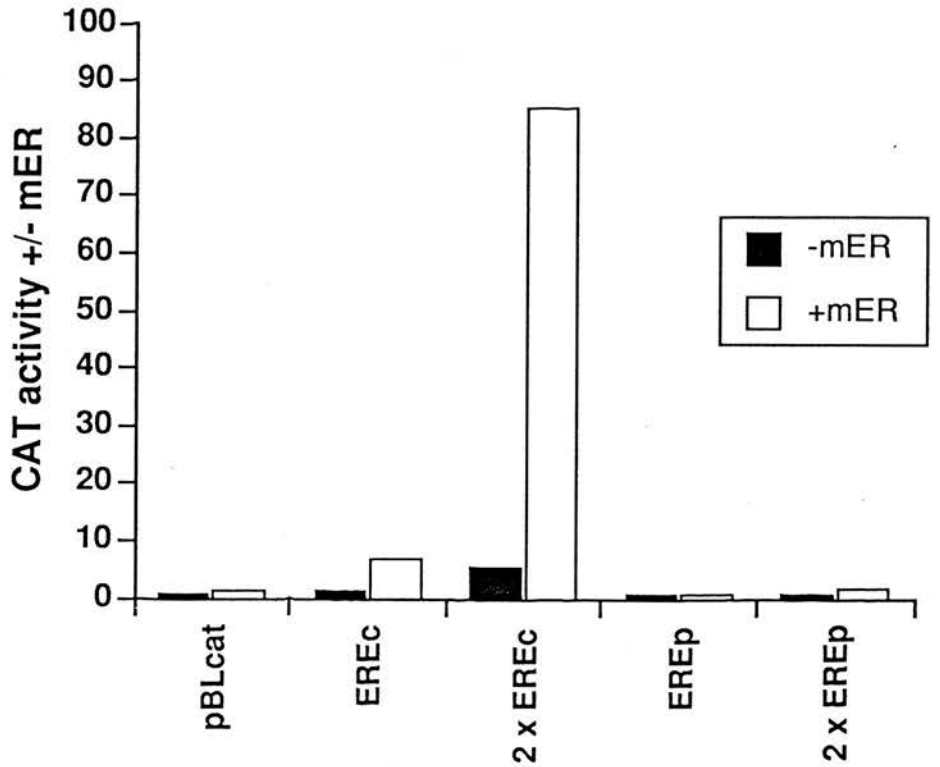
Top Panel: Open bars indicate CAT activity in the presence of mER (cells cotransfected with expression plasmid, pJ3MOR). Reporter plasmids were pBLCAT2 (pBLcat), pBLCAT2 containing one copy (ERE_c) or two copies (2 x ERE_c) of the ERE_{cons} oligonucleotide, or pBLCAT2 containing one copy (ERE_P) or two copies (2 x ERE_P) of the ERE_{PRL} oligonucleotide. Filled bars indicate CAT activity in the absence of cotransfected pJ3MOR.

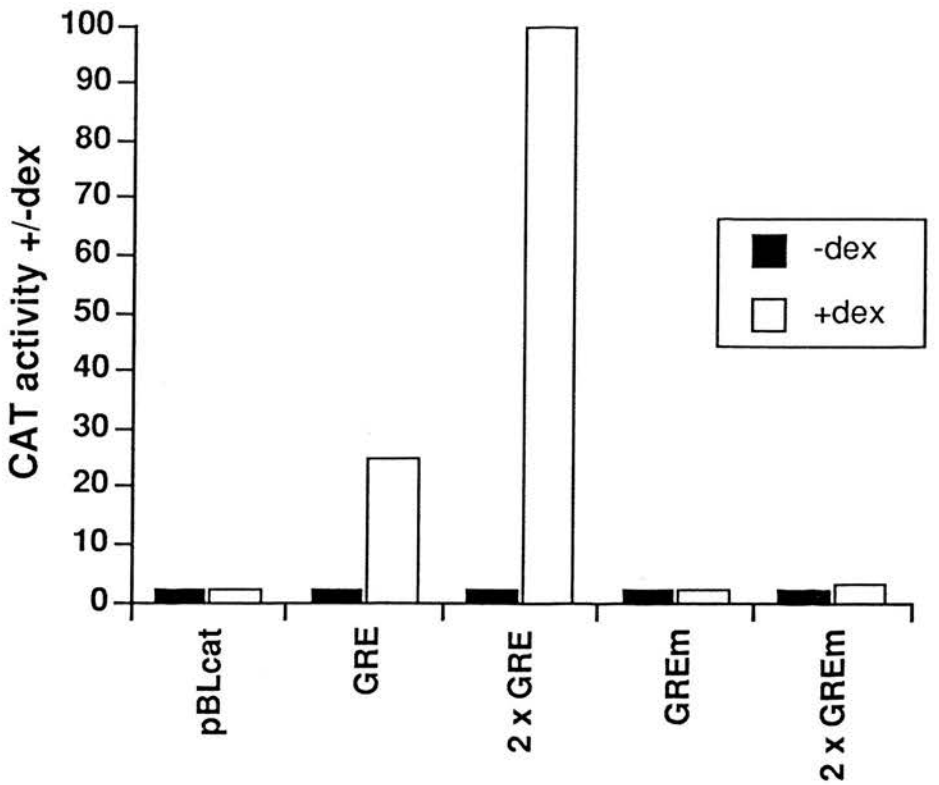
Bottom panel: filled bars and open bars indicate CAT activity in the absence or presence of 10^{-6}M dexamethasone respectively. Reporter plasmids were pBLCAT2 (pBLcat), one copy (GRE) or two copies (2 x GRE) of the GRE oligonucleotide, or one copy (GRE_M) or two copies (2 x GRE_M) of the GRE_M oligonucleotide

Methods oligonucleotides (one copy or two copies) were cloned into the polylinker of pBLCAT2. Each electroporation (carried out in duplicate) used 2 x 10^6 HeLa cells, transfected with 5μg reporter DNA and 5μg pCH110 (Pharmacia; included as an internal control for transfection efficiency). For measurement of oestradiol responsiveness, 1μg of pJ3 or pJ3MOR (a gift from J. Lees & M. Parker, ICRF) was co-transfected with the reporter construct. pGEM3 was added to give a total of 15μg of DNA.

Oestradiol responsiveness was measured in transfected cells plated in medium containing complete serum and to which 10^{-8}M 17β-oestradiol was added. Dexamethasone responsiveness was measured in transfected cells plated in medium containing charcoal-stripped serum. Where appropriate, dexamethasone was added at a final concentration of 10^{-6}M.

Each value represents an average from at least two experiments.


CAT activity +/- mER
100
90
80
70
60
50
40
30
20
10
0
-mER
+mER
pBLcat
EREc
2 x EREc
EREp
2 x EREp


CAT activity +/-dex
100
90
80
70
60
50
40
30
20
10
0
-dex
+dex
pBLcat
GRE
2 x GRE
GREm
2 x GREm

3.2 RESULTS

3.2.1 Identification of a nuclear protein which binds selectively to both an ERE_{con} and a GRE.

Identification: Gel retardation assays were used to detect the presence of DNA-binding protein(s) in WCE of HeLa, GH_3 and CV-1 cells which bind to a double-stranded oligonucleotide containing the ERE_{con} sequence (ERE_{con}; Fig. 3.1a). 10μg or 20μg of WCE, prepared according to Manley et al., (1980) (section 2.6.1) were incubated with approximately 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide and 4μg poly [d(I-C)], under standard band shift assay conditions (section 2.8.1). Bound labelled oligonucleotide was resolved from unbound oligonucleotide by 4% native polyacrylamide gel electrophoresis (section 2.2.2.2); complexes formed were detected by overnight autoradiography (section 2.3). HeLa, GH_3 (Fig. 3.3) and CV-1 (Fig. 3.4a) WCE all contained a cellular factor which bound to the ERE_{con} oligonucleotide in the presence of 4μg poly [d(I-C)], and formed a complex with identical retarded electrophoretic mobility and signal intensity (Fig. 3.3). This complex formation was not due to endogenous ER or GR as neither HeLa nor CV-1 cells express oestrogen receptor (Kumar & Chambon, 1988; Klein-Hitpass et al., 1989), nor do CV-1 cells express functional glucocorticoid receptor (Giguere et al., 1986; Umesono & Evans, 1989).

To rule out the possibility that the identified factor expressed in these cells is a unique characteristic of transformed cells, WCEs were prepared from normal female rat liver tissue. 741mg liver tissue were homogenised in TED buffer (section 2.6.1), and WCE were then made accordingly (section 2.6.1). Either 10μg or 20μg of liver WCE were included in a standard band shift reaction (section 2.8.1) with ^{32}P-labelled ERE_{con} oligonucleotide and 4μg poly [d(I-C)]. Liver WCE contained a factor which bound to ERE_{con} and exhibited identical electrophoretic mobility to the complex formed with HeLa WCE (Fig. 3.4b, compare lanes 3 & 4 with 1 & 2). This factor is, therefore, present in both

Figure 3.3 **Analysis of DNA binding activity present in whole cell extracts of HeLa and GH_3 rat pituitary tumour cells**

Whole cell extracts of HeLa and GH3 cells were prepared (section 2.6.1). Increasing amounts of whole cell extracts (as indicated in micrograms above each lane) were incubated with 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide in the presence of 4μg poly [d(I-C)]. Free and bound oligonucleotide were separated on a 4% polyacrylamide gel by electrophoresis (section 2.2.2.2). An arrow indicates the position of bound oligonucleotide.

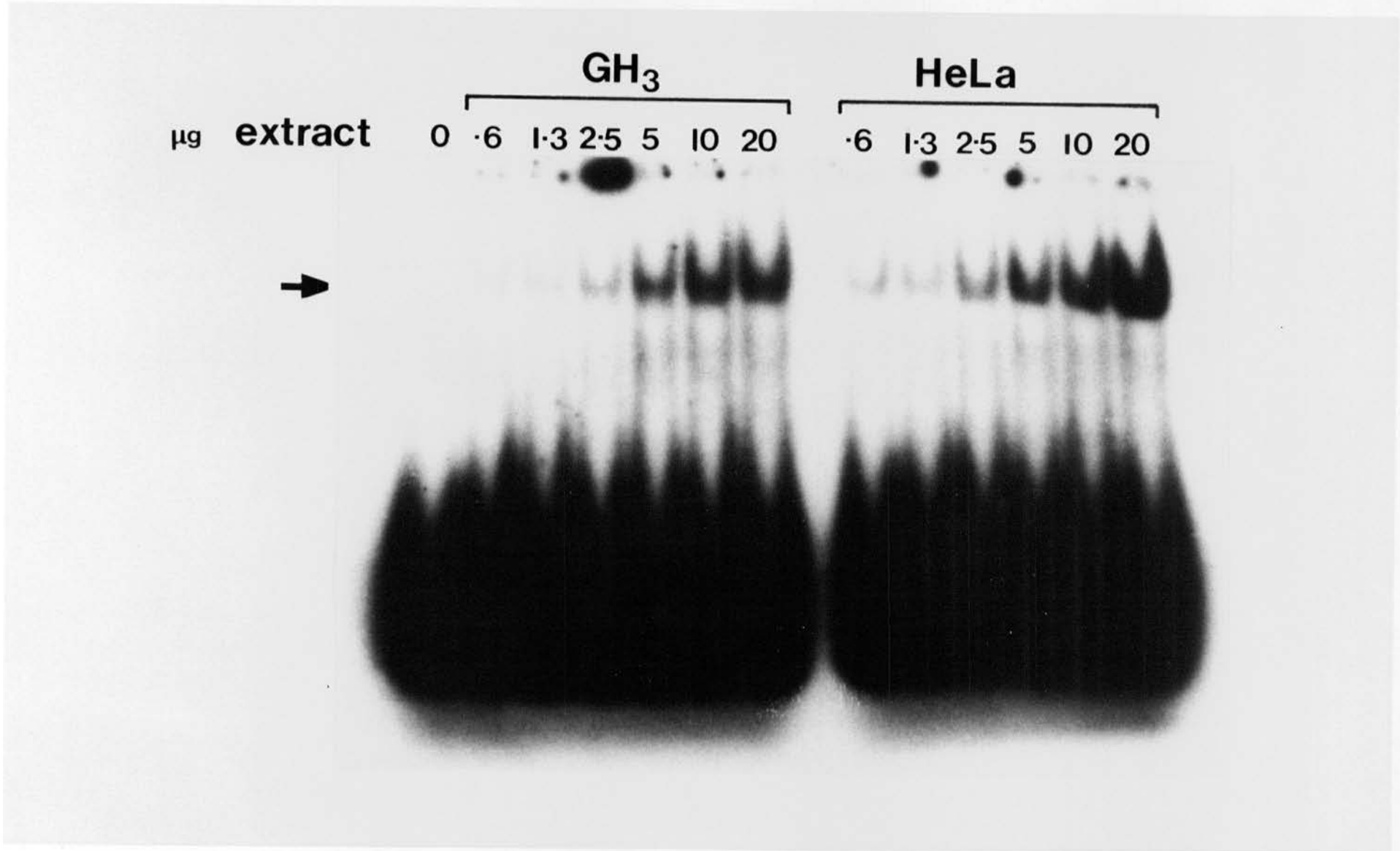
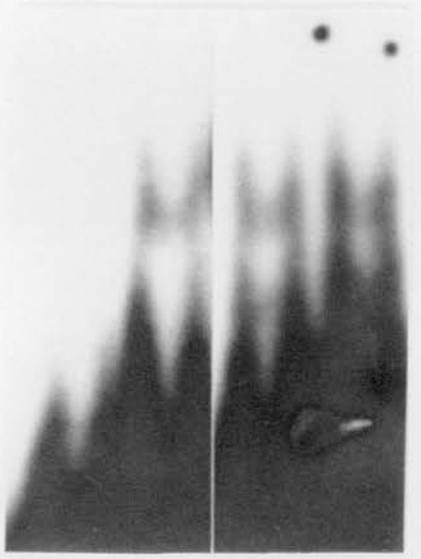
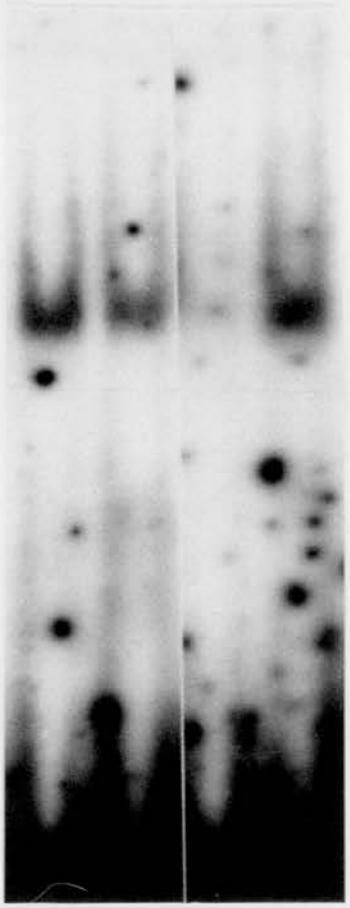

GH3
HeLa
µg extract
0 ·6 1·3 2·5 5 10 20
·6 1·3 2·5 5 10 20

Figure 3.4 **Analysis of DNA-binding activity present in WCE of HeLa, CV-1 monkey kidney tumour cells and rat liver tissue**

WCE of HeLa, CV-1 and Liver were prepared as described in section 2.6.1 and incubated with 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide in the presence of 4μg poly [d(I-C)]. (a) Lane 0, no protein added; lane 1, 10μg HeLa (H) WCE added; lane 2 and 3, 10μg and 20μg CV-I (C) WCE added respectively (b) lanes 1 and 2, 10μg and 20μg HeLa (H) WCE added respectively; lanes 3 & 4, 10μg and 20μg liver (L) WCE added, respectively. Free and bound oligonucleotide were separated on a 4% native polyacrylamide gel as described (section 2.2.2.2).



a



b

untransformed mammalian liver tissue and several transformed cell lines, and is therefore not an artifact of malignant cell lines.

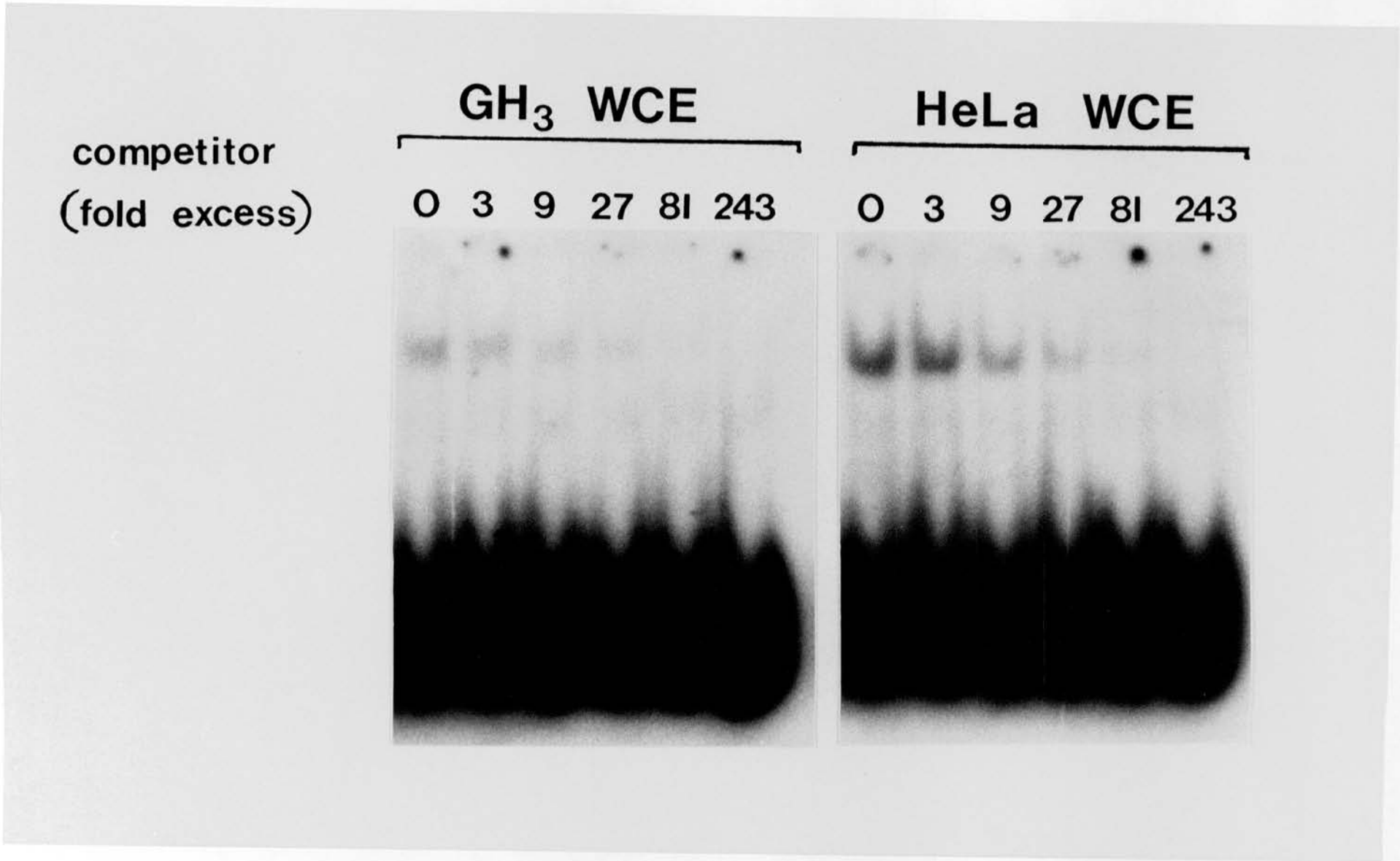
Factor Binding is Specific: If factor binding to a labelled DNA sequence is specific, then an excess of unlabelled homologous DNA sequence (competitor) included in the band shift reaction, will compete for factor binding, whereas heterologous unrelated sequences (non-specific competitor) will not. Thus, signal intensity of the labelled DNA-factor complex will decrease in the presence of increasing quantities of specific unlabelled competitor and will be unaltered with non-specific competitor.

To determine whether formation of the identified complex represented a sequence-specific interaction with the ERE_{con} oligonucleotide, competition band shift assays were carried out (section 2.8.1). Increasing quantities, from 3-fold to 243-fold molar excess of unlabelled ERE_{con} oligonucleotide (Fig. 3.1a) were included in a series of band-shift reactions each with 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide, 4μg poly [d(I-C)] and 10μg of HeLa or GH_3 WCE (Fig. 3.5). Significant competition for factor binding was observed at 9-fold molar excess of competitor oligonucleotide over labelled oligonucleotide (Fig. 3.5). As competitor concentration increased, signal intensity of the DNA-factor complex decreased until complete competition (no complex visualised) was apparent between 81-fold and 243-fold molar excess of competitor (Fig. 3.5), implying complex formation was sequence-specific.

To confirm that factor binding was specific, the ability of SRE-unrelated sequences, encoded within digested plasmid DNA, to compete for factor binding, was examined. Plasmid DNA used was pBLCAT2 (Luckow & Schutz, 1987) which contains both prokaryotic derived DNA and eukaryotic DNA sequences. Eukaryotic sequences include transcription factor binding sites present in the tk promoter of HSV (Jones et al., 1985). Plasmid DNA was initially titrated; 3μg, 6μg and 9μg (Fig. 3.6a) or 1μg, 2μg, 3μg and 4μg (Fig. 3.6b) of AluI digested pBLCAT2 were included in band shift reactions

Figure 3.5 **Analysis of specificity of factor binding to the ERE_{con} oligonucleotide: ERE_{con} oligonucleotide as competitor**

10μg of HeLa or GH_3 WCE were incubated with 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide and 4μg poly [d(I-C)]. Increasing amounts of unlabelled ERE_{con} oligonucleotide were added, as indicated in fold molar excess over labelled oligonucleotide, above each lane. Free and bound labelled oligonucleotide were separated by 4% native polyacrylamide gel electrophoresis as described (section 2.2.2.2).


competitor
(fold excess)
GH3 WCE
HeLa WCE
0 3 9 27 81 243
0 3 9 27 81 243

(section 2.8.1) containing 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide (Fig. 3.1a) and 4µg poly [d(I-C)]. As can be seen in Fig. 3.6a, 3µg of plasmid (3rd lane) competed less well, and 6µg of plasmid (4th lane) competed more strongly for factor binding, than did 4µg poly [d(I-C)] (2nd lane) . In Fig. 3.6b, 4µg plasmid competitor (6th lane) exhibited competition at a similar level to 4µg poly [d(I-C)] (2nd lane). It was, therefore, decided to replace 4µg poly [d(I-C)] with 4µg AluI digested plasmid DNA as the non-specific competitor in the assay described below.

Either pBLCAT2 (Luckow & Schutz, 1987) or pLC3, a derivative of pBLCAT2 that differs by the presence of an ERE_{con} sequence cloned into the pBLCAT2 polylinker, was used in this competition band shift assay. pLC3 was constructed by inserting a 140bp DNA fragment of M13 mp19 DNA, in which the *lac* operator had been converted into an ERE_{con} sequence (K. Chapman, personal communication), into the polylinker of pBLCAT2 (pLC3 construction and insert sequence described in section 2.4.2). 4µg of AluI digested pLC3 plasmid DNA (approximately equivalent to a 10-fold molar excess of competitor ERE_{con}) competed for complex formation between HeLa WCE and ^{32}P-labelled ERE_{con} oligonucleotide (Fig. 3.7, lanes 2 & 4), whereas 4µg of AluI digested pBLCAT2 did not (Fig. 3.7, lanes 3 & 5). This demonstrated that the factor detected bound specifically to an ERE_{con} sequence embedded in a fragment of non-specific DNA and did not bind to the transcription factor binding sites (Sp1, CTF and TATA factor binding sites) in the HSV tk promoter region (Jones et al., 1985) of pBLCAT2.

Factor has affinity for different, related oligonucleotide sequences: To investigate DNA sequence alteration tolerated by the DNA-binding activity of the identified factor, its ability to recognise a SRE related to the ERE_{con}, although functionally distinct from it, was examined. Introduction of four base-pair changes within the conserved bases of the ERE_{con} converts this sequence into a functional GRE (Klock et al., 1987;

Figure 3.6 **Analysis of DNA-binding activity in the presence of competitor plasmid DNA**

10μg HeLa WCE (H) were incubated with 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide, except for the left-most lane in panels (a) and (b) where WCE was not included. Increasing amounts of unlabelled, AluI digested pBLCAT2 were added to reactions as indicated (μg comp.) above each lane. 4μg poly [d(I-C)] was added only to band shift reactions marked (–) Free and bound oligonucleotide were separated by 4% native polyacrylamide gel electrophoresis (section 2.2.2.2). Panels (a) and (b) represent different band shift assays.

