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A Nucleosome Determines the DNA Binding Specificity of the Glucocorticoid Receptor for the Mouse Mammary Tumor Virus Long Terminal Repeat

by

Geoffrey Parsons

**DISSERTATION**

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Acknowledgments

A lot can happen in six years. The pursuit of a Ph.D. can be a very solitary experience, so much so that all aspects of your life can become intimately tied to it. Graduate school seemed a nearly impossible thing when, during my third year, my life seemingly fell apart. There are many people I would like to thank for getting me through that time as well as later on, when other lesser problems would often pile up into large ones. Firstly, I would like to thank Bonnie Maler, maven of quality foot wear, who always seemed to be able to sense how I was feeling and made the lab a comforting place to be during all of my years there. Tanya and Aaron really made it possible for me to get through the dreaded third year. I'd like to thank Ken & Heather and Kurt & Pratima for the gifts of their sofas. Lastly, I'd like to thank Nicole for teaching me so much about fun over the last three years and who I'm sure will continue to do so in the future.

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**A Nucleosome Determines the DNA Binding Specificity of the Glucocorticoid Receptor For the MMTV LTR**

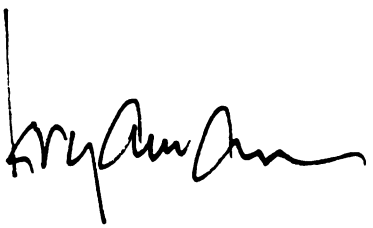
**Geoffrey Parsons**

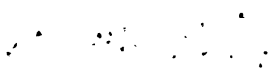
Thesis Abstract

DNA exists within the cell packaged into nucleosomes. The compaction of the DNA into a nucleosomal structure restricts the access of transcription factors and the general transcription machinery to the DNA. The mechanisms by which the repressive effects of nucleosomes are not known. Current models argue that nucleosomes are altered by an active process study during gene activation. An alternative explanation is that nucleosomes compete for binding with transcription factors and are altered as a passive consequence of factor binding.

This study seeks to understand how a transcriptional regulatory factor, the glucocorticoid receptor (GR), gains access to its binding site within a nucleosome. The mouse mammary tumor virus (MMTV) long terminal repeat (LTR) contains a promoter that is induced by GR and contains four glucocorticoid response elements (GREs) in the promoter proximal region. These GREs are comprised in a single nucleosome, termed nucleosome B. Purified core histones and a 166 bp DNA fragment from the nucleosome B region were used to reconstitute nucleosome B *in vitro*. The ability of the DNA binding domain of GR to occupy the nucleosomal GREs was examined by DNase I footprinting and by a gel mobility shift assay. The DNA binding domain of GR had a similar affinity for all four GREs as naked DNA, differing

by no more that 5 fold. In contrast, GR only occupied a single GRE, termed GRE 1, within the nucleosome. Furthermore, the DNA binding domain of GR was able to co-occupy the DNA with the nucleosome. This difference in DNA binding is attributed to the rotational position of the individual GREs on the surface of the histone octamer. These results are consistent with nucleosomes having a repressive role in transcription by competing for binding with transcription factors.





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# Introduction

The eukaryotic cell nucleus should be a mess. It faces the daunting task of accommodating an entire organism's genome within a space as small as 5 microns across. The human genome, for example, contains tens of thousands of genes and is billions of base pairs long. The amount of DNA within one human cell, if fully extended, would be over 5 feet in length. Compounding this storage problem, the nucleus must also manage the orderly replication of DNA and ensure its proper segregation during cell division. This would be a hopeless undertaking without some means of organizing the genome within the nucleus.

The solution to the genome packaging problem can be seen during mitosis. Visible, highly condensed chromosomes are composed of DNA that is complexed with specific proteins and folded into a structure known as chromatin. DNA can be folded to differing extents; the mitotic chromosome is the most condensed and represents a nearly 10,000 fold degree of compaction. Chromatin is a dynamic structure that goes through many changes during the cell cycle. Following mitosis, the cell must unfold the tightly coiled chromosomes in a process that occurs to differing extents throughout the genome, often in a cell-type specific manner. Some chromosomal regions, known as heterochromatin, never decondense and remain tightly compacted throughout the cell cycle.

When the decision is made to divide, the cell must carry out a single round of DNA replication. Chromatin helps to facilitate the orderly duplication of the DNA by regulating the timing of when different regions of the genome initiate replication. Upon completion of DNA replication the

chromosomes must again be condensed so that the copied DNA can be properly segregated. Clearly, chromatin does more than enable the genome to fit into the nucleus, it also ensures that a variety of nuclear processes occur in a congruous fashion.

The smallest unit of chromatin structure is the nucleosome core, which is composed of 145 base pairs of DNA and two copies of each of the 4 core histone proteins. The DNA is tightly wrapped 1.8 times around the histone octamer to form the nucleosome core. Nucleosomes are repeating units, spaced about 200 base pairs apart throughout the genome and thus represent an extremely abundant nuclear structure. Despite over 20 years of research since its discovery, the nucleosome is still the only universally accepted chromatin structure.

Nucleosomes arrayed along the DNA are further wrapped upon each other to form a more condensed fiber, the 30 nm filament, which is about 40 fold more compacted than naked DNA. This folding is thought to be mediated by the linker histone H1 which binds the linker DNA between nucleosome cores. The organization of the nucleosomes in the 30 nm fiber is still controversial: a common view is that they are wound into a solenoid structure (Finch and Klug, 1976) while more recent evidence shows them to be arranged in an irregular zigzag manner (Horowitz et al., 1994). In a still poorly characterized structure, the 30 nm filaments are condensed a further ten-fold in interphase chromosomes. A frequently sited model is that 30 nm fibers are folded into large loops of varying size, ranging from 50 kb to 2 Mb (Kitsberg et al., 1991), and are attached to a poorly characterized chromosome axis or possibly to a "nuclear scaffold" (Laemmli et al., 1992). The highest degree of compaction occurs during mitosis when the DNA is condensed

another 25-fold into metaphase chromosomes. Very little is known about the role of nonhistone components of chromosomes or the mechanisms by which the chromosomes are condensed during mitosis.

The highly compact state of DNA *in vivo* presents an obvious obstacle to any process that requires access to the DNA. For example, the strands of DNA in the coding region of a gene must be separated to allow transcription by RNA polymerase, a process that seems incompatible with a compact chromatin structure. It is because of this apparent conflict that researchers have long assumed that chromatin has a repressive effect on gene expression.

A paradox of modern biology is that vastly different cell types contain the same complement of genes. How do cells establish differing patterns of gene expression? A well supported model is that specific cell types contain different sets of transcriptional regulators and as a result of the integration of their influences, a unique pattern of gene expression is established. Another model to explain cell-type specific gene expression is that just as different cell types have unique morphologies, they also have unique chromatin structures. By tightly packing different regions of the genome, the set of genes available for expression becomes restricted. While these two models are by no means mutually exclusive, the amount of molecular evidence for the influence of chromatin has been slow in coming.

The many functions of chromatin remain poorly understood partly because it is still so ill-defined. Experimental approaches geared towards understanding the three-dimensional conformation of large chromatin complexes have traditionally relied on solubilized chromatin isolated from nuclei. None of the conventional methods of chromatin preparation reliably preserve the native state. For example, chromatin conformations are highly

sensitive to changes in ionic conditions and can also be altered by fixation techniques used in imaging studies. *In situ* approaches are limited in their level of detail and often expose the chromatin to extreme changes in temperature, salt, pH and hydration. New methodologies for the study of chromatin *in situ* in the fully hydrated and unfixed state will hopefully overcome previous experimental limitations (Swedlow et al., 1993). Still, the nucleosome remains the best understood chromatin structure and, consequently, is the object of most studies on the effects of chromatin on transcription.

**The Control of Gene Expression**

RNA polymerase II is responsible for the transcription of protein encoding genes but itself is incapable of binding a promoter and initiating transcription. Instead, the polymerase binds the promoter in concert with a set of general transcription factors (GTFs), forming a pre-initiation complex. It is in this context that the polymerase initiates transcription from the proper chromosomal locations. Thus, pre-initiation complex assembly represents a key step in determining gene expression.

Establishment of a pre-initiation complex is an inherently inefficient process and requires the action of one or more activator proteins. A large number of these regulatory factors are site specific DNA binding proteins that bind in various locations around a gene promoter and are thought to function by a number of different mechanisms. A simple model is that activators, through protein-protein contacts, can recruit GTFs to the promoter, thereby stimulating transcription (Stringer et al., 1990; Lin and Green, 1991). Pre-initiation complexes have been proposed to assemble at the promoter in a step-wise fashion; however, the number of stages of assembly is

controversial. RNA polymerase II has been isolated from mammalian cells in association with all of the GTFs, a complex denoted holoenzyme (Ossipow et al., 1995). By this view, pre-initiation complex assembly would be achieved in a single protein-DNA binding step. Holoenzyme isolated from yeast is lacking the protein complex known as TFIID, which contains the TATA binding protein TBP (Thompson et al., 1993). In this scenario, pre-initiation complex assembly would occur in two steps, binding of TFIID to the TATA box, followed by association of the holoenzyme. Alternatively, experiments performed *in vitro* with individual purified GTFs show that pre-initiation complexes can assemble in a specific, ordered, step-wise manner (Buratowski et al., 1989). By either model, transcriptional activators can function by interaction with the GTFs, stimulating a limiting step in the assembly of a multiprotein complex or recruitment of the entire complex in a single binding event (Lin and Green, 1991; Barberis et al., 1995).

A theme central to all models on the repression of transcription by chromatin is that nucleosomes inhibit the association of GTFs with the promoter. This is supported by the observation that nucleosomes inhibit activatorless, also known as basal, transcription *in vitro* (Lorch et al., 1992; Workman et al., 1991) and that a TATA box located on a nucleosome cannot be bound by TBP (Imbalzano et al., 1994). Therefore, it is likely that nucleosomes inhibit transcription by blocking pre-initiation complex assembly. It is now a commonly held view that nucleosomes are removed by an active process during the initiation of transcription and that activators must perform two functions; they must disrupt nucleosomes and then recruit GTFs to the promoter (Grunstein, 1992). An alternative model that explains gene regulation in a nucleosomal context is that the interactions of the GTFs with transcriptional activators are sufficient to overcome the repressive

effects of nucleosomes. That is, nucleosomes and GTFs compete for binding to the promoter. This model allows for inhibition of transcription by nucleosomes but argues that activators function by the same mechanism regardless of whether nucleosomes are present. Activators can also stimulate transcription by acting on events subsequent to pre-initiation complex assembly but such models will not be considered here since nucleosomes have not been demonstrated to inhibit these steps.

I will argue that the popular notion that activators function by two separate mechanisms is far from proven and that the simplest model to explain the regulatory effects of nucleosomes is that activators allow the general transcription factors to successfully compete with nucleosomes for binding to promoter.

**The Glucocorticoid Receptor is a Transcriptional Regulator**

The glucocorticoid receptor (GR), a member of the intracellular receptor superfamily, is one of the earliest described cellular regulatory factors for RNA polymerase II. In the absence of hormone, the apo-GR resides in the cytoplasm of nearly all cell types. Upon binding of its cognate hormone, GR translocates to the nucleus where it binds to specific genomic sites, termed glucocorticoid response elements (GREs), and modulates initiation from nearby promoters. GR, like many transcriptional regulators, can function either as an activator or repressor (Diamond et al., 1990). In nearly all glucocorticoid regulated genes examined to date, GR functions in concert with other families of regulatory factors to modulate gene expression. GR has also been shown to disrupt nucleosomes positioned at gene promoters (Richard-Foy and Hager, 1987).

Many of the functional properties of GR can be mapped to discrete domains of the protein. For example, the C-terminal third of the receptor is responsible for hormone binding and is also the site of interaction with a multiprotein molecular chaperone complex that serves to maintain GR in a high-affinity ligand binding state (Howard et al., 1990). Included in the N-terminal half of GR is a potent transcriptional activation domain as well as sites of phosphorylation (Godowski et al., 1988). The DNA binding domain is situated in the middle of the protein and is comprised in a 70 amino acid zinc binding motif. This domain also mediates receptor dimerization, nuclear localization, and is capable of interacting with various nonreceptor regulatory factors (Diamond et al., 1990). It is not known what domains of the receptor, if any, are important for interactions with chromatin.

The molecular mechanisms by which intracellular receptors regulate the initiation of transcription remain unclear. Efforts to identify a discrete region of GR capable of directing transcriptional repression have failed; to date, the intact protein is required. In contrast to repression functions, several domains of GR have been found to be capable of stimulating transcription. Fusion of an isolated activation domain from the N-terminus, termed ENH2, to the DNA binding domain results in a potent transcriptional activator both *in vivo* and *in vitro* (Godowski et al., 1988; Freedman et al., 1989). In cell-free transcription reactions on naked DNA ENH2 is capable of stimulating the rate of pre-initiation complex assembly, presumably through interactions with the general transcription machinery (Freedman et al., 1989). These results indicate that GR is capable of enhancing transcription in the absence of nucleosomes and may represent the sole mechanism by which GR activates transcription. It is also possible that, *in vivo*, GR stimulates transcription by additional mechanisms, such as perturbing nucleosome structure.

**Nucleosome Positioning**

Consistent with the idea that the cell uses nucleosomes as a means of regulating gene expression, positioned nucleosomes have been demonstrated to exist over the regulatory regions of a number of genes (Carr and Richard-Foy, 1990; Richard-Foy and Hager, 1987; Almer and Horz, 1986). The positioning of these nucleosomes is inferred from experiments showing the DNA to be protected, in nucleosome sized blocks, from cleavage by nucleases. Activation of transcription is accompanied by increased accessibility of nucleases to the underlying DNA. This is interpreted to mean that the previously positioned nucleosomes have been removed from the DNA or have been severely altered in structure such that nucleases now have access to the underlying DNA.

Since the frequency of positioned nucleosomes is not known it is difficult to assess their significance. Identification of positioned nucleosomes is determined solely by protection of the DNA from cleavage by nucleases. Lack of a nucleosome sized protection is nearly impossible to interpret as there are multiple factors that can affect a nuclease cleavage pattern: nonhistone proteins can alter nuclease accessibility, there may exist an overlapping set of nucleosome positions or the topology of the DNA may be altered. Furthermore, it is important to appreciate that a nucleosome can have two different types of positioning: the translational position, which indicates what stretch of base pairs actually contact the histone core, and the rotational position, which describes what face of the DNA helix is oriented towards the histone octamer. While nucleosome positioning usually refers to translational position, both types of positioning have the potential for

regulatory functions. Therefore it is important to ask, are there mechanisms that the cell uses to place nucleosomes in specific locations?

There are three basic theories for how nucleosomes can be specifically positioned on the DNA. The first, and simplest, explanation for nucleosome positioning is that the histone octamer inherently prefers some DNA sequences over others. While histones are primarily nonspecific DNA binding proteins, some DNA sequences display elevated affinities for histone octamers. This higher binding affinity is thought to be due to the intrinsic flexibility of a particular DNA sequence, favoring its bending around the histone octamer. The majority of identified sequences are derived from the small 5S RNA genes of a number of species (Buttinelli et al., 1993; Simpson and Stafford, 1983). Since no specific region of the 5S DNA has been found to be important for positioning, it is assumed that it is the sum of histone-DNA contacts that are important (FitzGerald and Simpson, 1985). High affinity for histone octamers has also been observed with artificial positioning sequences that are specifically designed for optimal bending into a nucleosome (Shrader and Crothers, 1989). This DNA is incorporated into nucleosomes 100 times more strongly than bulk nucleosomal DNA. However, these *in vitro* measurements were made using reconstitution techniques at elevated ionic strength and may not be relevant at physiologic salt concentrations. This is born out by the observation that these bending sequences fail to translationally position nucleosomes *in vivo* (Tanaka et al., 1992). However, like the 5S DNA sequences, the DNA of artificial phasing sequences is rotationally phased around the histone octamer.

Second, exclusion of histones from specific regions of the DNA, so called "boundaries", can also result in positioned nucleosomes by restricting possible random positions (Kornberg and Stryer, 1988). In principle,

nucleosomes would be precisely positioned close to the boundary, becoming less precise with increasing distance. Furthermore, two separate boundaries would be capable of setting the nucleosomal phasing of the intervening DNA. Boundaries could also be DNA sequences that bind histones poorly, such as poly(dA)•poly(dT) tracts, or site specific DNA binding proteins that compete with nucleosomes for the DNA. Transcription factors often create nucleosome free regions in chromatin (Gross and Garrard, 1988), observed as DNase I hypersensitive sites, and may also act as boundaries. The idea of a boundary has been invoked to explain nucleosome positioning in a variety of contexts: over the region of unknown function in the TRP1/ARS1 plasmid of yeast (Thoma, 1986), in the regulatory regions of a number of genes, such as the tyrosine aminotransferase gene (Carr and Richard-Foy, 1990), or over the actual coding region of a gene, such as the yeast URA3 gene (Tanaka et al., 1996). However, rigorous tests of the boundary idea have failed in that they do not seem to be moveable elements and rarely function when isolated from their normal chromosomal context. For example, DNA inserted on one side of a boundary will often influence nucleosomal positioning on the other side (Thoma, 1986) and manipulation of transcription factor binding sites also fails to reliably reproduce positioned nucleosomes. Therefore, some additional interactions or activities must also be important.

Lastly, phasing studies done on yeast minichromosomes that cannot account for positioning by histone-DNA interactions or the influence of boundaries invoke higher-order chromatin folding as being the determining feature (Thoma and Zatchej, 1988). Histones are highly conserved across species yet DNA sequences that yield positioned nucleosomes in one species often fail to position in another (Bernardi et al., 1992). Thus, nucleosomal positioning is a complex phenomenon which probably involves more than

just favorable or unfavorable histone-DNA contacts. In contrast to translational positioning, rotational positioning is better understood and can be explained by the flexibility of the DNA. However, this has mostly been looked at *in vitro* since rotational phasing is difficult to study *in vivo*.

Clearly, the rules governing nucleosome positioning remain to be defined and will probably not be fully understood until higher-order chromatin structures are better described. It is important to keep in mind that most studies of eukaryotic gene expression employ rearranged test promoters, almost certainly altering nucleosome positioning, yet still manage to recapitulate the expression patterns of the authentic gene. It would seem that the nucleosomal structure of a gene promoter can be severely altered with few regulatory consequences (Archer et al., 1992). This argues that if activators do, in fact, function through the disruption of nucleosomes, it plays a very minor role at most genes examined to date. How relevant, then, are these nucleosomes to the regulation of gene expression? In the following sections, I will present evidence consistent with the idea that nucleosomes function by blocking binding of the general transcription factors to the promoter and that they are removed as a consequence of pre-initiation complex assembly.

**Genetic Analysis of the Yeast Core Histones**

The four core histones have distinct functions within the nucleosome. Histones H3 and H4 associate with DNA as a tetramer while histones H2A and H2B do not associate with DNA on their own. The individual histones vary in their rates of evolutionary divergence, with histone H4 being the most conserved. Genetic, biochemical and structural studies on individual histones have identified important functional domains . All four core

histones have a predominantly α-helical, hydrophobic C-terminus that provides the structural basis for octamer assembly (Arents et al., 1991). Each histone also has an unstructured, hydrophilic N-terminus that extends from the nucleosome core. These N-terminal "tails" can be proteolytically removed from nucleosomes *in vitro* with little change in stability (Hayes et al., 1990); N-termini of H2A and H2B are much less conserved than those of H3 and H4. The N-termini are the sites for extensive post-translational modifications, including acetylation, phosphorylation and methylation (van Holde, 1989). Modifications of the N-termini are associated with different cellular events; for example there is a correlation between the acetylation of histone H4 and transcriptionally active chromatin (Turner and O'Neill, 1995).

Yeast strains have been constructed in which synthesis of specific histones is regulatable , causing nucleosome loss *in vivo* under non-induced conditions. Nucleosomes loss causes G2 cell cycle arrest (Han et al., 1987), chromosome loss during mitosis and results in defects in chromatin structure and nuclear segregation (Kim et al., 1988). Nucleosome loss also has varying effects on the transcription of a number of genes. The HIS3 and CUP1 promoters are activated to fully induced levels while PHO5, GAL1 and CYC1 are induced to moderate levels of activity (Durrin et al., 1992). While these studies indicate an important role for nucleosomes in gene regulation such drastic alterations of essential genes are difficult to interpret as specific effects.

