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CHARACTERIZATION OF THE REPRESSION OF GLUCOCORTICOID-INDUCED TRANSCRIPTION OF THE MOUSE MAMMARY TUMOR VIRUS THROUGH NEGATIVE REGULATORY ELEMENT 1

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Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Doctor of Philosophy

University of Ottawa
Ottawa, Ontario, Canada
January, 2001

ABSTRACT

The physiological effects of steroid hormones are mediated by their cognate receptors which regulate expression of target genes in response to binding of their steroid ligand. The mouse mammary tumor virus (MMTV) promoter is a widely used system for studying the effects of steroid hormones due to its strong activation by several classes of steroids. MMTV induces mammary tumors in female mice following lactation. T-cell lymphomas are also induced at much lower frequency and all cases of lymphoma that have been examined have deletions spanning a region of the long terminal repeat (LTR) from –421 to –364. This region has a transcriptional negative regulatory element called NRE1. Characterization of NRE1 revealed nuclear factors binding to the double-stranded (ds) form, as well as each of the single strands (up, lo – upper and lower strands respectively). A truncated element (MT) was also recognized but only the ds form. Kinetic studies showed binding occurred to the ds, up, lo and MT with $t_{1/2}$s of 11, 1.5, 3 and 4 min respectively. The off-rates ($t_{1/2}$) were determined to be 60 min, 30 min, 12 h and 4 h respectively. Four factors were observed to bind NRE1 in southwestern analysis, while only one was observed binding to MT, that corresponded in mobility to the smallest NRE1 binding factor. The four factors appeared to cross-react with an octamer motif. The dsNRE1 binding factor was confirmed to recognize an octamer motif in EMSA. The dsNRE1 binding factor was identified as the Ku autoantigen heterodimer. It was demonstrated to bind directly to NRE1 with an affinity of K_d = 0.84 +/- 0.24 nM. Ku also bound upNRE1 with an affinity of K_d = 3.5 +/- 1.3 mM. In kinetic analyses of purified Ku binding to upNRE1 the on-rate was determined to be $t_{1/2}$ = 1.6 min, while the

off rate was $t_{1/2}$ = 68 min. Transient transfection assays using MMTV reporters either containing or lacking NRE1 were performed. Using cell lines not expressing functional Ku or its associated factor DNA-PK it was shown that both factors were required for the NRE1 mediated repression of glucocorticoid-induced transcription of MMTV. DNA-PK was shown to specifically phosphorylate a myc-tagged glucocorticoid receptor (GR) on serine 527 (S527) *in vitro*. A second phosphorylation site was determined to result from the addition of the myc tag, while a native receptor was phosphorylated only on S527. Transient transfections using S527 mutants of GR demonstrated that subsitution of S527 for alanine abrogated NRE1 mediated repression. The same effect was observed for glutamic acid and aspartic acid substitutions. We therefore conclude that binding of DNA-PK to NRE1 through its DNA binding subunit, Ku, allows it to phosphorylate GR on serine 527 resulting in a repression of glucocorticoid-induced transcription.

DEDICATION

To my father John Rodda who may have sparked my interest in science through his demonstrations with nitrogen triiodide and liquid mercury when I was a child.

And to my mother Glenda Majkot who taught me that life is too short to take too seriously and yet too important to not take seriously enough.

Thank you both for your love and support.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor, Robert Haché for his guidance during my time in his lab. His teaching and support were essential in what proved to be a challenging project. From Rob I gained great insight into how to interpret and critically analyze experimental data, both my own and that of others. I'm very grateful for his mentoring and I'm certain that it will continue to influence me throughout my career.

I also wish to acknowledge the members of my thesis advisory committee: Drs. Mark Ekker and Johnny Ngsee for contributing their time and advice.

I wish to express my deepest thanks to Ward Giffin who contributed either directly or indirectly to many of the experiments conducted in this thesis. Ward conducted some of the repetitions of the DNA binding studies both with crude extracts and purified Ku as well as some repetitions of the original transient transfections in V79 and V15B cells. He also assisted greatly in the mapping of the DNA-PK phosphorylation sites on GR.