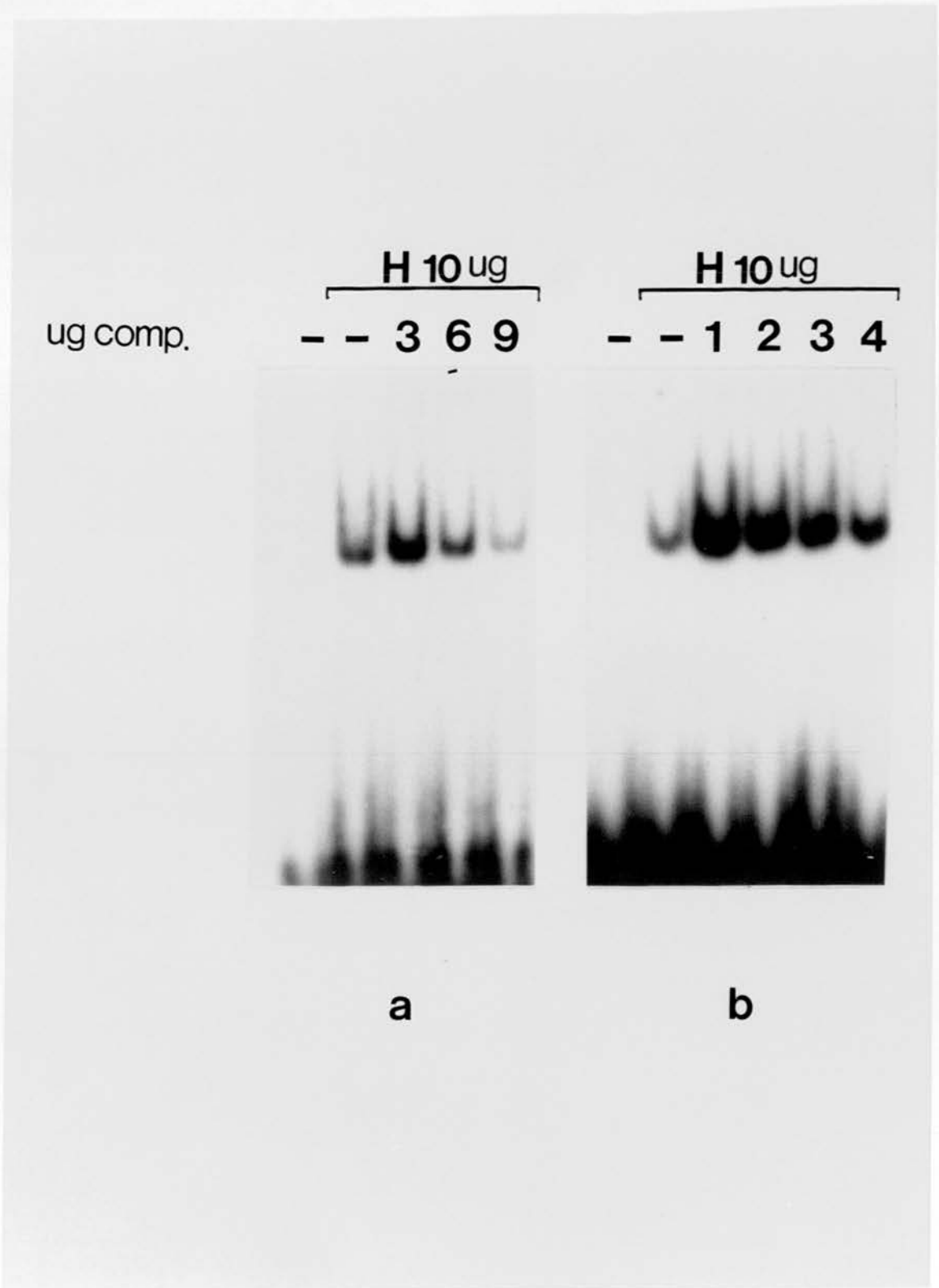
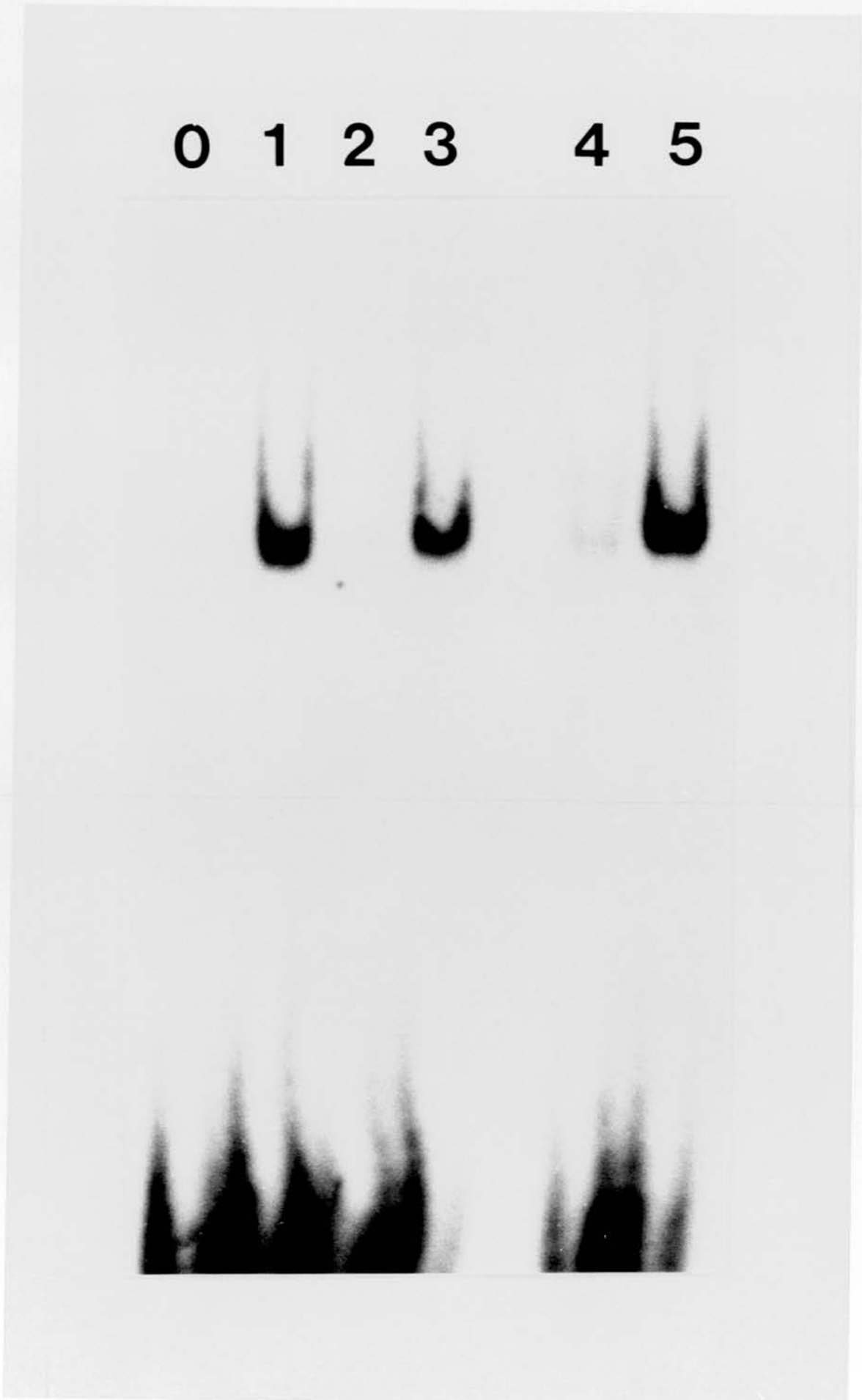

H 10 ug
H 10 ug
ug comp.
– – 3 6 9
– – 1 2 3 4
a
b

Figure 3.7 **Analysis of specificity of factor binding to ERE_{con} oligonucleotide: Alu I digested plasmid DNA as competitor**

10μg (lanes 1-3) or 20μg (lanes 4-5) HeLa WCE were incubated with 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide. Lane 0 does not contain WCE. Competitor unlabelled DNA was 4μg poly [d(I-C)] (lanes 0 and 1), 4μg AluI digested pLC3 (lanes 2 and 4) or 4μg AluI digested pBLCAT2 (lanes 3 and 5). Free and bound oligonucleotide were separated by 4% native polyacrylamide gel electrophoresis (section 2.2.2.2).

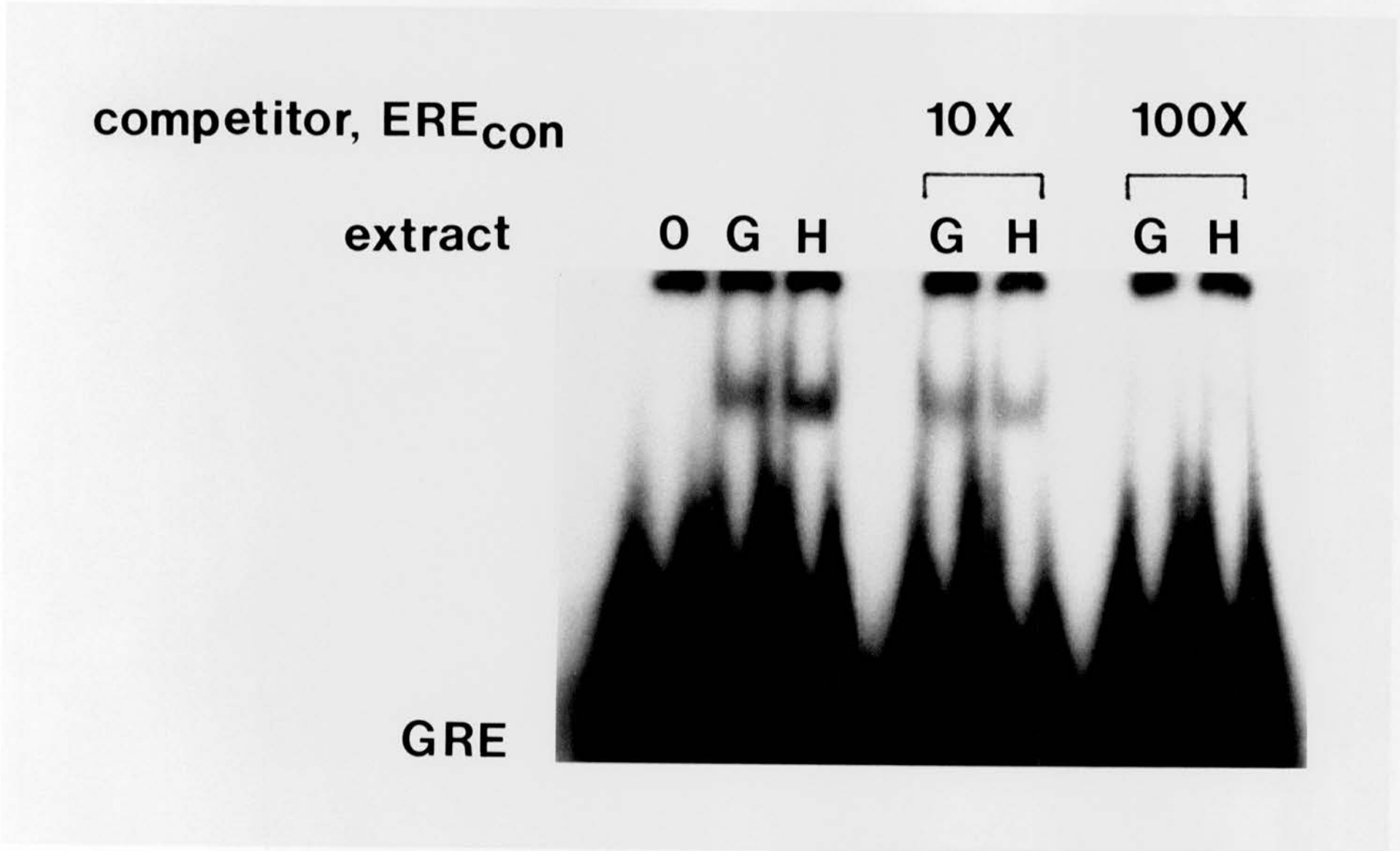

0 1 2 3 4 5

Martinez et al., 1987; Fig. 3.1) (GRE, Fig. 3.1c). Surprisingly, complex formation was observed between 0.1pmol ^{32}P-labelled GRE oligonucleotide and 10μg of either HeLa or GH_3 WCE (Fig. 3.8). Once more, complex formation was specific; complete competition by 100-fold molar excess of unlabelled ERE_{con} oligonucleotide was observed (Fig. 3.8, 6th & 7th lanes). Since this identified factor bound specifically to two functionally distinct SREs, it was named the Steroid Response Element-Binding Protein (SRE-BP).

Having determined that SRE-BP binding to ERE_{con} and GRE oligonucleotides was specific, SRE-BP affinity for oligonucleotides containing ERE_{con} and GRE related sequences was examined. ERE_{PRL} differs from the conserved nucleotides of the ERE_{con} by two base-pairs (Maurer & Notides, 1987; Waterman et al., 1988) (ERE_{PRL}; Fig. 3.1b). A mutant GRE (GRE_M) was created by increasing the spacing between the conserved nucleotides of a GRE (GRE_M; Fig. 3.1d), and a TRE was created by decreasing the spacing between the conserved nucleotides of a consensus ERE (Glass et al., 1988) (TRE; Fig. 3.1e). The ability of each oligonucleotide to compete for SRE-BP binding in 10μg HeLa WCE to 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide in a competition band shift assay (Fig. 3.9, left panel), was examined. A 100-fold molar excess of unlabelled ERE_{PRL} or GRE oligonucleotide competed for SRE-BP binding at a level comparable to that observed when 100-fold molar excess of ERE_{con} oligonucleotide was used (Fig. 3.9, compare lanes 2, 3 & 4 with lane 1). A 100-fold molar excess of unlabelled GRE_M oligonucleotide competed less well (compare lane 5 with lanes 1 & 2) whereas a 100-fold molar excess of unlabelled TRE oligonucleotide competed weakly, if at all, for SRE-BP binding (compare lane 6 with lanes 1 & 2). In the reciprocal band shift assay (Fig. 3.9, right panel), a similar competition pattern was seen in which 100-fold molar excess of unlabelled ERE_{con}, ERE_{PRL} or GRE oligonucleotide competed with 0.1pmol ^{32}P-labelled GRE oligonucleotide for SRE-BP binding (compare

Figure 3.8 **Specificity of factor binding to the GRE oligonucleotide: ERE_{con} oligonucleotide as competitor**

10µg of GH_3 (G) or HeLa (H) WCE were incubated with 0.1pmol ^{32}P-labelled GRE oligonucleotide in the presence of 4µg poly [d(I-C)]. Lane 0 does not contain WCE. Excess unlabelled ERE_{con} oligonucleotide was added as indicated in fold molar excess (10 x and 100 x) above the four right-most lanes. Free and bound oligonucleotide were separated by 4% native polyacrylamide electrophoresis (section 2.2.2.2).


competitor, ERE$_{con}$
10X
100X
extract
0 G H
G H
G H
GRE

lanes 2, 3 & 4 with lane 1); 100-fold molar excess of unlabelled GRE_M oligonucleotide competed poorly (compare lane 5 with lane 1 & 2) and the TRE did not compete (compare lane 6 with lanes 1 & 2). It was also noted that GRE_M was consistently a slightly better competitor for binding to a labelled GRE than to a labelled ERE_{con}.

As GRE_M and TRE oligonucleotides displayed consistently poor competition for SRE-BP binding, it followed that little or no SRE-BP-DNA complex would be detected when either labelled GRE_M or TRE oligonucleotide was used in a band shift assay. SRE-BP binding to either 0.1pmol ^{32}P-labelled GRE_M or TRE oligonucleotides was not detected after incubating with 10μg HeLa WCE and 4μg poly [d (I-C)] (Fig. 3.10, 8th & 6th lanes, respectively). In order to detect any weak complex formation, the gel was exposed to autoradiographic film for 10 times longer than usual; minimal SRE-BP GRE_M binding was detected (Fig 3.10, 12th lane) but no SRE-BP TRE-binding was observed (Fig. 3.10,10th lane). The SRE-BP therefore, binds with highest affinity to oligonucleotides which contain either an ERE or GRE sequence; the SRE-BP also binds to a GRE_M oligonucleotide although with reduced affinity, and does not bind at all to a TRE under band shift assay conditions. The SRE-BP, therefore, has varied affinity for different, related oligonucleotide sequences, although SRE-BP binding is more selective for either an ERE or a GRE, but preferential to neither.

3.2.2 The SRE-BP is located in the nucleus

The cellular location of the SRE-BP was investigated; DNA-binding proteins must be localised in the cell nucleus with DNA substrate in order to have a biological effect. Nuclear extracts of HeLa cells were prepared (section 2.6.2) and assayed for SRE-BP binding activity using 0.1pmol ^{32}P-labelled GRE oligonucleotide under standard band shift assay conditions (section 2.8.1). 4μg HeLa nuclear extract gave rise to a complex (Fig. 3.11) with identical

Figure 3.9 **Specificity of factor binding to both an ERE_{con} oligonucleotide or a GRE oligonucleotide**

10μg of HeLa whole cell extract were incubated with 4μg poly [d(I-C)] and either 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide (left panel) or 0.1pmol ^{32}P-labelled GRE oligonucleotide (right panel). Lane 0 does not contain WCE; lane 1 does not contain competitor; lanes 2-6 show binding in the presence of a 100-fold molar excess of competitor oligonucleotide as follows: lane 2, ERE_{con}; lane 3, ERE_{PRL}; lane 4, GRE; lane 5, GRE_M; lane 6, TRE. Free and bound labelled oligonucleotide were separated on a 4% polyacryalmide gel by electrophoresis (section 2.2.2.2).

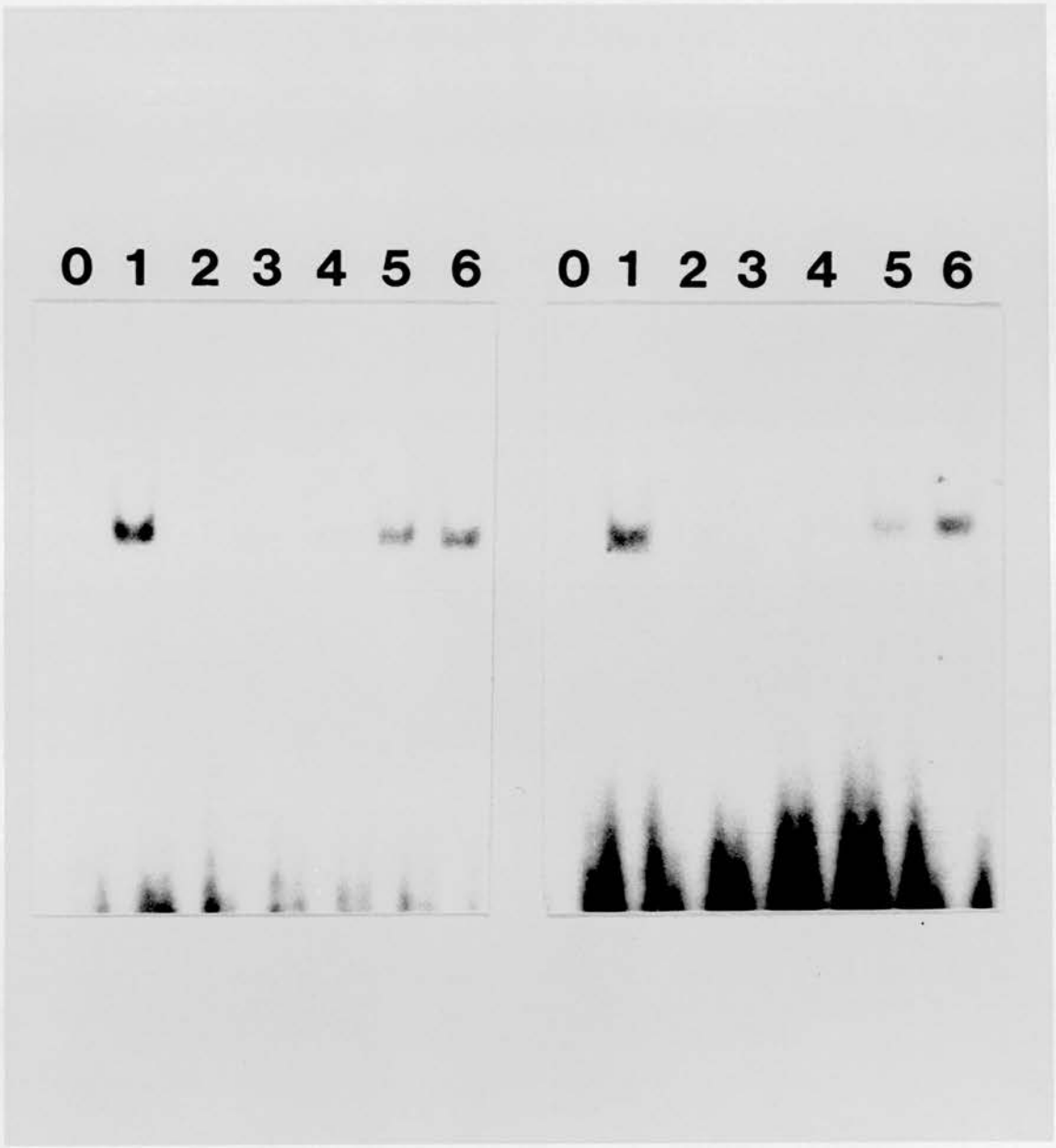
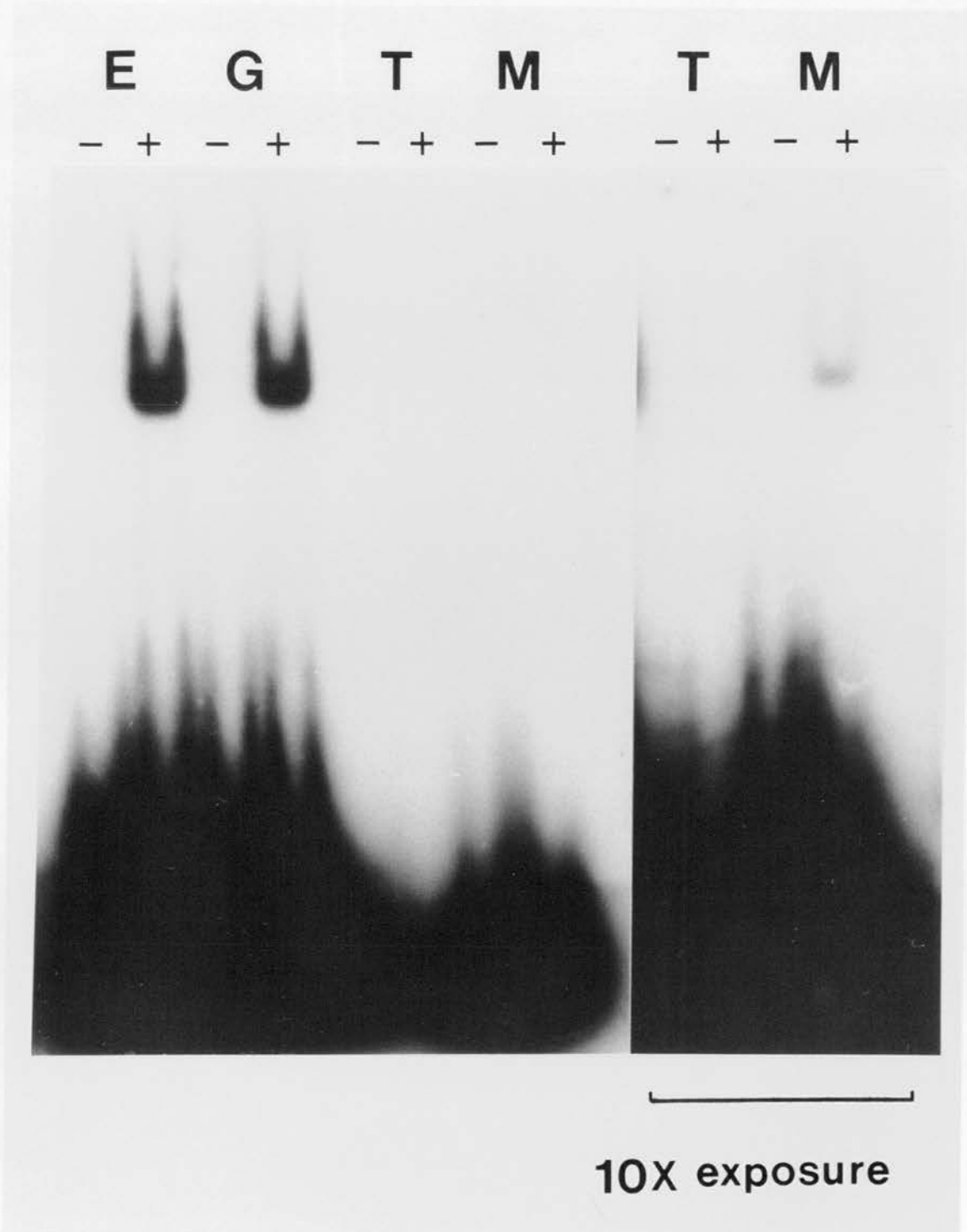

0 1 2 3 4 5 6
0 1 2 3 4 5 6

Figure 3.10 **Specificity of factor binding to radiolabelled ERE_{con}, GRE, TRE and GRE_M oligonucleotides**

10μg of HeLa WCE was (+) or was not (-) incubated with 0.1pmol each of ^{32}P-labelled ERE_{con} oligonucleotide (E), GRE oligonucleotide (G), TRE oligonucleotide (T), or GRE_M oligonucleotide (M) in the presence of 4μg poly [d(I-C)]. The right-most four lanes represent a 10 times longer exposure of the adjacent 4 lanes. Free and bound oligonucleotide were separated by 4% native polyacrylamide gel electrophoresis (section 2.2.2.2).


E G T M T M
− + − + − + − + − + − +
10X exposure

characteristics to SRE-BP complex formed by 10μg HeLa WCE; 100-fold molar excess of ERE_{con} and GRE oligonucleotide competed for complex formation (Fig. 3.11, lanes E & G), whereas GRE_M and TRE oligonucleotides did not (Fig. 3.11, lanes M & T). The sequence preference of nuclear factor binding and its identical electrophoretic mobility to SRE-BP in HeLa WCE (data not shown) indicated that this activity was SRE-BP and that SRE-BP was, therefore, localised in the cell nucleus. However, the possibility that SRE-BP activity is also found in the cytoplasm cannot be ruled out.

3.2.3 The SRE-BP is a protein

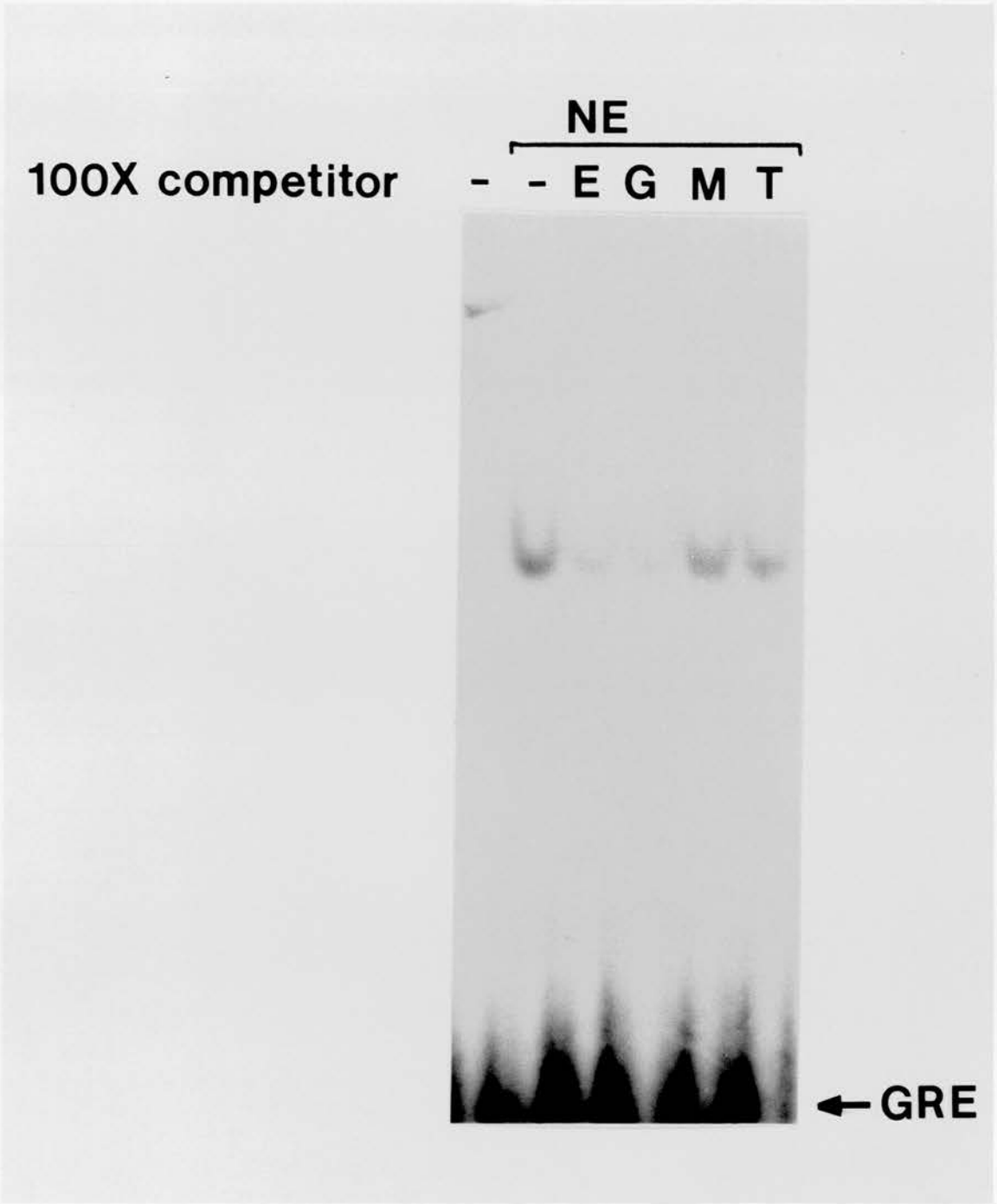
Extracts of whole cells have been used in many of the experiments detailed in this thesis. The method by which whole cell extracts are prepared (section 2.6.1) will yield a solution highly enriched for protein substrates. It is, therefore, extremely likely that the identified SRE-BP is a protein species. The major fat component in cells is phospholipid which is found in cellular membranes. It is assumed that these membranes are removed after cell lysis, along with contaminating nucleic acids, by high speed centrifugation; presumably, the resulting WCE has negligible content of fatty acid and nucleic acid. In this section, experiments are described in which various treatments which inactivate proteins were administered to HeLa WCE or to partially purified SRE-BP derived from HeLa WCE (see Chapter 4, section 4.2.1.1 and section 2.9) to reinforce this premise. Treated protein extracts were assayed for SRE-BP activity.

Most proteins are inactivated by either heating to temperatures above 60°C or exposure to high (usually 6-8M) concentrations of urea. Heat and urea denaturation of proteins is caused by disruption of non-covalent bonds necessary to maintain the three dimensional conformation of a protein.

10μg HeLa WCE or 1μg partially purified SRE-BP (see Chapter 4, section 4.2.1.1 and section 2.9) were exposed either to 60°C for 10 mins or

Figure 3.11 **Analysis and specificity of SRE-BP activity in nuclear extracts of HeLa cells**

Nuclear extracts of HeLa cells were prepared as described in section 2.6.2. 10μg of HeLa nuclear extract were incubated with 0.1pmol ^{32}P-labelled GRE oligonucleotide and 4μg poly [d(I-C)]. The left-most lane in the band shift reaction did not contain nuclear extract. Excess (100-fold) unlabelled competitor oligonucleotide was added as follows: ERE_{con} oligonucleotide (lane E), GRE oligonucleotide (lane G), GRE_M oligonucleotide (lane M), TRE oligonucleotide (lane T). Free and bound oligonucleotide were resolved by 4% native polyacrylamide gel electrophoresis (section 2.2.2.2).