Mutational analysis of subdomains of the histones has provided a wealth of functional information. Deletion of both N-terminal tails of histones H3 and H4 is lethal event, but individual deletions remain viable. Removal of either N-terminus causes the loss of gene silencing mediated by heterochromatin-like structures at the yeast silent mating loci and telomeres (Thompson et al., 1994). Specific mutation of the four acetylated lysines at the

H4 N-terminus reduces GAL1 activation 20-fold and alters nucleosome positioning at the promoter (Durrin et al., 1991; Fisher-Adams and Grunstein, 1995). PHO5 induction is also impaired in these strains, although to a lesser extent. Surprisingly, mutation of the corresponding lysines in the tail of H3 results in the opposite phenotype; GAL1 induction is stimulated 3-4 fold (Mann and Grunstein, 1992). Furthermore, the hyperactivation seen in the H3 mutants is dependent on the binding site for the transcriptional activator GAL4 while the impaired activation in H4 mutants is dependent on the GAL1 TATA box and initiation site (Wan et al., 1995). It would appear that the N-termini of H3 and H4 give nucleosomes functional complexity; the effects of these mutations are restricted to specific promoter elements.

A region of the N-terminal tails between the acetylated lysines and the α-helical core region is required for repressing basal transcription (Lenfant et al., 1996). Mutation of this region increases the uninduced expression of a GAL1-URA3 reporter construct, whose activity is measured as cellular viability in the presence of the drug 5-fluoroorotic acid (5-FOA). Removal of just four amino acids in any of the four histones increases sensitivity to 5-FOA by nearly six orders of magnitude. These deletions also decrease plasmid superhelical density, implying a smaller amount of DNA folded around nucleosome core particles *in vivo*. A similar decrease in superhelical density is also observed *in vitro* on nucleosome cores that have been stripped of the histone N-terminal tails by trypsinization (Garcia-Ramirez et al., 1992). This restriction of nucleosome size is proposed to cause a more open chromatin structure. Since these mutations cause an increase in basal transcription, they may allow greater access of the general transcription machinery to gene promoters.

In summary, mutation of the core histones results in a range of effects on transcription, some positive some negative. Whether these mutations directly affect the mechanism of transcription initiation remains to determined; a biochemical analysis of DNA templates containing nucleosomes composed of mutant histones will allow more specific models to be generated. While it is certainly possible that these histone mutations define sites of interaction for destabilization by transcriptional activators, a simpler explanation is that they alter nucleosome stability, competing better or worse for the binding of the general transcription machinery.

**A Nucleosome Destabilizing Machine?**

Aside from the histone genes, genetic analysis has also identified novel factors thought to be important for gene induction in a chromatin context. The SWI and SNF gene products were identified in genetic screens in yeast as positive regulators of the HO and SUC2 genes (Stern et al., 1984; Carlson et al., 1981). Upon further study, SWI1, SWI2 and SWI3 were shown to be required for the induction of multiple genes (Peterson and Herskowitz, 1992). SNF2, SNF5 and SNF6 were also shown to be required for expression of the same set of genes, with the SWI2 and SNF2 gene products being identical (Laurent et al., 1991). Homology searches have failed to discern any DNA binding domains in the SWI/SNF proteins but have identified SWI2/SNF2 as a member of a conserved family of proteins with similarity to helicases and DNA-dependent ATPases (Laurent et al., 1993). Further genetic analysis has also revealed a link between the SWI/SNF proteins and chromatin. Transcriptional defects in SWI/SNF mutant yeast strains can be relieved by deletion of HTA1-HTB1, one of the gene pairs encoding histones H2A and H2B (Hirschhorn et al., 1992). Point mutations in the genes encoding

histones H3 and H4 can also partially relieve the requirement for SWI/SNF (Kruger et al., 1995) Finally, mutations in SWI/SNF are suppressed by mutations in the SIN1 gene, encoding a protein similar to the nonhistone chromatin component HMG1 (Kruger and Herskowitz, 1991), and by mutations in SPT6, encoding a protein that interacts with histone H3 and is important for nucleosome assembly (Bortvin and Winston, 1996).

The SWI/SNF proteins can be purified from yeast cells as part of a multiprotein complex that includes all five SWI/SNF gene products along with five additional subunits (Peterson et al., 1994). The purified SWI/SNF complex can disrupt nucleosome structure *in vitro* in an ATP dependent manner (Cote et al., 1994). This disruption is inferred from a SWI/SNF induced change in the DNase I cleavage pattern of the nucleosomal DNA and from the fact that SWI/SNF is capable of facilitating the binding of GAL4 to a nucleosome (Cote et al., 1994; Kwon et al., 1994). Consistent with its proposed role as a global regulator of transcription, SWI/SNF homologues have been found in *Drosophila*, mice and humans (Tamkun et al., 1992; Khavari et al., 1993). Furthermore, the SWI/SNF proteins are also part of a multiprotein complex in human cells and it too has the ability to destabilize nucleosomes *in vitro* (Wang et al., 1996).

A biochemical study has identified a *Drosophila* homologue of SWI2, termed ISWI (Elfring et al., 1994), as a component of a nucleosome remodeling activity purified from *Drosophila* embryos (Tsukiyama et al., 1995). This complex, known as NURF, is different from the SWI/SNF complex in that it contains fewer subunits and does not contain the fly SWI2 homologue brahma. Furthermore, the ATPase activity of NURF is stimulated by nucleosomes while the ATPase activity of SWI/SNF is stimulated by naked DNA (Tsukiyama and Wu, 1995). The picture has grown

more complicated with the discovery that yeast and human cells contain multiple homologues of SWI2, existing in different complexes within the cell. It appears then that cells contain multiple complexes capable of destabilizing nucleosomes and that different cell types in higher eukaryotes may contain different sets of complexes (Wang et al., 1996).

The mechanisms that govern gene regulation are highly conserved between yeast and humans. Transcriptional regulators from a variety of species will function when introduced into yeast. The glucocorticoid receptor (GR), the *Drosophila* regulator *ftz*, and various LexA fusion proteins all function in yeast and have been shown, in some promoter contexts, to be SWI/SNF-dependent (Yoshinaga et al., 1992; Peterson and Herskowitz, 1992). By current models, these factors use SWI/SNF to overcome repressive chromatin.

Has the identification of the SWI/SNF complex finally provided proof for the elusive chromatin dependent step of transcriptional activation? Recall that this model stipulates that one of the ways that activators function is through the disruption of chromatin structure (Grunstein, 1992). An alternative view of SWI/SNF is that these proteins are part of a family of transcription factors, termed coactivators, that interact with both the GTFs and the activation domains of activators, mediating recruitment of the GTFs by the activator. Coactivators are broadly defined as being required for activated transcription, serving as a "bridge" between a DNA bound transcriptional activator and the general transcription machinery. Although this has yet to be directly demonstrated, coactivators can be viewed as being required for stable assembly of a pre-initiation complex by an activator. The non-TBP components of TFIID, termed TAFs, are the prototypical members of

this class (Gill and Tjian, 1992). The idea that the SWI/SNF proteins are coactivators is supported by the fact that they are required for the activation of a broad spectrum of genes and that the coactivator TAF30 is a component of the SWI/SNF complex (Cairns et al., 1996). Furthermore, SWI/SNF has been demonstrated, in a number of contexts, to be important for activation in the absence of an observable nucleosomal structure. Antibodies to the SWI3 component of the SWI/SNF complex will inhibit the activation mediated by GR *in vitro* (Yoshinaga et al., 1992). While the crude transcription extracts used in these experiments contain histone H1 and other non-histone chromatin components, it is unlikely that the naked DNA templates added to these extracts were packaged into any normal nucleosomal structure.

Consistent with results from yeast, GR mediated activation is impaired in human cell lines lacking the human SWI2/SNF2 homologue (Muchardt and Yaniv, 1993). Introduction of the gene encoding either of the human SWI2 homologues increases the activation potential of GR 5-10 fold(Muchardt and Yaniv, 1993; Wang et al., 1996). These experiments were performed using transient transfection assays. Nucleosomes can not be observed on DNA templates introduced into cells by this method (Archer et al., 1992; Jeong and Stein, 1994). These results show that SWI/SNF can mediate the activation of transcription in the absence of nucleosomes. It is possible that in these experiments, transcription occurs on an undetectably small percentage of templates that are packaged into nucleosomes.

The suppression of transcriptional defects in *swi/snf-* yeast strains by mutations in histones may not be specific to SWI/SNF. They may simply lessen the inhibitory effects of chromatin, thus allowing a crippled transcription apparatus to function. To test this hypothesis other mutations that weaken transcriptional activation need to be examined for suppression

by these histone mutations. Consistent with this idea, the suppressing *sin1* mutations in histone H3 will restore activity to a SUC2 gene that has been crippled by removal of the upstream activating sequence (Prelich and Winston, 1993). Furthermore, using the previously described 5-FOA sensitivity assay to measure basal transcription, Lenfant et al. show that these suppressing histone H3 mutations alter sensitivity to 5-FOA by four orders of magnitude (Lenfant et al., 1996). Suppression by histone mutations support the idea that chromatin has a repressive effect on transcription in general as much as they support the idea that the SWI/SNF complex functions by antagonizing chromatin.

Transcriptional activators have been proposed to recruit the SWI/SNF complex in order to disrupt nucleosomes at gene promoters (Yoshinaga et al., 1992; Peterson and Tamkun, 1995). This is an important point since the SWI/SNF complex is not very abundant; there are only about 150 complexes per yeast cell. This is hard to reconcile with the 1:1 stoichiometry of SWI/SNF to nucleosomes needed to observe nucleosome disruption *in vitro* (Imbalzano et al., 1996). Recruitment is not observed in studies showing that SWI/SNF activity stimulates transcription factor binding to nucleosomes. That is, in the presence of SWI/SNF DNA binding domains fused to activation domains do not bind nucleosomes any better than DNA binding domains on their own (Cote et al., 1994).

The recruitment problem may be explained with the claim that the SWI/SNF complex is part of the yeast RNA polymerase II holoenzyme (Wilson et al., 1996). By this scenario, an activator's interaction with the general transcription machinery would also result in the recruitment of the SWI complex, resulting in nucleosome disruption at the promoter. This is supported by the observation that preparations of holoenzyme are able to

stimulate binding of TBP to a nucleosomal TATA box (Wilson et al., 1996). In contrast to previous work, however, ATP is no longer required for this stimulation. This again raises the possibility that the role of SWI/SNF in transcriptional activation is not specific to chromatin. Disruption of nucleosome structure *in vitro* has been achieved by a number of proteins that do not necessarily have this activity *in vivo* : An elongating SP6 RNA polymerase can displace nucleosomes (Clark and Felsenfeld, 1992), the DNA binding domain of a transcription factor can, in high concentrations, destabilize nucleosomes (Workman and Kingston, 1992) and it is conceivable that factors responsible for assembling nucleosomes *in vivo* could, in sufficient quantity, stimulate the reverse reaction *in vitro*. Consistent with this idea, the nucleosome assembly protein nucleoplasmin can stimulate nucleosome disruption by the activator GAL4 (Chen et al., 1994). The *in vitro* destabilization activity of the SWI/SNF complex may be an artifact of being present in such high concentrations. The pre-initiation complex contains many surfaces that contact DNA (Goodrich et al., 1996) and SWI/SNF may provide additional DNA interactions (Laurent et al., 1993) that, together, alter nucleosome structure sufficiently to enable occupancy of a nucleosomal promoter by the GTFs.

**Studies of Intact Promoters**

Given the complex nature of the factors governing chromatin structure and nucleosome positioning, it may prove too challenging to tease apart their transcriptional roles in the reductionist systems common to many gene expression labs. Some of the most convincing evidence describing the role of nucleosomes in the mechanism of transcriptional activation has come from the study of native gene promoters. The yeast PHO5 promoter and the

MMTV LTR represent two well-studied inducible promoters where nucleosomes are thought to play a role in the mechanism of transcriptional activation.

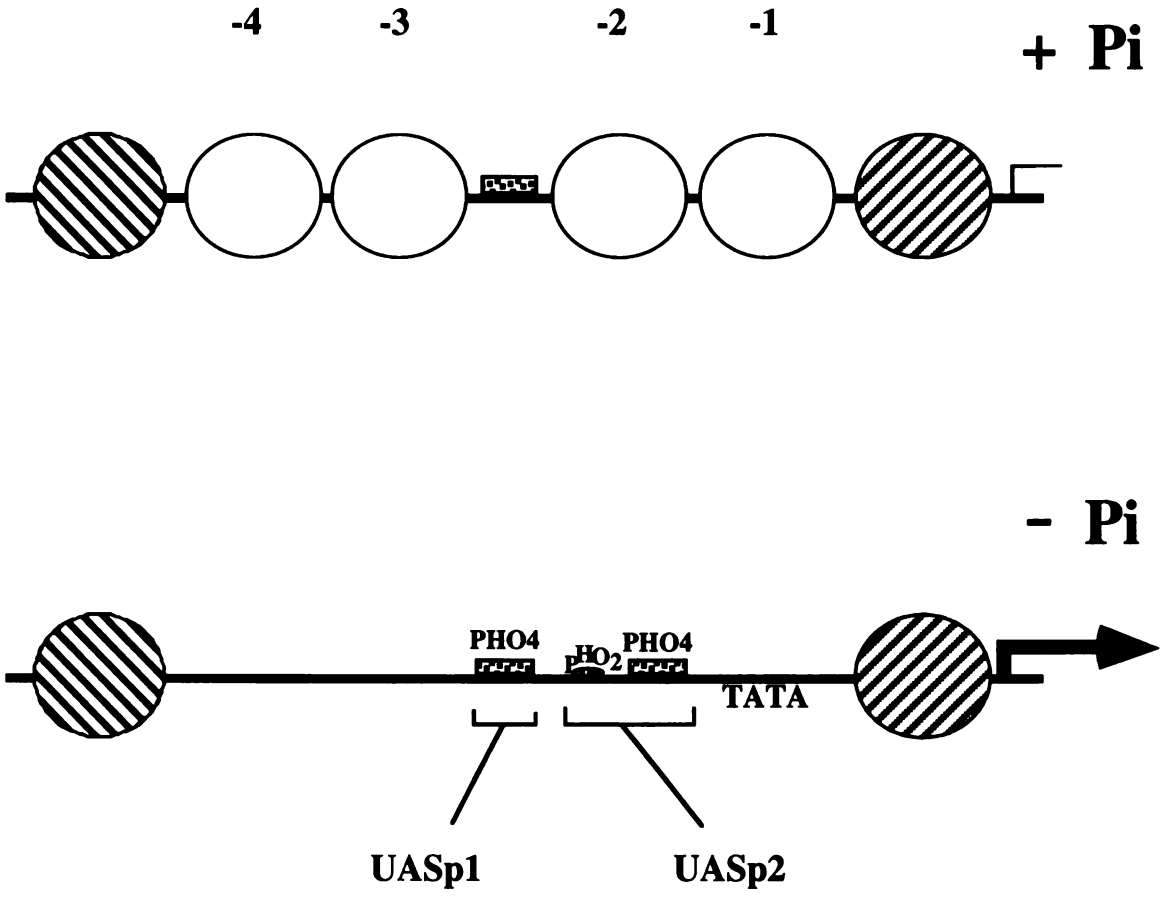
The yeast PHO5 gene, encoding a secreted acid phosphatase, is repressed when cells are grown in conditions of high phosphate. In low-phosphate conditions transcription of PHO5 is induced over 50-fold (Svaren et al., 1994). This induction requires the function of two transcriptional activators, PHO2 and PHO4 (Vogel et al., 1989). Under high-phosphate conditions, PHO4 is preferentially exported from the nucleus and does not become stably nuclear until the cells are introduced into low phosphate media. In the repressed state, positioned nucleosomes cover the gene promoter (figure 1). Induction of transcription is accompanied by an alteration of four of these nucleosomes (Almer et al., 1986). Binding sites for PHO4 exist in the linker region between nucleosomes -2 and -3 and within nucleosome -2. This nucleosome also contains a binding site for PHO2.

Once again, the key question is whether the alteration of nucleosome structure is a determinant of activation or occurs as a consequence of factor binding to the promoter. Mutation of the PHO5 TATA box results in a transcriptionally dead gene, yet this mutated promoter still undergoes nucleosome remodeling in inducing conditions (Fascher et al., 1993). This shows that chromatin disruption and transcription can be separated and may, in fact, represent two distinct processes. The PHO5 transcriptional regulators, PHO2 and PHO4, are still required for this remodeling event and it is possible they may still be recruiting some or all of the general transcription machinery to the promoter. There are several examples of transcriptionally silent genes that still bind GTFs. For example, *in vivo* footprinting experiments on the CYC1 gene showed that the TFIID complex was bound to a transcriptionally

**Figure 1:** The PHO5 promoter.

The inactive yeast PHO5 promoter contains 4 positioned nucleosomes that are altered during gene induction, -4, -3, -2, -1. UASp1 contains a binding site for the transcriptional activator PHO4 and UASp2, located within nucleosome -2, contains binding sites for PHO4 and an additional activator, PHO2. The TAT box is contained within nucleosome -1.

# PHO5 PROMOTER



silent promoter (Chen et al., 1994). Given that many different sequences can substitute for a TATA box (Singer et al., 1990), it is entirely possible that the general transcription factors, or possibly the RNA polymerase II holoenzyme, could still be recruited to these mutant promoters. Interestingly, mutations in the SWI/SNF complex do not affect PHO5 regulation, indicating that nucleosome remodeling at the PHO5 promoter can occur in the absence of SWI/SNF (Schneider, 1995).

As mentioned previously, genetic studies in yeast have implicated nucleosomes as having a regulatory role in PHO5 induction. Yeast strains engineered to repress histone synthesis show partial induction of the PHO5 gene independent of regulatory factors. Recall also that mutations in the N-terminal tail of histone H4 can inhibit PHO5 induction about 5-fold (Durrin et al., 1991). The nucleosomes of the PHO5 promoter also influence the accessibility of the PHO4 binding site, UASp2 (figure 1). In the absence of the PHO4 binding site (UASp1) positioned between nucleosomes -2 and -3, PHO4 cannot bind to its site within nucleosome -2 , UASp2 (Venter et al., 1994). However, when overexpressed, it can now bind UASp2, indicating that the nucleosome lowers the affinity of PHO4 for its site. Cooperative binding of PHO4 to these sites has not been reported and it is possible for PHO4 to occupy UASp1 in the absence of binding to UASp2 (Svaren et al., 1994). Thus occupancy of PHO4 at UASp1 is required for the binding of PHO4 to UASp2, probably through the destabilization of the adjacent nucleosome. By this view, the nucleosome plays a key regulatory role in that it must be altered in order to allow all of the required UAS's to be used. How PHO4 goes about facilitating the disruption of nucleosome -2 is a mystery, although it is known that its transcriptional activation domain is required (Svaren et al., 1994).

A similar story has emerged from the study of transcription from the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) promoter, which is activated by the glucocorticoid receptor (GR). In the absence of hormone, the promoter is organized into an array of six positioned nucleosomes (figure 2). The TATA box and transcriptional start site are covered by nucleosome A, and four glucocorticoid response elements (GREs) as well as a binding site for the transcription factor NF-1 reside on an adjacent nucleosome, nucleosome B. This nucleosomal structure is disrupted upon the addition of hormone, with DNA sequences in nucleosome B becoming accessible to nucleases (Richard-Foy and Hager, 1987). *In vivo* footprinting has revealed that the NF-1 site and TATA box are occupied only in the presence of hormone (Cordingley et al., 1987). Thus, the binding of GR is the initial event that results in the formation of an active promoter.