I would also like thank other members of the Haché laboratory for their contributions to this work. Louise Pope generated the HeLa cell line stably expressing wild type native rat GR. She also prepared the baculovirus-expressed recombinant Ku used in the DNA binding studies. Gratien Préfontaine constructed the pTL2-mycGR plasmid which was integral to this project. Dr. Joanne Savory constructed the pTL2-rGR plasmid. Claudia Bayer constructed the pTL2-rGR$_{S527A}$ and pTL2-rGR$_{S527E}$ plasmids as well as the corresponding mycGR plasmids. Dr. Joanna Kwast-Welfeld conducted the

preliminary studies that mapped the DNA-PK dependent phosphorylation site on GST-GR_{X568}. In addition she taught me most of the methodology used to identify the phosphorylation site on the full-length GR. Dr. Maya Traykova-Andonova constructed the pGEX-2T-X568 plasmid.

I am grateful to Dr. Keith Yamamoto who provided the p6RGR plasmid and Dr. Gilbert Chu who provided the pBJ6-Ku80 plasmid.

Finally I would like to thank all the members of the Haché and Lefebvre laboratories, as well as members of the Hormones, Growth and Development group of the Loeb Health Research Institute, both past and present, for their friendship, technical advice, encouragement and support.

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ABBREVIATIONS

AF ..... Activation function domain

AR ..... Androgen receptor

ATM ..... Ataxia-telengiectasia protein

β-gal ..... β-galactosidase

bp ..... Base pairs

BSA ..... Bovine serum albumin

CAT ..... Chloramphenicol acetyltransferase

CBP ..... CREB binding protein

CDK ..... Cyclin-dependent kinases

C/EBP ..... CCAAT enhancer binding protein

CHO ..... Chinese hamster ovary

CKII ..... Casein kinase II

CREB ..... Cyclic AMP response element-binding protein

DBD ..... DNA binding domain

DEAE ..... Diethylaminoethyl

DMEM ..... Dulbecco's modified Eagle medium

DNA ..... Deoxyribonucleic acid

DNaseI ..... Deoxyribonuclease I

Dex ..... Dexamethasone

ds ..... Double-stranded

DSB ..... Double-stranded break

DTT ..... Dithiothreitol

ECL ..... Enhanced chemiluminescence

EGF ..... Epidermal growth factor

EMSA ..... Electrophoretic mobility shift assay

ER ..... Estrogen receptor

EtBr ..... Ethidium bromide

FBS ..... Fetal bovine serum

GM-CSF ..... Granulocyte-macrophage colony-stimulating gactor

GR ..... Glucocorticoid receptor

GRE ..... Glucocorticoid response element

GRIP ..... Glucocorticoid receptor interacting protein

GST ..... Glutathione S-transferase

h ..... hours

HDAC ..... Histone deacetylase

HNF-4 ..... Hepatocyte nuclear factor-4

HRE ..... Hormone response element

HRP ..... Horse radish peroxidase

HTLV ..... human T cell leukemia virus

IκB ..... Inhibitor of NF-κB

JNK ..... c-Jun N-terminal kinase

kD ..... Kilodalton

LB ..... Leuria broth

LBD ..... Ligand binding domain

LSC ..... Liquid scintillation counting

LTR ..... Long terminal repeat

MAPK ..... Mitogen activated protein kinase

MHC ..... Major histocompatiblity complex

min ..... Minutes

mM ..... Millimolar

MMTV ..... Mouse mammary tumor virus

MR ..... Mineralocorticoid receptor

NCoR ..... Nuclear receptor corepressor

NF1 ..... Nuclear Factor 1

NF-κB ..... Nuclear factor kappa B

NHEJ ..... Non-homologous end joining

NLS ..... Nuclear Localization Signal

NP-40 ..... Nonidet P-40

OD ..... Optical density

ONPG ..... O-nitrophenyl-β-D-pyranogalactoside

PCAF ..... p300/CBP associated factor

PKA ..... Protein kinase A

PMSF ..... Phenylmethylsulfonyl fluoride

PPAR ..... Peroxisome proliferator-activated receptor

PR ..... Progesterone receptor

PRF ..... Plasmacytoma repressor factor

PVDF ..... Polyvinylidene fluoride

RAR ..... Retinoic acid receptor

RHD ..... Rel homology domain

RNA ..... Ribonucleic acid

RNA pol II ..... RNA polymerase II

rpm ..... Revolutions per minute

RPMI 1640 ..... Rosewell Park Memorial Institute tissue culture media 1640

RSV ..... Rous sarcoma virus

s ..... Seconds

SAg ..... Superantigen

scid ..... Severe combined immunodeficient

SDS ..... Sodium dodecyl sulphate

SDS-PAGE ..... SDS polyacrylamide gel electrophoreis