NE
100X competitor
- - E G M T
GRE

to 6M urea for 10 min at 0°C (section 2.6.1). Treated WCE or treated partially purified SRE-BP were incubated with 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide and 4μg poly [d(I-C)] in a band shift assay (section 2.8.1). Figure 3.12 demonstrates that SRE-BP DNA-binding activity was inactivated by heat treatment (Fig. 3.12a, compare lane 1 & 2 with 4 & 5) and exposure to 6M urea (Fig. 3.12b, compare lane 1 with lanes 3 & 4). SRE-BP binding activity was also denatured after leaving HeLa WCE at 4°C for more than six weeks, or at room temperature for more than two weeks; protein-DNA complexes with faster electrophoretic mobility were observed after 10μg of these old HeLa WCEs were incubated with ERE_{con} oligonucleotide (Fig. 3.12b, lane 2). This was probably due to proteolytic attack on SRE-BP.

3.3 DISCUSSION

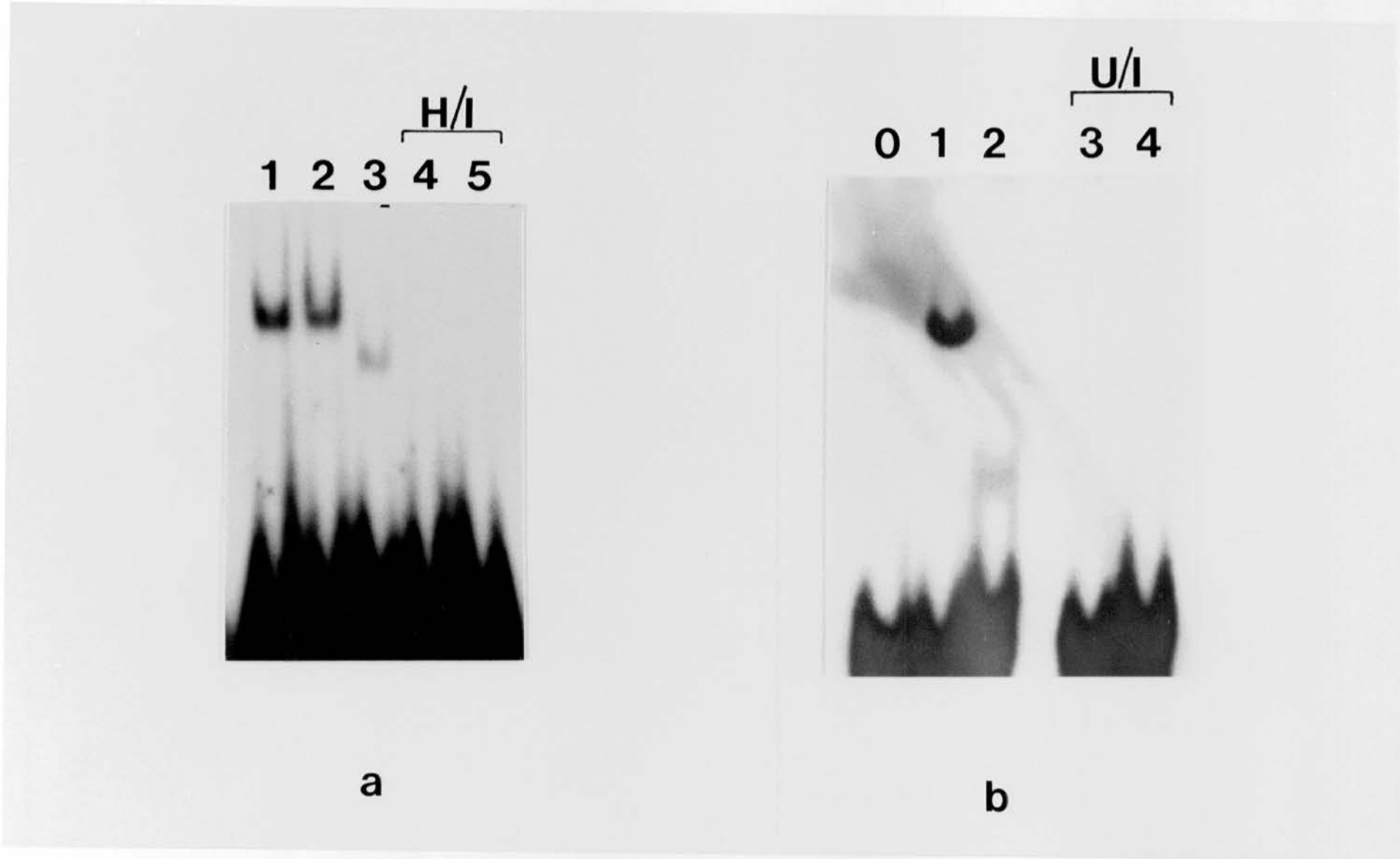
The results presented in this Chapter clearly show the existence of a cellular protein in WCE of HeLa, GH_3 and CV-1 cells, and in rat liver tissue, which exhibits preferential DNA binding to both ERE and GRE containing oligonucleotides. This cellular protein is named the Steroid Response Element-Binding Protein (SRE-BP).

The SRE-BP binds preferentially to EREs and a GRE *in vitro*. Transcription factor binding sites present within the truncated HSV tk promoter of pBLCAT2 (TATA, CTF and Sp1 binding sites) (Jones et al., 1985) are unable to compete for SRE-BP binding to an ERE_{con} oligonucleotide when digested pBLCAT2 DNA is included in the band shift reaction. Plasmid DNA only competes for complex formation if a 140bp DNA fragment containing an ERE_{con} sequence has been cloned into the pBLCAT2 polylinker. All of the DNA sequence flanking the ERE_{con} on the 140bp insert of pLC3 are derived from pUC (K.E. Chapman, personal communication). Exactly the same DNA sequences are present in the

Figure 3.12 **Analysis of SRE-BP activity after heat or urea denaturation**

Heat or urea denatured WCE was prepared as described (2.6.1) (a) 10μg and 20μg HeLa WCE (lanes 1 and 2); 10μg HeLa WCE which had been stored at 4°C for ~6 weeks (lane 3), or 10μg and 20μg heat denatured HeLa WCE (lanes 4 & 5 respectively) were incubated with 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide and 4μg poly [d(I-C)] (b) No WCE added to lane 0. 10μg HeLa WCE (lane 1); 10μg HeLa WCE having been left at room temperature for over two weeks (lane 2); 10μg and 20μg urea denatured HeLa WCE (lanes 3 and 4, respectively) were incubated as above.

Free and bound oligonucleotide were separated by 4% native polyacryalmide gel electrophoresis (section 2.2.2.2).


H/I
1 2 3 4 5
a
U/I
0 1 2 3 4
b

pBLCAT2 which contains the entire 2.7kb of pUC18 DNA (Luckow & Schutz, 1987). The observed competition is, therefore, due to the encoded ERE_{con} and not to flanking DNA sequences.

Three SREs; ERE_{PRL}, ERE_{con} and GRE, compete for SRE-BP binding to either an ERE or GRE oligonucleotide. Each SRE can function as a transcriptional enhancer in cell transfection studies. ERE_{PRL} contained within a 228bp fragment of rat prolactin DNA is able to confer oestradiol-dependent transcriptional enhancement on a linked promoter *in vivo* (Mauer & Notides, 1987; Waterman et al., 1988). Oligonucleotides encoding either a 13bp ERE consensus sequence or a 15bp GRE consensus sequence are independently able to confer transcriptional enhancement from a linked promoter *in vivo* in the presence of oestradiol and dexamethasone, respectively (Klock et al., 1987; Martinez et al., 1987; Klein-Hitpass et al., 1988; Strahle et al., 1987; Ham et al., 1988, Fig. 3.2). However, SRE-BP complex formation is competed for only weakly by a GRE_M oligonucleotide and undetectably by a TRE oligonucleotide. This weak competition is reflected in the low affinity binding of SRE-BP to a radiolabelled GRE_M oligonucleotide and in its total lack of binding to radiolabelled TRE oligonucleotide. GRE_M and TRE oligonucleotides share the same conserved nucleotides as the symmetrical GRE and ERE_{con} oligonucleotides, respectively. However, both GRE_M and TRE have mutated spacer regions with respect to the GRE and ERE_{con} respectively; the GRE_M has a 4bp spacer, whereas the TRE has no spacer. It therefore appears that the relative orientation and spacing of the SRE conserved arms is important for SRE-BP DNA-binding activity.

The DNA sequence at either end of ERE_{con} and GRE are identical to each other, although different from those of the TRE or GRE_M oligonucleotide. It can be argued that neither GRE_M nor TRE oligonucleotides compete for SRE-BP binding because they do not have

identical flanking DNA sequences to the ERE_{con} and GRE oligonucleotides. This is unlikely, since the ERE_{con} sequence encoded in pLC3 competes efficiently for SRE-BP binding, although its flanking DNA is different from ERE_{con} and GRE oligonucleotides. Also, the TRE and GRE_M oligonucleotides are only 20bp long whereas the ERE_{con} and GRE oligonucleotides are 21bp in length. As the capabilities of either TRE or GRE_M to bind SRE-BP are significantly different (see Fig. 3.10), it is unlikely that simply being a shorter oligonucleotide could account for the differential SRE-BP DNA-binding affinity exhibited.

Several lines of evidence indicate that the SRE-BP is unlikely to be either ER or GR. i) The SRE-BP is present in a number of cell-types, including HeLa cells and CV-1 cells. As neither HeLa nor CV-1 cells contain endogenous ER (Kumar & Chambon, 1988; Klein-Hitpass et al., 1989), SRE-BP activity cannot be due to the presence of contaminating ER. CV-1 cells do not respond to glucocorticoids (Giguere et al., 1986; Umesono & Evans, 1989) and it is generally assumed that they do not contain endogenous functional GR; it is, therefore, unlikely that SRE-BP activity is due to functional GR. It is, however, noted that Hoeck & Groner (1990), who also observed non-responsiveness of CV-1 cells to glucocorticoids, additionally demonstrated immunoprecipitation of a protein by polyclonal antiserum directed against the DNA-binding and ligand binding domains of rat GR. The relatedness of this protein to GR is not defined; nor is it known if this protein is peculiar to the CV-1 cell-line used in that particular study or present in all CV-1 cells lines. ii) In contrast to the sequence-specific binding of ER to an ERE, or GR to a GRE (Kumar & Chambon, 1988), the SRE-BP binds to either an ERE or a GRE without sequence preference for either. Beato (1989) claims that his laboratory detected significant binding of ER to the MMTV promoter, which contains a GRE, *in vitro*. However, no evidence of this has been published , and

conditions under which this apparent ER-GRE interaction is detected are not available for scrutiny. iii) GR is located cytoplasmically in its uninduced state (Wilkstrom et al., 1987). However, SRE-BP GRE-binding activity is detected in nuclear extracts of HeLa cells which were not exposed to high levels of hormone. Although some endogenous HeLa GR may be activated by residual glucocorticoids in the serum added to tissue culture medium (see section 2.5 for tissue culture condition), taking into account all three arguments it is unlikely that observed SRE-BP activity is due to either ER or GR activity.

It is also unlikely that the SRE-BP activity observed is due to TR. Although TR binds to an ERE with high affinity, TR does not bind to a GRE (Glass et al., 1988). Additionally, CV-1 cells do not contain endogenous TR (Glass et al., 1988; Umesono & Evans, 1989), although they do contain SRE-BP.

It is widely accepted that SRs bind to their specific SRE with high affinity *in vivo* and enhance transcription from a linked promoter. In support of this, Becker et al. (1986) have reported hormone-dependent changes in reactivity of guanine residues in the GRE of the tyrosine aminotransferase gene which reflect GR binding to its GRE *in vivo* in a hormone-dependent manner. In the absence of hormone, Becker et al. (1986) report some changes in reactivity of guanine residues within the GRE *in vivo*, compared to reactivity within the same region of naked DNA, *in vitro*. This data is consistent with the possibility that a protein (possibly SRE-BP) is bound to the GRE *in vivo* in the absence of hormone, although with lower affinity than activated GR. The data do not rule out the possibility that other proteins, in addition to activated GR, may bind to the GRE during hormone activation.

In contrast to the situation *in vivo*, *in vitro* studies which have examined the ability of purified SR to discriminate between a specific SRE and non-specific DNA have largely failed to demonstrate high specificity of

binding. In many cases, the purified receptor binds with less than 10-fold higher affinity to SRE-containing DNA than non-specific DNA (Maurer & Notides, 1987; Payvar et al., 1981; Compton et al., 1983; Bailly et al., 1986; von der Ahe et al., 1985; Jantzen et al., 1987; Peale et al., 1988; Härd et al., 1990b). Higher specificity could be achieved, in part, by accessory proteins. A number of reported experiments directly, or indirectly, suggest the involvement of accessory proteins in increasing sequence-specific binding of SRs to target DNA sequences (Feavers et al., 1987; Wrange et al., 1986; Payvar et al., 1983; Edwards et al., 1989) and of TR (Murray & Towle, 1989; Chin, 1990). Only one such accessory protein has been shown to bind directly and specifically to a SRE; Feavers et al. (1987) have identified a 70kD protein, NHP-1 (which is not ER) which binds specifically to the ERE of chick vitellogenin gene and which increases ER binding to the ERE. SRE-BP and NHP-1 have various features in common; neither is tissue- nor species-specific and neither binds to an ERE which retains the conserved nucleotide arms but has the 3bp spacer region deleted. Feavers et al. (1987) report a chick hepatoma cell line, DU249/2, which does not contain NHP-1. It would be interesting to examine whether this cell line also lacks SRE-BP DNA-binding activity.

Electron microscopic resolution of interaction between SR in nuclear extract and SRE demonstrate that these complexes are large and probably include additional proteins (Heggeler-Bordier et al., 1987). Furthermore, several reports demonstrate preferential binding of purified SR to DNA already complexed with protein, rather than to naked DNA (Thrall & Spelsberg, 1980; Feavers et al., 1987). This suggests the involvement of DNA-bound accessory proteins (possibly SRE-BP) in increasing the specificity of the SR-SRE interaction.

In contrast to SRs themselves, the SRE-BP interacts with two classes of functionally distinct SRE, binding to an ERE and GRE with similar affinity.

EREs and GREs direct oestrogen and glucocorticoid regulation of gene transcription, respectively. The GRE is also capable of directing transcriptional control in response to progestins, androgens and mineralocorticoids (Ham et al., 1988; Arizza et al., 1987). A GRE with identical conserved nucleotides to the symmetrical GRE used in this study can direct glucocorticoid and progesterone transcriptional regulation in cell transfection studies (Strahle et al., 1987). In this respect, the SRE-BP may be analogous to the 90kD heat shock protein which plays a global role in steroid hormone action. Hsp90 interacts with ER, GR, AR and PR (Renoir et al., 1990) and probably with MR, in the absence of hormone, and maintains the SR in a conformation which cannot bind to DNA (Denis et al., 1988b) but which can efficiently bind steroid hormone (Dalman et al., 1989; see section 1.6.3 for review). I propose that the SRE-BP described in this thesis whose DNA-binding activity is not confined to one class of SRE or one type of tissue, also plays a global role in steroid hormone regulation of gene transcription by modulating DNA-binding affinity of SR for SRE.

CHAPTER 4

MOLECULAR CHARACTERISATION OF THE STEROID RESPONSE ELEMENT-BINDING PROTEIN

4.1 INTRODUCTION

In the previous Chapter, the SRE-BP was identified and its DNA-binding affinity for different, related SREs was assessed by band shift analysis. For this chapter, the techniques of gel filtration chromatography, pore gradient gel electrophoresis, and UV-crosslinking were employed to investigate the relative mol. wt. of SRE-BP in its soluble or DNA-bound state, and the SRE-BP subunit composition, respectively.

Gel filtration chromatography commands separation of molecules based on their ability to be absorbed on Sephacryl beads, which, in turn, is dependent on molecule size and shape. Small protein molecules are easily absorbed on sephacryl beads and, therefore, their progression through the column is impeded, whereas large protein molecules are less easily absorbed and proceed through the column faster than smaller molecules. Thus, proteins of different mol. wts. are separated from each other and collected in fractions which elute from the column.

The size of the Sephacryl bead determines the mol. wt. parameters within which molecules can be separated. For the purpose of this study, a Sephacryl S300 column was used (precalibrated by Dr. S. Chapman, Department of Chemistry, Edinburgh University) which allowed absorption, and so separation of proteins of mol. wt. below 300kD. Proteins larger than the 300kD exclusion limit would travel through the column unhindered with the void buffer volume.

In pore gradient gel electrophoresis, protein-DNA complexes are electrophoresed adjacent to protein standard mol. wt. markers through a polyacrylamide gel with a pore size gradient (Rodriguez & O'Malley, personal communication; Andersson et al., 1972); the largest pores are nearest the loading wells of the gel and the smallest pores are furthest away from the wells. Complexes and markers eventually reach a pore size in the gradient gel through which they cannot proceed any further, as the pore size

becomes too small to accommodate the size of the molecule. Consequently, when the gradient gel is electrophoresed for a sufficient length of time, electrophoretic mobility of protein-DNA complexes and of protein markers is ultimately determined by their respective mol. wts. and is not affected by electrostatic charge (except for very positively charged proteins which are drawn out of the gel loading wells into the reservoir buffer). The mol. wt. of a protein-DNA complex can be determined by comparing its electrophoretic mobility to that of protein mol. wt. markers. Characterised mol. wts. published in this thesis represent mean and range calculations.

UV-crosslinking uses short-wave (312nM, for this study) UV-light to covalently crosslink protein to DNA (or RNA). Using this technique, specific interactions between protein and nucleic acid can be stabilised for further characterisation and analysis.

The chemistry behind UV-crosslinking is only partly understood. UV-irradiation of protein-nucleic acid complexes forms a covalent linkage between nucleotide and amino acid side chain. Nucleotide-amino acid crosslinks are specific as neither nucleic acid-nucleic acid nor protein-protein covalent crosslinks occur (Smith, 1976; Woppmann et al., 1988). Since UV light has a short effective crosslinking range, protein-nucleic acid adducts are formed only when interacting groups are in close proximity, within one bond length apart (Smith, 1976). Therefore, protein-nucleic acid molecules which become crosslinked are assumed to have been in direct contact in the stable non-covalent protein-DNA complex.

In this study, UV-crosslinking of cellular proteins to DNA containing a bromodeoxyuridine (BdU) nucleotide was employed. BdU is a derivative of thymine, which is particularly sensitive to irradiation by UV to form covalent BdU-protein crosslinks.

Two derivatives of the ERE_{con} oligonucleotide (Fig. 3.1a) were used for crosslinking analysis, each incorporating a single thymine to BdU substitution (Fig. 4.7). BdU-ERE1 contains a substitution within the ERE_{con}

spacer region, whereas BdU-ERE2 has a substitution within the conserved nucleotides of the ERE_{con}. In addition, the BdU substitutions are 4bp apart in their respective oligonucleotide. Hence, in their double-stranded conformation, each BdU substitution will almost be on opposite sides of the DNA helix, assuming 10.5bp per turn of B-form DNA. BdU-ERE1 and 2, therefore, provide differential positioning and orientation of UV-sensitive BdU molecules which will maximise the chance of generating crosslinked SRE-BP-BdU complexes.

This chapter describes mol. wt. characterisation of the SRE-BP as an approximately 200kD protein in both its soluble and DNA-bound state. Preliminary evidence is also presented which suggests the SRE-BP is not a single ~200kD polypeptide but probably a multisubunit protein complex.

4.2 RESULTS

4.2.1 Molecular weight characterisation of the SRE-BP

4.2.1.1 The SRE-BP is stable in solution as a 205kD (±20kD) protein:

The relative mol. wt. of the SRE-BP, as it exists in solution, was determined by gel filtration chromatography (section 2.9). HeLa WCE resuspended in column buffer (section 2.9) exhibited SRE-BP ERE-binding activity in a band shift reaction, although at a reduced level compared to WCE resuspended in extract buffer (section 2.6.1) (Fig. 4.2, compare complexes in lanes B and A, respectively). Approximately 7mg HeLa WCE (with blue dextran and cytochrome C added as described in section 2.9) were loaded onto the precalibrated, preequilibrated Sephacryl S300 column and allowed to travel through the column at 4°C for 24h-48h (section 2.9). In one such experiment, a total of 47 fractions each of 3.5ml eluted from the column within the mol. wt. range 300kD-12.5kD. 18µl of each fraction was assayed for binding to 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide in a

20µl band shift reaction containing 4µg poly [d(I-C)] (Fig. 4.2). Most of the ERE-binding activity was contained in fractions 20 and 21 (Fig. 4.2) with a lower amount of ERE-binding activity detected in fractions 19 and 22. Fractions 15 to 27 were subsequently assayed for binding to 0.1pmol ^{32}P-labelled GRE oligonucleotide in a similar band shift assay (Fig. 4.3). GRE-binding activity was also predominantly detected in fractions 20 and 21 with a lower amount observed in fractions 19 and 22 (Fig. 4.3). Thus, ERE and GRE binding activities co-eluted from the Sephacryl S300 column, supporting the theory that one cellular protein, the SRE-BP, contains both activities.

SRE-BP mol. wt. was determined relative to the protein mol. wt. markers (described in section 2.9). The average of the volumes at which fractions 20 and 21 eluted was divided by the void volume; the result was plotted on the calibration graph (Fig. 4.1). As can be seen from Fig. 4.1, SRE-BP eluted in a buffer volume at which proteins of approximately 200kD elute. Analysis of results provided by two different gel filtration chromatography experiments revealed the soluble form of SRE-BP had a relative mol. wt. of 205kD (± 20kD).

The SRE-BP has been partially purified: In addition to characterising the relative mol. wt. of the SRE-BP, gel filtration chromatography of HeLa WCE also partially purified SRE-BP activity from other cellular proteins of different mol. wt. The two fractions containing the most SRE-BP DNA-binding activity(20 and 21 in the described experiment) were pooled (total volume 7mls) and concentrated using an Amicon filter to a final volume of ~800µl (section 2.9). Concentrated partially purified SRE-BP was 1µgµl^{-1} as determined by Biorad Protein Assay (section 2.7).

To estimate the degree to which SRE-BP had been purified by gel filtration chromatography, the amount of partially purified SRE-BP required to give a protein-DNA complex of equivalent signal intensity to 10µg of HeLa

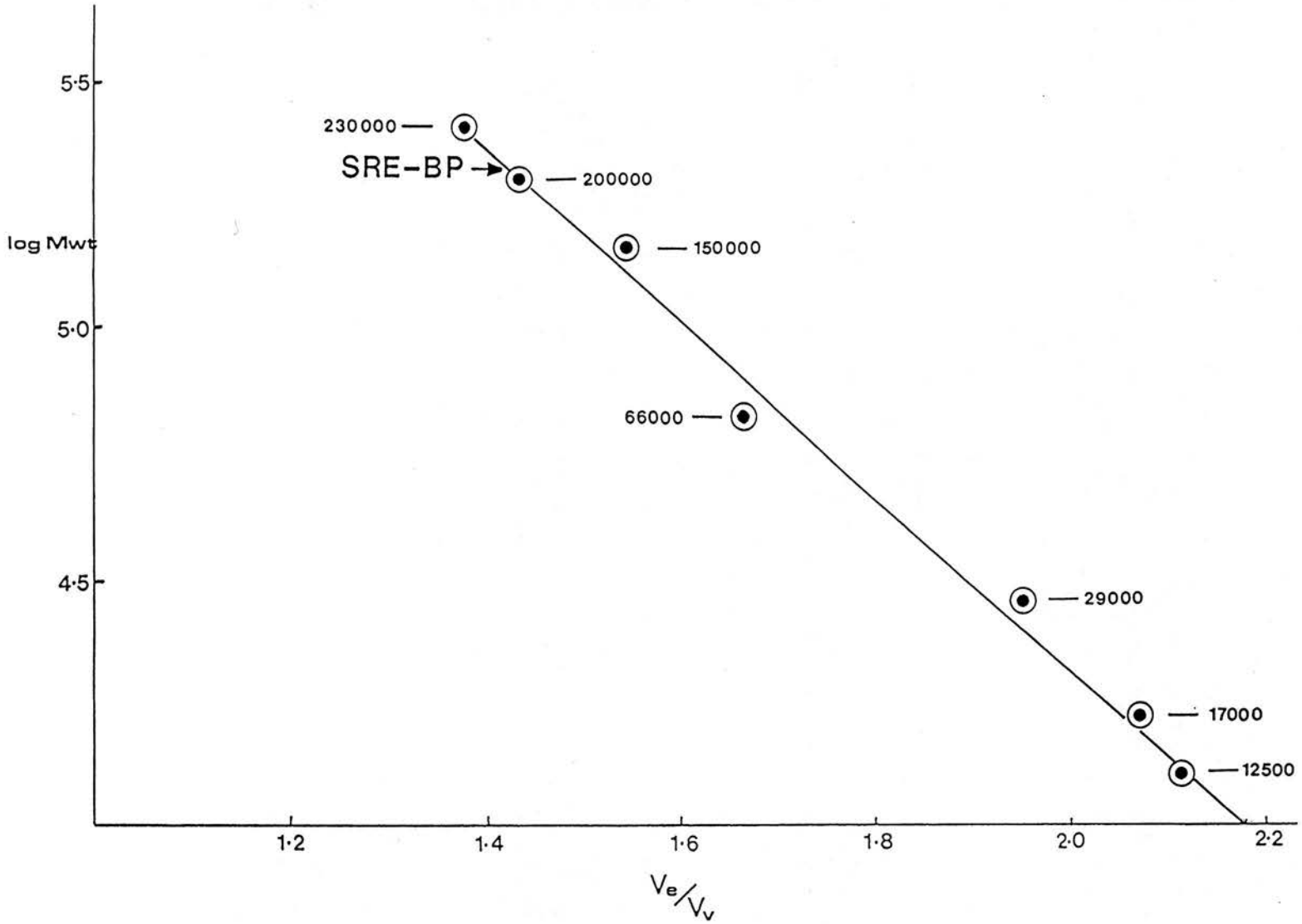


Fig 4.1 Calibration of gel filtration column. Arrow indicates the volume at which SRE-BP activity elutes relative to the proteins used to calibrate the column

Figure 4.2 **Gel filtration chromatography: Elution profile of ERE-binding activity**

HeLa WCE was fractionated over a Sephacryl S300 column; fractions were collected for band shift analysis. Each reaction contained 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide and 4µg poly [d(I-C)]. Reactions were as follows: no protein added (lane A), 10µg HeLa WCE finally resuspended in either extract buffer (lane B) or column buffer (lane C), or 18µl of fractions 1-26 (numbered above each lane). Free and bound oligonucleotide were separated by 4% native polyacrylamide gel electrophoresis (section 2.2.2.2).