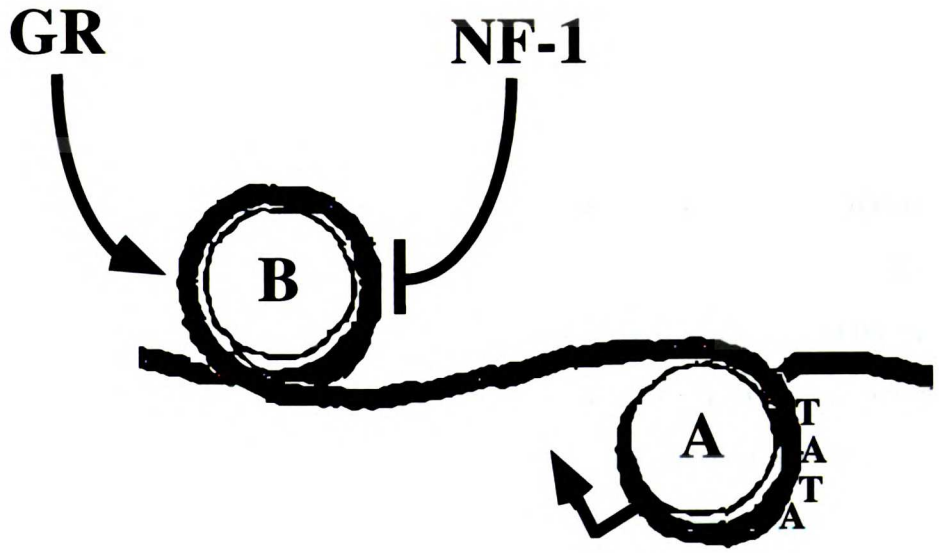
NF-1 is constitutively present in the nucleus, yet only binds to its site after the addition of hormone. This is not because of cooperative binding with GR since the two factors actually compete for occupancy of their sites *in vitro* (Bruggemeier et al., 1990). Reconstitution of nucleosome B *in vitro* has shown that GR is capable of binding this nucleosome while NF-1 is not (Archer et al., 1991; Pina et al., 1990). This has led to the model that GR does more than just interact with the general transcription machinery to activate transcription; it also disrupts nucleosome structure so that another essential transcription factor, NF-1, can occupy its site.

It would appear, then, that nucleosome B plays a key role in the regulation of transcription of the MMTV LTR. However, proper regulation of this gene can occur in the absence of nucleosome B. When introduced into cells by transient transfection, the DNA in the nucleosome B region is not packaged into a nucleosome and, as expected from the *in vitro* data, NF-1 is

**Figure 2:** A Promoter Within the MMTV LTR Contains Positioned Nucleosomes.

In the absence of hormone, positioned nucleosomes cover the GR and NF-1 binding sites as well as the TATA box and transcriptional start site. GR is capable of binding to nucleosome B while NF-1 is not.

# MMTV LTR



constitutively bound to the promoter (Archer et al., 1992). Yet, in the absence of hormone, the promoter is transcriptionally silent and is strongly induced by hormone treatment. Furthermore, occupancy of the TATA box is observed only in the presence of hormone. These results show that the function of GR is not just to remodel chromatin (Rigaud et al., 1991) to allow NF-1 binding; GR must also be present to interact with the general transcription machinery. Even though the disruption of nucleosome B is an obligate step (because NF-1 must be allowed to bind) in the activation of the MMTV LTR, proper regulation can still occur in its absence.

How does GR alter nucleosome B to enable NF-1 to bind its site? GR binding to nucleosome B *in vitro* does not visibly disrupt nucleosome structure (Pina et al., 1990). What events subsequent to GR binding alters nucleosome B? Does GR recruit a nucleosome destabilizing activity or is the interaction of GR with the general transcription machinery sufficient for nucleosome B disruption? If a nucleosome destabilizing activity is in fact part of the mammalian RNA polymerase holoenzyme, these models may not be mutually exclusive. An understanding of how GR binds nucleosome B is critical to identifying the nature of the chromatin alterations that are hypothesized to result in NF-1 binding. By determining the nature of the alterations of nucleosome B it will be possible to sort out whether the nucleosomes on the MMTV LTR are actively removed or are destabilized as a consequence of transcription initiation.

# CHAPTER ONE

## Nucleosome Assembly

### Introduction

Eukaryotic DNA exists *in vivo* packaged into chromatin. The simplest unit of chromatin structure is the nucleosome: a repetitive, periodic structure found throughout the genome. The nucleosome core consists of 146 base-pairs of DNA wrapped 1.8 times around a histone octamer, containing two each of the four core histones. A fifth histone, the linker histone H1, binds to an additional 20 base pairs of DNA between nucleosome cores and is thought to facilitate further compaction of the DNA into the 30 nm fiber. Higher order chromatin structure is non-uniform and is bound by a diverse set of non-histone proteins.

Most nuclear processes involving DNA are carried in the context of nucleosomes. The roles of nucleosomes in nuclear events such as transcription, replication and recombination are only just beginning to be described. Mutations in these essential proteins can perturb many nuclear processes or lead to cell death, making it difficult to determine the precise role of nucleosomes in a given process. It would be invaluable, for example, if the particular effects of histone mutations could be targeted to genomic locations of interest. Furthermore, the fact that nucleosome positioning is poorly understood has meant that manipulation of nucleosome positions and structures *in vivo* is still a matter of trial and error. This means that changes in nucleosome positioning or structure that accompany certain physiologic events may, in fact, be due to some other unrecognized process. While the techniques for the study of nucleosomes *in vivo* have been slowly

improving, the bulk of current knowledge has come from the manipulation of nucleosomes *in vitro*.

Most chromatin components described to date have been identified by the isolation of chromatin from cells. Yeast plasmids (Roth and Simpson, 1991) and viral episomes (Griffith and Christiansen, 1978) are packaged by cells into minichromosomes. These minichromosomes can be purified from cells and have been used to probe various aspects of nucleosome positioning and transcription. While techniques for the isolation of minichromosomes may perturb native chromatin structure, these chromatin complexes can be used as a means for enrichment of factors that interact with chromatin.

Three general strategies are commonly used for the reconstitution of nucleosomes *in vitro*. First, nucleosome assembly has been achieved using crude cellular extracts containing the core histones and assembly factors. The most active extracts are prepared from embryonic sources such as *Xenopus* oocytes and *Drosophila* embryos (Shimamura et al., 1989; Becker and Wu, 1992). These extracts are capable of assembling arrays of physiologically spaced nucleosomes on double-stranded DNA or, in conjunction with DNA replication, on single-stranded DNA templates. A drawback of assembly systems using whole cell extracts is that the system is poorly defined. Although the assembled templates can be purified by sucrose gradient sedimentation, they are likely to contain non-nucleosomal components. Of course the crude nature of this system can be advantageous for studies that seek to identify novel biochemical activities relating to chromatin structure (Tsukiyama and Wu, 1995). The ability of these extracts to recapitulate nucleosome phasing has not been reported.

Second, chromatin isolated from cells can also be a good source of histones for nucleosome assembly. Treatment of nuclei with micrococcal

nuclease cleaves cellular chromatin, facilitating its release from nuclei. The chromatin is then size fractionated at high ionic strength. Nucleosomes are quite stable and will remain intact at salt concentrations that dissociate most other bound proteins, including histone H1, from the DNA. Salt-stripped chromatin can then serve as an effective histone source for nucleosome assembly. Mixing of a radiolabeled DNA fragment of interest with the donor chromatin at high salt followed by dialysis to low salt results in the exchange of histones between DNA fragments. Since the labeled DNA is usually added to a large excess of donor chromatin there is little change in the overall DNA:Histone ratio, resulting in the efficient assembly of nucleosomes on the labeled DNA. This technique is capable of assembling both long arrays of nucleosomes as well as phased nucleosomes. Nucleosomal arrays assembled using this technique are not physiologically spaced, although spacing can be specified using reiterated phasing sequences (Owen-Hughes and Workman, 1996). As with cellular assembly extracts, salt-stripped chromatin likely includes DNA binding proteins other than histones, which may complicate the interpretation of results.

Third, nucleosomes have been reconstituted from purified components. A drawback of this strategy is the difficulty in achieving appropriate DNA/histone ratios, which are key to the assembly of soluble chromatin. This approach is very effective for assembling mono- and di-nucleosomes and can be used to assemble phased nucleosomes; however, it has not proved useful for assembling arrays of nucleosomes. Purified core histones and DNA are mixed at high salt followed by slow dialysis to low salt. Unbound DNA is separated from nucleosomal DNA by sucrose gradient sedimentation. Nucleosome assembly can be achieved at physiological ionic strength using a polyanionic assembly factor, poly-glutamic acid being the

most commonly used. This negatively charged polymer serves to prevent histone aggregation and can be removed by sucrose gradient sedimentation. The assembley of phased nucleosomes using these factors has not been explored.

In an effort to understand how the glucocorticoid receptor (GR) binds to glucocorticoid response elements (GREs) in a nucleosome, a system for the reconstitution of nucleosomes from purified components was developed. Salt gradient reconstitution with purified core histones was employed to assemble a rotationally phased nucleosome and to avoid contamination by non-histone proteins. By using this reductionist approach we hoped to understand the intrinsic ability of the DNA binding domain of GR to bind to nucleosomal GREs.

## Materials and Methods

The purification of the core histones, nucleosome reconstitution, and DNase I footprinting are described in chapter 2.

**Surplus Histone Competitions.** Nucleosomes containing ~4 ng of DNA were mixed with competitor DNA in 20 mM Tris-pH7.5, 0.2% NP-40, 10 % glycerol in a total volume of 8 µl. Samples were incubated for 2 hr at 37°C. 3 µl of each sample was loaded onto a 5 % polyacrylamide gel, 1X TGE.

## Results

### Purification of the Core Histones

Fresh calf thymus was frozen in liquid nitrogen and stored at -80°C. Nuclei were prepared by homogenization of frozen tissue in a Waring blender followed by partial digestion with micrococcal nuclease to yield chromatin fragments. Small scale test digests were performed on nuclei

containing 5 mg of DNA for various times to optimize production of chromatin fragments approximately 10 nucleosomes in length (figure 3A). Following large scale micrococcal digestion, the nuclei, containing 200 mg of DNA, were lysed by EDTA treatment and centrifuged to remove insoluble material.

The chromatin fragments were then fractionated by sucrose gradient sedimentation at 600 mM NaCl. Gradient fractions were analyzed for both DNA and histone content (figures 3B and 3C). The four core histones were observed in equal stoichiometries and appeared only in DNA containing fractions. Histone H1 appeared as a doublet band and was apparently not bound to the DNA; however, the trailing edge of the histone H1 peak did overlap with the mono-di- and tri-nucleosome containing fractions. Sucrose gradient fractions containing only trace amounts of histone H1 were pooled, with the fractions from the very bottom of the gradients also being excluded.

The core histones were then purified using hydroxylapatite chromatography. Nucleosomal fragments were bound to a hydroxylapatite column at 80 mM sodium phosphate and 600 mM NaCl. The chromatin bound to the column through the DNA, allowing dissociation of the histones at high salt. Core histones were eluted in a single step to 2.5 M NaCl; the DNA remained bound to the column. The core histone-containing fractions were pooled, glycerol was to added to a final concentration of 20%, and the sample was concentrated to 2 mg/ml by centrifugation through a centricon concentrator and stored at -20°C. The purified material contained stoichiometric amounts of each of the core histones (figure 3D) and < 1% histone H1.

**Figure 3:** Purification of Calf Thymus Core Histones.

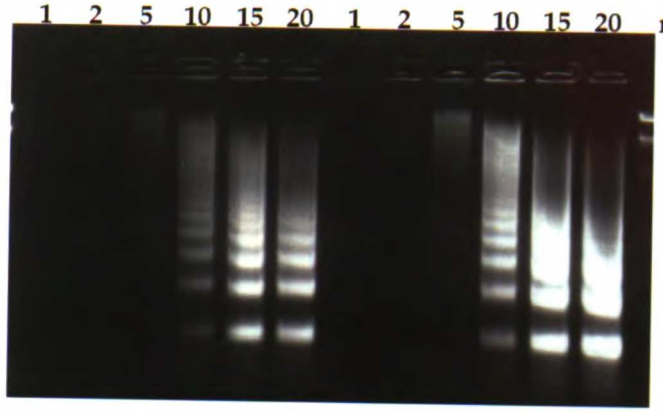
**Panel A.** DNA was prepared from nuclei that had been digested with micrococcal nuclease for 1, 2, 5, 10, 15 or 20 minutes. The DNA was resolved on a 1.8% agarose gel and stained with ethidium bromide.

**Panel B.** DNA was prepared from sucrose gradient fractions and resolved on a 1.8 % agarose gel and stained with ethidium bromide.

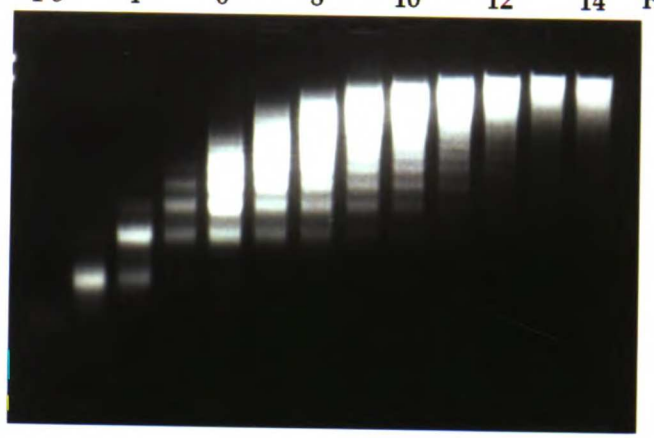
**Panel C.** Samples from sucrose gradient fractions were resolved on 18% SDS-PAGE gels and stained with coomasie blue.

**Panel D.** Total protein eluted from hydroxylapatite column. Lanes 1, 2, 3 contain 1, 2 or 5 μl of protein, respectively.

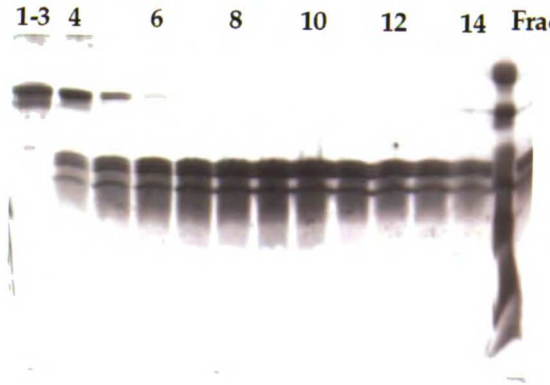
A


1 2 5 10 15 20 1 2 5 10 15 20 minutes

B


1-3 4 6 8 10 12 14 Fraction #

C


1-3 4 6 8 10 12 14 Fraction #

D


1 2 3

**Nucleosome Reconstitution**

Nucleosomes can be assembled by mixing the core histones and DNA in high salt followed by a gradual lowering of the ionic strength. Aggregation of the histones and DNA is significant if the salt concentration is lowered too rapidly or if the components are mixed at low ionic strength. Reconstitutions must also be carried out at appropriate histone:DNA ratios, generally 0.6 to 0.9, in order to maintain solubility of the reconstitute. Histones and DNA interact very little at 2 M NaCl; as the salt is lowered to 500 mM NaCl, the histones and DNA will assemble into nucleosomes. The remaining free DNA is then separated from the nucleosomes on a sucrose gradient.

A 200 base pair DNA fragment spanning positions -232 to -36 from the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) was isolated from plasmid DNA and end-labeled. This fragment encompasses a positioned nucleosome, as defined by protection from micrococcal nuclease in isolated nuclei (Richard-Foy and Hager, 1987), and also contains 3 or 4 binding sites for GR, depending on the virus strain (3 sites for C3H strain, 4 for GR strain). Since it is difficult to achieve a proper DNA:histone mass ratio using only trace amounts of labeled DNA, unlabeled carrier DNA is added to achieve the necessary histone:DNA ratios. DNA and histones were mixed at 2 M NaCl and dialyzed at 4°C for 2 hr against 2 M NaCl buffer. Using a peristaltic pump, the same buffer containing no salt was slowly added over 15 hr, lowering the salt to 500 mM NaCl. The ionic strength was lowered further by dialysis for 2 hr against 250 mM NaCl buffer. If an excess of histones were used or the salt was lowered too rapidly a precipitate often appeared at this stage. Aggregates were removed by centrifugation at 16,000 x g. Typically, >90% of the labeled probe remained soluble following dialysis.

Assembled nucleosomes were separated from free DNA by velocity gradient sedimentation. The unlabeled carrier DNA was greater than 500 bp in length and, when complexed with histones, migrated to the bottom of the gradient. Linear DNA functioned as a carrier better than circular DNA. Sheared calf-thymus or herring sperm DNA were typically used and linearized plasmid DNA also worked well. Figure 4 shows the sedimentation profile of the radiolabeled MMTV DNA in a 5-30% sucrose gradient. A small peak of free DNA ran near the top of the gradient while a much larger peak, representing histone bound DNA, ran just past the center of the gradient. Nucleosomes containing the minimal 146 bp of DNA migrate as a 10.6S complex whereas nucleosomes containing 200 bp of DNA sediments as an 11.2S complex (Noll and Noll, 1989). DNA extending beyond the core 146 bp is not tightly bound the histone octamer and is more open to nucleolytic attack, thus reconstitution with DNA fragments longer than 146 bp will likely result in a less compact structure. As a result of this change in shape, there is only a small change in the sedimentation rate between nucleosomes associated with 200 bp of DNA and the 146 bp core particle. In the experiments described here, nucleosomes reconstituted with 200 bp or 166-bp of DNA sedimented at similar rates, both comigrating with the 11.3S standard, bovine catalase.

**Analysis of Reconstitution**

Equal numbers of cpms from each sucrose gradient fraction were loaded onto 5 % nondenaturing polyacrylamide gels in order to resolve the reconstituted material. Figure 5 shows that the reconstitution contained several different complexes, as well as free DNA. It was expected that assembly of a homogeneous population of mononucleosomes would result

**Figure 4:** Sedimentation Profile of MMTV DNA.

MMTV DNA content was monitored by liquid scintillation counting of 1 µl from each gradient fraction.

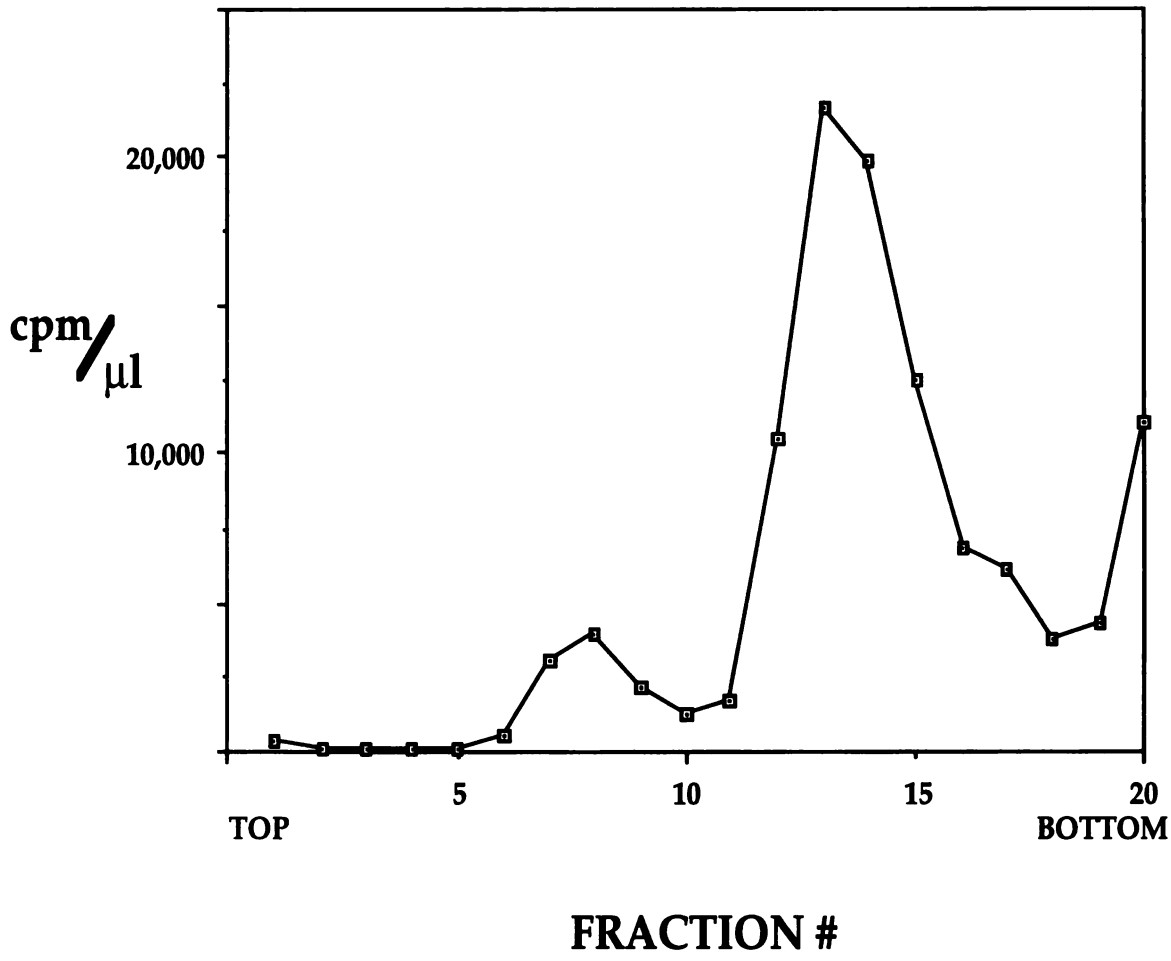
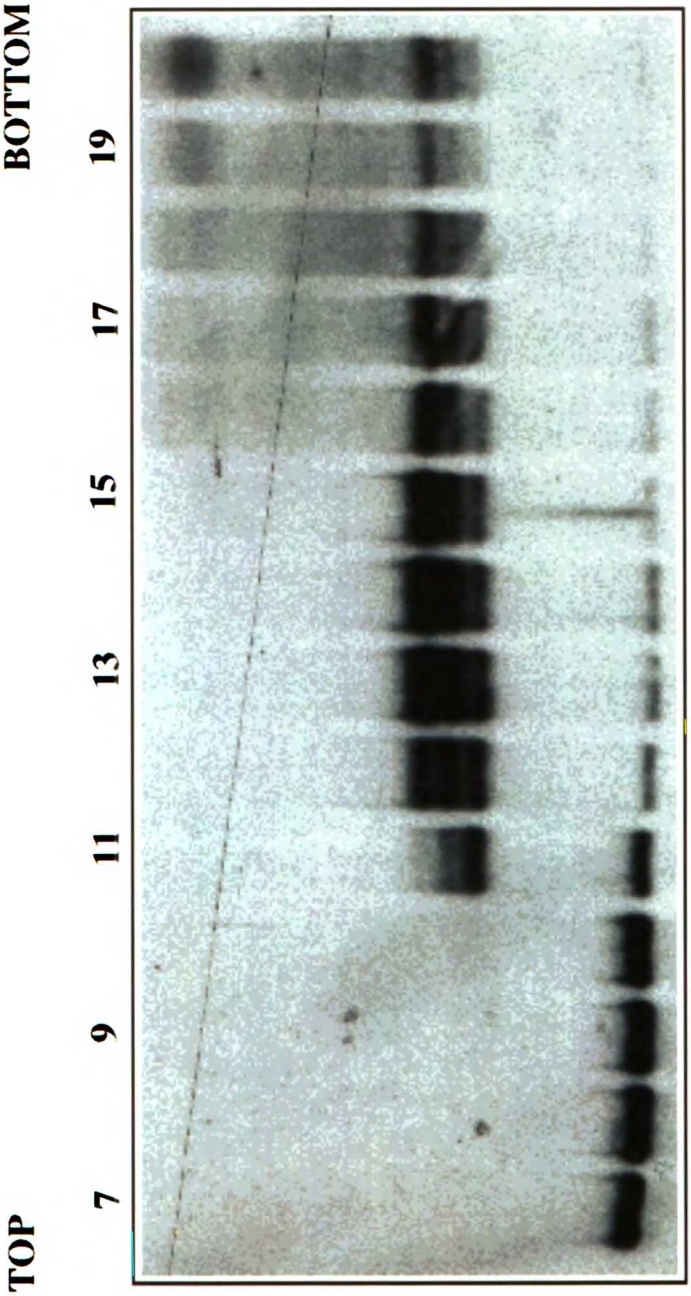