SMRT ..... Silencing mediator of retinoic acid and thyroid hormone

SNF ..... Sucrose non-fermentable proteins

SRC-1 ..... Steroid receptor coactivator 1

SRF ..... Serum response factor

STAT ..... Singnal transducer and activator of transcription

ss ..... single-stranded

SV40 ..... Simian Virus 40

SWI ..... Switch proteins

T-Ag ..... Simian Virus 40 large tumor antigen

TAF ..... TBP associated factor

TBE ..... Tris-HCl, Boric Acid, EDTA buffer

TBS-T ..... Tris buffered saline with Tween-20

TCR ..... T-cell receptor

TFA ..... Trifloro acetic acid

TGF ..... Transforming growth factor

TLC ..... Thin layer chromatography

TNF ..... Tumor necrosis factor

TPA 12-o-tetradecanoylphorbol-13-acetate

TPCK ..... L-1-tosylamido-2-phenylethyl chloromethyl ketone

TR ..... Thyroid hormone receptor

w/w ..... Weight per weight

w/v ..... Weight per volume

I. INTRODUCTION

1. Mouse mammary tumor virus

Mouse mammary tumor virus (MMTV) is a type B retrovirus that induces carcinomas of the mammary gland in female lactating mice. Premalignant lesions and malignant tumors result from insertional mutagenesis adjacent to cellular proto-oncogenes (Li et al., 2000; van Leeuwen and Nusse, 1995). Mammary gland specificity derives from the high viral replication rate and reinfection rate in mammary epithelial cells, a result of stimulation of these cells by pregnancy related hormones (Callahan and Smith, 2000). This response to hormones is driven by a steroid-hormone responsive promoter located within the viral LTR. This MMTV promoter transmits one of the strongest responses to steroid hormones observed to date (Beato, 1989; Ringold et al., 1975; Ucker and Yamamoto, 1984). When stably incorporated into the host genome the MMTV LTR is arranged in a regular array of 6 positioned nucleosomes (Richard-Foy and Hager, 1987). The second nucleosome upstream of the viral genes, called nucleosome B, contains DNA binding sites for steroid hormone receptors and the transcription factor NF1 (Cordingley et al., 1987; Hager, 1988). In the absence of hormone the chromatin is 'closed' and transcription factor access is prevented (Archer et al., 1991; Richard-Foy and Hager, 1987). Hormonal stimulation results in a remodeling of nucleosome B allowing transcription factors to assemble on the promoter and a concurrent activation of transcription (Archer et al., 1991; Cordingley et al., 1987; Truss et al., 1995). These properties of MMTV have made it one of the best-investigated model

systems in the study of mammary tumor development, the control of transcription by steroid hormones and the regulation of transcription by chromatin structure.

In this thesis I will study a negative regulatory element located within the MMTV LTR called NRE1 which specifically represses glucocorticoid-induced transcription. Initially I will characterize binding to this element by factors in crude nuclear protein extracts. Subsequently, I will characterize binding to NRE1 by purified Ku autoantigen, a factor that has been demonstrated to bind specifically to NRE1 (Giffin et al., 1996). Following this I will establish the involvement of both Ku and its associated factor, DNA-PK, in the repression of glucocorticoid-induced transcription of MMTV. Moreover, I will investigate the mechanism by which Ku and DNA-PK mediate this repression. First, I will identify GR as a phosphorylation target of DNA-PK on the MMTV LTR. I will then identify DNA-PK phosphorylation sites within GR. I will then provide evidence that DNA-PK dependent phosphorylation of GR leads to a repression of glucocorticoid-induced transcription of MMTV.

(i) The MMTV life cycle

MMTV can be transmitted either horizontally as milk-borne infectious particles or vertically, as integrants into the germ line (Acha-Orbea et al., 1999; Luther and Acha-Orbea, 1997). During primary infection MMTV is passed from the milk of a viremic mother to the newborn where it is taken up in the intestine. Within the first day the virus infects B-cells in lymphoid tissues of the intestine, such as Peyer's patches, which initiates a lymphocyte dependent transmission to the mammary gland (Acha-Orbea et al., 1999).