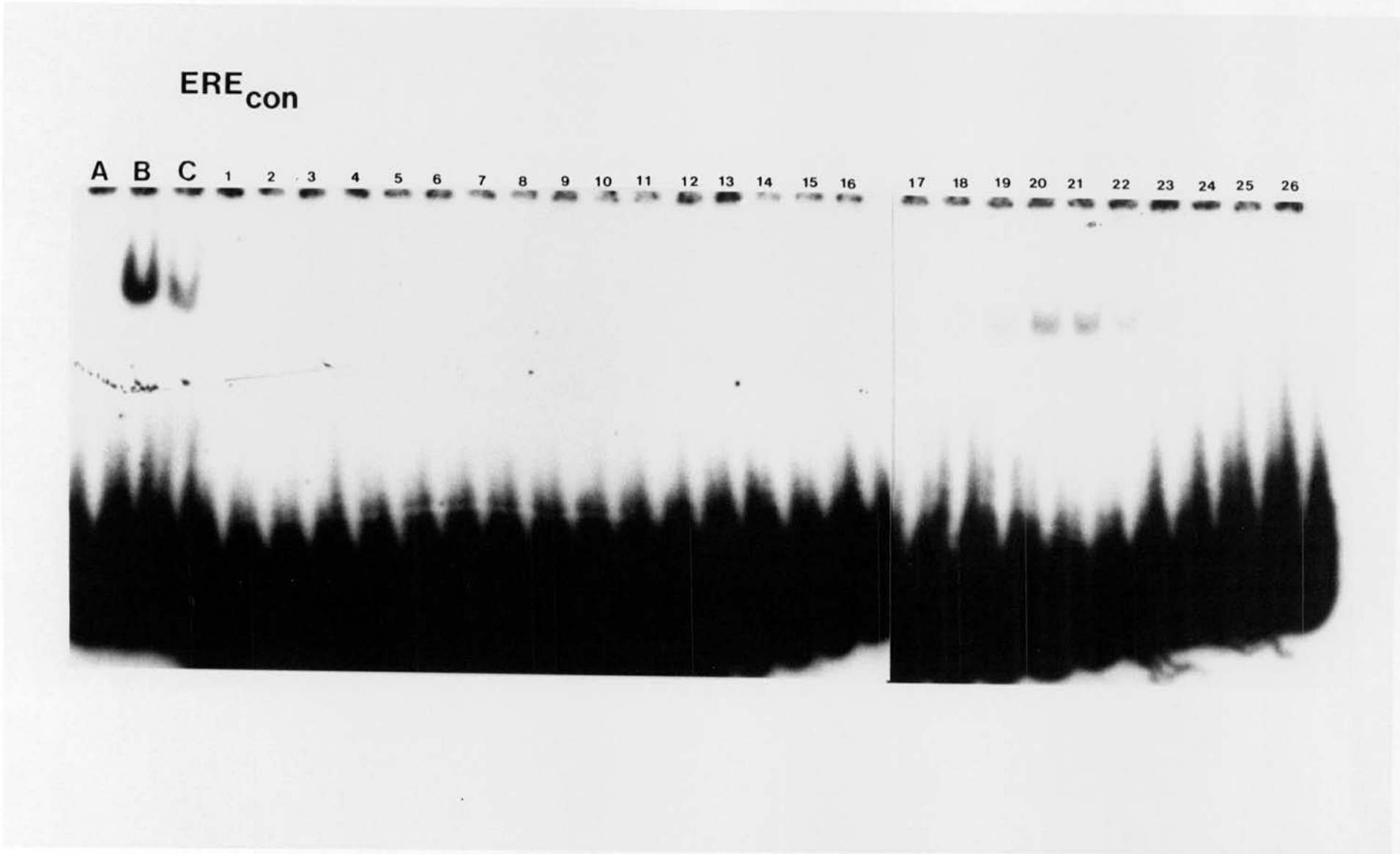
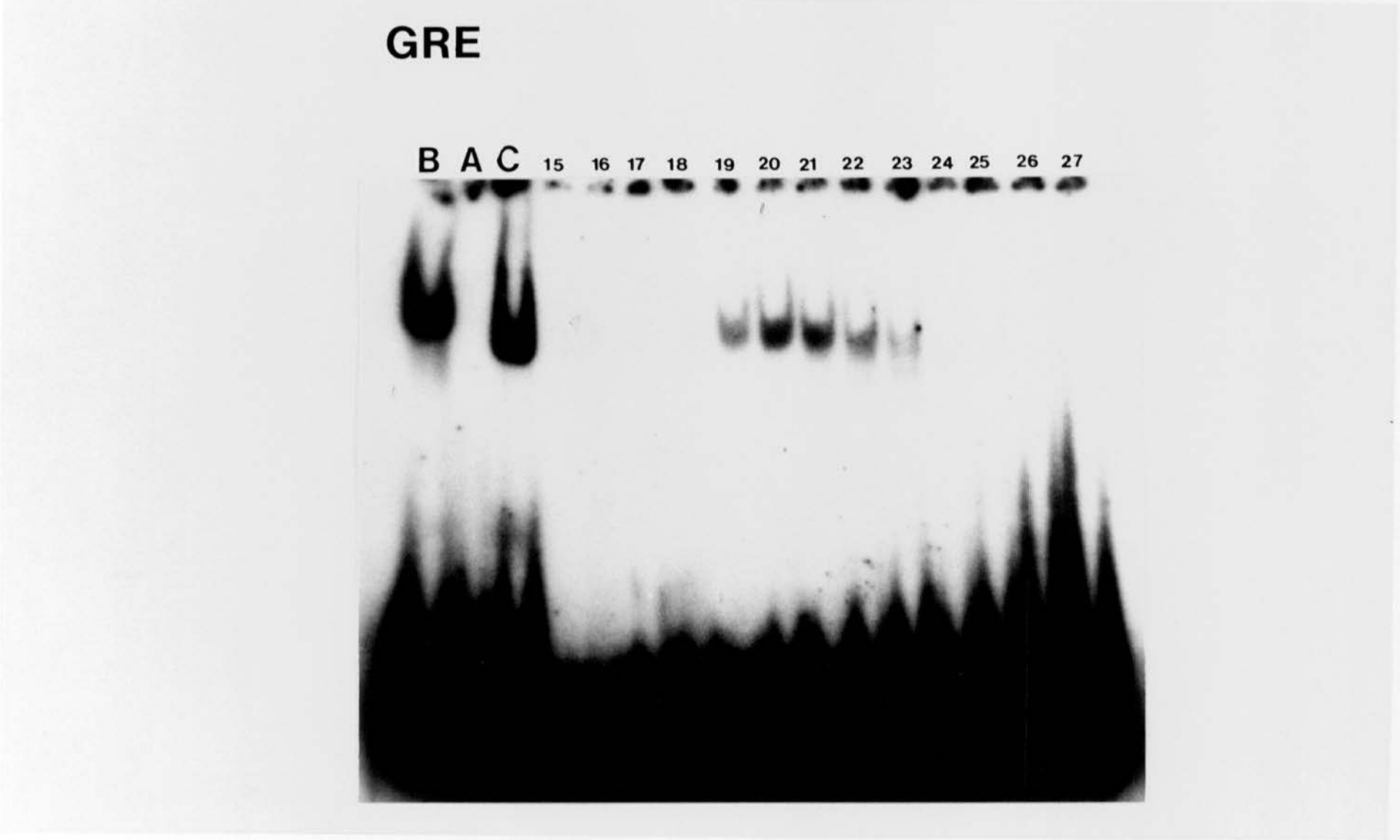

ERE con
A B C 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

Figure 4.3 **Gel filtration chromatography: Elution profile of GRE-binding activity**

HeLa WCE was fractionated over a Sephacryl S300 column; fractions were collected for band shift analysis. Each reaction contained 0.1pmol ^{32}P-labelled GRE oligonucleotide and 4μg poly [d(I-C)]. Reactions were as follows: no protein added (lane A), 10μg HeLa WCE finally resuspended in either extract buffer (lane B) or column buffer (lane C), or 18μl of fractions 15-27 (numbered above each lane). Free and bound oligonucleotide were separated by 4% native gel electrophoresis (section 2.2.2.2).


GRE
B A C 15 16 17 18 19 20 21 22 23 24 25 26 27

WCE was determined in a band shift assay. Either 1μg, 2μg or 3μg of partially purified SRE-BP or 10μg of HeLa WCE were mixed with 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide and 4μg poly [d(I-C)] in a standard band shift reaction (section 2.8.1). DNA-binding activity in 1μg of partially purified SRE-BP was at least equal to that contained in 10μg of HeLa WCE (Fig. 4.4, compare lanes 2 and 3). Thus, the partially purified SRE-BP represented at least a 10-fold purification of SRE-BP from HeLa WCE.

When 3μg of partially purified SRE-BP was included in the band shift, a second complex exhibiting faster electrophoretic mobility, but the same signal intensity compared to the SRE-BP/ERE complex, was observed which was not seen when only 1μg partially purified SRE-BP was used (Fig. 4.4, compare lane 3 with 1). The reason for the formation of this second complex remains unknown.

To verify that partially purified SRE-BP retained its previously characterised DNA-binding preference for both EREs and a GRE (described in Chapter 3), a competition band shift assay was performed. In a 20μl band shift reaction containing 4μg poly [d(I-C)] (see section 2.8.1), 100-fold molar excess of unlabelled ERE_{con}, ERE_{PRL} or GRE oligonucleotides (oligonucleotide sequences detailed in Fig. 3.1) competed efficiently for binding of 1μg of partially purified SRE-BP to 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide (Fig. 4.5, lanes 2-4), whereas 100-fold molar excess of GRE_M competed poorly (Fig. 4.5, lane 5). This competition pattern was identical to that previously characterised for SRE-BP DNA-binding activity present in 10μg HeLa WCE (compare Fig. 4.5 to Fig. 3.9). Hence, partially purified SRE-BP, retained the ability to bind preferentially to oligonucleotides containing an ERE or a GRE sequence.

Figure 4.4 **Analysis of DNA-binding activity in partially purified SRE-BP**

Sephacryl S300 column fractions containing SRE-BP activity (fractions 20 and 21 in Figs. 4.2 and 4.3) were pooled and concentrated using an Amicon filter. Concentrated partially purified SRE-BP was used for band shift analysis as shown: 10μg HeLa WCE (H) or 1μg, 2μg, and 3μg of partially purified SRE-BP (S300) (as indicated above relevant lanes) were incubated with 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide and 4μg poly [d(I-C)]. Lane O did not contain any protein. Free and bound oligonucleotide were separated by 4% polyacryalmide gel electrophoresis (section 2.2.2.2).

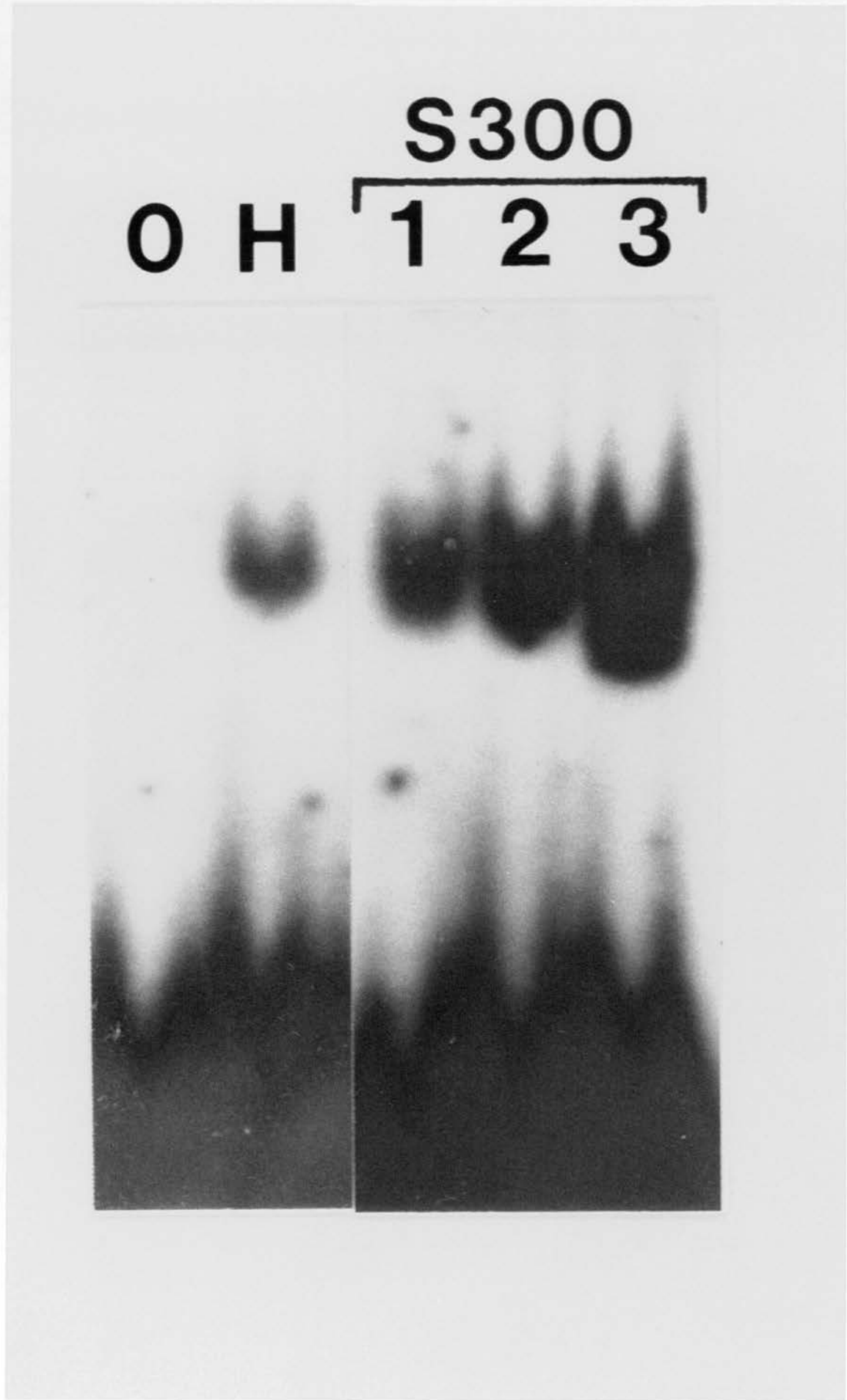
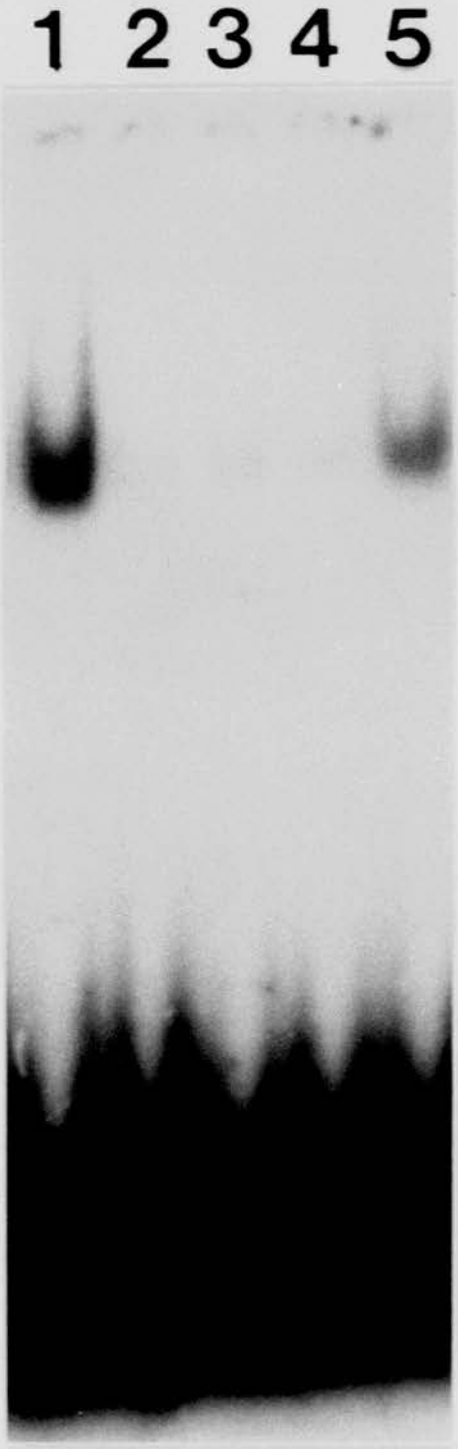

S300
O H 1 2 3

Figure 4.5 **Specificity of partially purified SRE-BP binding to the ERE_{con} oligonucleotide**

Fractions 20 and 21 containing partially purified SRE-BP were concentrated using an Amicon filter. 1μg of partially purified SRE-BP was incubated with 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide and 4μg poly [d(I-C)]. A 100-fold molar excess of competitor ERE_{con} (lane 2), ERE_{PRL}(lane 3), GRE (lane 4), and GRE_M (lane 5) oligonucleotide was added; competitor was not added to lane 1. Free and bound labelled oligonucleotide were separated on a 4% polyacrylamide gel by electrophoresis (section 2.2.2.2).


1 2 3 4 5

4.2.1.2 The SRE-BP forms a 213kD ±27kD complex with the ERE_{con} oligonucleotide

Some transcription factors exist in solution in a different state to their DNA-bound form. For example, the steroid hormone receptors are complexed with a 90kD heat shock protein (hsp90) in their soluble form, but hsp90 is dissociated from SR which is bound to DNA (reviewed in section 1.6.3.1).

To examine whether SRE-BP binds to DNA in solution in a similar or different form to its soluble state (determined in 4.2.1.1), native pore gradient gel electrophoresis was used (section 2.2.2.3).

Protein extract, either 10μg of HeLa WCE or 1μg of partially purified SRE-BP, was incubated with 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide and 4μg poly [d(I-C)] in a standard band shift assay (section 2.8.1). Reactions were loaded onto a 3-25% pore gradient polyacrylamide native gel (prepared as described in section 2.2.2.3), adjacent to protein standard mol. wt. markers (bovine serum albumin, 67kD; Myosin, 200kD; *E.coli* galactosidase tetramer, 464kD), and electrophoresed for 1500 volt-hours. The gel was stained with Coomassie Brilliant Blue R (section 2.6.4.1) to visualise protein markers, dried and exposed to autoradiographic film overnight.

Figure 4.6 shows that SRE-BP/ERE complex formed with either HeLa WCE or partially purified SRE-BP migrated to a position near to the 200kD mol. wt. marker. By relating the electrophoretic mobility of the SRE-BP/DNA complex to that of protein mol. wt. markers, the SRE-BP/ERE complex was shown to have a relative mol. wt. of 213kD. Analysis of four such experiments, each correcting for inclusion of the ERE_{con} oligonucleotide (approximately 13kD), demonstrated that the active SRE-BP DNA-binding form was 200kD ± 27kD. Thus, the relative mol. wts. of the soluble (~205kD,

Figure 4.6

Pore gradient gel electrophoresis of ERE/SRE-BP complexes

Either 1μg of partially purified SRE-BP (lane 1) or 10μg HeLa WCE (lane 2) were incubated with 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide and 4μg poly [d(I-C)]. Lane 3 contained 10μg HeLa WCE previously left at 4°C for longer than six weeks. Free and bound oligonucleotide were resolved by electrophoresis through a 3-25% native gradient polyacrylamide gel (section 2.2.2.3). Protein mol. wt. markers were electrophoresed in parallel lanes: bovine serum albumin (67kD), myosin (200kD), and *E. coli* β-galactosidase (464kD).

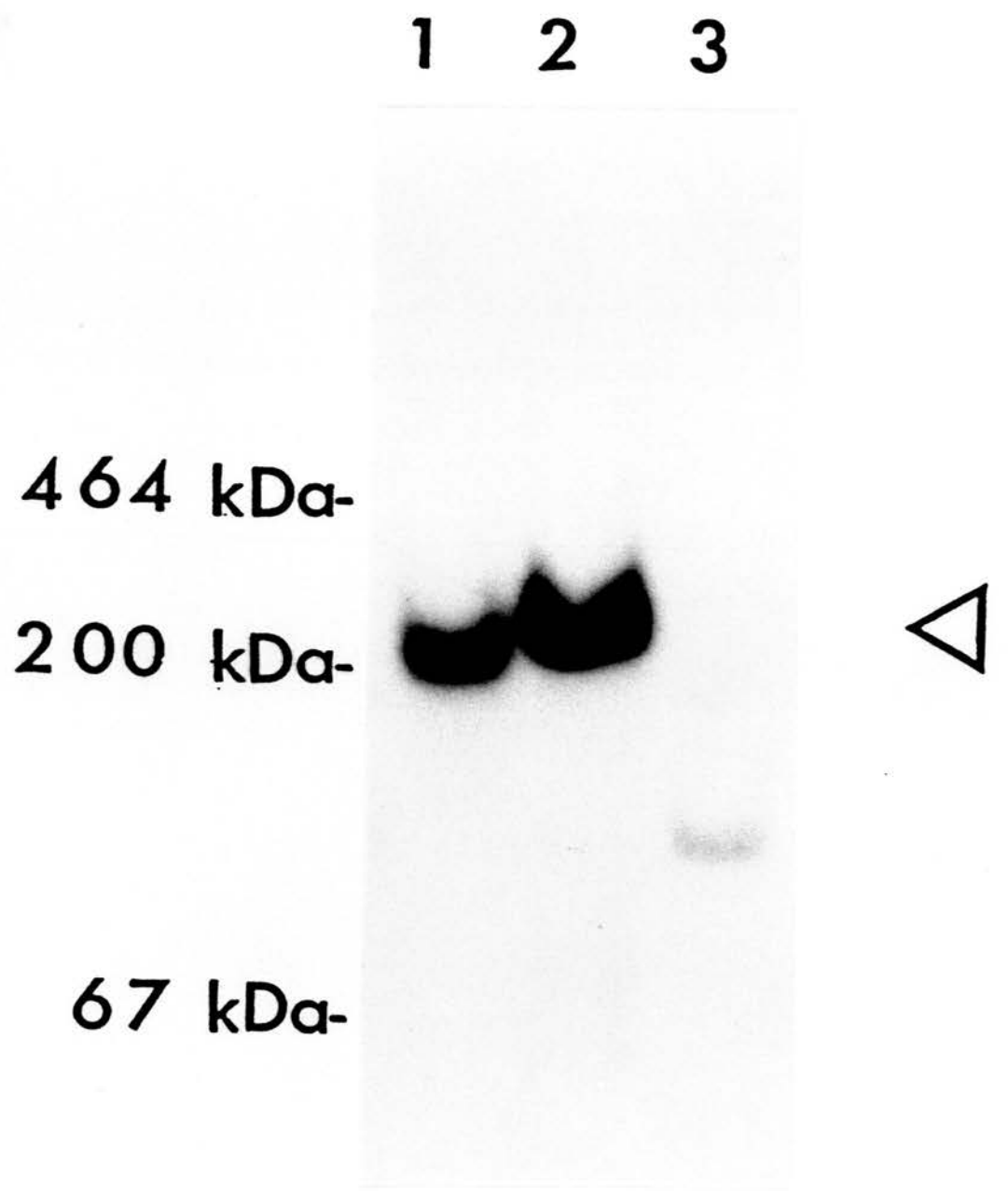

1 2 3
464 kDa-
200 kDa-
67 kDa-

Fig. 4.1) and DNA-bound forms (~200kD, Fig. 4.6) of SRE-BP are very similar. It is likely that these forms represent the same protein species.

4.2.2 Characterisation of subunit composition of SRE-BP

The SRE-BP activity identified (Chapter 3) may represent either a single DNA binding, 205kD polypeptide, or a multiple subunit protein complex with a total mol. wt. of approximately 205kD, of which one subunit, the DNA-binding subunit, binds to DNA. To explore the subunit composition of SRE-BP, UV-crosslinking experiments were implemented.

DNA-binding reactions to be crosslinked (section 2.8.2) contained 100μg HeLa WCE, 40μg poly [d(I-C)] and 1pmol of either ^{32}P-labelled BdU-ERE1, BdU-ERE2, or EREcon oligonucleotide (see Figs. 4.7 and 3.1a for oligonucleotide sequences) in a final volume of 100μl; reactions were exposed to UV light for either 20 or 40 minutes, as described in section 2.8.2.

To confirm that SRE-BP-DNA complexes had formed in UV -irradiated reactions, a 10μl aliquot from each reaction was electrophoresed through a native 4% gel (section 2.2.2.2), adjacent to a set of standard band shift reactions (section 2.8.1). These standard band shift reactions, which had not been irradiated, contained 10μg HeLa WCE, 4μg poly [d (I-C)] and 0.1pmol of ^{32}P-labelled BdU-ERE1, BdU-ERE2 or ERE_{con} oligonucleotide. SRE-BP binding to each labelled oligonucleotide was detected before and after irradiation (Fig. 4.8); irradiated reactions and their counterpart non-irradiated control reactions exhibited similar SRE-BP activity with respect to SRE-BP-DNA complex intensity and electrophoretic mobility. This indicated that BdU-substitutions in BdU-ERE1 and BdU-ERE2 did not interfere with detection of a SRE-BP-DNA complex. The decreased signal intensity of SRE-BP-BdU-oligonucleotide complexes compared to SRE-BP-ERE_{con} oligonucleotide complex probably reflected the lower

Figure 4.7 **Sequence of oligonucleotides used in bromodeoxyuridine crosslinking experiments**

(a) BdU-ERE 1

GCAGGTCACAGTGACCTGGAC
CGTCCAGTGBCACTGGACCTG

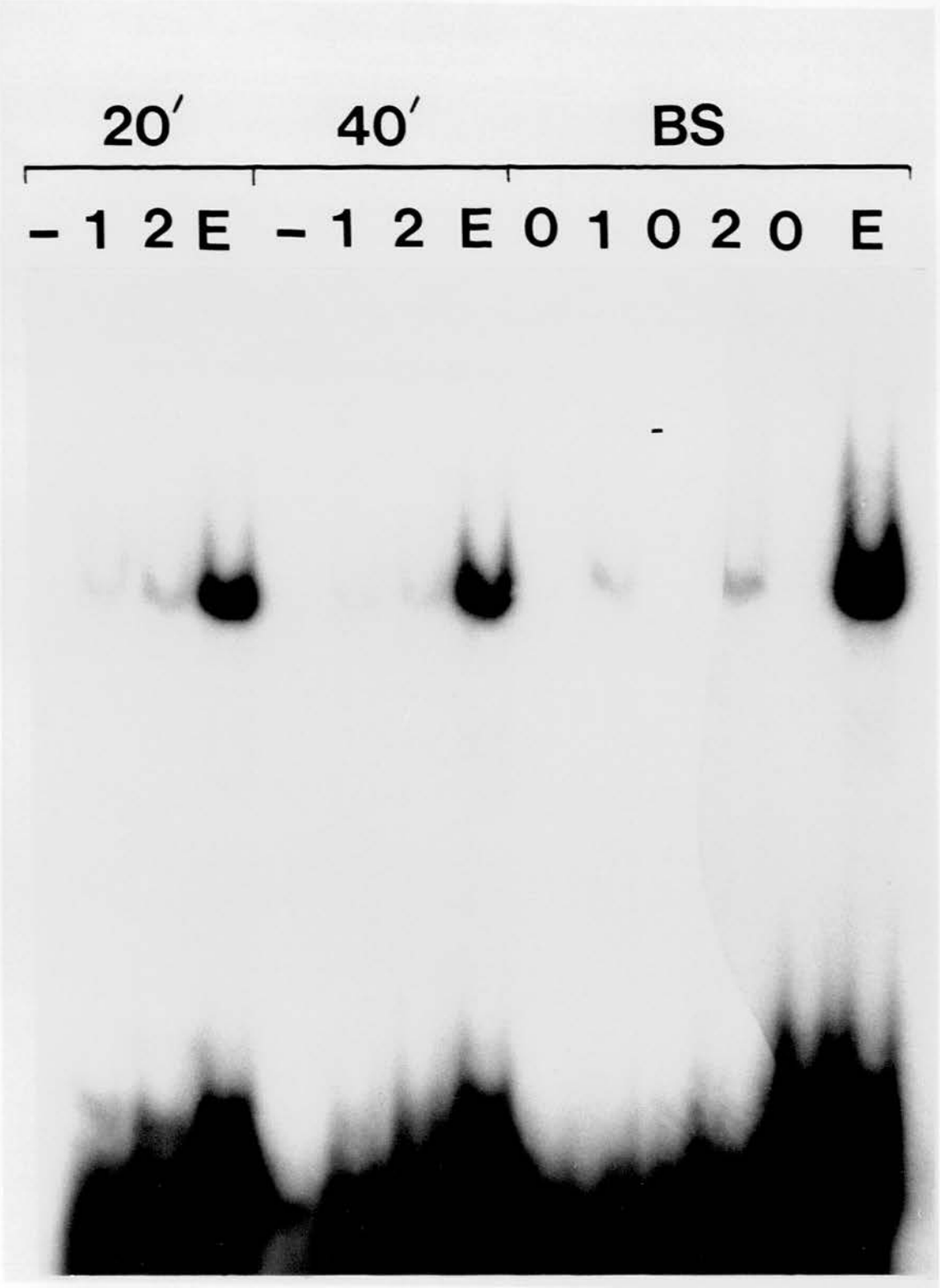
(b) BdU-ERE 2

GCAGGTCACAGTGACCTGGAG
CGTCCBGTGTCACTGGACCTC

Horizontal lines indicate the conserved nucleotides within ERE. The letter B marks the position of a single thymine to bromodeoxyuridine substitution in the ERE_{con} sequence. The BdU substitution of ds oligonucleotide (a) and (b) exist within the spacer region and conserved nucleotides of the ERE_{con} respectively (ERE_{con} oligonucleotide sequence is presented in Fig. 3.1a). Oligonucleotides were synthesised by Oswell DNA Services, Department of Chemistry, Edinburgh University.