20,000
cpm/µl
10,000
5
10
15
20
TOP
BOTTOM
FRACTION #

**Figure 5:** Polyacrylamide Gel Electrophoresis of Sucrose Gradient Fractions.

In order to visualize the histone-DNA complexes, equal numbers of cpms from each sucrose gradient fraction were resolved on a 5% polyacrylamide gel, 1X TGE.


TOP
7
9
11
13
15
17
19
BOTTOM

in the production of a single complex. The multiple complexes detected might represent either a single mononucleosome located in different positions on the DNA fragment, the DNA bound to an octamer, hexamer and tetramer of histones, or a mononucleosome bearing "surplus" bound histones.

Digestion of nucleosomal DNA with DNase I yields a cleavage pattern distinct from that of naked DNA. If the nucleosome is rotationally positioned, with the major and minor grooves of the DNA always being oriented the same with respect to the histone octamer, then a 10-bp ladder of cutting can be observed. This is due to the fact that the DNA is bound to the histones through the minor groove and that DNase I cleaves DNA in the minor groove; thus a rotationally phased nucleosome presents a discrete set of cleavage sites to DNase I. DNase I digestion of different sucrose gradient fractions yielded similar patterns of cleavage (figure 6). Small differences could be detected at one end of the fragment but the overall pattern was basically the same. This result makes it unlikely that the fastest migrating complexes contained an incomplete complement of histones since tetramers of histones H3 and H4 protect only 72-bp of DNA. It is also unlikely that the nucleosome occupies dramatically different positions on the DNA fragment. A change in position on the order of 20- to 40-bp would be expected if the overall shape of the complex were to be altered. It was observed that gradient fractions containing higher proportions of the slower migrating complexes had a larger amount of undigested DNA, indicating they were cleaved less efficiently by DNase I. This was consistent with surplus histones being bound on top of a mononucleosome.

If the slower migrating complexes did, in fact, contain surplus histones, then it might be possible to compete them onto exogenously added

**Figure 6:** DNase I Digestion of Sucrose Gradient Fractions.

The presence of nucleosomes was determined by DNase I digestion of sucrose gradient fractions 11, 14 and 16. The DNA was isolated and run out on an 8% denaturing polyacrylamide gel. The dashes indicate the ~10 bp ladder of cleavage by DNase I.


Fraction #
11
14
16

competitor DNA. Sucrose gradient fractions containing two different DNA/histone complexes were incubated with increasing concentrations of 200- to 600-bp DNA fragments. As shown in figure 7A, the amount of the faster migrating complex increased at the expense of the slower migrating one. This occurred with little change in the amount of free DNA. Nucleosomes are very stable, at this salt concentration (<10 mM NaCl) and nucleosomal histones do not exchange between DNA fragments. This result supported the idea that the slow migrating complexes contained surplus histones and that the fastest migrating complex was a mononucleosome. The transfer of histones was facilitated at higher temperatures, as shown in figure 7B. Even at temperatures as high as 55°C the nucleosome was still quite stable, with only a small increase in the amount of free DNA.

**GR 440-525 Does Not Bind the Slower Migrating Complexes**

The DNA fragment from the C3H strain of the MMTV LTR used in this study contains three binding sites for GR. These sites, termed GREs, are spaced throughout the fragment, with one GRE located near the promoter distal end and the others situated immediately adjacent to each other proximal to the promoter. An 86 amino acid fragment from GR (440-525) is sufficient for binding to GREs both *in vivo* and *in vitro*. The ability of GR 440-525 to bind to the MMTV GREs in the DNA/histone complexes was determined using a DNase I footprinting assay (figure 8). On naked DNA, GR 440-525 protected all three GREs. Binding to sucrose gradient fractions containing slower migrating complexes showed no protection of the GREs by GR. Sucrose gradient fractions that contained predominantly the fastest migrating complex showed protection of one of the GREs. This difference in GRE occupancy will be explored in detail in chapter 2.

**Figure 7:** Competition of Surplus Histones Onto Competitor DNA.

**Panel A.** Autoradiogram of histone-DNA complexes after incubation with competitor DNA. 1 µl of the sucrose gradient fraction was incubated with 0, 4, 8, 12, 16 or 20 µg of competitor DNA. The position of free DNA is indicated by the arrow.

**Panel B.** Autoradiogram of histone-DNA complexes after incubation with competitor DNA at different temperatures. 1 µl of sucrose gradient fraction was incubated with 16 µg of competitor DNA.

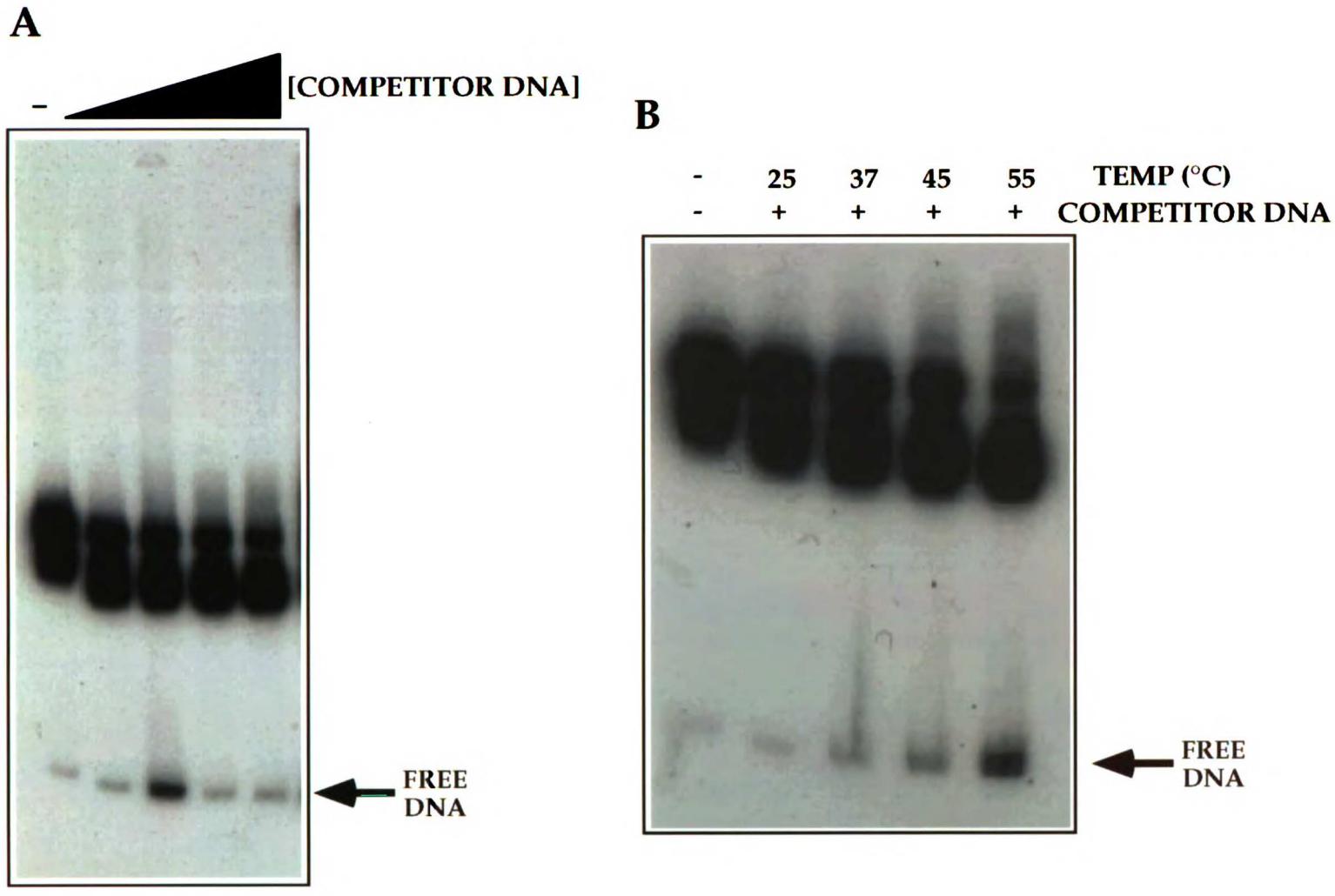
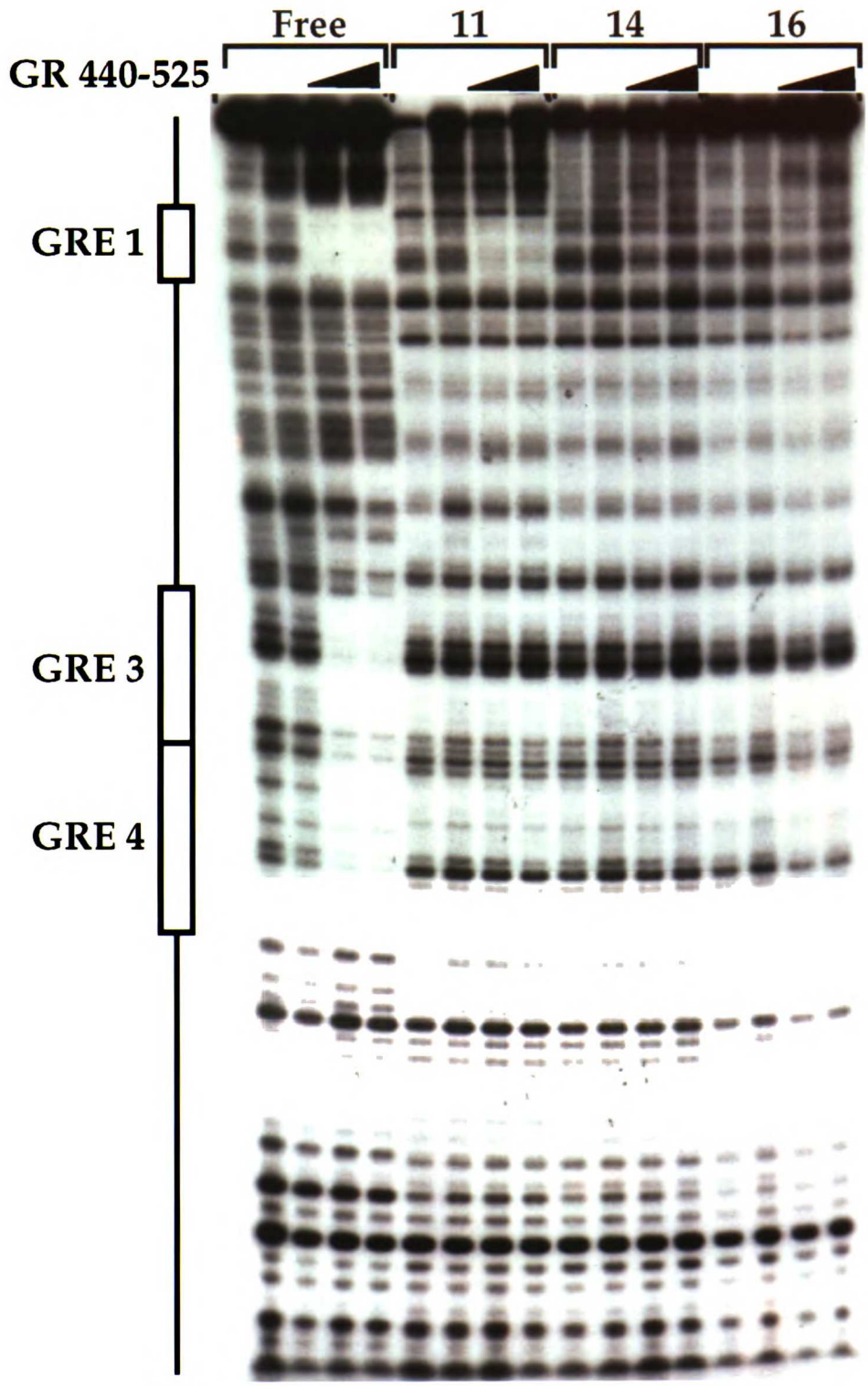

A
-
[COMPETITOR DNA]
FREE
DNA
B
-
25
37
45
55
TEMP (°C)
-
+
+
+
+
COMPETITOR DNA
FREE
DNA

**Figure 8:** GR 440-525 Binding to Free DNA or DNA-Histone Complexes.

An MMTV DNA fragment labeled on the top strand was bound to 20 or 50 ng of GR 440-525 either as free DNA or reconstituted into nucleosomes. Binding was analyzed on three different sucrose gradient fractions by DNase I footprinting. The open boxes indicate the location of the GRE footprints as naked DNA.

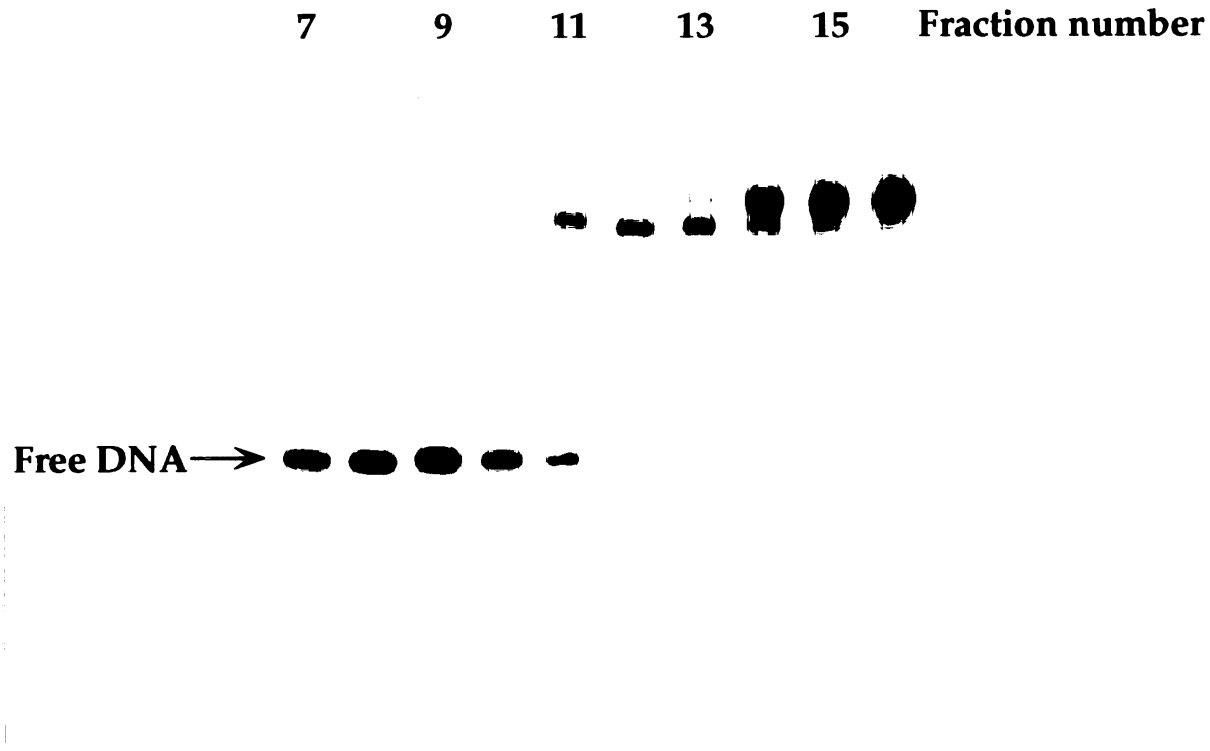

Free
11
14
16
GR 440-525
GRE 1
GRE 3
GRE 4

**Purification of Mononucleosomes**

Analysis of reconstitutes on 5-30% sucrose gradients revealed that mononucleosomes and the slower migrating complexes sedimented at similar rates. The complexes were separated only at the edges of the peak of bound DNA. Furthermore, fractions containing predominantly mononucleosomes often contained a large amount of free DNA. Mononucleosomes could be separated from the complexes containing surplus histones on shallower (5-20%) sucrose gradients (figure 9). A higher success rate was also achieved by reconstituting nucleosomes onto a 166-bp fragment spanning MMTV sequences -213 to -54. Fragments of this size generally gave only one other slower migrating complex and since the free DNA was ~40-bp smaller, the mononucleosome fractions usually had a smaller amount of contaminating free DNA.

**Figure 9:** Purification of Mononucleosomes.

Polyacrylamide gel electrophoresis of nucleosomes reconstituted onto a 166 bp MMTV DNA fragment and resolved on 5-20% sucrose gradients. Equal numbers of cpms from each fraction were loaded on the gel. Free DNA is indicated by the arrow.


7
9
11
13
15
Fraction number
Free DNA

## Discussion

Mononucleosomes were reconstituted using core histones purified from calf thymus and 200- or 166-bp DNA fragments from the MMTV LTR. The histones were assembled into nucleosomes on the DNA using the salt gradient dialysis technique. Reconstituted material was sedimented on sucrose gradients followed by analysis of the gradient fractions on nondenaturing polyacrylamide gels. Multiple complexes were found in the gradient fractions of bound DNA, not the single complex that would be expected of a homogeneous population of mononucleosomes. It is most likely that the slower migrating complexes contained a mononucleosome bound to additional histones. Consisitent with the idea that the different complexes all contained a mononucleosome, DNase I digestion of different sucrose gradient fractions showed that even though the slower migrating complexes were cleaved less efficiently by DNase I, all had a very similar pattern of cleavage. Furthermore, the fastest migrating complex was stable to challenge with an excess of competitor DNA whereas the amount of slower migrating complex decreased in the presence of competitor DNA. In fact, as the amount of slower migrating complex decreased, it gave rise to the faster migrating species. The surplus histones present in the slower migrating complexes impeded cleavage by DNase I and completely blocked binding by GR 440-525.