Upon infection of B-cells the virus is reverse transcribed and integrates into the B-cell genome (Luther and Acha-Orbea, 1997). In the U3 region of the MMTV 3'-LTR is an open reading frame which encodes a superantigen (SAg) (see figure 1A) that is expressed following proviral integration (Acha-Orbea and MacDonald, 1995). The SAg is integral to the transmission of the virus to the mammary epithelium. The MMTV viral SAgs are transmembrane glycoproteins having an extracellular carboxy terminus (Choi et al., 1992; Korman et al., 1992). The SAgs have a high degree of homology in their primary structure except for a C-terminal polymorphic region in the extracellular domain (Brandt-Carlson et al., 1993). Within the first 24 hours post ingestion the primarily infected B-cells are activated but do not proliferate appreciably (Ardavin et al., 1997). The expressed SAg is presented on the cell surface by MHC class II proteins (Held et al., 1993a; Held et al., 1993b). The presented SAg interacts with the Vβ domains of the TCRs on specific subsets of T-cells. The C-terminal polymorphic region of the SAg specifies which subclass of Vβ domain is able to interact with it (Yazdanbakhsh et al., 1993).

Unlike classical antigen responses, recognition of the SAg is carried out almost solely by the TCR Vβ domain and has little requirement of other polymorphic regions of the TCR (Acha-Orbea and MacDonald, 1995). Therefore entire subclasses of T-cells expressing the cognate Vβ domain within their TCRs are able to recognize the presented SAg. Up to 30% of the entire T-cell population can be activated by the SAg in this manner (Acha-Orbea and MacDonald, 1995; Scherer et al., 1993). The cognate T-cells are activated and secrete lymphokines initiating a second phase of B-cell proliferation that occurs 30 to 48 hours post infection (Ardavin et al., 1997). This T-cell-dependent B-