Figure 4.8 **Analysis of SRE-BP activity after UV-irradiation**

Reactions were exposed to UV radiation for 20 (panel 20') and 40 (panel 40') mins (section 2.8.2). A 10µl aliquot of each UV-irradiated reaction, equivalent to 10µg HeLa WCE, 4µg poly [d(I-C)] and 0.1pmol ^{32}P-labelled BdU-ERE1 (lane 1), BdU-ERE2 (lane 2) or ERE_{con} (lane E) oligonucleotide was analysed by 4% native polyacrylamide gel electrophoresis (section 2.2.2.2). No labelled oligonucleotide was added to lanes marked (-). Standard 20µl band shift reactions (panel BS) (section 2.8.1) containing 10µg HeLa WCE 0.1pmol ^{32}P-labelled BdU-ERE1 (lane 1), BdU-ERE2 (lane 2), or ERE_{con} (lane E) oligonucleotide and 4µg poly [d(I-C)] were electrophoresed in parallel lanes. Lanes marked O did not contain protein extract.


20′
40′
BS
– 1 2 E – 1 2 E 0 1 0 2 0 E

specific activity of the former oligonucleotides; only the BdU oligonucleotide was end labelled and not the complementary oligonucleotide to which it had been annealed (see 2.8.2).

The remainder of each 100µl irradiated reaction was subjected to 10% SDS gel electrophoresis (section 2.2.2.4); protein standard mol. wt. markers (detailed in Fig. 4.9 legend) were electrophoresed in parallel.

Several crosslinked complexes were detected, none of which represented a complex of around 200kD; all complexes were smaller than 92.5kD (Fig. 4.9). These complexes were due to protein DNA interactions, since no complex formation was detected when 1pmol ^{32}P-labelled oligonucleotide was exposed to UV radiation in the absence of WCE (Fig. 4.10, right-most three lanes). Six crosslinked complexes were observed with both BdU-ERE1 and BdU-ERE2, of mol. wts. 88kD, 74kD, 60kD, 55kD, 48kD, and 42kD. These complexes were most apparent with BdU-ERE1 oligonucleotide after 40 mins exposure to UV radiation (Fig. 4.9; panel 40'; lane 1). Similar crosslinked complexes detected with the labelled ERE_{con} were less intense, except for a prominent 88kD complex observed most readily after 40 mins UV-irradiation (Fig. 4.9; panel 40; lane E).

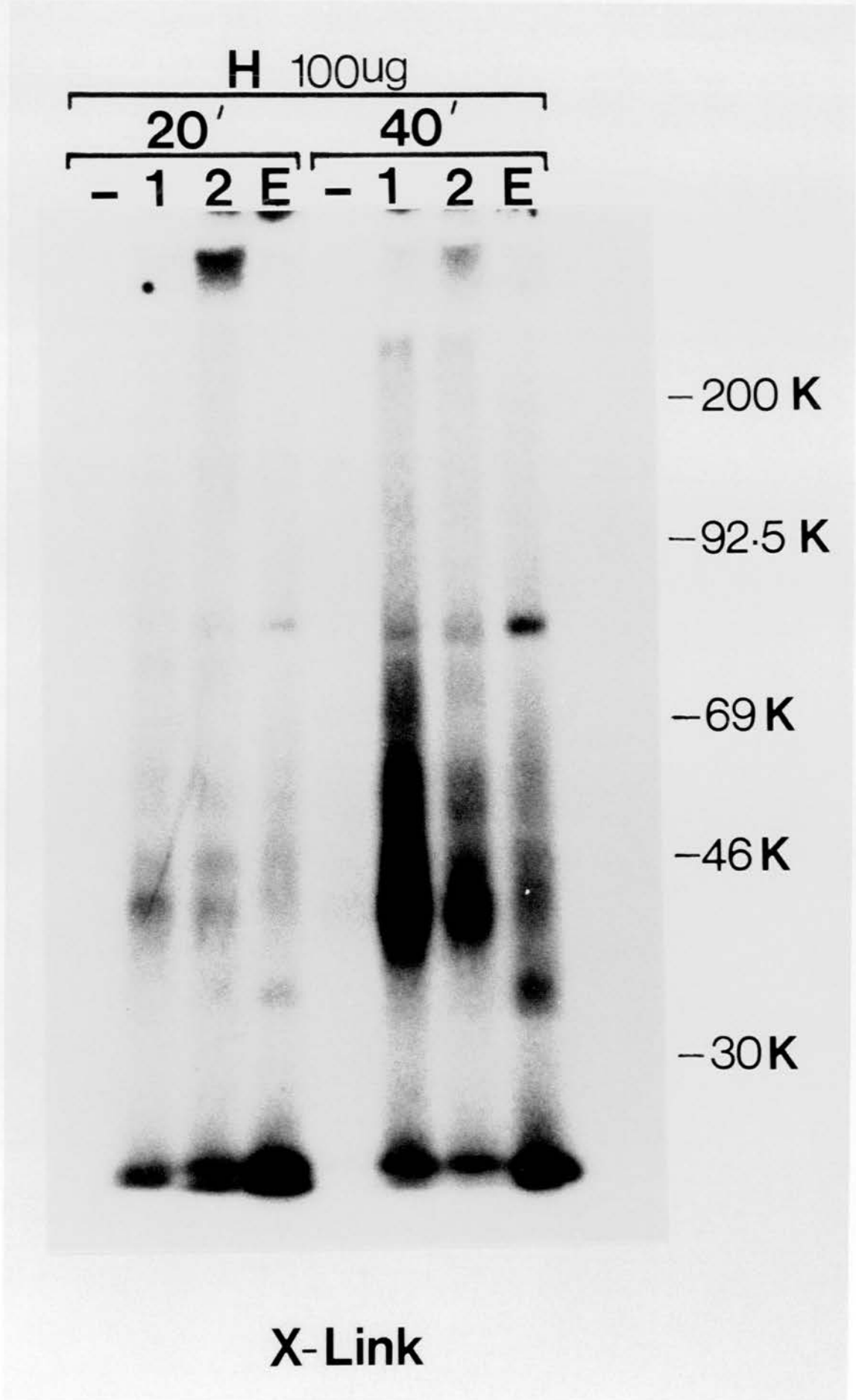
To examine whether any of the crosslinked complexes formed specifically with an ERE sequence, a competition experiment was carried out. A 100-fold molar excess of ERE_{con} or TRE oligonucleotide was included into a 100µl crosslinking reaction (section 2.8.2) containing 100µg HeLa WCE, 40µg poly [d(I-C)] and either 1pmol ^{32}P-labelled BdU-ERE1 or ERE_{con} oligonucleotide. Reactions were exposed to UV radiation for 30 mins then analysed by SDS polyacrylamide gel electrophoresis (section 2.2.2.4) (Fig. 4.10).

The six complexes previously observed were reproducibly formed with BdU-ERE1 and ERE_{con} oligonucleotides (Fig. 4.10; panel BdU1,

Figure 4.9

Analysis of ERE/HeLa WCE UV-crosslinked complexes

Reactions were exposed to UV-radiation for 20 (panel 20') and 40 (panel 40') mins (section 2.8.2). 90µl of each 100µl reaction, which contained 100µg HeLa WCE, 40µg poly [d(I-C)], and approximately 1pmol ^{32}P-labelled BdU-ERE1 (lane 1), BdU-ERE2 (lane 2) or ERE_{con} (lane E) oligonucleotide, were analysed by 10% SDS polyacrylamide gel electrophoresis (section 2.2.2.4). Oligonucleotide was not added to lanes marked (-). Protein mol. wt. markers were electrophoresed in parallel lanes: carbonic anhydrase (30kD), ovalbumin (46kD), bovine serum albumin (69kD), phosphorylase b (92.5kD), and myosin (200kD).


H 100ug
20′
40′
– 1 2 E – 1 2 E
–200 K
–92·5 K
–69 K
–46 K
–30 K
X-Link

lane (–) and panel ERE, lane (–)) although the 88kD protein/ERE_{con} complex was much less prominent (Fig. 4.10; compare panel ERE, lane (–) with Fig. 4.9; panel 40; lane E). Proteins crosslinked to BdU-ERE1 were competed neither by excess ERE_{con} nor TRE oligonucleotide (Fig. 4.10). In contrast, protein-ERE_{con} oligonucleotide crosslinked complexes were competed by excess of either ERE_{con} or TRE oligonucleotide (Fig. 4.10).

Although a single crosslinked complex was not detected, assuming that SRE-BP was involved in crosslinked complex formation, the absence of a complex of about 200kD makes it unlikely that the SRE-BP is a single polypeptide of approximately 200kD. It is more likely that SRE-BP is a multi-subunit protein complex with a DNA-binding subunit of between 88kD and 42kD.

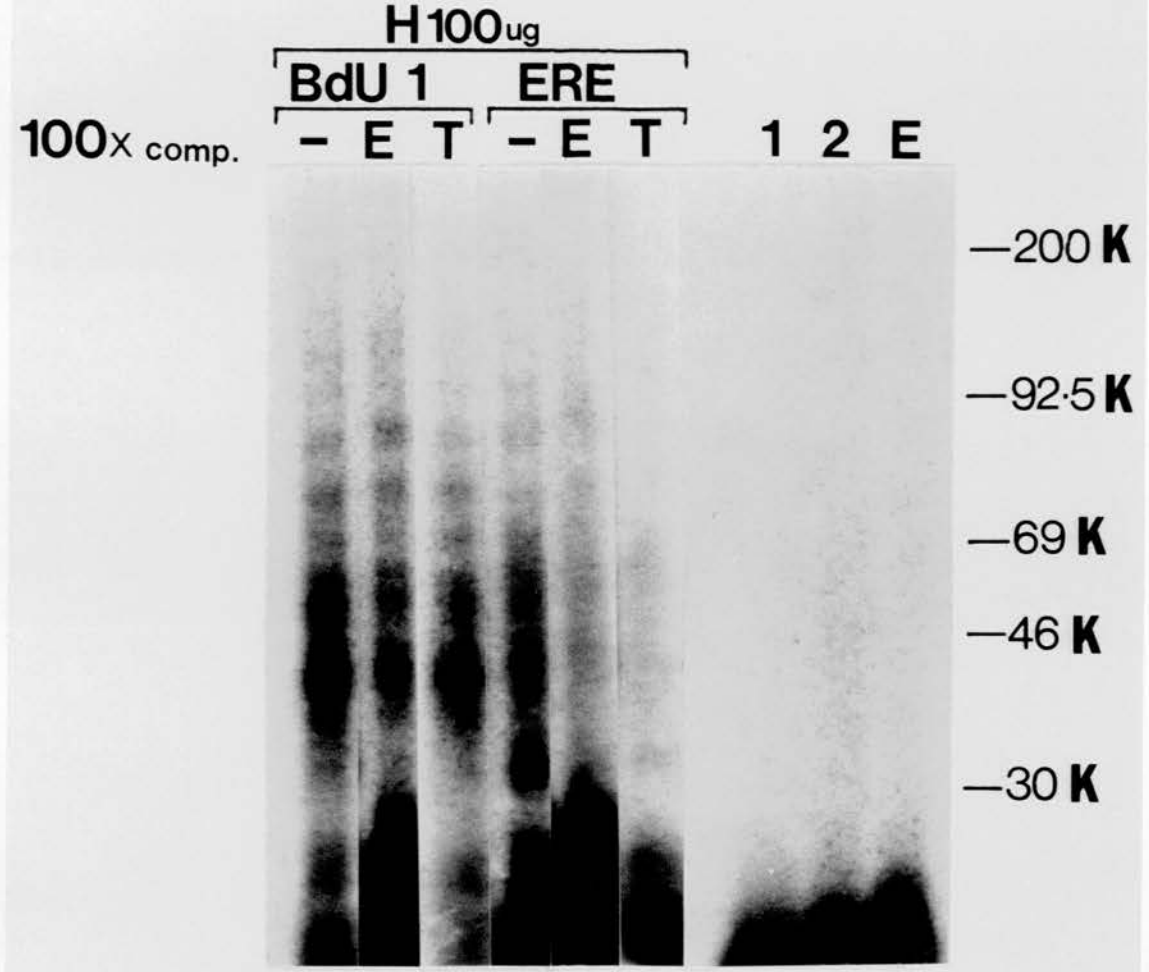
4.4 DISCUSSION

In Chapter 3, I report the identification of a protein present in extracts prepared from several different cell types, which specifically binds to two classes of functionally distinct SRE (Chapter 3; Crawford & Chapman, 1990). Using the techniques of gel filtration chromatography and pore gradient gel electrophoresis, I now demonstrate that the mol. wt. of SRE-BP in solution and as it binds to DNA respectively, is approximately 200kD. These data strongly imply that the stable form of SRE-BP in solution is also the active DNA-binding species. Additionally, preliminary evidence from crosslinking experiments indicates that SRE-BP is not a single polypeptide of approximately 200kD, but more likely a multisubunit protein complex; evidence suggests that the mol. wts. of constituent SRE-BP subunits are probably between 88kD and 42kD.

UV-irradiation of protein-DNA complex formed between BdU-containing oligonucleotides and HeLa WCE creates six crosslinked complexes. As native gel electrophoresis of an aliquot of UV-irradiated

Figure 4.10 **Specificity of ERE/HeLa WCE UV-crosslinked complexes**

Reactions were exposed to UV radiation for 30 mins (section 2.8.2) . Either a 100-fold molar excess of ERE_{con} (lane E) or TRE (lane T) oligonucleotide was included in a 100µl crosslink reaction (section 2.8.2) containing 100µg HeLa WCE, 40µg poly [d(I-C)] and either 1pmol ^{32}P-labelled BdU-ERE1 (panel BdU 1) or ERE_{con} (panel ERE); oligonucleotide competitor was not added to lane marked (–). The right-most control lanes marked 1, 2 and E represent control reactions containing UV-irradiated ^{32}P-labelled BdU-ERE1, BdU-ERE2, and ERE_{con} oligonucleotides respectively, to which no WCE was added. Crosslinked complexes were analysed by 10% SDS polyacrylamide gel electrophoresis (section 2.2.2.4). Protein standard mol. wt. markers were electrophoresed in parallel: carbonic anhydrase (30kD), ovalbumin (46kD), bovine serum albumin (69kD), phosphorylase b (92.5kD), and myosin (200kD).



Comp. X-Link

crosslinked reactions only indicates the presence of the SRE-BP-ERE complex, one would imagine that the observed crosslinked complexes are SRE-BP derived. However, since crude WCE was used to generate these complexes, the possibility that crosslinked complexes resulting from DNA interaction with cellular proteins other than SRE-BP cannot be excluded.

In native gel electrophoresis, SRE-BP binds to BdU-ERE1 and BdU-ERE2 with equal affinity. However, crosslinked complexes are most readily observed with BdU-ERE1 which contains a BdU substitution within the ERE spacer region (see Fig. 4.7). Assuming crosslinked complexes are SRE-BP derived, the higher level of BdU-ERE1-mediated crosslinking suggests that the SRE-BP more closely approaches the BdU within the spacer region of the ERE than BdU within the conserved arm (BdU-ERE2). It is noteworthy that, as demonstrated in Chapter 3, SRE-BP DNA binding to an ERE is abolished when the 3bp spacer region is deleted (see Fig. 3.10, 6th & 10th lanes).

The inability to demonstrate sequence specificity of BdU-oligonucleotide crosslinked complex formation was disappointing. If crosslinked complexes are due to SRE-BP binding and are, therefore, sequence-specific (see Chapter 3), one would expect to see competition by excess unlabelled ERE competitor and not by excess TRE. If, on the other hand, complex formation is sequence-independent, competition by both excess ERE and TRE sequences would occur. However, neither pattern of competition is seen. Possibly SRE-BP binds preferentially to EREs containing a BdU substitution. Alternatively, covalent crosslinking of BdU-ERE1 or 2 to SRE-BP, which would prevent SRE-BP dissociation from the oligonucleotide would, in turn, lead to an overall decrease in SRE-BP-DNA 'off' rate. Subsequently, a reduction in the effectiveness of competition by excess ERE_{con} oligonucleotide is predicted. Inclusion of excess BdU-containing competitor ERE oligonucleotide, instead of excess

ERE_{con} oligonucleotide into a competition crosslinking reaction, may help resolve this peculiarity.

The data presented in this chapter provides further support that the identified SRE-BP activity (Chapter 3) is neither ER nor GR. Firstly, the mol. wt. of the SRE-BP is approximately 200kD; ER is smaller and has a monomer mol. wt. of 65kD and a dimer mol. wt. of 130kD (Kumar & Chambon, 1988; Fawell et al., 1990). Secondly, the mol. wt. of activated GR [a dimer of 190kD (Giguere et al., 1986; Tsai et al., 1988)] is close to that of SRE-BP, and its elution profile from a Sephacryl S300 column may overlap with that of SRE-BP. However, as ERE and GRE binding activities coelute from the Sephacryl S300 column, SRE binding activity is unlikely to be due to ER or GR, which would be expected to elute in distinct fractions according to their mol. wt. Finally, crosslinking experiments do not show a complex which reflects the monomer mol. wt. of either ER or GR which are 65kD and 94kD respectively.

As discussed in Chapter 3, Feavers et al. (1987) have identified and partially purified a 70kD protein which binds specifically to an ERE and is not ER. Payvar & Wrange (1983) have shown that fractions which contain a 72kD protein increase the DNA-binding activity of purified GR, although it is not known whether this 72kD protein can also bind to DNA. The mol. wts. of these two proteins were defined under denaturing conditions and, therefore, cannot be compared to the native mol. wt. derived for SRE-BP. Preliminary crosslinking experiments did, however, indicate that SRE-BP is probably not a single polypeptide of approximately 200kD, and that its DNA binding subunit is less than 92.5kD. In fact, crosslinking allowed detection of six complexes, one of which is approximately 74kD. Whether this complex is related to the proteins discussed above remains to be determined. Ideally, the SRE-BP should be purified in order to allow elucidation of its subunit composition and subunit relationship to other proteins implicated in steroid

hormone regulation. The use of Fast Protein Liquid Chromatography (FPLC) is an attractive method by which SRE-BP purification could be initiated.

CHAPTER 5

EVIDENCE FOR INVOLVEMENT OF ACCESSORY PROTEINS IN ER DNA-BINDING ACTIVITY

5.1 INTRODUCTION

The SRE-BP binds preferentially to EREs and a GRE and has a relative mol. wt. under non-denaturing conditions of ~200kD, in both its soluble and active DNA-binding state (Chapters 3 and 4; Crawford & Chapman, 1990a; Crawford & Chapman, 1990b). In Chapter 3, I proposed that SRE-BP is involved in steroid hormone regulation of gene expression by modulating SR-SRE interaction *in vivo*.

Binding of SRE-BP to a SRE could positively or negatively modulate SR-SRE interaction; alternatively, it may not affect SR binding. Assuming SRE-BP does modulate SR-SRE interaction, it may elicit its effect by 1) regulating access of SR to its target DNA sequence, 2) binding to the SRE and creating a more or less favourable DNA conformation which could potentially increase or decrease SR affinity for SRE, respectively, or 3) by binding together with SR to provide protein-protein contact in addition to SR-DNA contact and secure SR-SRE interaction.

In this chapter, I examine the effect of protein extract, which either does or does not contain SRE-BP activity, in modulating ER affinity for DNA, *in vitro*. ER used in this study was prepared and kindly donated by Dr. S. Fawell, with consent from Dr. M. Parker, both from Imperial Cancer Research Fund (ICRF) in London. ER was prepared by synthesis of cRNA transcribed from a linearised mouse ER cDNA template and *in vitro* translated in reticulocyte lysate as described in Fawell et al. (1990). Control reticulocyte lysate in which ER had not been expressed was also supplied. The protein concentration of reticulocyte lysate was calculated (section 2.6.4.2) to be $100\mu g\mu l^{-1}$.

ER polyclonal antibodies generated against synthetic peptides from the non-conserved N-terminal region of ER (MP16) and preimmune serum were also a gift from Dr. M. Parker's laboratory. Generation of antibody has been described elsewhere (Fawell et al., 1990).

This chapter describes preliminary evidence that accessory proteins, possibly SRE-BP, are involved in ER DNA-binding activity. ER binding to DNA *in vitro* is increased when HeLa WCE, which contains SRE-BP, is present, but not by bacterial lysate, which does not have SRE-BP activity. Additionally, pore gradient gel electrophoresis demonstrates ER binding to DNA as part of a macromolecular protein complex and not simply as a 130kD homodimer.

5.2 RESULTS

5.2.1 *In vitro* translated ER does not bind to a 21bp ERE_{con} oligonucleotide

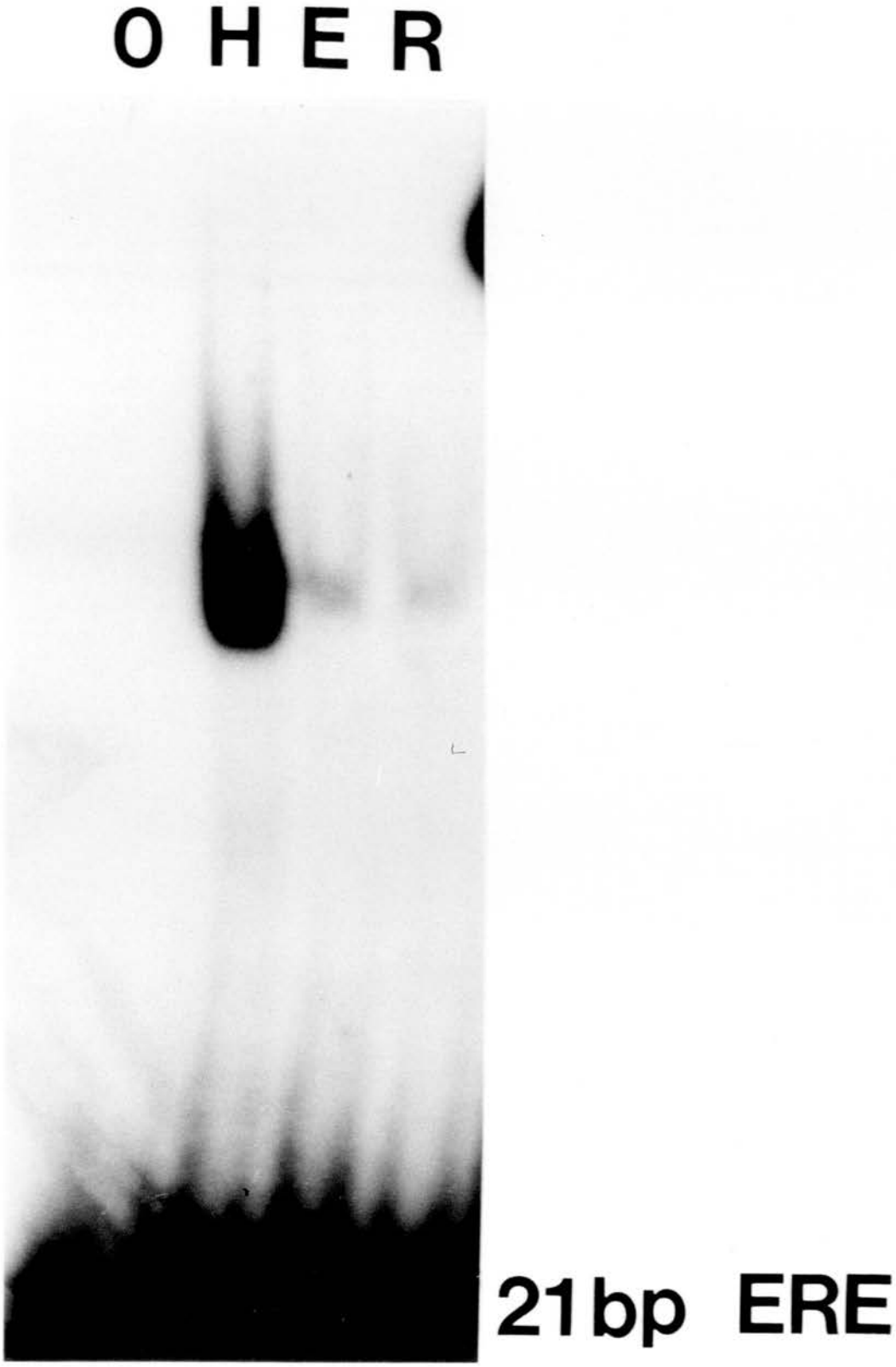
In vitro translated (IVT) ER binding to the 21bp ERE_{con} oligonucleotide (Fig. 3.1a) was not detected (Fig. 5.1). In a band shift assay (section 2.8.1) either 0.5µl IVT ER (containing 50µg protein) or 0.5µl (50µg) control reticulocyte lysate were incubated with 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide and 4µg poly [d(I-C)]. No complex uniquely generated by IVT ER was observed (Fig. 5.1, compare lanes E and R). However, IVT ER and reticulocyte lysate gave rise to complexes with similar electrophoretic mobility and intensity to each other (Fig. 5.1, lanes E and R, respectively), and similar electrophoretic mobility to the SRE-BP-ERE_{con} complex formed with 10µg HeLa WCE (compare lanes E and R to lane H). Therefore, complex formation with either IVT ER or reticulocyte lysate is probably due to residual SRE-BP activity in reticulocyte lysates.

5.2.2 Identification of ER-DNA complexes; correlation between number of EREs present and complexes formed

Since ER binding to the 21bp ERE_{con} oligonucleotide was not observed (Fig. 5.1), longer DNA probes (Fig. 5.2) were used to detect ER DNA-binding activity. DNA fragment two (DF2) encodes two ERE_{con}

FIGURE 5.1 **Analysis of ER DNA-binding activity to the 21bp ERE_{con} oligonucleotide**

Either 10μg HeLa WCE (H), 0.5μl ER (E), or 0.5μl control reticulocyte lysate (R) were incubated with 0.1pmol ^{32}P-labelled ERE_{con} oligonucleotide and 4μg poly [d(I-C)]. WCE was not added to lane O. Free and bound oligonucleotide were resolved by 4% native polyacrylamide gel electrophoresis.


O H E R
21bp ERE

sequences within an 89bp fragment; DF1 has one ERE_{con} on a 77bp fragment and DFO is a 92bp fragment with no EREs.

HeLa WCE binding to each DNA probe in Fig. 5.2 was initially examined. In a band shift assay (section 2.8.1), when 10μg HeLa WCE was incubated with 4μg poly [d(I-C)] and approximately 3fmol of ^{32}P-labelled DF2 or DF1, the number of complexes formed reflected the number of EREs encoded on each probe (Fig. 5.3, lanes 7 to 10). HeLa WCE generated two complexes with DF2 (Fig. 5.3 complexes c and d) and one complex (complex c) with DF1. Presumably, complex (c) represented protein binding to one ERE of DF1 or DF2 and complex (d), which had slower electrophoretic mobility and decreased signal intensity, represented occupancy of both EREs encoded on DF2. Unexpectedly, four poorly defined complexes were generated by HeLa WCE incubation with labelled DFO (Fig. 5.3, lanes 11 and 12) which did not contain any EREs.

In a similar band shift assay, 0.5μl IVT ER was incubated with 4μg poly [d(I-C)] and 3fmol of ^{32}P-labelled DF2, DF1, or DFO (Fig. 5.3, first six lanes, marked E). As with HeLa WCE, the number of complexes generated with IVT ER reflected the number of EREs encoded on either DF1 or DF2 probe. Two complexes (Fig. 5.3, complexes a and b) formed with DF2 and only one complex (complex a) formed with DF1. Since complex formation between ^{32}P-labelled DF2 and 0.5μl reticulocyte lysate was not observed (see Fig. 5.4 and data not shown), complexes (a) and (b) were, therefore, ER derived. Presumably, complex (a), which exhibited fastest electrophoretic mobility and increased signal intensity represented IVT ER binding to one ERE of DF1 or DF2 and complex (b) represented IVT ER binding to both EREs of DF2 probe. As expected, IVT ER binding to DFO, which did not contain any EREs, was not observed.