The main complication of this reconstitution technique was the binding of surplus histones to an already assembled nucleosome. Additional histone binding was observed even in reconstitutions carried out with an excess of DNA, indicating that nucleosomes and free DNA have a similar affinity for histones. High histone/DNA stoichiometries have been reported

in a number of other studies (Tatchell and Van Holde, 1977; Voordouw and Eisenberg, 1978; Stein, 1979; Royer et al., 1992). Stein has demonstrated that the additional histones bind as a complete histone octamer and that a single nucleosome core can bind as much as 12 additional octamers (Stein, 1979). Furthermore, the standard free energy difference of a histone octamer binding to a nucleosome or to free DNA was determined to differ by only 2.6 kcal. Using fluorescence anisotropy, it has been shown that a 200-bp DNA fragment can bind histones with a stoichiometry that varied between 4 and 16 histone octamers/ DNA 200mer (Royer et al., 1992). Voordouw and Eisenberg (1978) demonstrated that a nucleosome can bind one additional equivalent of histones with no apparent alteration of shape or structure, as measured by velocity gradient sedimentation. The excess histones were thought to displace bound water molecules, resulting in little change in the hydrodynamic volume of the particle.

The fact that competition of the excess histones was dependent on the concentration of the competitor DNA, even at high molar excess, indicates that the competitor is probably not functioning as a "sink" that traps dissociating histones. Rather, the competitor may function in a more active manner, perhaps facilitating the direct transfer of histones from one DNA molecule to the other. Plasmid DNA functioned poorly as a competitor, often resulting in the smearing of the complexes in the gel. DNA cellulose also was an inefficient competitor for the excess histones. These results indicate histone transfer may not occur as diffusion from one DNA to another but rather as a direct exchange between DNAs with some forms of competitor DNA functioning better than others.

Finally, it is worth noting that nucleosomes reconstituted onto DNAs with lengths closer to the canonical 146-bp show less absorption of excess

histones. The DNA protruding from the ends of the nucleosome may serve as binding sites for additional histone octamers. Linker DNA is thought to be bound by histone H1 *in vivo* and it is not clear whether the binding of excess core histones, as described here, occurs *in vivo*. This phenomenon probably represents an artifact of assembly from purified components at elevated ionic strength. Nucleosome assembly occurs *in vivo* tightly linked to DNA replication and in the presence assembly factors (Kaufman and Botchan, 1994). Newly deposited histones are also post-translationally modified at specific residues (Brownell and Allis, 1996). The cell may employ these mechanisms to circumvent nonproductive modes of histone binding.

# CHAPTER TWO

## A Nucleosome Determines the DNA Binding Specificity of the Glucocorticoid Receptor for the MMTV LTR

### Introduction

The packaging of the eukaryotic genome into chromatin performs many important functions. For example, the folding of DNA into chromosomes ensures the proper segregation of genetic information during cell division. This high degree of compaction, which is nearly 10,000-fold during mitosis, presents an obvious problem for gene expression and its regulation. Regulatory factors must gain access to their binding sites in chromatin and must subsequently stimulate the initiation of transcription from core promoter elements that also exist as chromatin. For example, the regulatory regions of many genes are covered by positioned nucleosomes and activation of transcription is accompanied by an alteration of this nucleosomal structure (Almer et al., 1986; Carr and Richard-Foy, 1990; Richard-Foy and Hager, 1987). It is unclear whether these chromatin structures are components of the regulatory mechanisms *per se*, or whether chromatin alterations are a passive consequence of regulatory factor binding and the assembly of the pre-initiation complex.

The glucocorticoid receptor (GR) is part of a large superfamily of transcriptional regulators termed the intracellular receptors. GR is a cytoplasmic protein that, upon binding of its cognate hormone, translocates the nucleus where it binds to specific DNA sequences and modulates the transcriptional activity of nearby promoters (Beato et al., 1995). The mouse mammary tumor virus (MMTV) long terminal repeat (LTR) includes a viral

promoter that has a well studied chromatin structure and is strongly induced by glucocorticoid treatment. In the absence of hormone, the transcriptionally silent promoter is organized in an array of six positioned nucleosomes. Induction by glucocorticoid treatment results in nuclease hypersensitivity in the positioned nucleosome covering the glucocorticoid response elements (GREs) (Richard-Foy and Hager, 1987).

The GRE containing nucleosome, termed nucleosome B, contains a set of receptor binding sites that have been shown to be critical for glucocorticoid induction. GR has a high affinity for these sites as free DNA (Perlmann et al., 1990) and can bind to them in a nucleosome as well (Perlmann and Wrange, 1988). Nucleosome B also contains a binding site for NF-1, a constitutively nuclear transcription factor. The NF-1 site is critical for transcriptional activation (Bruggemeier et al., 1990) but, in contrast to GR, NF-1 is incapable of binding its site when incorporated into a nucleosome (Archer et al., 1991; Pina et al., 1990). Furthermore, *in vivo* Exo III footprinting shows that, in the absense of hormone, the NF-1 site is unoccupied (Cordingley et al., 1987; Archer et al., 1992). This is not due to cooperative binding of GR and NF-1 since they compete for binding on naked DNA (Bruggemeier et al., 1990). Thus, it is thought that GR causes a disruption of nucleosome B that enables NF-1 to bind its site.

The DNA binding domain of GR is comprised in a 70 amino acid zinc binding motif, situated in the middle of the protein. This domain also mediates receptor dimerization, nuclear localization, and interacts with non-receptor regulatory factors (Luisi et al., 1991; Picard and Yamamoto, 1987; Diamond et al., 1990). The DNA binding domain is also an important site of interaction with nucleosomes. In order to understand in more detail how GR recognizes and binds to nucleosomal GREs , an *in vitro* binding assay using

purified components was developed. By utilizing just the DNA binding domain of GR and nucleosomes reconstituted from purified core histones we hoped to understand the intrinsic properties of the DNA binding domain that enable GR to bind nucleosomal GREs.

## Materials and Methods

**Construction and Preparation of DNA Fragments.** Labelled DNA fragments were prepared from the plasmid pMTV.GBX or pMTV.GB. The pMTV.GBX was constructed by amplifying MMTV sequences -213 to -54 using PCR. pMTV.GB contained MMTV sequences from -234 to -36. The primers contained 17 bases of homology to MMTV (GR strain) with the remaining sequence containing Not I and Kpn I sites for the 5' primer or Not I and EcoR I sites for the 3' primer. The amplified fragments were then subcloned into the Not I site of pBluescript (Stratagene). Fragments for reconstitution were prepared by sequential digestion with either Kpn I or Eco RI followed by digestion with Not I. The DNA was then radiolabeled with ($\alpha$-$^{32}$P)dGTP followed by isolation from a 5% polyacrylamide gel. The DNA was then eluted into 500 µl of 50 mM Tris-pH8.0, 0.2% SDS, 300 mM NaOAc, 4 mM EDTA.

Mutation of GRE1 (-184 to -170) was constructed by PCR using a primer extending from the Kpn I cloning site to 5 bases beyond GRE1. The primer altered the GRE1 sequence to GTTACCAACTACTAT. Mutations in GREs 3 (-107 to -93) and 4 (-92 to -78) were constructed using a primer extending from the internal Sst I site at position -108 down through GREs 3 and 4, altering the TGTTCT motifs to TACTAT. The mutation of GREs 3 and 4 to GRE 1 was constructed using a primer extending from the Tfi I site at -73 up through GREs 3 and 4 to position -113. The primer altered the non-conserved half-

sites to GTTACA. All contructs were sequenced to confirm that there were no mutations introduced by Taq polymerase.

**Histone Purification.** Histone purification was based on a previously described protocol (Laybourn and Kadonaga, 1991) with some minor modifications. Briefly, 25g of frozen calf thymus was pulverized with a hammer followed by cell lysis in a waring blender in 50 mM Tris-pH7.4, 5 mM $MgCl_2$, 1.2 M sucrose, 1 mM $Na_2S_2O_5$, 1 mM PMSF, 1 mM DTT, 1 mM benzamidine. Nuclei were strained through two layers of cheese cloth and then pelleted in a GSA rotor. Nuclei were washed twice with 10 mM Tris-pH7.4, 3 mM $MgCl_2$, 350 mM sucrose, 1 mM $Na_2S_2O_5$, 1 mM PMSF, 1 mM benzamidine. Test micrococcal nuclease (Sigma) digestions were performed on aliquots of nuclei containing 5 mg DNA to determine enzyme concentrations that would yield DNA fragments carrying ~10 nucleosomes. Large scale micrococcal nuclease digestion was performed on nuclei containing 200 mg DNA. The nuclei were lysed with EDTA and resulting lysate fractionated on 5-30% sucrose gradients containing 10 mM Tris-pH7.5, 1 mM EDTA, 500 mM NaCl, 0.3 mM PMSF. Gradients were spun at 26,000 rpm, 16 hr at 4°C. Nucleosome containing fractions that lacked histone H1 were pooled and dialyzed against 50 mM Tris-pH7.9, 1 mM EDTA. The sample was then loaded onto a hydroxyapetite column equilibrated with 80 mM $Na_2HPO_4$-pH6.8, 500 mM NaCl, 1 mM DTT, 0.2 mM PMSF and washed with 5 column volumes. The core histone were eluted with a single step to 2.5 M NaCl. The eluted protein was visualized by coomasie staining and observed to have equal amounts of the four core histones with <1% contamination by histone H1.

**Nucleosome Reconstitution.** Reconstitution was carried out by salt-gradient dialysis as described (Workman and Kingston, 1992) with some modifications.

Radiolabeled MMTV probe was ethanol precipitated followed by resuspension in reconstitution mix. Herring sperm DNA (>500 bp) and histones were mixed in a 0.6 to 0.8 mass ratio, NaCl was added to a concentration of 2 M and BSA to 1.7 mg/ml. Samples contained 40 µg of DNA and were mixed in a total volume of 30 µl, with the remaining volume made up with 50 mM Hepes, pH7.5 and 1 mM EDTA. The probe was resuspended in all of the reconstitution components except for the histones and then placed on ice. After addition of the histones, the sample was dialyzed for 2 hr against 200 mls of 10 mM Hepes, pH7.5, 1 mM EDTA, 2 M NaCl. The salt was lowered over a 15 hr period to 500 mM NaCl by adding the same buffer -NaCl with a peristaltic pump. The reconstitute was then dialyzed for 2 hr against 200 mls of buffer with 250 mM NaCl. Large aggregates were removed by a 5 min spin in a microfuge at 4°C. The reconstituted nucleosomes were then purified on 5-20% sucrose gradients containing 10 mM Hepes, pH7.5, 0.1% NP-40, 1 mM EGTA. Gradient were spun for 20 hr in a SW50.1 rotor at 34,500 rpm, 4°C. 250 µl fractions were collected from the top and counted in a scintillation counter. Equal numbers of counts were run out on 5% polyacrylamide gels. Nucleosomes reconstituted from 160 bp fragments migrated to the same position in the gradient as bovine catalase, an 11.3 S sucrose gradient standard.

**DNase I Digestion.** Free DNA and reconstituted nucleosomes were incubated in 10 mM Tris, pH7.5, 10% glycerol, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mg/ml BSA and 1 mM DTT. Samples were incubated for 10 min at 30°C followed by digestion with DNase I for 30 sec. Free DNA was digested with 0.02 units (Boehringer Mannheim)/µl and nucleosomal DNA with 0.05 units/µl. The reaction was stopped by addition of 0.3 vol of 1.3 M NaAc., 40 mM EDTA and 0.75 mg/ml tRNA followed by phenol-chloroform extraction. The DNA was

then precipitated with 2.5 volumes of ethanol. Samples were dried in a speed-vac and resuspended in formamide dye and run out on 1X TBE 8% polyacrylamide/8M Urea gels. Gels were dried and exposed to film at -80°C with an enhancing screen. Quantitation was done using a Storm Phosphorimager™ equipped with Image-Quant (Molecular Dynamics) software. The $K_d$ for each GRE was determined as the amount of GR 440-525 required to produce half-maximal footprint.

**Electrophoretic Mobility Shift Assay.** Reconstituted nucleosomes or free DNA were mixed with varying amounts of GR in 20 mM Tris-pH7.5, 0.2% NP-40, 10 % glycerol, 100 mM KCl, 0.1 mg/ml BSA and 3 mM DTT in a total volume of 8 µl. Samples were incubated for 10 min at room temperature followed by separation on 5% polyacrylamide gels, 1X TGE. Gels were dried and then exposed to film.

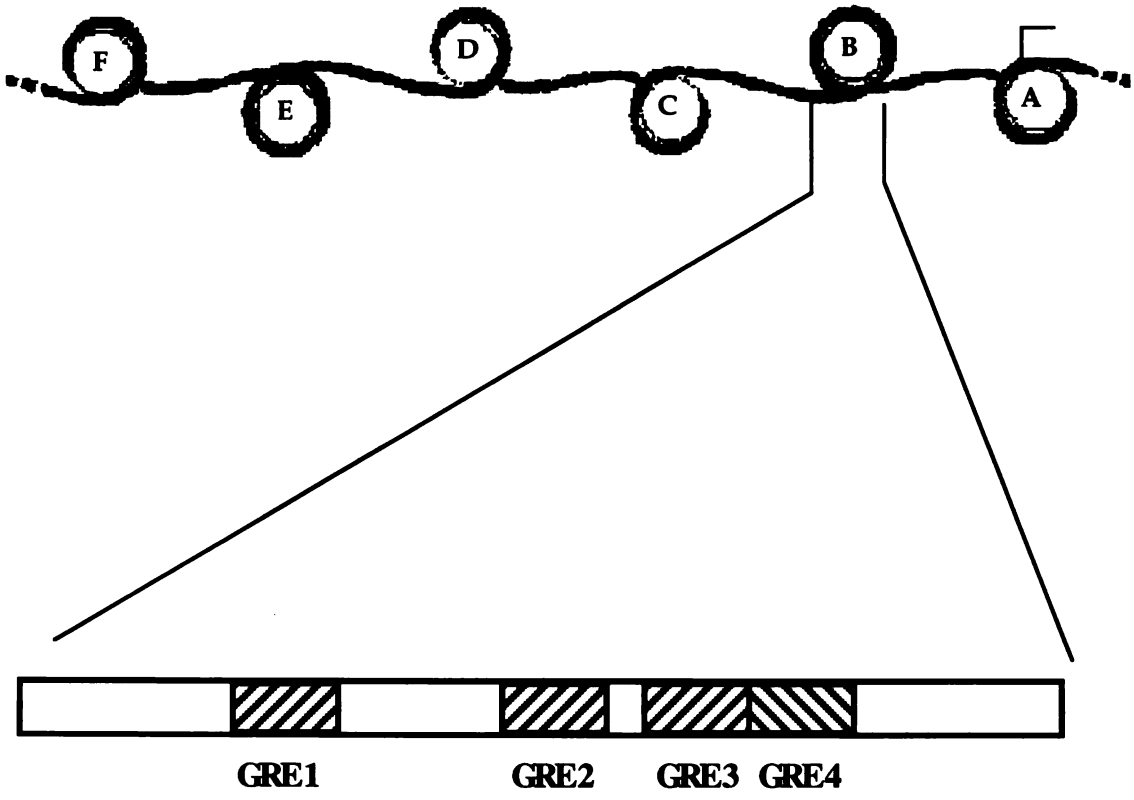
## Results

### Nucleosome Assembly

The region of the MMTV LTR critical for transcriptional activation by the glucocorticoid receptor (GR) lies between positions -184 and -78 relative to the transcriptional start site. These glucocorticoid response elements (GREs) are encompassed by the region defined as nucleosome B (Richard-Foy and Hager, 1987). The MMTV LTR has 4 GREs in the nucleosome B region (figure 10): the 5' most GRE, here termed GRE1, lies between positions -184 and -170 while 2 other GREs, GRE3 and GRE4, are positioned immediately adjacent to each between positions -107 and -78. An additional GRE, GRE2, is situated between positions -128 and -114. Each of these sites contains one consensus half-site TGTTCT and mutation of any of these sites reduces hormone

**Figure 10:** The Nucleosome B Region of the MMTV LTR.

Nucleosome B from the MMTV LTR is broadly defined as covering sequences from -250 to -60 relative to the transcriptional start site. Nucleosome B has recently been shown to occupy multiple positions within this region (see discussion). The positions of the GREs within the fragment are indicated by the hatched boxes. The sequences of the GRE half-sites are compared to that of an indealized palindromic GRE and are drawn in bold.




| | | | |
|---|---|---|---|
| **PALINDROMIC GRE** | **AGAACA** | NNN | **TGTTCT** |
| **GRE 1** | **GTTACA** | AAC | **TGTTCT** |
| **GRE 2** | **GGTATC** | AAA | **TGTTCT** |
| **GRE 3** | **AGCTCT** | GAG | **TGTTCT** |
| **GRE 4** | **ATTTTC** | CTA | **TGTTCT** |

responsiveness in transfection assays *in vivo* (Buetti and Kuhnel, 1986; Chalepakis et al., 1988).

Nucleosomes were reconstituted from core histones purified from calf thymus. Histones were assembled into nucleosomes on a radiolabeled 166 bp DNA fragment that included MMTV LTR sequences from -213 to -54 using the salt-gradient dialysis technique followed by purification on a sucrose gradient. Gradient fractions were monitored on nondenaturing polyacrylamide gels. Fractions containing nucleosomes and a minor amount of free DNA were used for GR binding experiments. The results of a typical assembly can be seen in the nucleosome containing lanes of figure 14. Identical results were also obtained using nucleosomes reconstituted from 200 bp DNA fragments, comprising MMTV LTR sequences from -234 to -36.

**Affinity of GR-DBD for Nucleosome B GREs**

DNase I footprinting was used to assess binding of GR to the nucleosomal GREs. An 86-amino-acid fragment from the rat glucocorticoid receptor, which spans amino acid residues 440-525, is sufficient for specific interaction with GREs both *in vivo* and *in vitro.* On naked DNA, the DNA binding domain of GR (GR-440-525) produced detectable DNase I footprints over all four MMTV GREs (figures 11 and 12). The affinity for these sites was determined by measuring the amount of GR 440-525 required to produce half-maximal protection from cleavage by DNase I. GR bound to these sites as naked DNA within a 5-fold range of affinities (Table 1); GREs 1 and 2 had the strongest affinity while GRE 4 had the weakest.

Digestion of nucleosomes with DNase I yielded a distinct DNA cleavage pattern , cutting at exposed minor grooves present on the surface of the nucleosome. This cleavage pattern resulted in an approximate 10 bp

**Figure 11:** DNase I Footprints of the Bottom Strand of the MMTV LTR.

The open boxes show the positions of the footprints of each of the MMTV nucleosome B GREs as free DNA. GR 440-525 was bound to free DNA at a concentration of 37 nM. GR 440-525 was bound to the nucleosome at 0.3, 1.24, 3.0, 12.4, 30, 124, 300 and 900 nM. The concentration of the DNA is ~20 pM. The positions of the ~10 bp ladder of DNase I cleavages on the nucleosome are indicated.