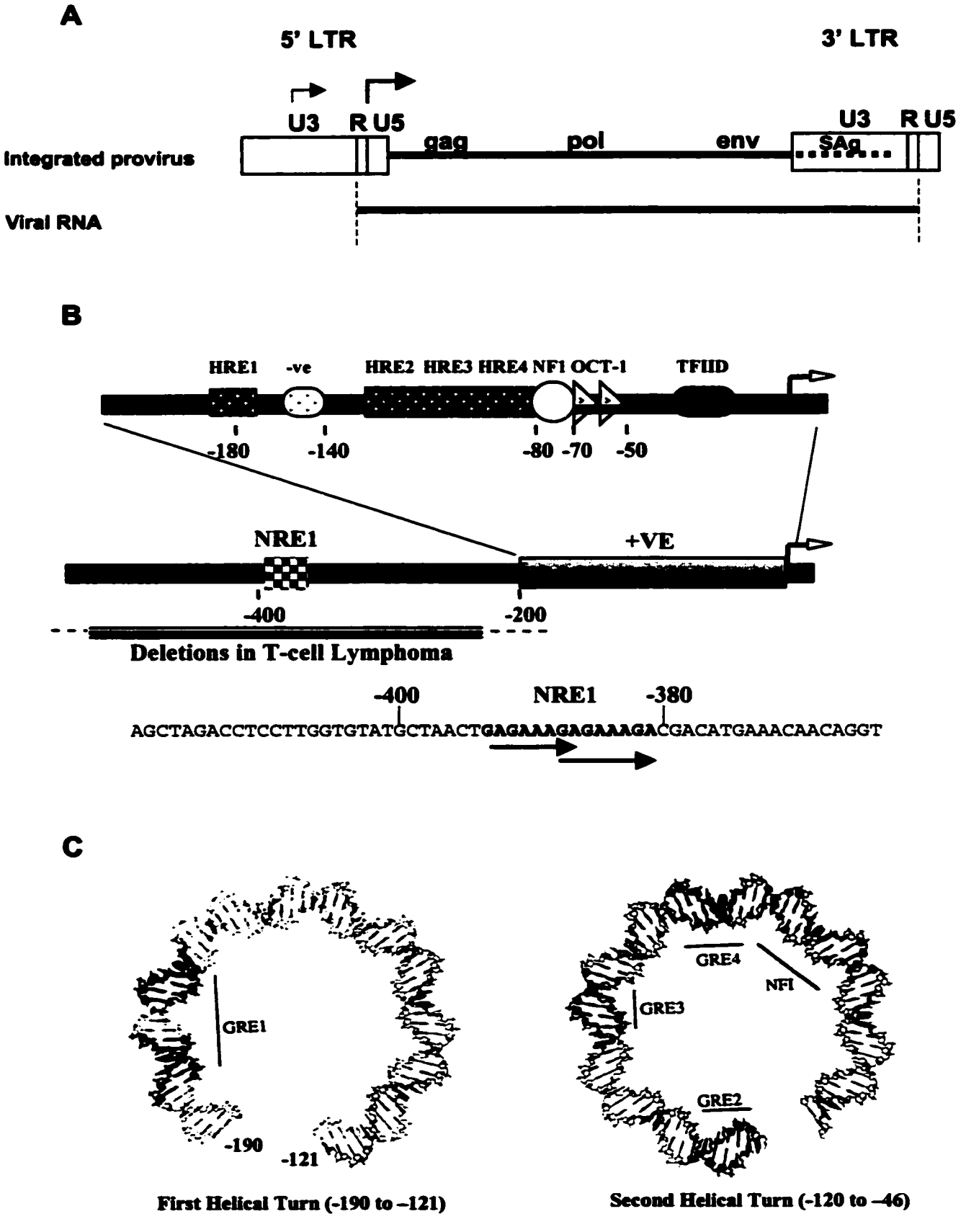

A
5' LTR
3' LTR
U3
R U5
U3
R U5
gag
pol
env
SAg
Integrated provirus
Viral RNA
B
HRE1
-ve
HRE2
HRE3
HRE4
NF1
OCT-1
TFIID
-180
-140
-80
-70
-50
NRE1
+VE
-400
-200
Deletions in T-cell Lymphoma
-400
NRE1
-380
AGCTAGACCTCCTTGGTGTATGCTAACTGAGAAAGAGAAAGACGACATGAAACAACAGGT
C
GRE1
-190
-121
First Helical Turn (-190 to -121)
GRE4
NF1
GRE3
GRE2
Second Helical Turn (-120 to -46)

Figure 1. Organization of the mouse mammary tumor virus.

Panel A: the mouse mammary tumor virus proviral DNA contains the viral gag, pol and env genes flanked by two long terminal repeats (LTRs). The LTRs are divided into U3, R and U5 regions. In the viral RNA the U3 region is unique to the 3' end of the virus while the U5 is unique to the 5' end. The R region is repeated on both ends. In the provirus the 3' LTR encodes a sugerantigen gene. The 5' LTR contains the transcriptional initiation sites as well as the promoter. Panel B: The organization of the promoter for the major transcription start site is shown. The proximal region within 200 bp from the start site (-200) is expanded at the top. It contains a TATA box (TFIID), octamer motifs (OCT), a nuclear factor 1 binding site (NF1) and four hormone response elements (HRE). The orientation of the HREs is indicated by the arrows. The two 3' sites are half-sites while the two 5' sites are imperfect palindromes. A negative regulatory element (-ve) has been reported between the two 5' HRE's. Another negative regulatory element (NRE1) is located between –380 and –395. A region spanning this element has been deleted in all T-cell lymphomas that have been examined. The NRE1 element is a polypurine:polypyrimidine sequence containing an overlapping direct repeat as indicated in the sequence at the bottom. Panel C: The two DNA loops around nucleosome B are shown indicating the relative positions of the glucocorticoid response elements (GRE) and the NF1 binding site (adapted from (Beato, 1991)).

cell activation and proliferation greatly increases the number of MMTV infected cells and is required for efficient infection of the virus (Golovkina et al., 1992; Held et al., 1993b). This increase in MMTV infected lymphocytes appears to be a result of clonal expansion of already-infected cells and not reinfection of new cells by the virus (Held et al., 1994; Held et al., 1993b). After infection and the initial expansion, the SAg reactive T-cells gradually undergo clonal deletion either in the thymus or peripherally (Held et al., 1992; Marrack et al., 1991; Papiernik et al., 1992; Webb et al., 1990). The cognate B-cell-T-cell interaction results in a vigorous SAg-induced immune response that is necessary for efficient spread and transmission of the virus (Champagne et al., 1996; Golovkina et al., 1992; Held et al., 1993b; Palmer et al., 1996). The mode of spread of the virus from the infected lymphocytes to the mammary gland is not known, and may involve viral particles or cell-cell contact (Acha-Orbea et al., 1999). Early conclusions that T-cells carried the virus to the mammary gland (Tsubura et al., 1988) are now in doubt and it remains unclear which lymphocyte population is responsible for mammary infection (Acha-Orbea et al., 1999; Waanders et al., 1993).