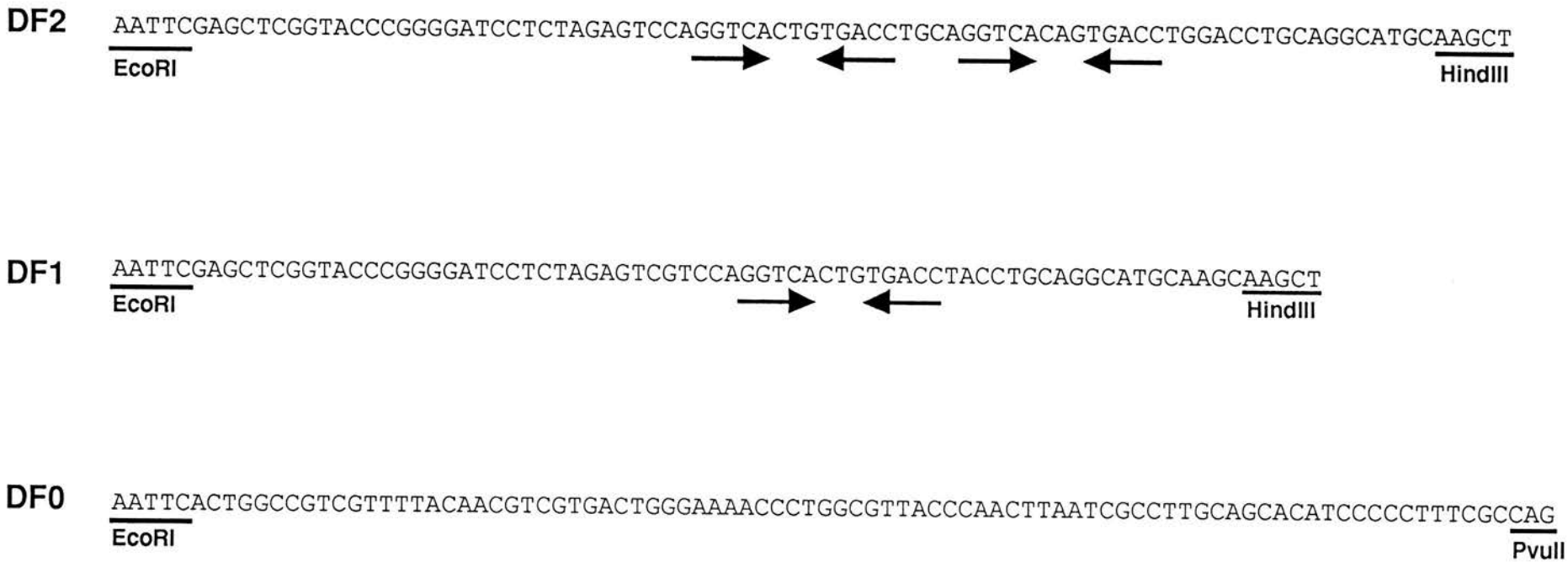
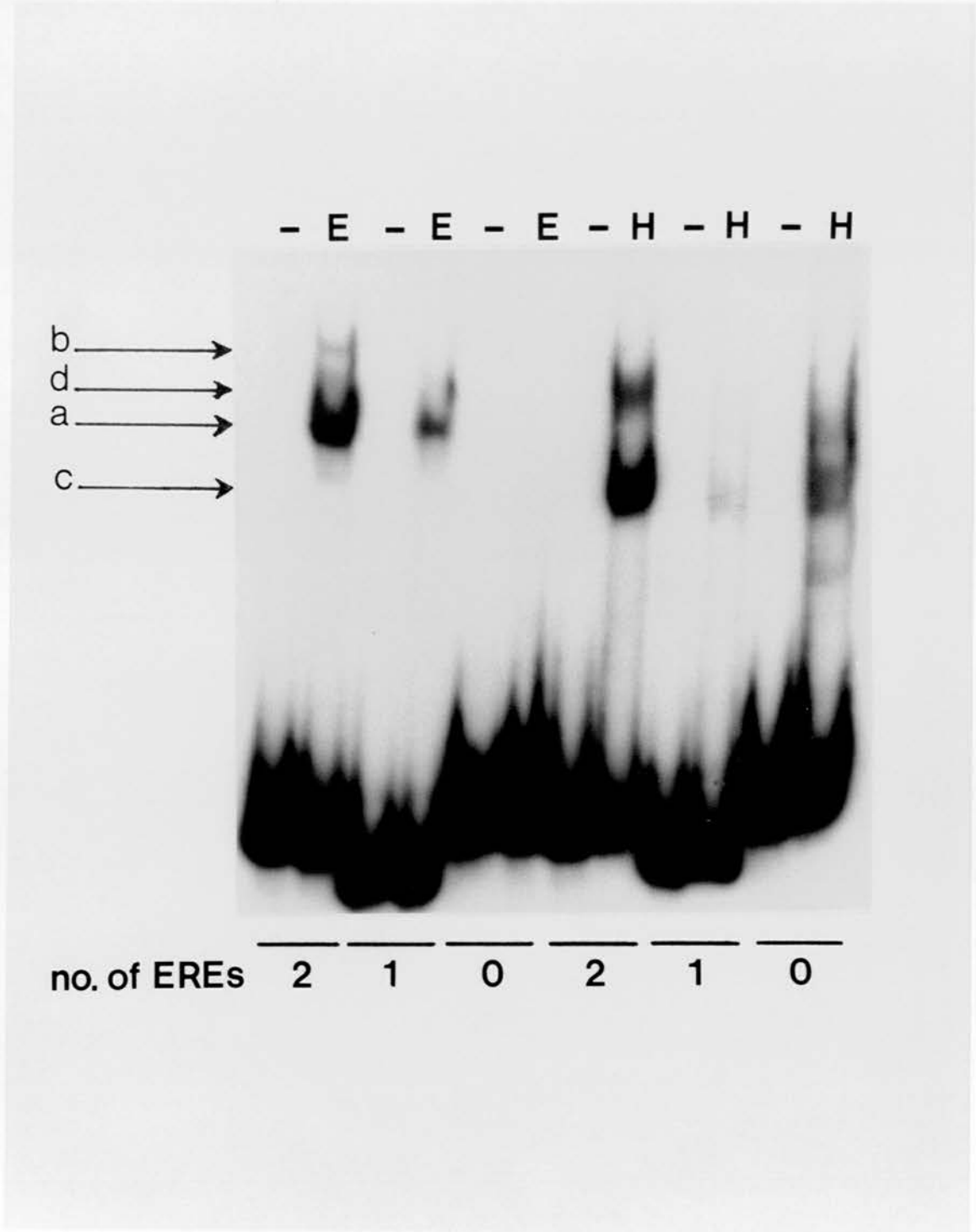


Figure 5.2 Nucleotide sequence of DNA probes used in Chapter 5.

One strand only is shown; horizontal arrows indicate the dyad symmetry of conserved nucleotides within the ERE_{con}. DF2, DF1 and DF0 were generated as described in section 2.7.2.; restriction sites used are underlined at either end of each fragment. DF2 contains two ERE_{con}; DF1 contains one ERE_{con}; DF0 does not contain any EREs. Although all fragments were derived from pUC19, flanking DNA of DF1 and DF2 are similar to each other but different from DF0.

Figure 5.3 **Analysis of ER and HeLa WCE DNA-binding activity to DF2, DF1, and DFO**

DF2, DF1, and DFO were prepared and end labelled as described in section 2.7.2. Either 0.5µl ER (lanes E) or 10µg HeLa WCE (lanes H) were incubated with 4µg poly [d(I-C)] and 3fmol of ^{32}P-labelled DF2, DF1, or DFO (depicted at bottom of figure as no. of EREs: 2, 1, or O, respectively). No protein was added to lanes marked (–). Free and bound DNA were resolved by 4% native polyacrylamide gel electrophoresis (section 2.2.2.2). Horizontal arrows indicate complexes a and b or c and d, as discussed in the text.


- E - E - E - H - H - H
b
d
a
c
no. of EREs 2 1 0 2 1 0

Since complex formation between DF2 and either IVT ER or HeLa WCE was significantly greater than with DF1 probe, DF2 was used for further experiments presented in the following sections.

5.2.3 ER DNA-binding is increased by the presence or absence of SRE-BP containing protein extract

The possibility that WCE containing SRE-BP activity could, in some way, modulate ER binding to target DNA, was investigated in a band shift assay. The approach taken for this investigation was to examine whether IVT ER bound differentially to naked DNA compared to DNA which had been preincubated with HeLa WCE.

In a band shift assay (section 2.8.1) 12 different reactions were set up, each containing approximately 3fmol ^{32}P-labelled DF2 and 4μg poly [d(I-C)]. In control reactions, 10μg HeLa WCE were incubated with DF2 for 20 mins and 0.5μl of either IVT ER or reticulocyte lysate were incubated with DF2 for 10 mins. Band shift reactions containing both WCE and either IVT ER or reticulocyte lysate were set up as follows: DF2 probe was preincubated for 10 mins with 10μg HeLa WCE before adding 0.5μl of IVT ER or reticulocyte lysate and incubating for a further 10 mins. Where appropriate, either 1μl ER polyclonal antibody or preimmune serum was added and incubation was continued for an extra 2 mins before resolving complexes by native gel electrophoresis (section 2.2.2.2).

Incubation of HeLa WCE with DF2 (Fig. 5.4, lane 2) generated two complexes. The most prominent complex was analogous to complex (c) described in section 5.2 (refer to Fig. 5.3). The second, less well defined complex, which exhibited slower electrophoretic mobility and decreased signal intensity compared to complex (c), probably corresponded to complex (d), defined in Fig. 5.3. Incubation of IVT ER with DF2 (Fig. 5.4, lane 3) only allowed detection of one complex, analagous to complex (a) defined in

Fig. 5.3. Inability to detect the weaker ER derived complex (b) (see Fig. 5.3) was probably due to the lower specific activity of ^{32}P-labelled DF2 used in this assay compared to labelled DF2 used in the Fig. 5.3 assay. No complex formation was observed between ^{32}P-labelled DF2 and either reticulocyte lysate (lane 4), ER polyclonal antibody (lane 5), or preimmune serum (lane 6).

Preincubation of DF2 probe with HeLa WCE increased the signal intensity of the ER generated complex (Fig. 5.4; compare signal intensity of ER-generated complex in lane 3 with that in lanes 7 and 9). Addition of ER antibody (lane 8), but not preimmune serum (lane 9), resulted in the disappearance of ER generated complex and formation of a supershifted ER-antibody complex (Fig. 5.4, complex S).

Addition of 0.5µl of reticulocyte lysate to HeLa WCE preincubated with DF2, with or without subsequent addition of ER polyclonal antibody or preimmune serum (Fig. 5.4, lanes 10,11,12 respectively), did not give rise to either the ER-generated complex or supershifted complex S.

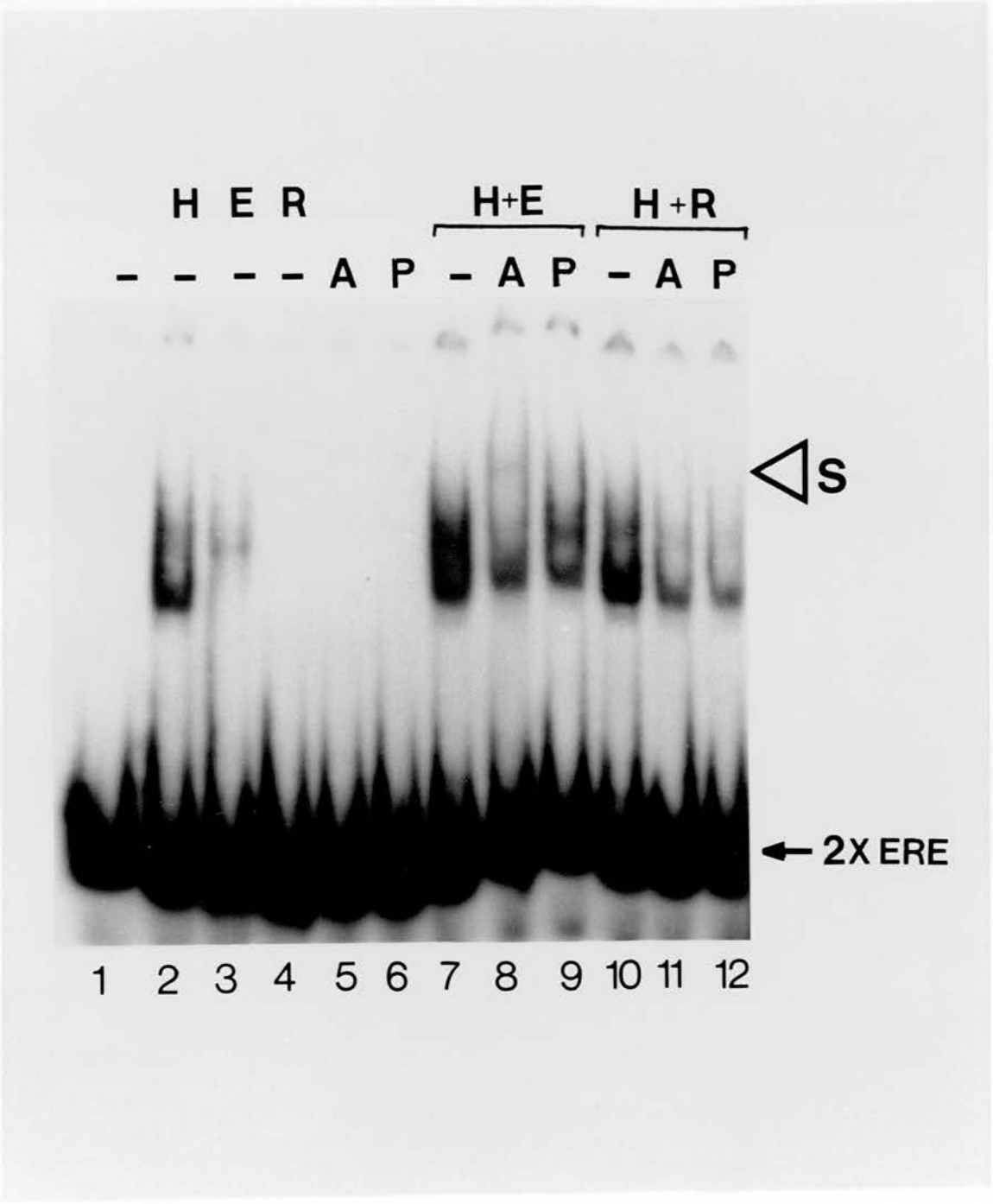
5.2.4 ER DNA-binding activity is not affected by protein extract which does not contain SRE-BP activity

It could be argued that a simple excess of DNA-binding proteins in HeLa WCE, binding non-specifically to poly [d(I-C)] in the band shift reaction, would leave more ERE-containing DNA available for ER binding. Thus, favourable conditions which drive ER-ERE interaction would be created and increased ER-DNA interaction would be observed. If this is so, the observed increase in IVT ER binding in the presence of HeLa WCE (Fig. 5.4) would not be due to the presence of accessory proteins modulating SR-SRE interaction.

To test this theory, DF2 probe was preincubated with either HeLa WCE (which contained SRE-BP activity) or protein extract (which did not

Figure 5.4 **Analysis of ER DNA-binding activity to DF2 in the presence and absence of HeLa WCE**

Protein binding to 3fmol ^{32}P-labelled DF2 probe in the presence of 4μg poly [d(I-C)] was assayed as follows: Lane 1 did not contain protein. Lane 2 and 7-12 each contained 10μg HeLa WCE; lanes 3 and 7-9 each contained 0.5μl ER, and lanes 4 and 10-12 each contained 0.5μl control reticulocyte lysate. Either 1μl polyclonal antibody was added to lanes 5, 8 and 11, or 1μl preimmune serum was added to lanes 6, 9, and 12. Free and bound DNA were separated by 4% native polyacrylamide gel electrophoresis (section 2.2.2.2). Hollow arrow represents position of supershifted complex S.


H E R
H+E
H+R
- - - - A P - A P - A P
S
2X ERE
1 2 3 4 5 6 7 8 9 10 11 12

contain SRE-BP activity) before addition of IVT ER. Ideally, eukaryotic WCE without SRE-BP activity should have been used. However, such cell lines were difficult to find (unpublished results). For this reason, lysate of *E.coli*, in which no SRE-BP activity was detected, was used. In addition to containing prokaryotic DNA-binding proteins, bacterial lysate used also expressed high levels of plasmid-encoded cAMP-receptor protein (Dr. K.E. Chapman, see section 2.6.3).

A band shift assay was used to examine two aspects of ER activity: 1) the effect of IVT ER concentration, together with 2) the effect of preincubating DF2 probe with either HeLa WCE or bacterial lysate on DNA-binding activity of IVT ER.

IVT ER was diluted to half, quarter and an eighth of its original concentration with 1 x binding buffer (recipe, 2.8.1). Twelve band shift reactions were carried out (section 2.8.1), each containing 3fmol ^{32}P-labelled DF2 and 4μg poly [d(I-C)]. Labelled DF2 was preincubated for 10 mins with either 10μg HeLa WCE or 10μg bacterial lysate before addition of 1μl of IVT ER dilution and incubation for a further 10 mins.

As seen previously (see Fig. 5.3 and 5.4), HeLa WCE incubated with DF2 probe created two complexes (Fig. 5.5, lane 2) equivalent to complexes (c) and (d) in Fig. 5.3 and IVT ER formed one complex (Fig. 5.5, lane 4) equivalent to complex (a) in Fig. 5.3. Inability to detect the less abundant ER-generated complex (b) (see Fig. 5.3) was probably due to the lower specific activity of the DF2 probe used in this assay. Incubation of DF2 probe with bacterial lysate produced one faint complex exhibiting very slow electrophoretic mobility (Fig. 5.5, lane 3) compared to complexes formed by either HeLa WCE or IVT ER (Fig. 5.5, lanes 1 and 4). This bacterial complex was not investigated further.

IVT ER diluted by half exhibited DNA-binding to naked DF2 probe (Fig. 5.5, lane 4) whereas dilution to a quarter or an eighth of the original

concentration prohibited complex detection (lanes 7 and 10). However, when DF2 probe was preincubated with HeLa WCE prior to addition of IVT ER, not only was DNA-binding activity of $^1/_2$ concentration IVT ER slightly increased, but more significantly $^1/_4$ concentration IVT ER then exhibited detectable DNA-binding activity (Fig. 5.5, compare lanes 5 with 4 and 8 with 7). One eighth concentration of IVT ER still did not show DNA binding activity to DF2 preincubated with HeLa WCE (lane 11). DNA-binding of $^1/_2$ concentration of IVT ER to DF2 probe was not increased by preincubation of DF2 with bacterial lysate (Fig. 5.5, lane 6). A slight increase in binding of $^1/_4$ concentration of IVT ER to bacterial lysate preincubated probe was observed (compare lane 9 to lane 7) although this increase was not as obvious as that mediated by DF2 preincubation with HeLa WCE.

Thus, preincubation of ERE-containing probe with HeLa WCE which contained SRE-BP activity increased DNA-binding of limiting amounts of IVT ER. DNA-binding of IVT ER was otherwise unaffected by preincubation of probe with bacterial lysate which did not contain SRE-BP activity.

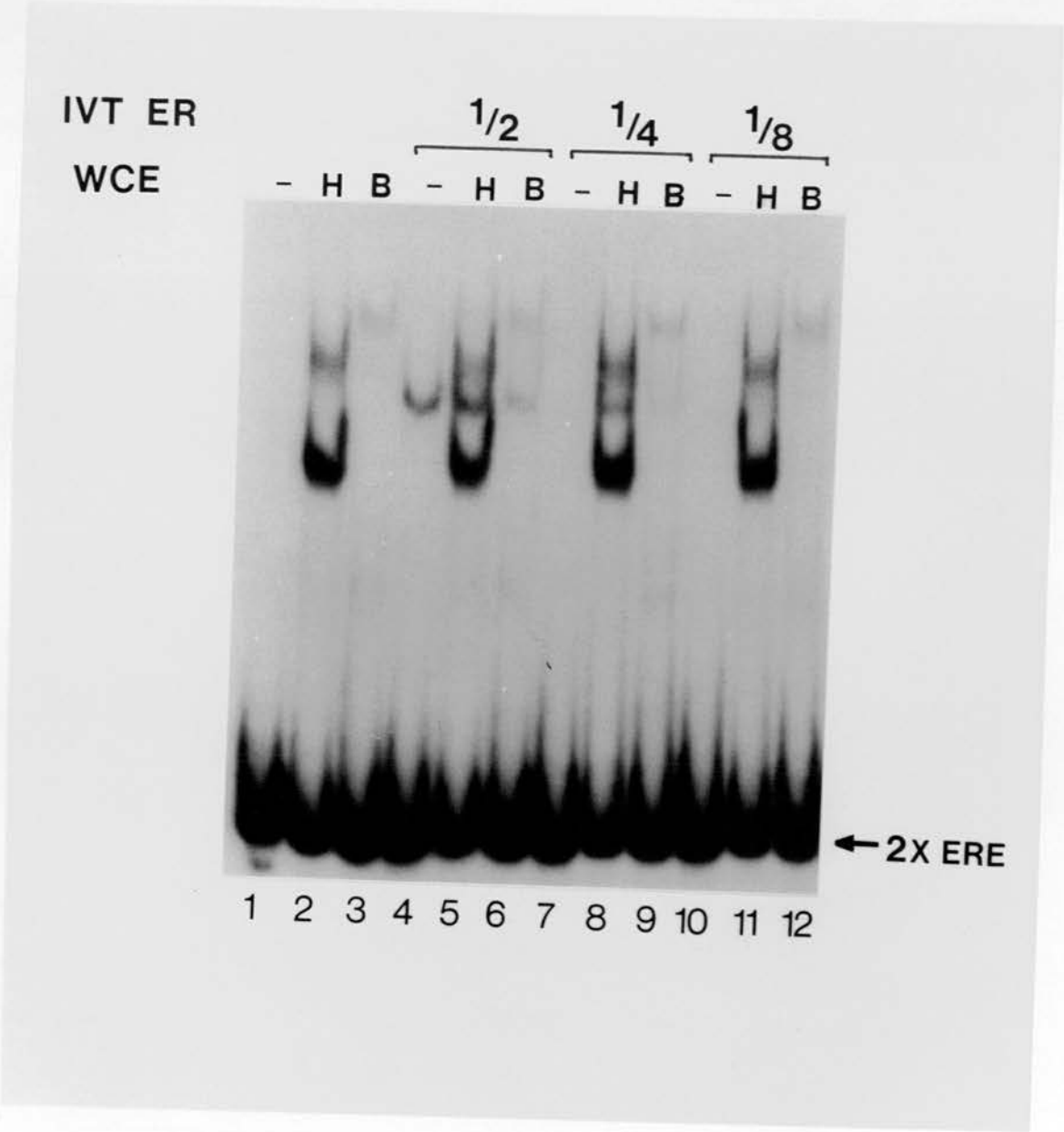
5.2.5 ER binds to DNA as part of a macromolecular protein complex

It has been demonstrated that efficient ER binding to target DNA requires dimer formation between two 65kD ER monomers (Kumar & Chambon, 1988; Fawell et al., 1990). To determine the mol. wt. of the active DNA-binding ER complex, native pore gradient gel electrophoresis was carried out (section 2.2.2.3) using IVT ER. The theory of pore gradient gel electrophoresis has already been discussed (section 4.1).

Three band shift reactions (section 2.8.1) were set up, each containing 3fmol ^{32}P-labelled DF2 probe and 4µg poly [d(I-C)]. DF2 was incubated with 10µg HeLa WCE for 20 mins (Fig. 5.6, lane H), 0.5µl IVT ER for 10 mins (Fig. 5.6, lane E), or 10µg HeLa WCE for 10 mins followed by

Figure 5.5 **Analysis of ER DNA-binding activity in the presence of protein extract which does not contain SRE-BP activity**

Bacterial lysate which does not contain SRE-BP activity was prepared as described (section 2.6.3). Protein binding to 3fmol ^{32}P-labelled DF2 probe in the presence of 4μg poly [d(I-C)] was assayed as follows: Lane 1 did not contain protein extract; lanes 2, 5, 8, and 11 contained 10μg HeLa WCE, and lanes 3, 6, 9, and 12 contained 10μg bacterial lysate. ER was diluted and 1μl of diluted ER was added to lanes 4, 5, 6 ($^1/_2$ dilution); lanes 7, 8, and 9 ($^1/_4$ dilution) and lanes 10, 11, and 12 ($^1/_8$ dilution) respectively. Free and bound DF2 probe were separated by 4% native polyacrylamide gel electrophoresis (section 2.2.2.2).


IVT ER
WCE
1/2
1/4
1/8
- H B - H B - H B - H B
2X ERE
1 2 3 4 5 6 7 8 9 10 11 12

0.5µl IVT ER for 10 mins (Fig. 5.6, lane H + E). Protein mol. wt. markers were electrophoresed in adjacent lanes (detailed in Fig. 5.6 legend).

The mol. wt. of the ER-DNA complex was calculated by relating its electrophoretic mobility to that of protein markers. The ER-DNA complex was found to be 420kD. By correcting for DNA content (DF2 probe, 58kD), it was demonstrated that ER bound to DNA as part of a 362kD protein complex. Also, after correcting for DNA content, the mol. wt. of the DNA-binding protein in HeLa WCE, which generated a complex equivalent to complex (c) in Fig. 5.3, was calculated to be approximately 200kD.

5.3 DISCUSSION

This chapter presents preliminary evidence which suggests that accessory proteins, present in HeLa WCE, increase ER affinity for target DNA. Also reported is evidence for the participation of additional proteins, besides an ER homodimer, in ER-DNA complex formation.

In vitro translated ER does not bind to the 21bp ERE_{con} oligonucleotide used for experimentation in Chapters 3 and 4. However, other studies demonstrate binding of either *in vitro* translated ER (Fawell et al., 1990) or ER present in crude cell extracts (Kumar & Chambon, 1988) to a 32bp or 35bp oligonucleotide, respectively, containing an ERE_{con} sequence. Chalepakis et al. (1988b) demonstrate that either purified GR or PR protect a region of the MMTV promoter which includes both arms of one SRE and additional flanking DNA from hydroxyl radical attack. It is, therefore, likely that the 21bp oligonucleotide provides too small a target for ER interaction and that DNA flanking a SRE is probably required to allow additional non-specific contact between SR and the phosphate backbone of DNA which will secure SR-SRE interaction.

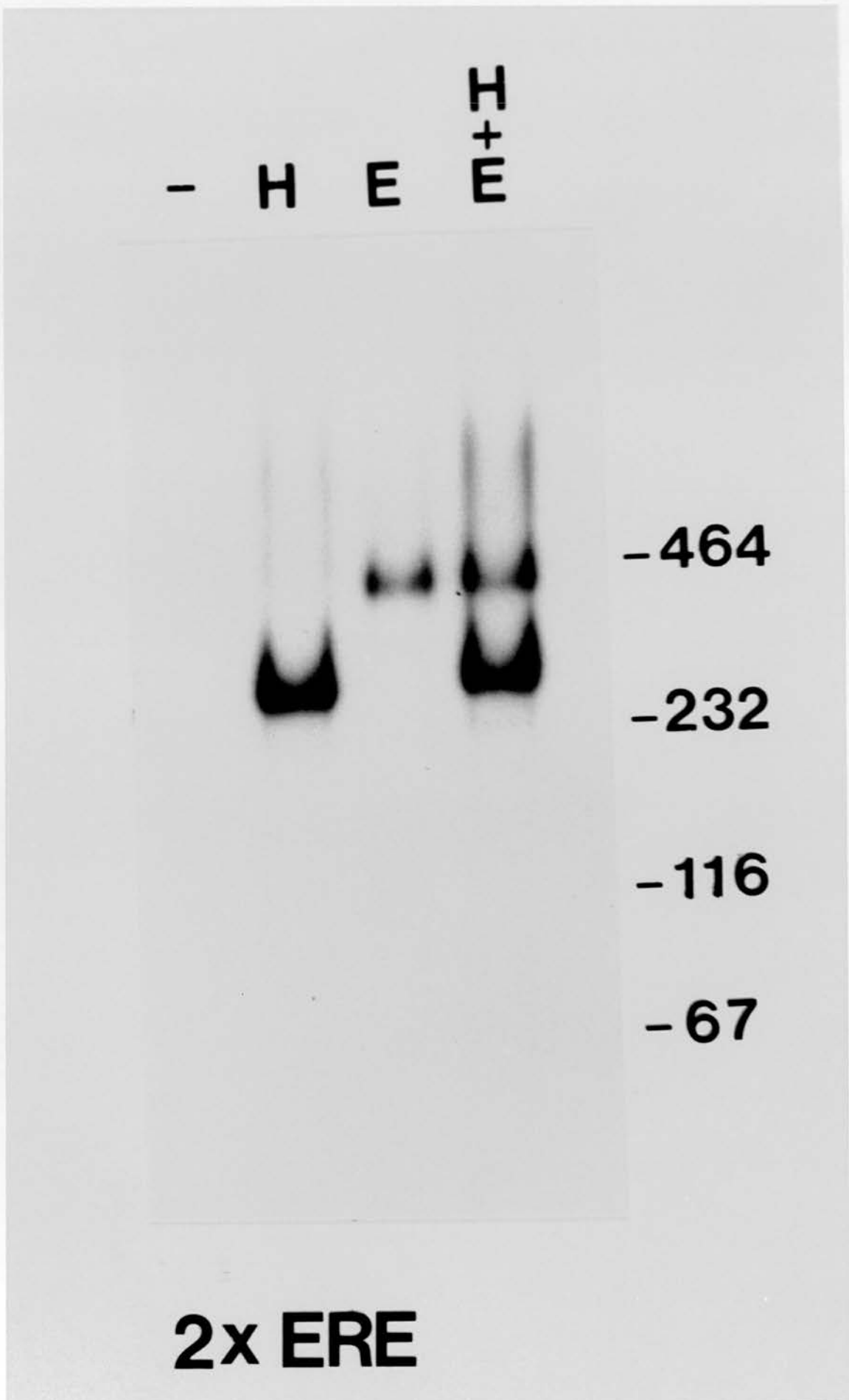
In vitro translated ER can bind to either an 89bp DNA fragment containing two consensus EREs (DF2) or a 77bp DNA fragment containing

Figure 5.6

Pore gradient gel electrophoresis of ER/DF2 complexes

Either 10μg HeLa WCE (H), 0.5μl ER (E), or both (H + E) were incubated with 3fmol ^{32}P-labelled DF2 and 4μg poly [d(I-C)]. Protein/DNA complexes were resolved by electrophoresis through a 3-25% native pore size gradient polyacrylamide gel (section 2.2.2.3). Protein mol. wt. markers were electrophoresed in adjacent lanes; bovine serum albumin (67kD), β-galactosidase (116kD-monomer, 232kD-dimer and 464kD-tetramer).