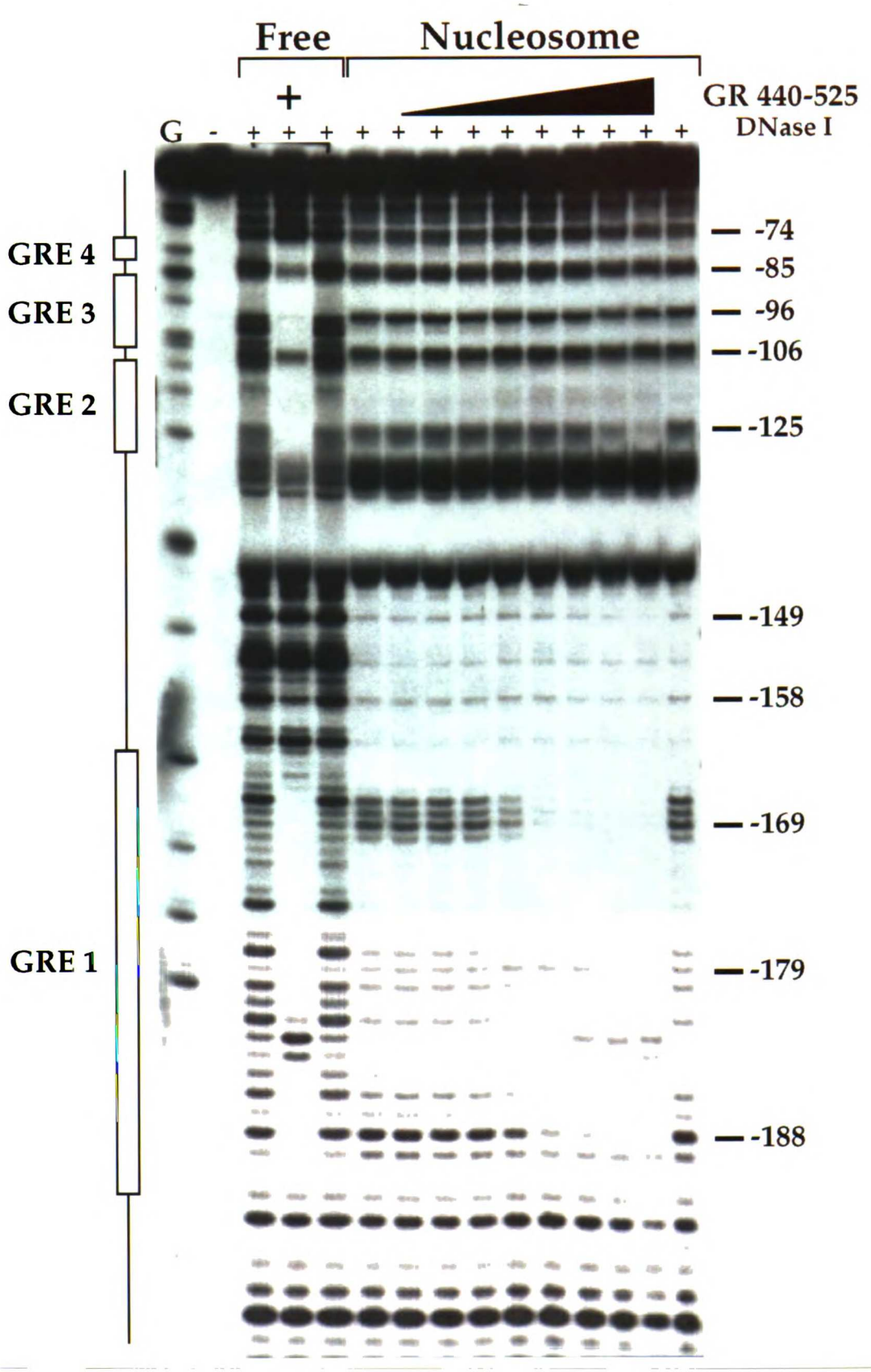

Free
Nucleosome
+
GR 440-525
DNase I
G - + + + + + + + + + + + +
GRE 4
GRE 3
GRE 2
GRE 1
-74
-85
-96
-106
-125
-149
-158
-169
-179
-188

**Figure 12:** DNase I Footprints of the Top Strand of the MMTV LTR.

The open boxes show the positions of the footprints of each of the MMTV nucleosome B GREs as free DNA. GR 440-525 was bound to free DNA at a concentration of 37 nM. GR 440-525 was bound to the nucleosome at 0.3, 1.24, 3.0, 12.4, 30, 124, 300 and 900 nM. The concentration of the DNA is ~20 pM. The positions of the ~10 bp ladder of DNase I cleavages on the nucleosome are indicated.

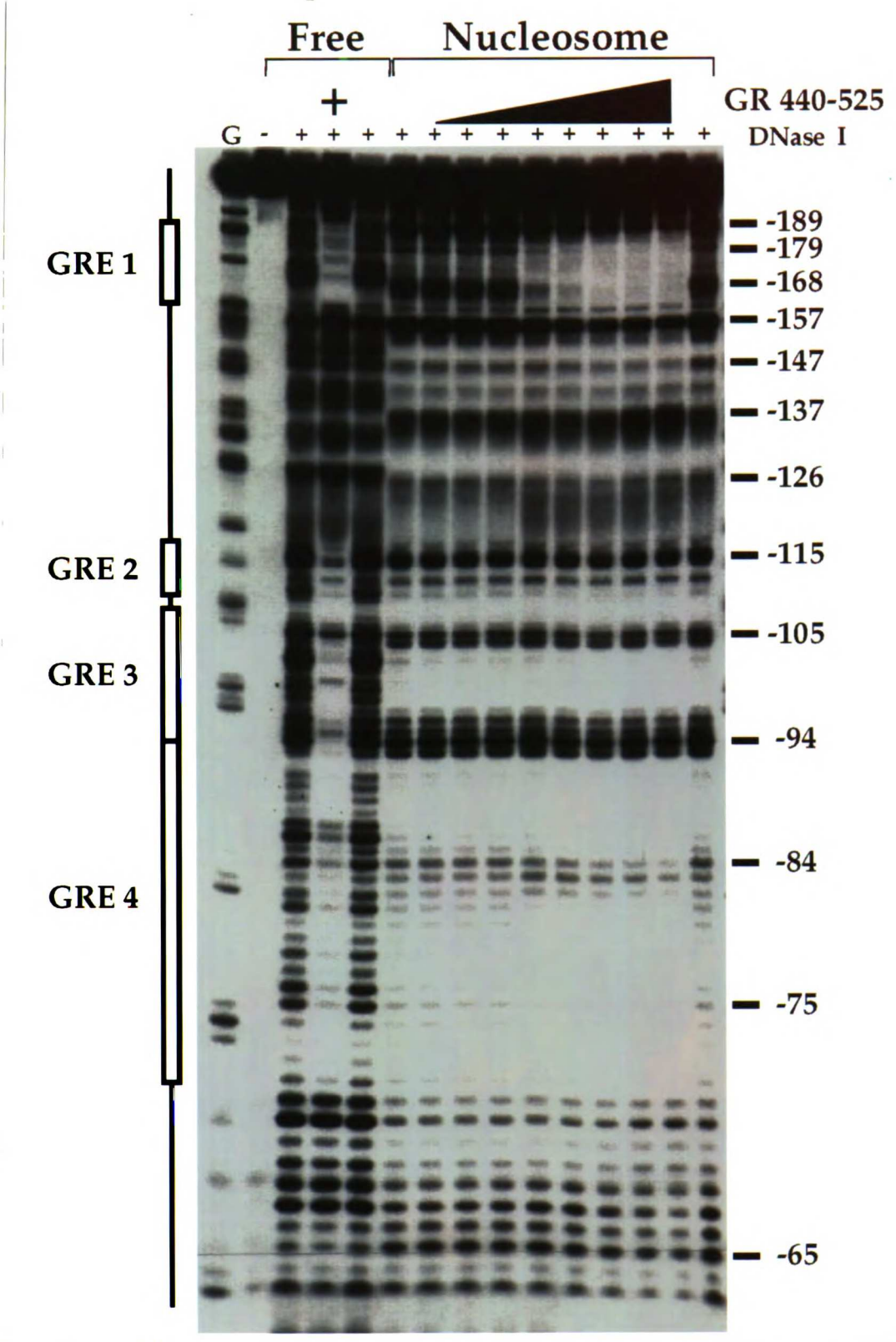

Free
Nucleosome
+
GR 440-525
G - + + + + + + + + + + + +
DNase I
GRE 1
GRE 2
GRE 3
GRE 4
-189
-179
-168
-157
-147
-137
-126
-115
-105
-94
-84
-75
-65

**Table I:** Apparent Affinity of GR 440-525 For the MMTV GREs.

Affinity for each nucleosomal GRE was determined from three different nucleosomal preparations and represents six independent binding experiments. The $K_d$ for each GRE as free DNA were determined from four independent binding experiments for GREs 1, 3 and 4 and two experiments for GRE 2.

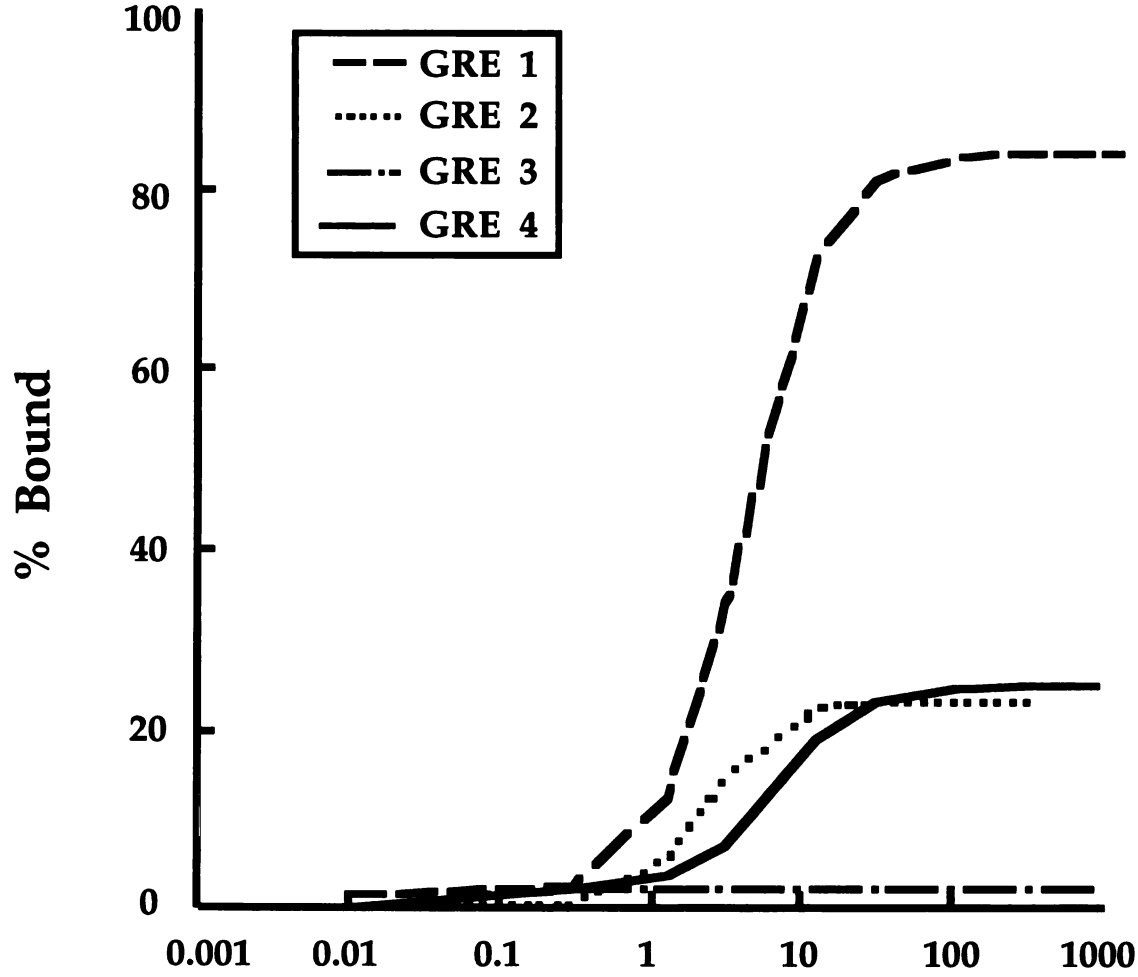
| GRE | $K_d$ (nM) |
|---|---|
| *Naked DNA* | |
| GRE1 | 3.2 ±0.4 |
| GRE2 | 2.4 ±0.7 |
| GRE3 | 6.0 ±0.8 |
| GRE4 | 17 ± 2 |
| *Nucleosomal DNA* | |
| GRE1 | 3.9 ±0.6 |

ladder, which can be seen in the -GR lanes of figures 11 and 12. On naked DNA, addition of GR resulted in detectable footprints over all four GR binding sites. Binding at GRE 1 also resulted in a DNase I hypersensitive site. Footprinting of the bottom strand of nucleosomal DNA produced strong protection of GRE 1 while no binding was observed at GREs 3 and 4. At very high concentrations of GR 440-525 some protection could be seen at GRE 2. One footprinting experiment of the top strand showed some protection of a few faint bands immediately adjacent to GREs 2 and 4; this titration is shown in figure 12.

The affinity of GR 440-525 for the MMTV GREs, as free DNA, varied over a 5-fold range, while their affinity was quite different on nucleosomal DNA. The affinity for GRE 1 was nearly unchanged compared to free DNA. A barely detectable percentage of nucleosomes are occupied at GREs 2 and 4 (figure 13). This low level of binding may represent binding to the small amount of free DNA in the sucrose gradient fraction and was well within the error range of the footprinting assay and thus did not yield a meaningful $K_d$. No protection was observed at all at GRE 3. In fact, after normalizing to a reference band, a very slight increase in DNase I cutting was observed at this site. Other studies examining GR binding to nucleosome B have reported a more dramatic hypersensitivity in this region (Perlmann, 1992) while another observed no change (Pina et al., 1990). Since we detected only a very slight increase in cutting at GRE 3, with no discrinable plateau, we concluded that the DNA binding domain of GR efficiently occupies nucleosome B only at GRE 1. Furthermore, occupancy of GRE 1 showed no change in the nucleosome induced 10 bp cutting pattern, indicating that the histones were probably still bound to the DNA.

**Figure 13:** Degree of Saturation of the MMTV GREs as a Function of GR 440-525 Concentration.

Binding curves for each of the nucleosomal GREs are shown. The curves represent pooled data from six separate binding experiments. The small amount of binding that can be measured at GREs 2 and 4 had a high degree of error and did not yield a meaningful $K_d$. Binding to GRE3 is drawn as a flat line since the slight increase in cleavage observed at this site is not a reliable measure of occupancy.


100
80
60
40
20
0
% Bound
GRE 1
GRE 2
GRE 3
GRE 4
0.001
0.01
0.1
1
10
100
1000
nM GR 440-525

**Gel Mobility Shift of Nucleosome B**

To confirm these results and to discard the possibility that GR may be able to occupy the nucleosome at GREs 2 and 4 without producing a discernible footprint, we examined the binding of GR to nucleosome B by an independent method. GR binding to either free DNA or nucleosomes can be determined by an electrophoretic mobility-shift assay. Recombinant GR 407-525 fused to ENH2 (107-318), an activation domain from the amino terminal region, was used for gel shift assays because this larger derivative of the receptor gave an easily visualized mobility change. This protein, termed EX525, bound naked MMTV GREs with comparable affinity to GR 440-525 in DNase I footprinting assays and also only bound GRE 1 on nucleosome B (data not shown).

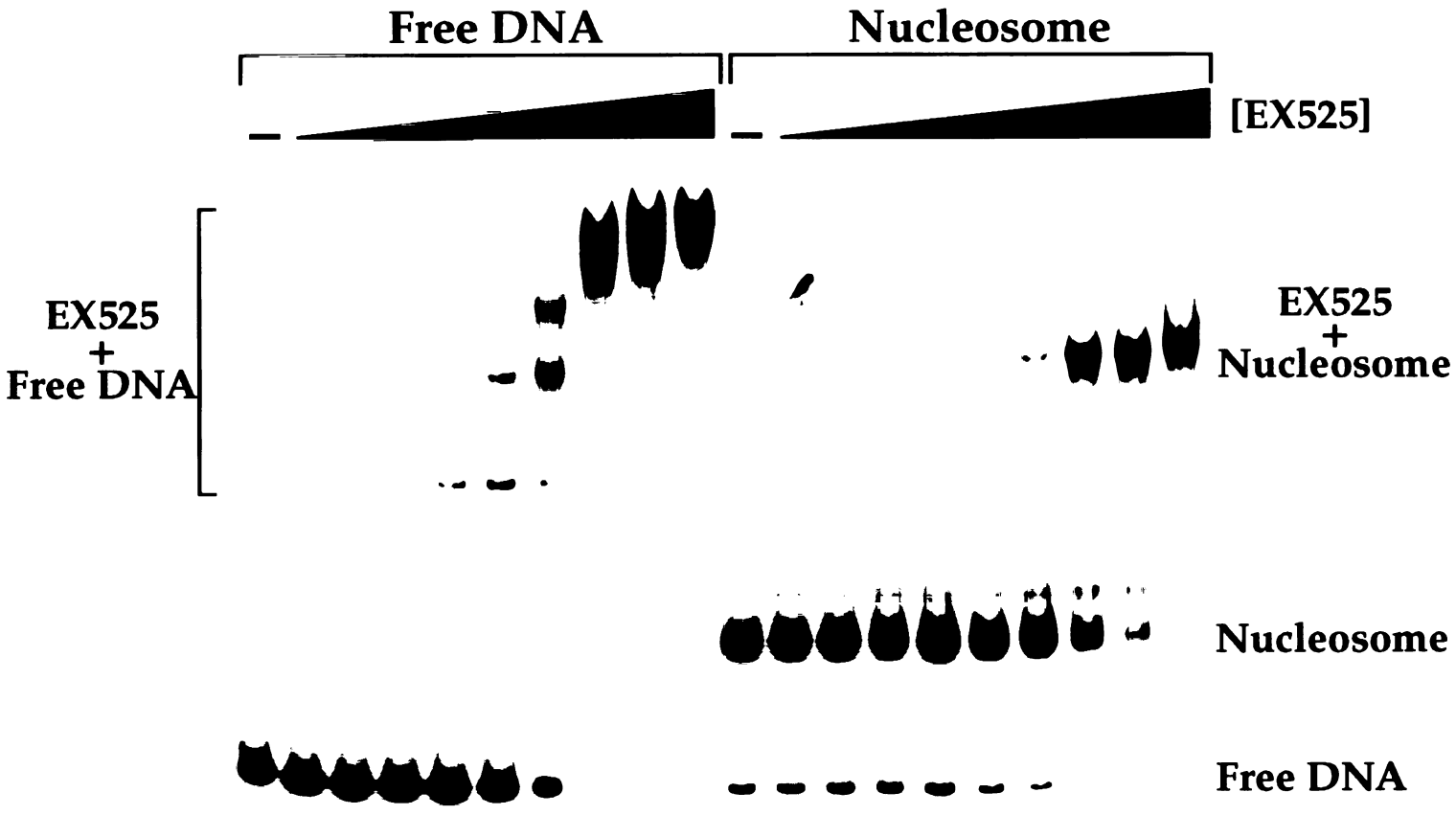
Incubation of EX525 with free DNA produced multiple shifted species, presumably representing binding to different combinations of the four GREs. At high concentrations of EX525 all of the fragment was shifted (Figure 14). Binding to the nucleosome occured with the same apparent affinity but, in contrast to the naked DNA, the nucleosome shifted to only one position. This can be taken as evidence that only one of the GREs was occupied. The results from the DNase I footprinting indicate that the single site occupied was GRE1. The fact that this position is different from any of the GR-DNA complexes again indicated that GR and the core histones can co-occupy the same piece of DNA.

**Gel Mobility Shift of Nucleosome B GRE mutants**

In order to rigorously determine which GRE(s) is occupied by GR in the nucleosome, two different sets of mutations were constructed. The conserved TGTTCT sequence of the GREs were mutated at positions where GR makes

**Figure 14:** Gel Mobility Shift of GR On Naked and Nucleosomal DNA.

Increasing concentrations of EX525 were incubated with end-labeled naked or nucleosomal DNA and the complexes were resolved on a 5% nondenaturing polyacrylamide gel, 1X TGE. EX525 was added in 2 to 2.5-fold increments ranging from 0.01 to 20ng.


Free DNA
Nucleosome
[EX525]
EX525
+
Free DNA
EX525
+
Nucleosome
Nucleosome
Free DNA

important base contacts, as determined from the protein crystal structure of GR 440-525 (Luisi et al., 1991). Two different mutations were made: one has the TGTTCT sequence of GRE 1 altered, and the other changing the TGTTCT sequences of GREs 3 and 4. Figure 15 shows that GR 440-525 was no longer capable of occupying the mutated GREs as free DNA.

If GRE 1 was in fact the only site occupied by GR in nucleosome B, then mutation of this site should abolish binding in the gel shift. As shown in figure 16, EX525 bound to the wild-type nucleosome as well as to the contaminating free DNA present in the nucleosome preparation. At GR concentrations where nearly all of the wild-type nucleosome was bound, no binding to the GRE 1 mutant nucleosome could be seen. The limited shifts that are observed in the mutant nucleosome lanes can be attributed to the small amount of contaminating free DNA present in the sucrose gradient fraction.

If GREs 3 and 4 are not occupied in nucleosome B, then nucleosomes with mutations in the TGTTCT sequences of GREs 3 and 4 should show binding similar to wild-type. Figure 17 shows that increasing amounts of EX525 incubated with either mutant or wild-type nucleosomal DNA yielded identical gel shifts. Thus, by both gel-shift assay and DNase I footprinting, GRE1 is the only site that GR-DBD is capable of occupying on nucleosome B.