Pore gradient gel electrophoresis of ER/DF2 complexes was carried out twice.


-
H
E
H+E
-464
-232
-116
-67
2x ERE

one consensus ERE (DF1). The number of complexes generated with either DF2 or DF1 reflects the number of EREs each fragment encodes. ER-generated complex (b) formed only with DF2 probe. The slower electrophoretic mobility of complex (b) indicated that it was larger than complex (a), which was generated by ER binding to either DF1 or DF2 probe. Complexes (a) and (b) most probably represent ER occupancy of one and two EREs respectively, although to verify this, DNA footprinting analysis is needed. Similarly, complex formation between HeLa WCE and either DF2 or DF1 reflects the number of EREs encoded. As SRE-BP in HeLa WCE binds preferentially to an ERE_{con} (Chapter 3), it is proposed that complex (c) represents SRE-BP interaction with one ERE and complex (d), formed only with DF2 and exhibiting slower electrophoretic mobility than complex (c), represents SRE-BP interaction with two EREs. This is supported by mol. wt. analysis which shows that complex (c) formation is due to DF2 interaction with a protein of approximately 200kD, identical to the characterised mol. wt. of SRE-BP (Chapter 4). HeLa WCE incubated with DFO probe which did not contain any EREs unexpectedly created four poorly resolved complexes. It is unlikely that such complex formation is SRE-BP derived; DFO originates from *lac* operator DNA, which is also present in pBLCAT2 and digested pBLCAT2 is incapable of competing for SRE-BP DNA-binding activity *in vitro* (Chapter 3, Fig. 3.7; Crawford & Chapman, 1990a).

In vitro translated ER binds with increased affinity to DF2 when DF2 is preincubated with HeLa WCE; the degree to which ER DNA-binding activity is increased appears to be inversely related to ER concentration. At lower concentration of ER, preincubation of DNA with bacterial lysate also increases ER DNA-binding activity although to a much lesser degree than WCE. It is unlikely that the enhancement of ERE binding by bacterial lysate is specific to ER as neither SRs nor steroid hormone responsiveness have

been reported in prokaryotes. More likely, non-specific prokaryotic DNA-binding proteins interact with, and therefore reduce, the quantity of unbound poly [d(I-C)] available for non-specific ER interaction, thus driving ER-ERE binding. Of course, this phenomenon will also occur when non-specific DNA-binding proteins in HeLa WCE titrate poly [d(I-C)] out of solution, thus driving ER-ERE interaction. However, HeLa WCE increases ER DNA-binding activity noticeably more than the same quantity of bacterial lysate. Assuming eukaryotic and prokaryotic cell extracts contain approximately the same amount of DNA-binding proteins per µg, then these results suggest the existence of eukaryotic proteins which positively modulate ER-DNA interaction. Since SRE-BP also interacts with EREs, I propose that this activity constitutes the positive modulator of ER DNA-binding activity. If this is so, the actual extent to which SRE-BP can increase ER affinity for target DNA will be masked by the residual SRE-BP present in the reticulocyte lysate in which ER was expressed. Purification of SRE-BP activity and/or obtaining a cell line devoid of SRE-BP activity will hopefully provide direct evidence to test this hypothesis.

Whether SRE-BP remains bound to DNA after ER interaction is not known. The ER-DNA complex has a mol. wt. of approximately 362kD which presumably contains the 130kD ER homodimer necessary for efficient and specific DNA-binding (Kumar & Chambon, 1988; Fawell et al., 1990). Inclusion of the approximately 200kD SRE-BP may account for, at least in part, the remaining 232kD of mol. wt. Indeed, electron microscopy studies of protein-DNA interaction at the ERE of the Xenopus vitellogenin (Heggeler-Bordier et al., 1987), indicate that protein-DNA complexes at the ERE are large and may include other proteins besides hormone receptor.

There are several reported experiments which demonstrate how SR affinity for target DNA is increased in the presence of additional proteins. As discussed in Chapter 3 and Chapter 4, Feavers et al. (1987) have identified

a protein, NHP-1, which specifically binds to an ERE_{con} sequences; preincubation of a 45bp oligonucleotide containing an ERE_{con} with HeLa nuclear extract containing NHP-1 increases the affinity of partially purified ER for this oligonucleotide more than 8-fold. Thrall & Spelsberg (1980) have demonstrated that activated PR has a higher affinity for DNA which is complexed with nuclear proteins than for naked DNA, and propose that the nuclear acceptor site for high affinity PR binding is complexed with other proteins. In addition, mixing cytosolic PR with nuclear extracts depleted of PR by immunoaffinity absorption increases PR affinity for target DNA more than 4-fold (Edwards et al., 1989). Wrange et al. (1986) have identified a 72kD protein (determined under denaturing conditions) that copurifies with activated rat GR. Although DNA-binding activity by this protein has not been demonstrated, fractions containing this protein increase the DNA-binding activity of GR (Payvar & Wrange, 1983).

Thus, there is mounting evidence that the SR molecule alone does not contain all the information required for high affinity, specific DNA-binding. This is ultimately reflected in the poor ability of purified receptors to discriminate between a specific SRE and non-specific DNA (see below for references). As discussed in Chapter 3, purified receptors often bind with less than 10-fold higher affinity to SRE-containing DNA than to non-specific DNA (Payvar et al., 1981; Compton et al., 1983; Bailly et al., 1986; von der Ahe et al., 1985; Maurer & Notides, 1987; Jantzen et al., 1987; Peale et al., 1988; Härd et al., 1990b).

The relationship between the SRE-BP and the accessory proteins discussed above also implicated in SR action, remains to be determined. I have previously proposed that SRE-BP plays a global role in steroid hormone regulation of gene transcription by modulating the DNA-binding affinity of SR for SRE (Chapter 3). In light of the finding that SRE-BP does not bind to a palindromic TRE (Chapter 3), and that accessory nuclear

factors which increase TR affinity for the rat growth hormone TRE have been reported (Murray & Towle, 1989), I also propose that accessory proteins (the SRE-BP) involved in positive modulation of SR DNA-binding activity are different from those involved in TR DNA-binding activity. Perhaps, for the steroid hormone receptor superfamily, there is a family of related accessory proteins which modulate receptor-DNA interaction.

CHAPTER 6

SUMMARY

This thesis clearly shows the existence of a cellular protein in HeLa, GH_3, CV-1 and liver tissue whole cell extracts, and in nuclear extracts of HeLa cells, that specifically binds to SREs. I have named this protein the steroid response element-binding protein (SRE-BP) and propose that, by modulating the interaction of SRs with target SREs, the SRE-BP plays a global role in gene regulation by steroid hormones.

The SRE-BP binds preferentially to two related, although functionally distinct, SREs. In a band shift assay, the SRE-BP binds with highest affinity to oligonucleotides containing either an ERE or asymmetrical GRE; it binds weakly to a mutant GRE and does not recognise a palindromic TRE. The mutant GRE and palindromic TRE encode conserved arms identical to those of the symmetrical GRE and consensus ERE, respectively. It therefore appears that the orientation of, and the spacing between, the conserved arms of the SRE are important to SRE-BP activity.

SRE-BP binding to a SRE is specific. When digested plasmid competitor DNA, which encodes transcription factor binding sites present in the HSV tk promoter (TATA, CTF and Sp1 binding sites), is included in a band shift assay, it is unable to compete for SRE-BP DNA-binding activity. Only when the plasmid DNA also encodes an ERE consensus sequence is it then able to compete for SRE-BP binding.

The mol. wt. of the SRE-BP as it exists in solution is approximately 200kD, as determined by native gel filtration chromatography. The mol. wt. of the DNA-bound form of SRE-BP is also approximately 200kD, as determined by native pore gradient gel electrophoresis. This strongly suggests that the stable form of SRE-BP in solution is the active DNA-binding species. Preliminary covalent crosslinking experiments suggest that the SRE-BP is not a single 200kD polypeptide, but probably a multisubunit protein complex.

Several lines of evidence indicate that the SRE-BP is unlikely to be either ER or GR. (1) The SRE-BP is present in cells that express neither ER nor GR; (2) The SRE-BP binds to either an ERE or GRE without sequence preference for either. In contrast, ER and GR display sequence-specific binding to an ERE and a GRE, respectively; (3) The mol. wt. of the SRE-BP is approximately 200kD which is larger than the 130kD ER dimer; formation of such a dimer is necessary for efficient ER DNA-binding. The SRE-BP mol. wt. is close to that of a GR dimer (190kD), however, since the SRE-BP contains both ERE and GRE binding activities, the SRE-BP is unlikely to be either ER or GR; (4) Protein-DNA complexes generated in preliminary crosslinking experiments suggest that the SRE-BP contains a DNA-binding subunit of between 42kD and 88kD; complexes which reflected the monomer mol. wt. of ER and GR (65kD and 94kD, respectively) were not apparent.

Circumstantial evidence reported in this thesis indicates that the SRE-BP present in HeLa whole cell extracts, behaves as an accessory protein and increases the affinity of *in vitro* translated ER for its response element. Additionally, the demonstration of *in vitro* translated ER binding to DNA as part of a 362kD complex, and not simply a 130kD receptor dimer, further substantiates my proposal that accessory proteins are involved in SR binding to DNA.

Ultimately, it is desirable to purify the SRE-BP; an attractive method by which SRE-BP purification could be achieved is by Fast Protein Liquid Chromatography. Purified SRE-BP could be used in many experimental applications. Perhaps the most informative path to follow would be to generate sufficient quantities of pure SRE-BP, thereby allowing adequate production of SRE-BP peptides for amino acid sequencing. Degenerate oligonucleotide probes would then be synthesised for use as primers for

DNA amplification by polymerase chain reaction, with a view to cloning the SRE-BP.

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APPENDIX 1

ABBREVIATIONS

LIST OF ABBREVIATIONS

A_{600}, A_{595}, A_{260}	Absorbance (number = wave length in nm)
Amp^r	ampicillin resistance
AR	androgen receptor
bp	base-pair
cAMP	cyclic adenosine monophosphate
CAT	chloramphenicol acetyltransferase
cDNA	complementary DNA
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dH_2O	distilled water
DNA	deoxyribonucleic acid
ds	double-stranded
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediamine tetra acetic acid
EGTA	ethylene glycol-bix (β-aminoethyl ether) N,N,N',N'-tetraacetic acid
ER	Oestrogen receptor
ERE	Oestrogen response element
EXAFS	extended X-ray absorption fine structure
GR	Glucocorticoid receptor
GRE	glucocorticoid response element
h	hour
HCl	hydrochloric acid
HEPES	N-(2-hydroxyethyl) piperazine-N-2-ethane
HSV	Herpes Simplex Virus
IVT	*in vitro* translated

kD	kilo Dalton
M	Molar
mA	milliamp
mg	milligramme
mins	minutes
ml	millilitre
mM	millimolar
MMTV	mouse mammary tumour virus
mol. wt.	molecular weight
MR	Mineralocorticoid receptor
nM	nanometer
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
pmol	picomoles
PMSF	phenylmethylsulphonyl fluoride
PR	progesterone receptor
PRE	progesterone response element
RAR	retinoic acid receptor
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SR	steroid receptor
SRE	steroid response element
TEMED	N,N,N',N'-Tetramethylethylene diamine
tk	thymidine kinase
TR	thyroid receptor
TRE	thyroid response element
U	international enzyme unit
UV	ultraviolet
V	volt

VDR	vitamin D_3 receptor
VLDL	very low density lipoprotein

APPENDIX 2

PUBLISHED PAPERS

Identification of a High Molecular Weight Steroid Response Element Binding Protein

Louise Crawford and Karen Chapman

MRC Brain Metabolism Unit
Royal Edinburgh Hospital
Morningside Park
Edinburgh, Scotland EH10 5HF

In this study we report the identification of a Steroid Response Element-Binding Protein (SRE-BP) present in whole cell extracts of HeLa cells and GH_3 pituitary tumor cells which specifically binds to two classes of functionally distinct SREs. In gel retardation experiments SRE-BP binds preferably to oligonucleotides containing an estrogen response element (ERE) or a symmetrical glucocorticoid response element (GRE); it binds less well to a mutant GRE and poorly, if at all, to a thyroid response element (TRE). The SRE-BP does not recognize transcription factor binding sites present in the promoter of the Herpes Simplex Virus thymidine kinase gene. We have shown, using gel filtration chromatography that the SRE-BP has a relative molecular weight under nondenaturing conditions of 205 K (±20 K). The SRE-BP is not a steroid receptor as evidenced by different DNA sequence specificity, cell type distribution, and molecular weight. We propose that by modulating the interaction of steroid receptors with target SREs, the SRE-BP plays a role in specificity of steroid hormone action. (Molecular Endocrinology 4: 685–692, 1990)

INTRODUCTION

The steroid hormone receptors are nuclear transcription factors which, upon activation by hormone binding, specifically bind to short DNA sequences in steroid regulated genes and alter the transcription rates of those genes (reviewed in Refs. 1–4). These DNA sequences, or steroid response elements (SRE) (5–8) are generally present in the 5′-flanking regions of steroid hormone responsive genes, although the existence of a glucocorticoid response element (GRE) in the first intron of the human GH gene has been reported (9). In most instances, binding of steroid receptor to a SRE leads to increased transcription rates of target genes; however, some examples of negative regulation by glucocorticoid receptor (GR) have also been described (10–13).

Steroid receptors share a common structural organization in which a hormone binding domain determines the ligand specificity of the steroid receptor and a highly conserved DNA binding domain determines the DNA sequence to which the steroid receptor will bind (2, 3 and references therein). Different steroid receptors may, however, elicit a functional response by binding to a common SRE. Receptors for glucocorticoids, mineralocorticoids, progesterone, and androgens can all act through a GRE/progesterone response element (GRE/PRE), whereas the receptor activated by estrogen acts through the similar, but distinct, estrogen response element (ERE) (5–7, 14–18).

The consensus GRE/PRE and ERE are related sequences which show dyad symmetry with 5 or 6-base-pair (bp) arms separated by a 3-bp spacer region. Each is independently able to confer hormone-dependent transcriptional enhancement from a linked promoter, as demonstrated by gene transfer experiments (5–7, 17,18). SREs which deviate from the consensus GRE/PRE or ERE may function as less efficient transcriptional enhancers *in vivo* or lose their regulatory capacity altogether (5–7, 18–22), although they may still retain the ability to be bound by steroid receptor *in vitro* (18, 20–23). For example, the PRL ERE (ERE_{PRL}), which differs from the consensus ERE by two nucleotide substitutions, confers a weak response to estradiol [~3-fold; (20, 21)] but can be directly bound by highly purified estrogen receptor (ER) *in vitro* (20, 24). Although purified steroid receptors directly bind to the respective SRE *in vitro,* the discrimination between a specific binding site and nonspecific DNA may be less than 10-fold (see for example Refs. 20, 25–30).

In this study, we wished to determine whether accessory proteins (which are not steroid receptors) could bind to a SRE and thus increase the specificity of the steroid receptor SRE interaction. Such accessory proteins might elicit an effect either by regulating access of steroid receptor to the SRE, or alternatively, by binding to the SRE together with steroid receptor, to provide protein-protein contact in addition to steroid receptor-DNA contact.

0888-8809/90/0685-0692$02.00/0
Molecular Endocrinology

In this paper, we report the identification and partial purification of a cellular factor which binds preferably to oligonucleotides containing either an ERE or a GRE. The identified factor is distinct from either ER or GR, is present in whole cell extracts of different cell types, and has a relative mol wt of 205 K (±20 K) under nondenaturing conditions.

RESULTS

Identification of a Factor which Binds Selectively to Both EREs and GREs

Gel retardation assays were used to detect the presence of DNA-binding protein(s) in whole cell extracts of HeLa and GH_3 cells which bind to a double-stranded oligonucleotide containing an ERE_{con} sequence (ERE_{con}; Fig. 1a). Both GH_3 and HeLa whole cell extract contained a cellular factor which bound to the ERE_{con} oligonucleotide in the presence of nonspecific competitor, poly[d(I-C)], to form a complex with identical retarded electrophoretic mobility and signal intensity (Fig. 2).

To determine whether the formation of this complex represented a sequence-specific interaction with the ERE_{con} oligonucleotide, competition experiments were carried out. A 100-fold molar excess of unlabeled ERE_{con} oligonucleotide efficiently competed for complex formation (Fig. 3a), demonstrating that complex formation is sequence specific.

In a similar competition assay, poly[d(I-C)] was replaced with plasmid DNA digested with *Alu*I, either pBLCAT2 (31) or pLC3, a derivative of pBLCAT2 that differs only by the presence of an ERE_{con} sequence cloned into the pBLCAT2 polylinker. Four micrograms of digested pLC3 plasmid DNA (~equivalent to a 10-fold molar excess of competitor ERE_{con}) competed for complex formation, whereas 4 μg pBLCAT2 did not (Fig. 3b), demonstrating that the factor detected could efficiently bind to an ERE_{con} sequence embedded in a fragment of nonspecific DNA. Furthermore, the identified factor did not bind to the transcription factor binding sites (Spl, CTF, and TATA factor binding sites) of the Herpes Simplex Virus (HSV) thymidine kinase (tk) gene promoter (32) present in pBLCAT2.

To investigate further the sequence preference of the identified factor, its ability to bind to oligonucleotides containing a SRE distinct from ERE_{con} was examined. ERE_{PRL} differs from the conserved nucleotides of the ERE_{con} by 2 bp (20, 21) (ERE_{PRL}; Fig. 1b). The introduction of 4 bp changes within the conserved bases of the ERE_{con} converts this sequence into a functional GRE (5); (GRE; Fig. 1c). A mutant GRE was created by increasing the spacing between the conserved nucleotides of a GRE (GRE_M; Fig. 1d), and a TRE was created by decreasing the spacing between the conserved nucleotides of a consensus ERE (33) [thyroid response element (TRE); Fig. 1e]. Each of these oligonucleotides

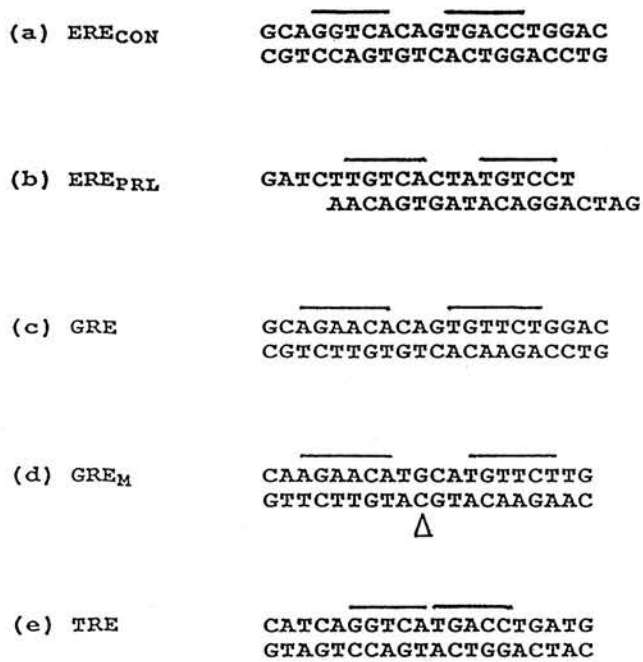

Fig. 1. Sequence of Oligonucleotides Used in the Gel Retardation Assays

Horizontal lines indicate the conserved nucleotides within the SRE. A *hollow arrow* head shows the position of the additional base-pair in the spacer region of oligonucleotide (d). (a) ERE consensus sequence derived from the estrogen-inducible vitellogenin genes of *Xenopus laevis* and chicken (5). (b) ERE_{PRL} sequence from the 5′ flanking region of the rat PRL gene (20, 21). (c) GRE sequence with perfect 2-fold symmetry (5). (d) Mutant GRE sequence. (e) Symmetrical TRE sequence (33)

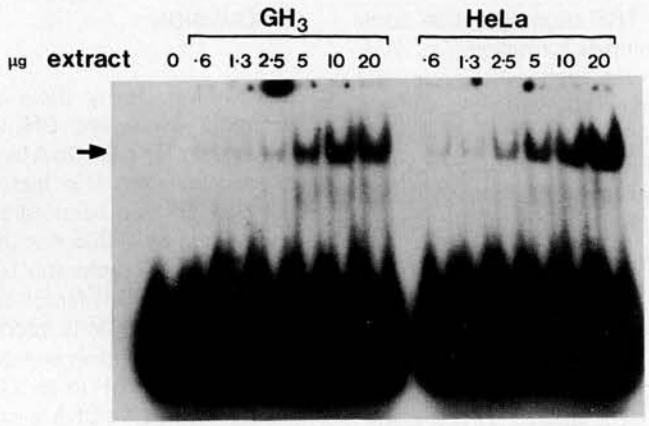

Fig. 2. Analysis of DNA Binding Activity Present in Whole Cell Extracts of HeLa and GH_3 Pituitary Tumor Cells

Whole cell extracts of HeLa and GH_3 cells were prepared. Increasing amounts of whole cell extracts (as indicated in micrograms above each lane) were incubated with 0.1 pmol labeled ERE_{con} oligonucleotide in the presence of 4 μg poly[d(I-C)]. Free and bound labeled oligonucleotide were separated on a 4% polyacrylamide gel by electrophoresis. An *arrow* indicates the position of bound oligonucleotide.

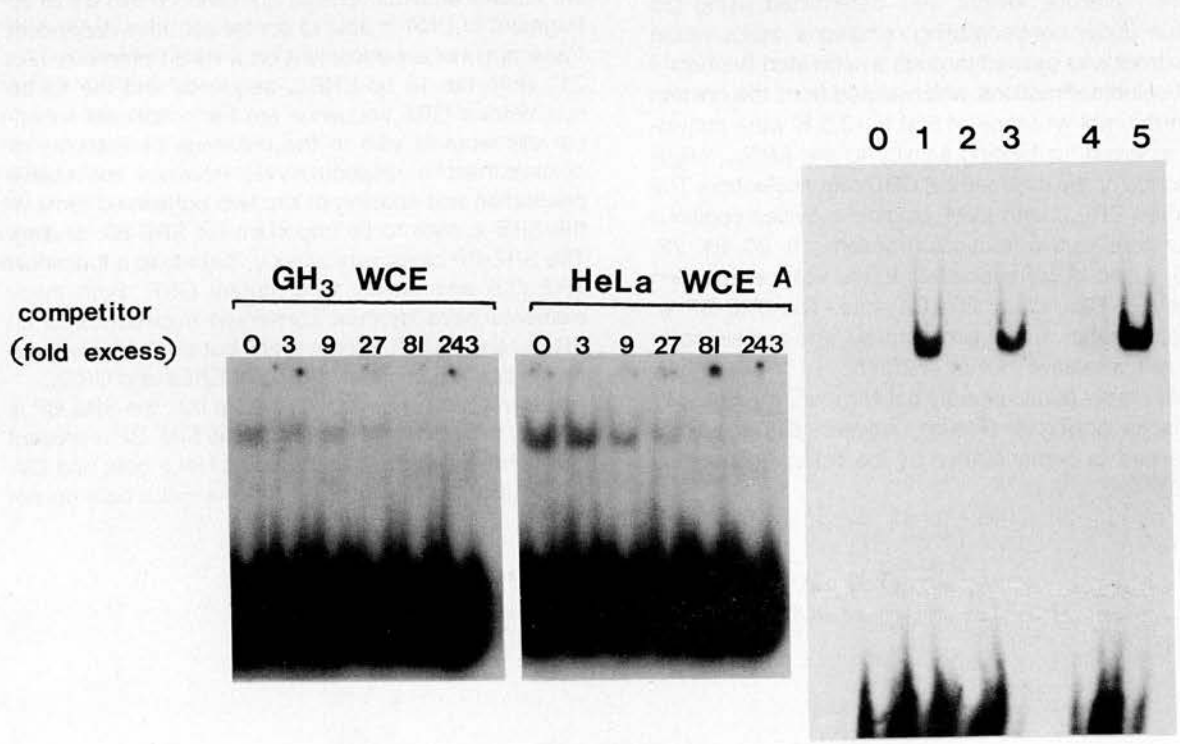

Fig. 3. Analysis of Specificity of Factor Binding to the ERE_{con} Oligonucleotide

(a) Ten micrograms of HeLa or GH_3 whole cell extract were incubated with 0.1 pmol labeled ERE_{con} oligonucleotide and 4 μg poly[d(I-C)]. Increasing amounts of unlabeled ERE_{con} oligonucleotide were added as indicated in fold excess over labeled ERE_{con} oligonucleotide. Free and bound labeled oligonucleotide were separated on a 4% polyacrylamide gel by electrophoresis. (b) Ten micrograms (lanes 1–3) or 20 μg (lanes 4–5) HeLa whole cell extract were incubated with 0.1 pmol labeled ERE_{con} oligonucleotide. Lane 0 does not contain whole cell extract. Competitor labeled DNA was 4 μg poly[d(I-C)] (lanes 0 and 1) 4 μg *Alu*I digested pLC3 (lanes 2 and 4) or 4 μg *Alu*I digested pBLCAT2 (lanes 3 and 5). Free and bound labeled oligonucleotide were separated on a 4% polyacrylamide gel by electrophoresis.

was tested for ability to bind the factor in competition assays. A 100-fold molar excess of unlabeled ERE_{PRL} or GRE oligonucleotide competed with ^{32}P-labeled ERE_{con} oligonucleotide for complex formation to an extent comparable to that observed using 100-fold excess of ERE_{con} oligonucleotide (Fig. 4). A 100-fold molar excess of unlabeled GRE_M oligonucleotide competed less well for complex formation and a 100-fold

molar excess of unlabeled TRE oligonucleotide competed weakly, if at all, for complex formation (Fig. 4). A similar competition pattern was seen in the reciprocal gel retardation assay in which a 100-fold molar excess of unlabeled ERE_{con}, ERE_{PRL}, or GRE oligonucleotides competed for binding to ^{32}P-labeled GRE oligonucleotide and a 100-fold molar excess of unlabeled GRE_M and TRE oligonucleotides competed poorly (Fig. 4).We have also noted the GRE_M is consistently a slightly better competitor for binding to a labeled GRE than to a labeled ERE_{con}. Thus, the identified factor has varied affinity for different, related oligonucleotide sequences. However, factor binding is more selective for either a GRE or an ERE, although preferential to neither. This factor has been termed the SRE-binding protein (SRE-BP).

The SRE-BP has a Relative Mol Wt of ≃205 K

Having identified a SRE-BP and confirmed that it binds with the same affinity to both an ERE and a GRE, its relative molecular weight was determined using gel filtration under nondenaturing conditions. HeLa whole cell extract was passed through a calibrated Sephacryl S300 column. Fractions which eluted from the column within the mol wt range of 300 K-12.5 K were individually assayed for binding activity to the ERE_{con} oligonucleotide or the symmetrical GRE oligonucleotide. The observed ERE_{con} and GRE binding activities coeluted in four consecutive fractions (fractions 19, 20, 21, 22; Fig. 5, a and b) corresponding to the volume at which proteins of 205 kDa ± 20 kDa elute. The SRE-BP is, therefore, either a very large protein or a protein complex, with a relative mol wt of 205 K.

After partial purification by gel filtration, the SRE-BP retains its ability to discern between different SRE sequences as demonstrated by the competition assay (Fig. 6).

DISCUSSION

Our studies clearly show the existence of a cellular protein in HeLa and GH_3 whole cell extracts, which exhibits preferential DNA binding to both ERE and GRE oligonucleotides. This factor, which we have termed the SRE-BP has been partially purified and shown to have a mol wt of 205 K ± 20 K.

The SRE-BP preferably binds to EREs and a GRE *in vitro*. Transcription factor binding sites present within the truncated HSV tk promoter of pBLCAT2 (TATA, CTF, and Spl binding sites) are unable to compete for binding of SRE-BP to an ERE_{con} oligonucleotide when pBLCAT2 plasmid DNA is included in the binding assay. Plasmid DNA will only compete for complex formation if an ERE_{con} sequence has been cloned into pBLCAT2, demonstrating the sequence-specific binding of the SRE-BP to the SRE and not to other regulatory DNA elements.

The SRE-BP binds with equal affinity to three different functional SREs. ERE_{PRL} contained within a 228 bp fragment of DNA is able to confer estradiol-dependent transcriptional enhancement on a linked promoter (20, 21). Both the 13 bp ERE_{con} sequence and the 15 bp symmetrical GRE sequence are transcriptional enhancer elements *in vivo* in the presence of estradiol or dexamethasone, respectively (5). However, the relative orientation and spacing of the two conserved arms of the SRE appear to be important for SRE-BP binding. The SRE-BP binds very weakly, if at all, to a functional TRE (33) and weakly to a mutant GRE. Both these elements have identical conserved nucleotides to an ERE_{con} and a GRE, respectively, but do not have the 3 bp spacer region characteristic of EREs and GREs.

Several lines of evidence indicate that the SRE-BP is unlikely to be either ER or GR. 1) The SRE-BP is present in a number of cell-types including HeLa cells and CV-1 cells (our unpublished results). As HeLa cells do not

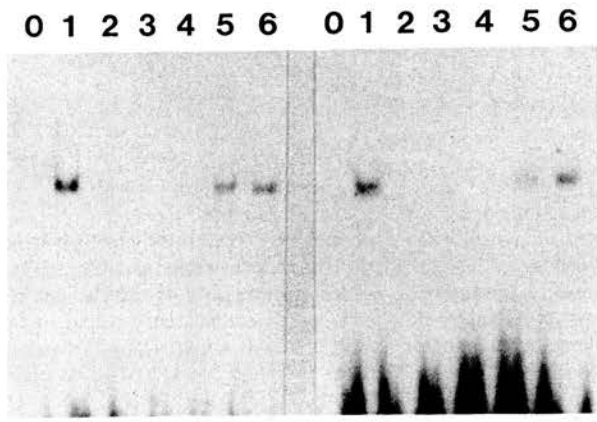

Fig. 4. Specificity of Factor Binding to Both an ERE_{con} Oligonucleotide or a GRE Oligonucleotide

Ten micrograms of HeLa whole cell extract were incubated with 4 μg poly[d(I-C)] and either 0.1 pmol labeled ERE_{con} oligonucleotide (*left panel*) or 0.1 pmol labeled GRE oligonucleotide (*right panel*). Lane 0 does not contain whole cell extract; lanes 2–6 show binding in the presence of a 100-fold molar excess of competitor oligonucleotide as follows: lane 2, ERE_{con}; lane 3, ERE_{PRL}; lane 4, GRE; lane 5, GRE_M; lane 6, TRE. Free and bound labeled oligonucleotide were separated on a 4% polyacrylamide gel by electrophoresis.

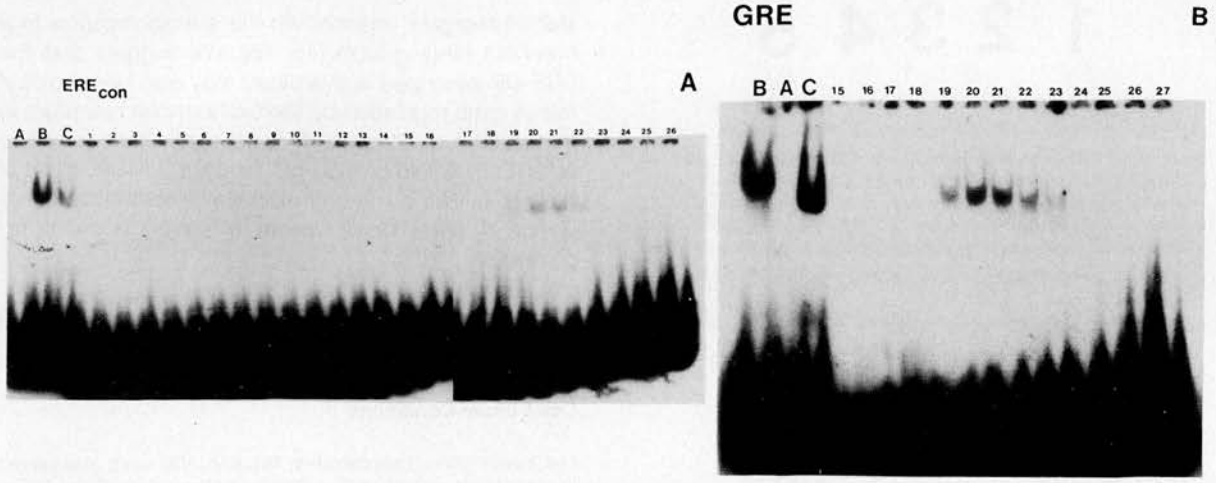

Fig. 5. Gel Filtration Chromatography of SRE-BP

Hela whole cell extract was fractionated over a Sephacryl S300 column (see *Materials and Methods*). Lane (A) has no protein added, lane (B) has 10 μg whole cell extract added in binding buffer (see *Materials and Methods*) and lane (C) has 10 μg HeLa whole cell extract added in column buffer (see *Materials and Methods*). (a) Fractions 1 to 26 (numbered above each lane) were incubated with 0.1 pmol labeled ERE$_{con}$ oligonucleotide in the presence of 4 μg poly[d(I-C)]. (b) Fractions 15 to 27 (numbered above each lane) were incubated with 0.1 pmol labeled GRE oligonucleotide in the presence of 4 μg poly[d(I-C)]. Free and bound labeled oligonucleotide were separated on a 4% polyacrylamide gel by electrophoresis.

contain ER (23) and CV-1 cells contain neither ER nor GR (23, 34, 35) SRE-BP activity present in whole cell extract of HeLa and CV-1 cells cannot be due to either ER or GR. 2) In contrast to the sequence-specific binding of ER or GR to an ERE or GRE, respectively, the SRE-BP binds to either an ERE or a GRE without sequence preference for either. 3) The mol wt of the SRE-BP is 205 K; ER is smaller and binds to DNA as a dimer (23) with a relative mol wt of 130 K. The mol wt of activated GR [a dimer of 190 K (35, 37)] is close to that of SRE-BP and its elution profile from the Sephacryl S300 column might overlap with that of the SRE-BP. However, as the ERE and GRE binding activities coeluted from the Sephacryl S300 column SRE binding activity is unlikely to be due to ER or GR, which would be expected to elute in distinct fractions according to their different molecular weights.

It is widely accepted that steroid receptors bind to their specific SRE *in vivo* to enhance transcription from a downstream promoter. In support of this, Becker *et al.* (38) have reported hormone-dependent changes in reactivity of guanine residues in the GRE of the tyrosine amino transferase gene which reflect the binding of GR *in vivo* to the GRE in a hormone-dependent manner. In the absence of hormone, Becker *et al*. (38) report some changes in reactivity of guanine residues within the GRE *in vivo,* compared to reactivity within the same region of naked DNA *in vitro.* This data is consistent with the possibility that a protein (possibly the SRE-BP) may be bound at the GRE *in vivo* in the absence of hormone, although with lower affinity than the activated GR.

In contrast to the situation *in vivo, in vitro* studies which have examined the ability of purified steroid receptor to discriminate between a specific SRE and nonspecific DNA have largely failed to demonstrate high specificity of binding. In many cases, the receptor binds with less than 10-fold higher affinity to SRE-containing DNA than nonspecific DNA (20, 25–30). Higher specificity could be achieved, in part, by additional cellular proteins. It has been suggested (24) that high affinity binding of ER to ERE$_{PRL}$ is achieved by a local structural alteration in the DNA allowing binding of ER to single stranded DNA. If local structural changes are involved, they could be facilitated by accessory proteins. There are several reported experiments which directly or indirectly suggest the involvement of accessory proteins which increase sequence-specific binding of steroid receptors to DNA. Feavers *et al.* (39) identified a 70 kDa protein which binds specifically to the ERE of the chick vitellogenin gene. In the presence of this 70 kDa protein, ER binding to the ERE was increased (39). Wrange *et al.* (40) have identified a 72 kDa nonsteroid binding protein that copurifies with the rat GR. Fractions containing this protein increased the DNA binding activity of the GR (41). Similarly, Edwards *et al.* (42) have identified an activity in nuclear extracts which is distinct from progesterone receptor and which increases the specific binding of progesterone receptor to DNA *in vitro*. There are also a number of reports of avian progesterone receptor binding to a class of nonhistone chromosomal proteins (43, 44). We have preliminary evidence that the SRE-BP is present in nuclear extracts of HeLa cells, and we are currently purifying the SRE-BP in order to further characterise the protein to determine its relationship to the above proteins.

In contrast to steroid receptors themselves, the SRE-BP interacts with two classes of SRE, binding to an

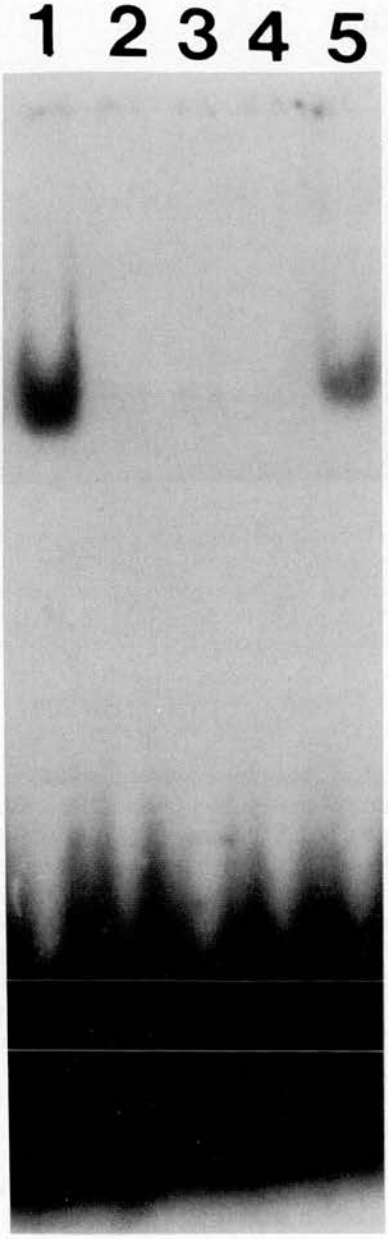

Fig. 6. Specificity of Partially Purified SRE-BP Binding to the ERE_{con} Oligonucleotide

Fractions 20 and 21 were concentrated using an Amicon concentrator. One microgram of pooled fractions 20 + 21 was incubated with 0.1 pmol ERE_{con} oligonucleotide in the presence of 4 μg poly[d(I-C)]. A 100-fold molar excess of competitor oligonucleotide was added as follows: lane 1, no competitor; lane 2, ERE_{con} lane 3, ERE_{PRL}; lane 4, GRE; lane 5, GRE_M. Free and bound labeled oligonucleotide were separated on a 4% polyacrylamide gel by electrophoresis.

ERE and GRE with similar affinity. In this respect, the SRE-BP may be analogous to the 90 kDa heat shock protein (HSP 90) which interacts with several different steroid receptors and which appears to play a global role in steroid hormone action. In the absence of steroid hormone, HSP 90 forms a complex with the unoccupied steroid receptor, maintaining the steroid receptor in a non-DNA binding form (45, 46). We suggest that the SRE-BP described in this paper may also play a global role in gene regulation by steroid hormone receptors in that its DNA binding activity is not confined to one class of SRE. The role of SRE-BP remains unclear, but it is likely to fulfill a common function in transcriptional regulation by many or all classes of steroid hormone receptors.

MATERIALS AND METHODS

Cell Culture Conditions

HeLa cells (Flow Laboratories, McLean, VA) were maintained in monolayer culture in 1 × RPMI 1640 medium (Flow Laboratories) supplemented with 10% fetal calf serum (Seralab, Crawley Down, UK), 2 mM L-Glutamine (Flow Laboratories), 100 IU ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (GIBCO, Grand Island, NY). Cells were grown in 75 cm^2 flasks in 5% CO_2 in air at 37 C. GH_3 cells (47) were maintained as above in supplemented Dulbecco's Modified Eagles Medium (Flow Laboratories).

Cell Extract Preparation

Whole cell extracts were made by harvesting 2 × 10^8 cells using 0.05% Trypsin, 0.02% EDTA (Flow Laboratories). The cells were washed twice in PBS, pelleted, and stored at −70 C. Cells were thawed slowly on ice and whole cell extracts prepared according to procedures outlined (48). Extracts were aliquoted and stored at −70 C for up to 3 months. Generally, the whole cell extracts contained 3–10 μg μl^{-1} protein.

Labeling of Oligonucleotide

Thirty picomoles of single stranded oligonucleotide (OSWEL DNA Service, University of Edinburgh) were labeled with 50 μCi [γ-^{32}P]ATP [SA, 3000 Ci/mmol (Amersham, Little Chalfont, UK)] in a total volume of 20 μl containing 100 mM Tris HCl (pH 8), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT) and 4.5 U T_4 polynucleotide kinase. ^{32}P-Labeled oligonucleotide was separated from unincorporated radionucleotide by passage through a sephadex G-50 column (49). For some experiments, ^{32}P-labeled oligonucleotide was further purified by excision from a 20% denaturing polyacrylamide (19:1) gel and extracted by freeze/thawing in 10 mM Tris HCl (pH8), 1 mM Na_2-EDTA. Oligonucleotides were made double-stranded by mixing equal amounts of complementary oligonucleotides and heating to 60 C for 10 min, then allowing the mixed solution to cool slowly to room temperature.

Gel Retardation Assay

Double-stranded, ^{32}P-labeled oligonucleotide (≈0.1 pmol) was added to binding buffer (100 mM NaCl, 10 mM Tris HCl (pH 8), 1 mM Na_2-EDTA, 12.5% glycerol) with 4 μg poly[d(I-C)] (except where stated), and with or without unlabeled competitor oligonucleotide. The binding reaction was carried out in a final volume of 20 μl and was initiated by addition of whole cell extract. The binding reaction was incubated at room temperature for 20 min; reactions were loaded immediately onto a 4% polyacrylamide (19:1) gel containing 6.7 mM Tris·HCl (pH 7.5), 3.3 mM Na-acetate, 1 mM Na_2-EDTA to separate DNA-protein complexes from free DNA. Electrophoresis was carried out at 30 mA for 30 min, with circulating reservoir buffer. The gel was vacuum dried and autoradiographed.

Fractions collected after gel filtration (see below) were as-

sayed by adding a maximum volume of each fraction to the poly[d(I-C)] and 0.1 pmol ^{32}P-labeled oligonucleotide in a final volume of 20 μl.

Gel Filtration

HeLa whole cell extract (≃7 mg), made according to Manley *et al.* (48), was finally resuspended in column buffer: 50 mM Tris HCl (pH 7.9), 6 mM $MgCl_2$, 100 mM NaCl, 0.2 mM Na_2-EDTA, 1 mM DTT, 10% glycerol. The molecular weight of the native SRE-BP was determined using a Sephacryl S300 column (150 cm × 3 cm) calibrated with the following molecular weight markers: flavocytochrome b_2 (230,000), β-amylase (200,000); alcohol dehydrogenase (150,000); BSA (66,000); carbonic anhydrase (29,000); myoglobin (17,500), and cytochrome *c* (12,500). A total of 47 3.5-ml fractions were collected at 4 C and assayed for binding activity as outlined above. Fractions containing SRE-BP activity were concentrated using an amicon filter. Approximately 1 ml (1 μg μl^{-1}) of concentrated protein was recovered.

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Address requests for reprints to: Dr. Karen Chapman, Royal Edinburgh Hospital, MRC Brain Metabolism Unit, Morningside Park, Edinburgh, Scotland EH10 5HF.

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The changes in ODC and SAM-DC activities seen here, when sheep adipose tissue slices were cultured for 48 h with various hormones, suggest that putative polyamine concentrations will fall when the tissue is cultured with insulin or dexamethasone, but spermidine and spermine concentrations will rise when the tissue is cultured in the presence of growth hormone.

These putative changes in polyamine concentrations do not parallel changes in the rate of lipolysis during tissue culture as this falls in the presence of dexamethasone and is unaffected in the presence of insulin; growth hormone alone has no effect, but prevents the fall seen in the presence of dexamethasone [9]. In addition, there is no relationship between the putative changes in polyamine concentrations and rates of basal or catecholamine-stimulated lipolysis measured after culture for 48 h with the various hormones (R. G. Vernon & E. Finley, unpublished work). Thus it seems unlikely that changes in polyamine concentration modulate the rate of lipolysis in sheep adipose tissue or mediate the chronic effects of insulin, growth hormone or glucocorticoids on lipolysis.

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A high molecular mass steroid response element binding protein forms a 213 kDa complex with an oestrogen response element

LOUISE CRAWFORD and KAREN CHAPMAN

M.R.C. Brain Metabolism Unit, Royal Edinburgh Hospital, Morningside Park, Edinburgh EH10 5HF, Scotland, U.K.

We have previously reported the identification of a DNA-binding protein present in whole-cell extracts of HeLa cells which specifically binds to two classes of functionally distinct steroid response elements. We have named this protein the steroid response element-binding protein (SRE-BP) [1]. In gel-retardation experiments, the SRE-BP bound specifically to oligonucleotides containing an oestrogen response element (ERE) or a glucocorticoid response element (GRE), but only poorly to a mutant GRE and not at all to transcription factor-binding sites present in the herpes simplex virus thymidine kinase promoter (Sp-1, CTF, TATA). Using gel-filtration chromatography, we have shown that the SRE-BP has a molecular mass under non-denaturing conditions of 205 kDa (±20 kDa) as it exists in solution. The SRE-BP is neither an oestrogen receptor nor a glucocorticoid receptor as evidenced by binding sequence specificity, cell type distribution and apparent molecular mass. We have proposed that by modulating the interaction of steroid receptors with target SREs, the SRE-BP is involved in steroid hormone regulation of gene expression.

We have now used pore-gradient gel electrophoresis [2, 3] to demonstrate that the form of SRE-BP that binds to an ERE has a molecular mass of 213 kDa ± 27 kDa.

HeLa whole-cell extract (WCE) was made as previously described [4]. SRE-BP was partially purified by gel-filtration chromatography [1]. WCE (10 μg) or 1 μg of partially purified protein were incubated with 0.1 pmol of double-stranded ^{32}P-labelled ERE oligonucleotide (5′-GCAGGTCACAGTGACCTGGAC-3′) in 20 μl of binding buffer containing: 100 mM-NaCl, 10 μM-Tris/HCl, pH 8.0, 1 mM-EDTA (sodium salt), 12.5% (v/v) glycerol, 4 μg of poly[d(I-C)] for 20 min at room temperature. Samples were loaded on to a 3–25% (w/v) gradient polyacrylamide gel (79/

Abbreviations used: SRE-BP, steroid response element-binding protein; ERE, oestrogen response element; WCE, whole-cell extract.

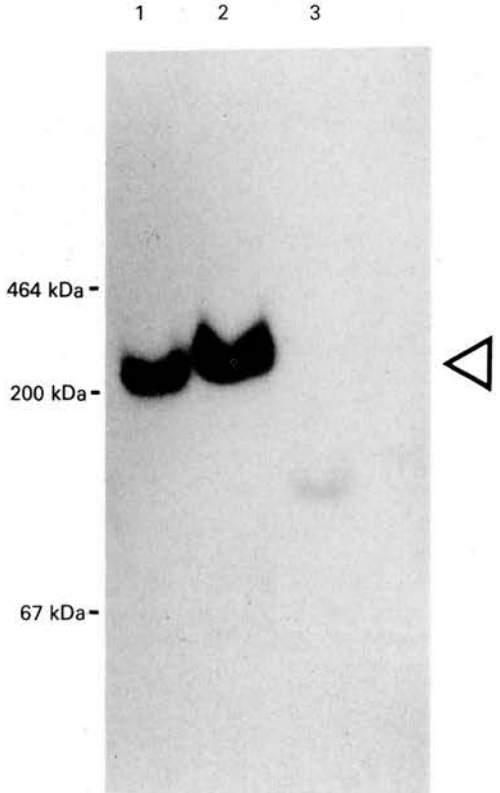

Fig. 1. *Pore-gradient gel electrophoresis of ERE/SRE-BP complexes*

The arrowhead indicates the ERE/SRE-BP complex formed in the presence of 1 μg of partially purified SRE-BP or 10 μg HeLa WCE: lane 1 and lane 2, respectively. Lane 3 shows non-specific complex formation with double-stranded ERE using HeLa WCE previously left at 4°C for longer than 6 weeks. Molecular mass markers were electrophoresed in parallel lanes: bovine serum albumin (67 kDa), myosin (200 kDa) and β-galactosidase (464 kDa).

0.8/0.2, acrylamide:bis:diallyltartardiamide, 0.5×TBE [1×TBE = 0.089 M-Tris base, 0.089 M-boric acid, 0.002 M-EDTA]) which had been pre-electrophoresed overnight at 75 V in 0.5×TBE buffer. Protein standard markers were loaded in parallel lanes and the gel was electrophoresed for 1500 V h. The gel was stained with Coomassie Brilliant Blue R to visualize the protein standards, dried and exposed to autoradiographic film overnight.

Fig. 1 shows that the ERE/SRE-BP complex migrates to a position where proteins of 213 kDa are prevented from further progression through the gradient gel. Lanes 1 and 2 demonstrate ERE/SRE-BP complex formation using partially purified SRE-BP and HeLa WCE, respectively. The third lane shows complex formation using HeLa WCE after storage at 4°C for longer than 6 weeks. We believe the complex formed in lane 3 is a proteolysed form of the SRE-BP which retains the DNA-binding activity of the SRE-BP, but loses the sequence specificity characteristic of the SRE-BP [1]. Taking into account the molecular mass of the double-stranded ERE oligonucleotide used for complex formation (13.6 kDa), we have determined that the sequence-specific SRE-BP binds to the ERE oligonucleotide as a 200 kDa ± 27 kDa protein complex.

These results indicate that the form of SRE-BP which is bound to a steroid response element has a molecular mass of 200 kDa ± 27 kDa. This is very similar to the molecular mass that we have determined by gel-filtration chromatography and we therefore conclude that the stable form in solution is the active DNA-binding species.

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On memory, morphogenesis and the hormonal control of transcription

YORAM SCHIFFMANN
Department of Applied Mathematics and Theoretical Physics, University of Cambridge, Silver Street, Cambridge CB3 9EW, U.K.

We refer to the 'substrate inhibition' model in [1, 2] and let a = [cyclic AMP] and h = [ATP] or a = cyclic AMP and h = ATP. A simple non-trivial model for morphogenesis involves the following non-linear reaction-diffusion system:

$$\dot{a} = V_m^{cyc} \cdot K_i^{cyc} \left[\frac{1}{h} \left(\frac{1}{C_0} + \frac{a}{C_0^2} + \frac{a^2}{C_0^3} + \frac{a^3}{C_0^4} + \dots \right) \right]^{1/2} - \frac{V_m^{PD} \cdot a}{K_m^{PD} + a} - k \cdot h \cdot a + D_a \Delta a \qquad 1(a)$$

$$\dot{h} = \frac{z(a)}{r(h)} - w(h) + D_h \Delta h \qquad 1(b)$$

where V_m^{cyc}, K_i^{cyc} represent the usual quantities for the M.E. [1] adenylate cyclase, and V_m^{PD}, K_m^{PD} for the cyclic AMP phosphodiesterase. $z(a)$, $r(h)$, $w(h)$ are increasing functions of their argument. Eqns 1(a) and 1(b) can be written more compactly and generally as:

$$\dot{a} = f(a, h) + D_a \Delta a, \qquad \dot{h} = g(a, h) + D_h \Delta h \qquad (2)$$

Upon hormone activation, K_i^{cyc} becomes large enough, the autocatalysis for a in eqn 1(a) becomes strong enough, and the production (positive) term in eqn 1(a) overcomes the loss (negative) terms in eqn 1(a), and we then have $f_a > 0$ (f_a is the partial derivative of f with respect to a). The mathematical expression in eqn 1(a) of the 'substrate inhibition' autocatalysis corresponds to the increase in the rate of the reaction $h \rightarrow a$ as the reaction progresses, i.e. as we move from right to left on a rate curve in Fig. 2 in [1]. The assumption that $a \ll h$ used in [1] and [2] and here is valid *in vivo* (see [3]). We also see from eqn 1(a) that h reduces the rate of production of a, both because of the substrate inhibition [the first term in eqn 1(a)] and also because readily oxidizable substrates such as glucose (which we assume will yield h) stimulate the release of intracellular a into the external milieu [4]. Indeed, Sutherland and co-workers suggest that a is actively pumped out of the cell (see reference 2 in [5]). h should be a major requirement for such active pumping. This active pumping is represented by $-k \cdot h \cdot a$ in eqn 1(a). Thus $f_h < 0$. $f_h < 0$ is important for long-range inhibition in order to keep the autocatalysis local, and the fact that there is an excess of h in relation to a and that the species ATP^{4-}, $ATPMg^{2-}$,

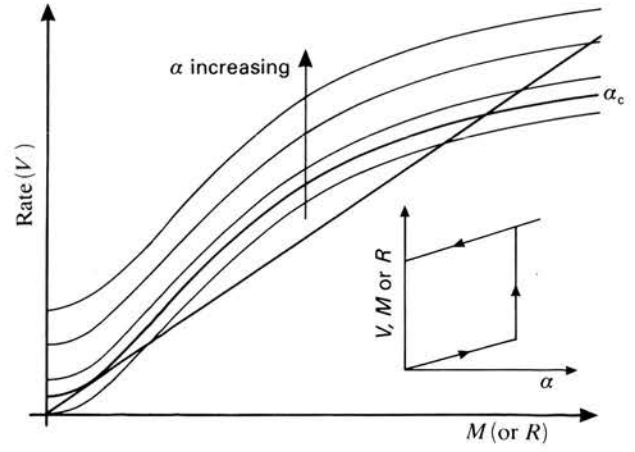

Fig. 1. *Establishment and persistence of lysogeny, segment-polarity and homoeotic selector gene transcription*

A non-linear curve represents the rate of transcription (M production) or the rate of translation (R production), i.e. it represents the sum of positive terms in eqn (5). The straight line represents the linear term in eqn (5) and the rate of loss of M (or R). An intersection of a non-linear curve with the linear curve represents a steady state. For the lower non-linear curve an unstable steady state separates two stable steady states. For increasing a the non-linear curves are displaced upwards. Beyond a critical a there is only the 'high' steady state and an abrupt transition occurs from the low to the high steady state. But if a is now decreased, the system remains in the high steady state. The irreversible 'hysteresis' is depicted in the inset.

Abbreviations used: PLC, phospholipase C; PK, protein kinase; PLA_2, phospholipase A_2; AC, adenylate cyclase.