High affinity natural GRE sequences are characterized by a partially palindromic structure (Jantzen et al., 1987). Of the four MMTV GREs, only GRE1 contains a second half-site that is partially symmetrical with the conserved TGTTCT sequence. We wished to determine whether it was because of the poorly matching half sites of the other GREs that GR 440-525 failed to bind them in a nucleosome. To test this idea, we mutated the non-

**Figure 15:** DNase I Footprinting of MMTV GRE Mutants.

GR 440-525 was incubated with end-labeled DNA at a concentration of 50 nM. Occupancy of the GREs was determined by DNase I footprinting. The open boxes show the locations of the footprints over the wild-type GREs. In the mutant GREs, the conserved half-sites have been changed from TGTTCT to TACTAT.

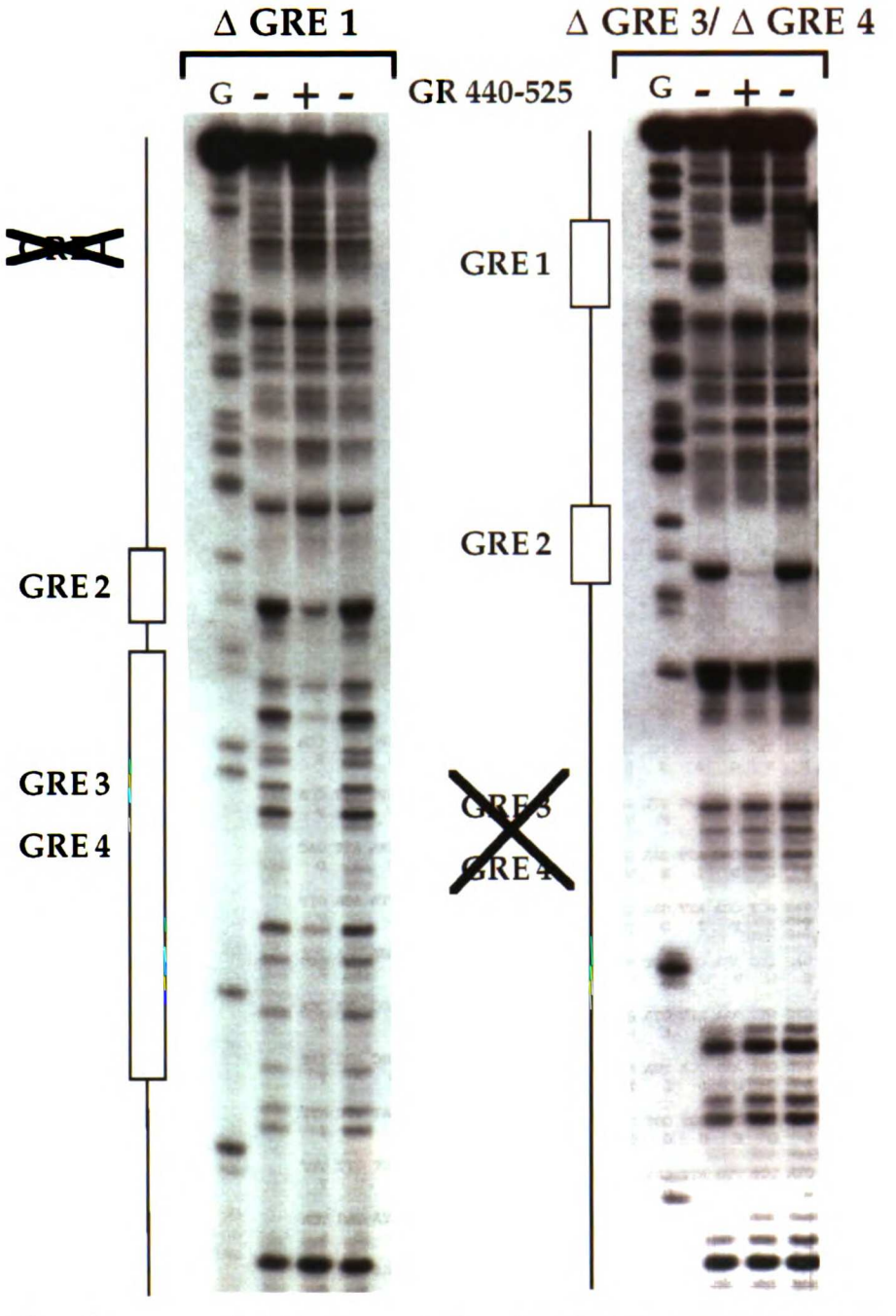

Δ GRE 1
G - + -
GR 440-525
Δ GRE 3/ Δ GRE 4
G - + -
GRE 1
GRE 2
GRE 3
GRE 4
GRE 2
GRE 3
GRE 4

**Figure 16:** Gel Mobility Shift of GRE 1 Mutants.

Increasing concentrations of EX525 were incubated with either wild-type nucleosomes or nucleosomes containing a mutation in GRE 1. The complexes were resolved on a 5% nondenaturing polyacrylamide gel, 1X TGE. Lanes 1 and 8 contain no EX525, while lanes 2-7 contain 0.05, 0.125, 0.3, 0.75, 1.5 and 3.0 ng of EX525. Lanes 9-11 contain 0.125, 0.75 and 3.0 ng of EX525.

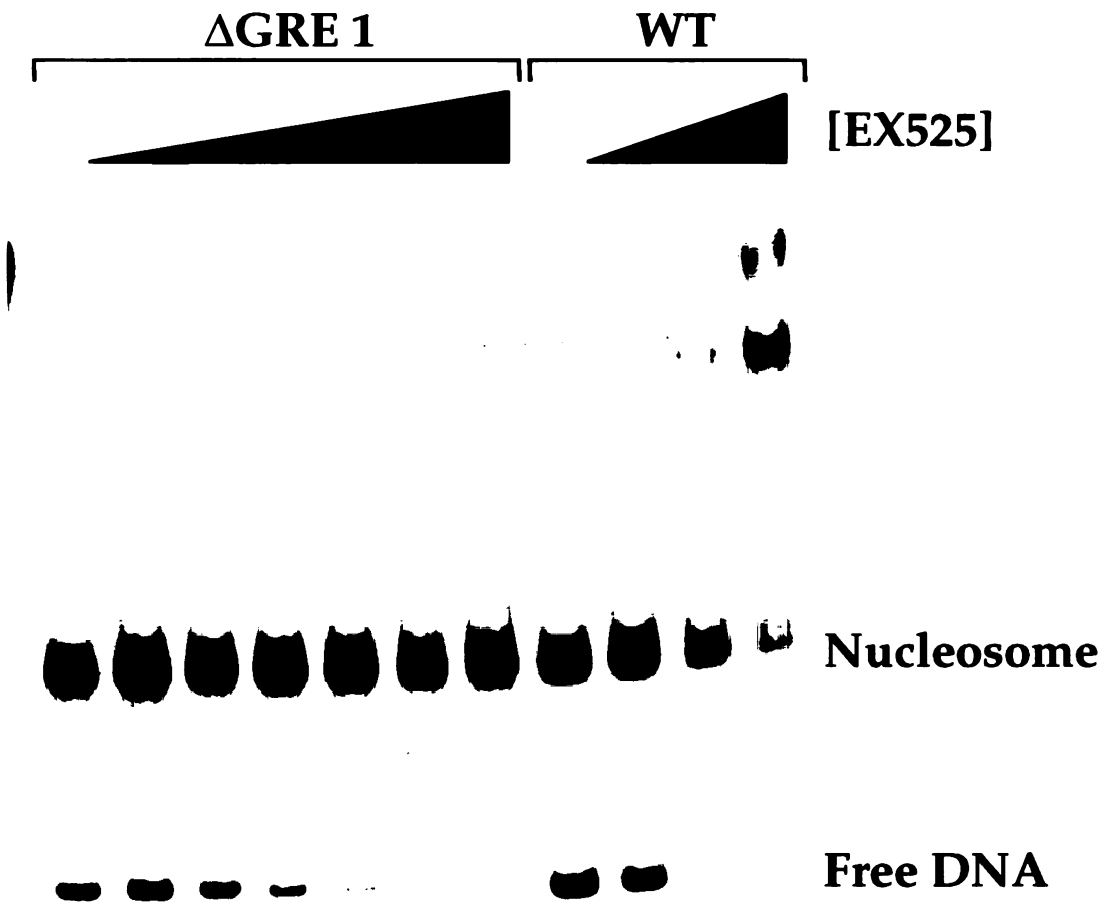
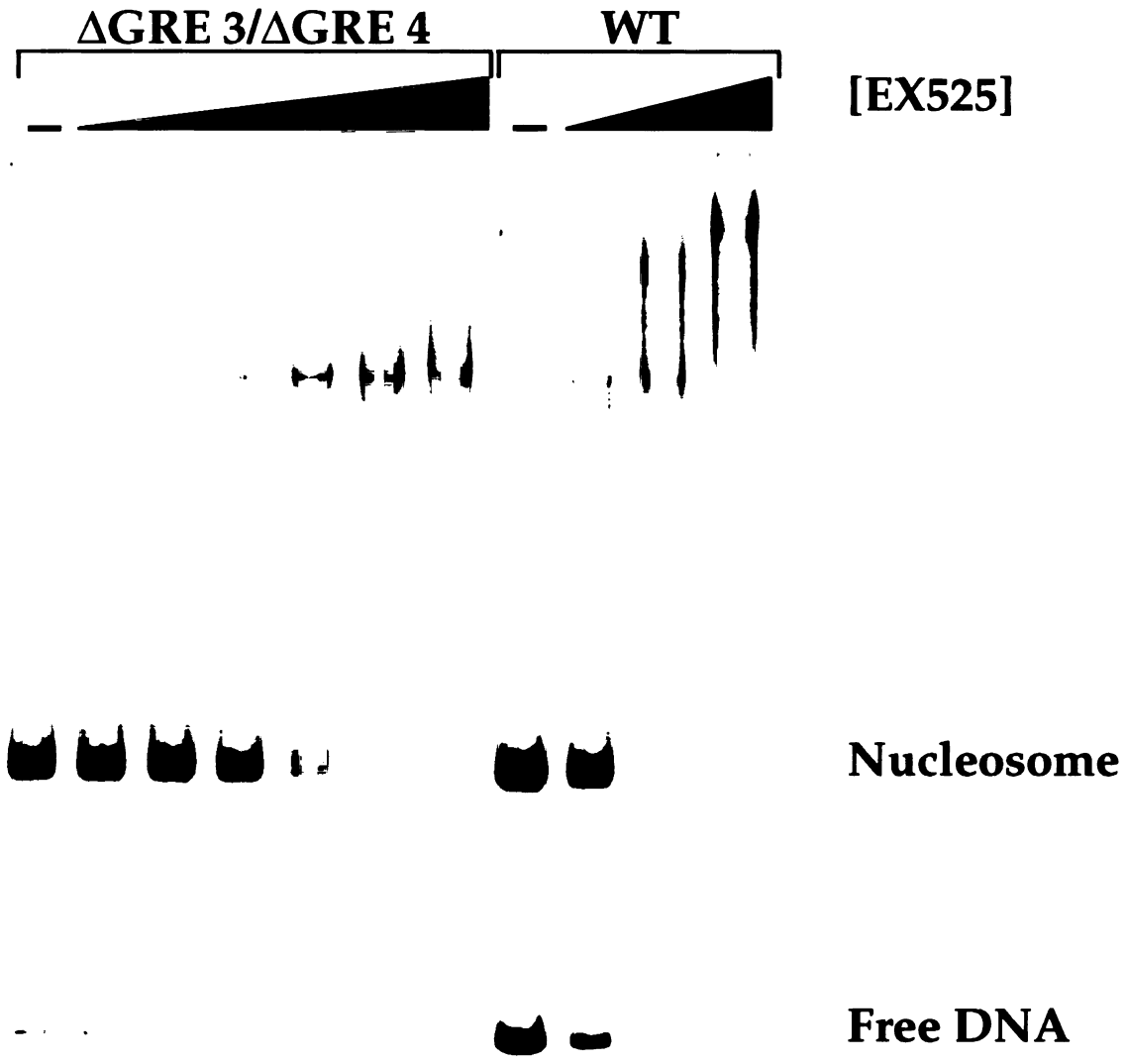

ΔGRE 1
WT
[EX525]
Nucleosome
Free DNA

**Figure 17:** Gel Mobility Shift of GRE 3 and 4 Mutants.

Increasing concentrations of EX525 were incubated with either wild-type nucleosomes or nucleosomes containing mutations in GREs 3 and 4. The complexes were resolved on a 5% nondenaturing polyacrylamide gel, 1X TGE. Lanes 1 and 8 contain no EX525, while lanes 2-7 contain 0.05, 0.125, 0.3, 0.75, 1.5 and 3.0 ng of EX525. Lanes 9-11 contain 0.125, 0.75 and 3.0 ng of EX525.


ΔGRE 3/ΔGRE 4
WT
[EX525]
Nucleosome
Free DNA

conserved half-sites of GREs 3 and 4 to that of GRE 1. If GR could now bind these GREs then a slower migrating complex should be observed in the gel shift.

Figure 18 shows that EX525 bound to both the wild-type and the mutant nucleosomes to a similar extent, indicating that it is not because of the sequence of the GREs that GR shows preferential binding to GRE1 in nucleosome B.

**Rotational Position of the Nucleosomal MMTV GREs**

Since GR 440-525 binds to one face of the DNA helix (Luisi et al., 1991) it is possible that the rotational position of the GREs on the surface of the histone octamer can account for the differences in binding affinity. The positions of the DNase I cuts within the GREs defines the face of the DNA helix that is oriented outward from the surface of the histone octamer (Drew and Travers, 1985). Figure 19A-C shows the orientation of the individual GREs on the surface of the nucleosome. GREs 2 and 3 have the same orientation, such that the face of the DNA helix that is bound by GR 440-525 overlaps extensively with the site of histone binding. GRE 1 is turned almost completely outward on the surface of the octamer. Figure 19A clearly shows how GR 440-525 can co-occupy GRE 1 with a nucleosome. The orientation of GRE4 is tilted slightly with respect to GRE 1, but it appears that the GRE is oriented such that the histone octamer would not occlude binding to this site by GR 440-525. Furthermore, the tilt orients the GRE away from the adjacent superhelical turn of DNA. Because DNase I shows sequence preferences in its sites of cleavage, this is not a rigorous proof of the rotational orientation of the GREs and, thus, only defines a small range of possible orientations. Therefore, additional studies will be needed before it is possible to determine

**Figure 18:** Gel Mobility Shift of GRE 3•1 and 4•1 Mutants.

The non-conserved half-sites of GREs 3 and 4 were altered to be the same as that of GRE1, GTTACA. Increasing concentrations of EX525 were incubated with either wild-type nucleosomes or nucleosomes containing mutations in GREs 3 and 4. The complexes were resolved on a 5% nondenaturing polyacrylamide gel, 1X TGE. 0.05, 0.125, 0.3, 0.75, 1.5 and 3.0 ng of EX525 was added to wild-type or mutant nucleosomes.

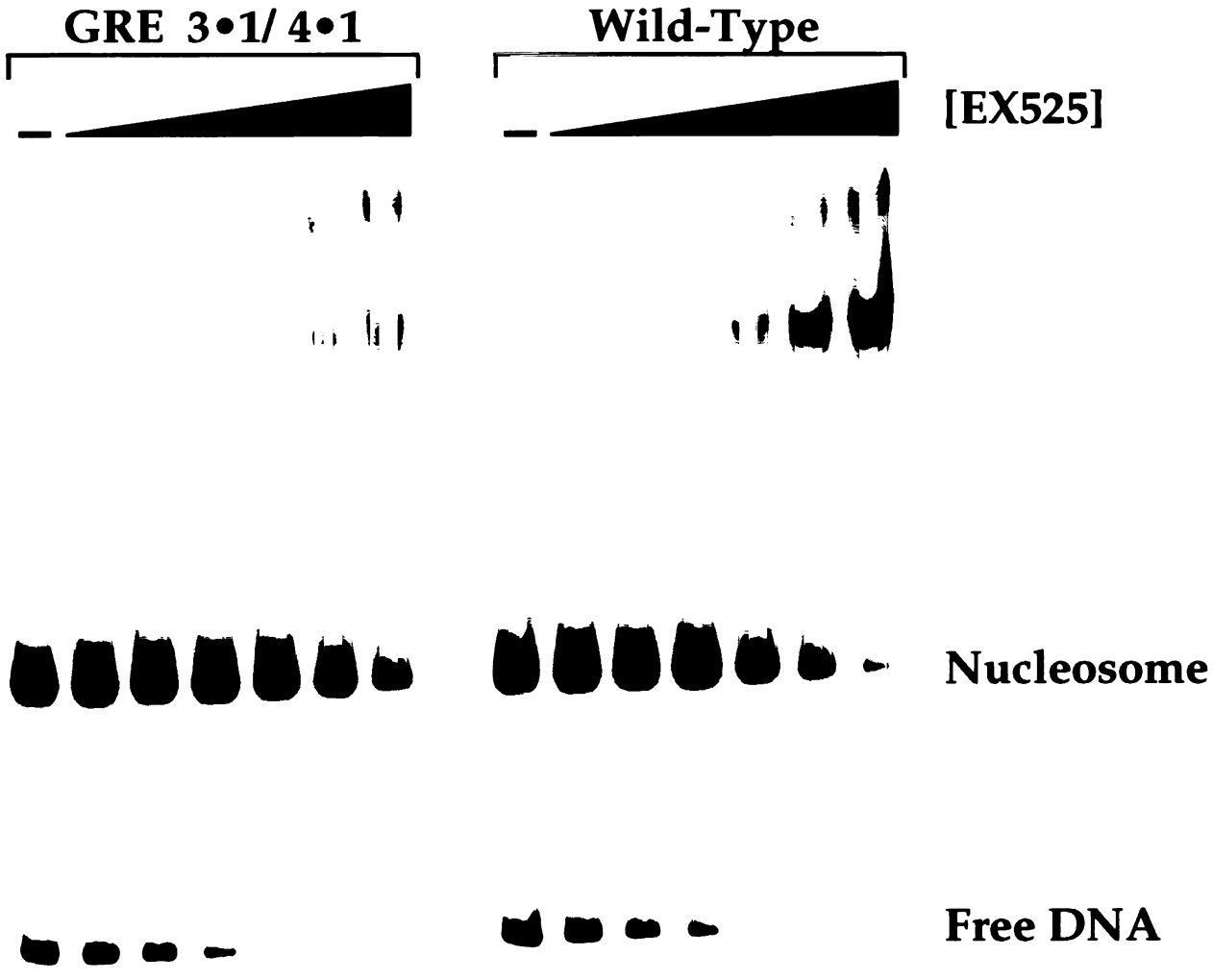

GRE 3•1/ 4•1
Wild-Type
[EX525]
Nucleosome
Free DNA

**Figure 19:** Rotational Position of the MMTV GREs.

An end view of the crystal structure of GR 440-525 (shown in purple) bound to an idealized palindromic GRE (shown in green) was used to show the orientation of the GRE on the surface of the nucleosome. The position of the DNase I cut within the GRE on the nucleosome is indicated by the red ball. The top of the figure indicates the face of the DNA helix that is oriented towards solution whereas the bottom indicates the face oriented towards the histone core.

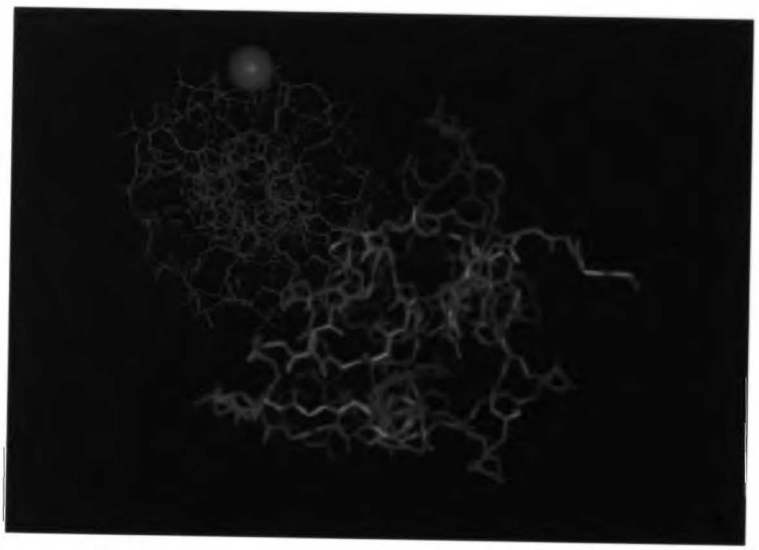
**Panel A.** Orientation of GRE 1.

**Panel B.** Orientation of GREs 2 and 3.

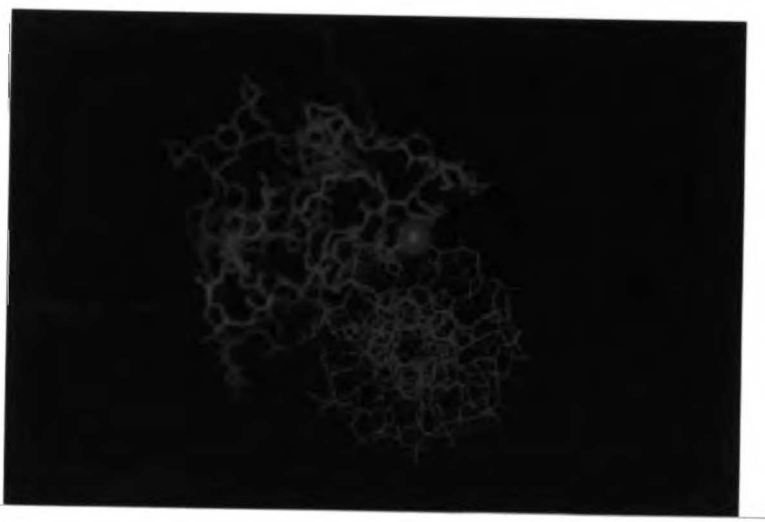
**Panel C.** Orientation of GRE 4.

A

B



C



whether the orientation of GRE 4 on the octamer is sufficient to block occupancy of this site by GR 440-525.

## Discussion

The MMTV LTR contains multiple binding sites for the glucocorticoid receptor. As naked DNA, these sites had a similar affinity for the DNA binding domain of GR; their $K_d$'s differed by no more than 5-fold. We have shown, using two different techniques, that when the DNA was packaged into a nucleosome GR 440-525 selectively bound only one of the possible GREs. The GR 440-525 bound to a nucleosomal GRE 1 with an affinity that was within 2-fold of the affinity for naked DNA. Even though the affinity for GREs 2, 3 and 4 was equal to or slightly weaker than GRE 1 as naked DNA, very little binding to these sites could be detected in a nucleosome.

Other reports using nucleosomes reconstituted from less defined sources and with full-length GR have arrived at differing conclusions. While one study reported binding to GREs 1 and 4 but not GREs 2 and 3 (Pina et al., 1990), others observed occupancy of all four sites (Perlmann and Wrange, 1988; Perlmann, 1992). The affinities of GR for the individual sites as naked DNA were not determined. In all of these studies, occupancy of the GREs was determined either by DNase I protection or hypersensitivity. While protection from DNase I provides a reliable measure of GRE occupancy, quantitation of DNase I hypersensitivity is not imformative in this regard. Given the varied effects of GR on DNase I cleavage of nucleosome B DNA we also assessed binding to nucleosomes using a gel-mobility shift assay. A nucleosome with mutations in GRE 1 showed no binding while mutations in sites 3 and 4 showed binding that is indistinguishable from that of wild type

nucleosomes. Interestingly, other studies examining GR binding to nucleosome B by mobility shift have also shown only one shifted complex (Pina et al., 1990; Archer et al., 1991).

Selective occupancy of GRE 1 is paradoxical given that all of the sites have been shown to be important *in vivo* for glucocorticoid mediated stimulation of transcription (Buetti and Kuhnel, 1986; Chalepakis et al., 1988). Consistent with our data, the one study to examine the transcriptional induction of MMTV LTR mutants in stably transfected cells has demonstrated a stronger dependence on GRE 1 (Buetti and Kuhnel, 1986). It is possible that occupancy of GREs 2, 3 and 4 requires domains of the receptor not present in the DBD. It is important to note that another study using the full-length receptor has also demonstrated a selective interaction with the MMTV GREs (Pina et al., 1990). Perlmann et al. (Perlmann et al., 1990) have reported that the full-length protein binds cooperatively to GREs 2-4 as naked DNA. Such interactions may be important for occupancy of these sites in nucleosome B.

The affinity of GR for a nucleosomal GRE is known to be very sensitive to the rotational and translational position of the GRE within the nucleosome (Li and Wrange, 1993; Li and Wrange, 1995). Other studies have indicated that the MMTV GREs are differentially situated on the surface of the histone octamer (Perlmann and Wrange, 1988; Pina et al., 1990). From the positions of the DNase I cleavages as shown in figures 11 and 12, our data is consistent with GREs 1 and 4 facing outward from the nucleosome core. DNA sequences flanking the GREs are known to be important for determining occupancy by GR (La Baer and Yamamoto, 1994) and may explain why binding of GRE 4 is not observed here.

Another consideration is that the nuclesome we have reconstituted *in vitro* may be different from what exists *in vivo*. A recent study using high

resolution mapping techniques has indicated that positioning of the MMTV nucleosomes may not be as precise as previously thought (Fragoso et al., 1995). Nucleosome B was shown to exist in various positions in a given population of cells, with some positions preferred over others. This would indicate that a given MMTV GRE can exist in a variety of translational, and possibly rotational, positions within nucleosome B. Thus, transcriptional induction in response to hormone may be restricted to cells with a specific phasing of nucleosome B (Fragoso et al., 1995).

Given that all four GREs are important for activation of transcription, it may be that GREs 2 -4 function similarly to the NF-1 site; they require modification of nucleosomal structure before occupancy can be achieved. Since it has not been possible to observe occupancy of MMTV by GR *in vivo* (Archer et al., 1992; Truss et al., 1995), there is no direct evidence for this model as of yet.

What is the mechanism by which GR elicits an alteration of nucleosomal structure *in vivo*? GR may directly destabilize the nucleosome upon binding, as has been shown for the yeast regulatory factor GAL4 (Workman and Kingston, 1992). We view this as unlikely given that five GAL4 dimers were needed to destabilize nucleosomes while only one GR dimer binds to nucleosome B. Furthermore, binding of the receptor to GRE 1 results in no apparent change in the nucleosome dependent DNase I protection pattern. Other studies using full-length GR also observed no change in the DNase I pattern, although one did detect an alteration of the nucleosome borders (Pina et al., 1990). A number of biochemical activities that are capable of nucleosome destabilization have recently been described (Cote et al., 1994; Tsukiyama and Wu, 1995). The most intensively studied is the SWI/SNF complex (Peterson and Tamkun, 1995). Given that in some

cellular contexts GR has been shown to be SWI/SNF dependent (Yoshinaga et al., 1992), GR bound at GRE 1 may recruit SWI/SNF or a SWI/SNF-like activity to facilitate occupancy of GREs 2-4 and to enable NF-1 binding. A simpler model is that interactions of GR with the general transcription machinery is sufficient to disrupt nucleosome structure and allow the binding of additional factors. With the observation that the yeast SWI/SNF complex may exist in a complex with the RNA polymerase holoenzyme (Wilson et al., 1996) these may not be mutually exclusive models.

The regulatory regions of most genes from higher eukaryotes are exceedingly complex and bind a multitude of transcriptional regulators. Our results illustrate that even though nucleosomes can dramatically alter the affinity of transcriptional regulators for their binding sites, the ability to bind nucleosomes need not be a requirement for a transcription factor to function. It may be that prior occupancy of another activator may be sufficient to initiate chromatin alterations that allow additional regulators to bind. This would allow the cell to build layers of transcriptional control without having to contend with the specific chomatin structure of a gene.

# PERSPECTIVES

Ever since it was observed that eukaryotic DNA is highly compacted *in vivo* there has existed the idea that chromatin plays a negative role in the regulation of gene expression. Highly condensed regions of the genome, known as heterochromatin, are transcriptionally inactive. Heterochromatin is thought to maintain the repressed state of select domains of the genome through multiple cell divisions and has been linked to the phenomenon of position effect variegation (Henikoff, 1990), mammalian X chromosome inactivation (Reuter and Spierer, 1992), and silencing at yeast telomeres and silent mating type loci (Thompson et al., 1993). Nucleosomes, the simplest unit of chromatin structure, are also thought to inhibit transcription (Felsenfeld, 1992). The idea that chromatin structure must be modified to allow gene expression is a theme central to current models on the regulation of transcription.

The transcription of protein encoding genes is carried out by RNA polymerase II. In order to initiate transcription from the proper genomic loci the polymerase must bind a gene promoter in concert with a host of general transcription factors, or GTFs. The regulation of transcription is achieved through the combined action of positively and negatively acting regulatory proteins. One class of positively acting factors, referred to here as activators, contain a site specific DNA binding domain as well as a transcriptional activation domain. Activators have been shown to stimulate transcription on naked DNA *in vitro*. This stimulation is thought to occur through protein-protein contacts between the activation domain of the activator and one or more of the GTFs, serving to recruit them to the promoter (Lin and

Green, 1991; Stringer et al., 1990). Activators are known to act at steps following the assembly of an initiation complex but such mechanisms will not be discussed here. These *in vitro* experiments indicate that some form of transcriptional control can be achieved in the absence of chromatin.

The popular view is that activators function by additional mechanisms separate from their ability to interact with GTFs. In a chromatin context, activation is thought to result from two effects: the disruption of nucleosomal structure and the recruitment of GTFs (Grunstein, 1992). There are many examples correlating the activation of transcription with the disruption of chromatin structure. The specific nucleosomal structure of gene promoters is often altered upon gene induction (Richard-Foy and Hager, 1987; Carr and Richard-Foy, 1990; Almer et al., 1986). Yeast strains that are constructed to regulate histone expression show nucleosome loss and partial, or full, activation of a number of genes when grown under non-inducing conditions (Durrin et al., 1992). Yeast strains carrying specific mutations in the histone genes also show defects in the regulation of transcription (Durrin et al., 1991). *In vitro* transcription reactions performed on nucleosomal templates show repression by nucleosomes and the presence on an activator can overcome this repression (Workman et al., 1991; Lorch et al., 1992). Examples such as these are invoked to support the commonly held view that transcriptional activators have the property of being able to alter nucleosomal structure, either directly or by the recruitment of a separate nucleosome destabilizing activity. By this view, nucleosomes play an active role in gene regulation and the cell has evolved specific mechanisms to overcome their repressive effects.

An alternative view, equally supported by the above observations, is that nucleosomes block the assembly of a pre-initiation complex but present no barrier to their recruitment by transcriptional activators. In this model

nucleosomes are ignored by the activator; the interactions of the activator with the general transcription machinery are sufficient to recruit GTFs to a nuclesomal promoter. For example, the fortuitous interaction of the GAL4 DNA binding domain with a mutant component of the RNA polymerase holoenzyme is sufficient to confer activation of the GAL1 gene (Barberis et al., 1995). However, this does not mean that nucleosomes have no regulatory role at the GAL1 promoter. Recall that mutations of the N-terminus of histone H4 can impair GAL1 induction as much as 20-fold (Durrin et al., 1991). It will be important to test whether these mutations will impair activation in the mutant yeast strains that enable the GAL4 DNA binding domain to function as an activator. Perturbation of nucleosomal structure may be a required event for induction of the GAL1 promoter and these mutant nucleosomes may be resistant to disruption by binding of the holoenzyme.

The mouse mammary tumor virus (MMTV) long terminal repeat (LTR) contains a promoter that is packaged into phased nucleosomes. One of these nucleosomes, nucleosome B, is thought to play a key role in the regulation of promoter activity (Pina et al., 1990; Archer et al., 1991). Nucleosome B contains four binding sites (GREs) for the glucocorticoid receptor (GR) and a single binding site for the transcription factor NF-1. Hormone treatment results in the activation of transcription and the disruption of nucleosome B, as measured by the accessibility of nucleases to the underlying DNA (Richard-Foy and Hager, 1987). Nucleosome B is important for regulation in that it blocks the binding of GR to three of the GREs as well as NF-1 binding. Mutational analysis has shown all of these sites to be important for activation (Buetti and Kuhnel, 1986; Chalepakis et al.,

1988). However, regulation appears normal in transient transfection assays even though nucleosome B can no longer be observed (Archer et al., 1992). Thus the interactions of GR with the general transcription machinery are the primary determinants of the activation of transcription at the MMTV LTR. Does this mean that nucleosome B is not required for regulation?

*In vivo* footprinting has revealed that in the presence of nucleosome B the NF-1 site is not occupied. Nucleosomes have also been shown to inhibit NF-1 binding *in vitro* (Pina et al., 1990; Archer et al., 1991; Blomquist et al., 1996). By this view, disruption of nucleosome B is an obligate step in the activation of transcription: unless there is some alteration to nucleosome B a key regulatory factor cannot occupy its site. From the results presented in chapter 2, the inhibitory effects of nucleosome B can be extended to include the blocking of binding of GR to three of the GREs. Thus, it may be that binding to GRE 1 is the initial event that results in the alteration of nucleosome B. A test of this model may be slow in coming since footprinting of the GREs *in vivo* has been inconclusive; exo III digestion (Archer et al., 1992) and DMS protection (Truss et al., 1995) have failed to show occupancy of the GREs by GR.

Identification of the mechanism of disruption of nucleosome B will provide a means of determining whether chromatin is altered by an active process during the initiation of transcription. There may exist some nucleosome destabilizing activity (such as SWI/SNF) that GR brings to nucleosome B, allowing it to occupy all four nucleosomal GREs. Alternatively, GR may bind to GRE 1 and interact with the general transcription machinery, resulting in the destabilization of nucleosome B and the subsequent occupancy of the other GREs and NF-1 site. In the former

model GR does something specific to nucleosome B; in the latter, disruption of nucleosome B occurs as a passive consequence of pre-initiation complex assembly. In both models, an alteration of nucleosome B is a required event in the activation of transcription.

If GR can bind to GREs 2, 3 and 4 *in vivo* in the absence of GRE 1, this will lend support to the idea that disruption of nucleosome B occurs by an active process. That is, some other activity helps GR occupy these nucleosomal GREs. There exists the important caviat that other portions of the receptor, not present in the DNA binding domain, may facilitate DNA, and thus nucleosome, binding. The rotational, and possibly the translational, position of the GREs within the nucleosome provides a likely explanation for how nucleosomes can inhibit DNA binding. It will be important to confirm that the inhibitory effects of the nucleosome observed in chapter 2 also occur with the full-length receptor. Using artificial phasing sequences, nucleosomal binding by the full-length receptor has been shown to be dramatically affected by the rotational and translational position of the GRE (Li and Wrange, 1993; Li and Wrange, 1995). In these cases, the inhibitory effects of the nucleosome can be extended to include more than just the DNA binding domain.

The inability of GR 440-525 to occupy GREs 2, 3 and 4 on the nucleosome *in vitro* could, in principle, provide a biochemical assay to search for cellular activities that facillitate occupancy of these sites by GR. Activities that can displace nucleosomes have been purified using these types of *in vitro* assays (Tsukiyama and Wu, 1995). The difficulty with this experimental approach is to show that activities identified *in vitro* have the same function *in vivo*. This caveat exists with all *in vitro* studies but the problems associated with nucleosome destabilization are more acute. Such a laborious

procedure could easily result in the identification of a nucleosome assembly protein, such as nucleoplasmin (Chen et al., 1994).

The notion that transcriptional activators posses two properties predicts that these properties should be genetically separable. That is, it should be possible to identify an activator mutant this is capable of stimulating transcription on a naked DNA template but not a nucleosomal one, or vice versa. A good first step towards this end would be to examine *in vivc* in yeast whether GR can occupy a nucleosome with the major grooves of the GRE half-sites facing the histone octamer. Such a result would indicate that GR can overcome a nucleosomal block in the absence of a promoter. However, as in studies with gene promoters carrying mutations in the TATA box, there is the caveat that the activator may still be recruiting some component(s) of the general transcription machinery. A positive result in this system would provide an assay with which to screen through a series of GR mutants that are known to be compromised for transcriptional activation. The ability of these mutants to function *in vitro* on naked and nucleosomal DNA would a provide important information on the nature of the transcriptional defects.

The merits of undertaking such a study are debatable since the mechanism of transcriptional activation on naked DNA is still not completely understood. The targets of transcriptional activators within the pre-initiation complex are only beginning to be described. It makes sense to understand this simpler situation before undertaking the study of more complex systems. With this information in hand, it will then be possible to determine whether the mechanism of activation that occurs on naked DNA is sufficient to explain what occurs in the presence of nucleosomes.

Nucleosome alterations and the activation of transcription are so intimately linked that it has been nearly impossible to sort out the cause/effect relationship between them. The identification of a transcriptional activator specifically defective in one of these putative pathways would provide the first conclusive evidence for a chromatin dependent step in the process of transcriptional activation.

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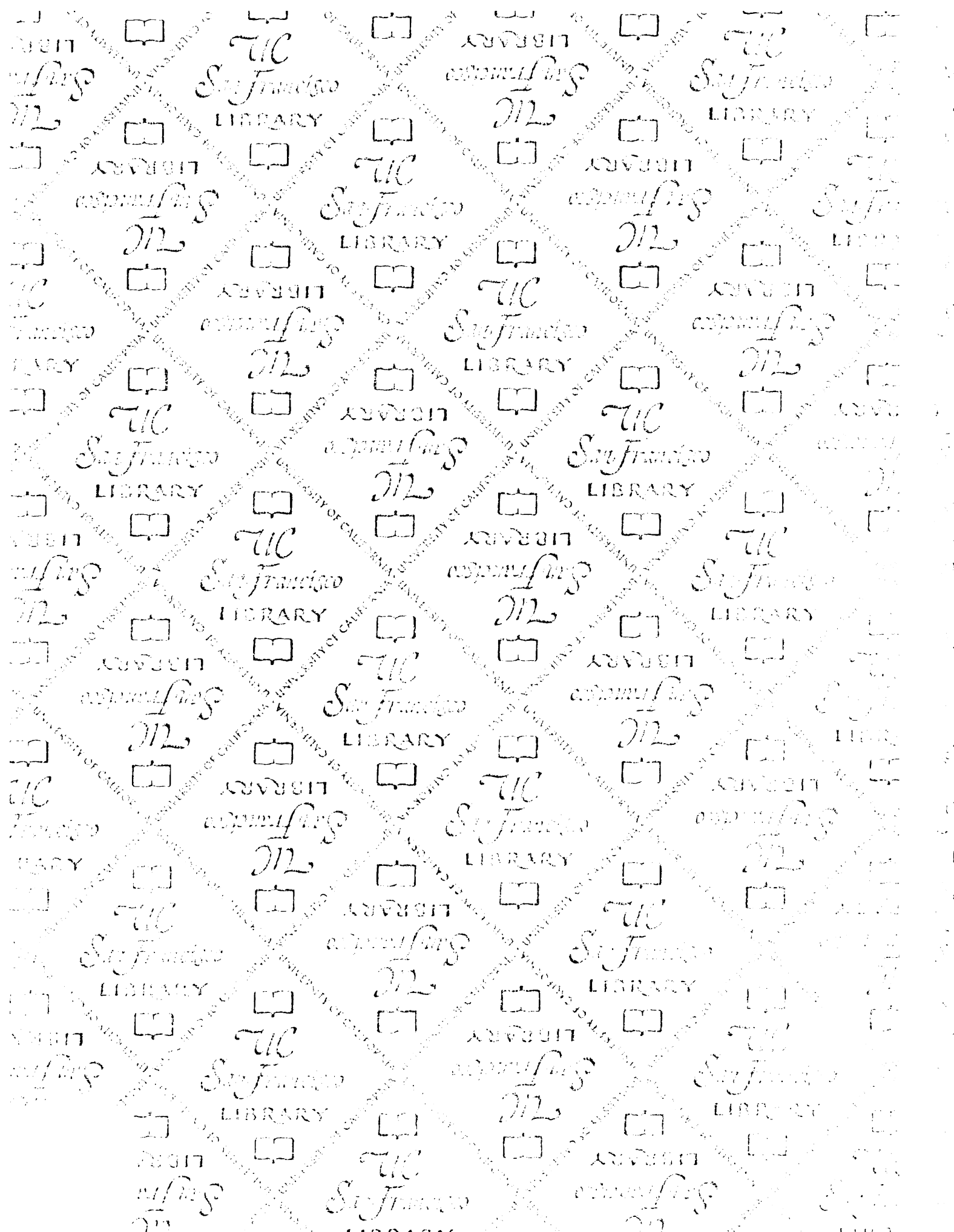
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