Transmission of MMTV also occurs through the germline (vertical transmission). This is a result of rare proviral integrations into germ cells which are transmitted by inheritance following normal Mendelian rules. Commonly studied inbred mouse strains have between 2 to 8 copies of the MMTV provirus, while wild mice carry 0 to14 (Acha-Orbea and MacDonald, 1995; Luther and Acha-Orbea, 1997). Only a few of the endogenous MMTV loci have retained the ability to form infectious particles (Imai et al., 1983; Michalides et al., 1978; van Nie and Verstraeten, 1975), the others having lost this ability most likely through the accumulation of mutations (Kuo et al., 1988; Salmons et

al., 1986). Most however, are able to express a functional SAg (Luther and Acha-Orbea, 1997). As with primary infections, expression of endogenous SAgs results in clonal deletion of T-cell subclasses carrying reactive Vβ domains (Acha-Orbea et al., 1991; Choi et al., 1991). This deletion has been suggested to protect these mice from a subsequent infection by exogenous MMTV encoding a SAg with the same Vβ specificity (Golovkina et al., 1992).

(ii) Genomic organization of MMTV

The MMTV retrovirus contains a 8.5 kb RNA genome as shown in Figure 1, panel A (Acha-Orbea and MacDonald, 1995; Luther and Acha-Orbea, 1997). Three overlapping genes common to all retroviral genomes are encoded: the internal structural proteins, the capsids (gag), the protease and polymerase (pol) and the envelope proteins (env). The capsids, protease and polymerase proteins are translated from full length mRNA which initiates in the U5 region of the LTR (Acha-Orbea and MacDonald, 1995; Luther and Acha-Orbea, 1997). These proteins are encoded in 3 different reading frames and therefore two frameshifts during translation are required for their expression. The env protein is translated from a splice variant of the full length mRNA lacking the gag and pol regions. Expression of the gag, pol and env genes yields precursor proteins which are proteolytically processed to the final protein products (Acha-Orbea and MacDonald, 1995; Luther and Acha-Orbea, 1997).

In the proviral form of MMTV the viral genes are located between two LTRs. Duplication of the LTRs occurs during the reverse transcription process prior to integration of the virus into the host genome (Acha-Orbea and MacDonald, 1995; Luther and Acha-Orbea, 1997). The LTRs are divided into 3 regions which are named based on

their occurrence in the MMTV viral RNA. There are two unique sequences, U5 and U3 which are each found once within the viral RNA at either the 5' or 3' end respectively. In addition there is a repeated region, R, that is integral to the reverse transcription process. The major transcription start site is located in the U5 region (Acha-Orbea and MacDonald, 1995; Luther and Acha-Orbea, 1997). The MMTV U3 region is much larger than corresponding regions of other retroviruses. The 3' U3 region contains the gene encoding the SAg which overlaps at its 5' end with the 3' end of the env gene (Acha-Orbea and MacDonald, 1995; Luther and Acha-Orbea, 1997). Transcription of the SAg gene can occur from a spliced mRNA which initiates at the major initiation site in the U5 region or from a second promoter located in the 5'U3 region (Gunzburg et al., 1993; Salmons et al., 2000). The 5'U3 region also contains a complex transcriptional promoter including hormone responsive elements (described in detail in section (iv)).

(iii) MMTV-induced tumorigenesis

Following infection of mammary epithelial cells MMTV randomly inserts its proviral DNA into the host cell DNA during its replicative cycle. Unlike other oncogenic retroviruses MMTV does not carry an oncogene within its genome. Instead carcinogenic transformation arises due to insertional mutagenesis at sites flanking host proto-oncogenes. In most cases the activation of the proto-oncogenes is an effect of enhancer sequences within the LTR of the integrated MMTV provirus on the promoter of the affected gene (Callahan and Smith, 2000). Mammary tumors in mice appear to be clonal populations, likely arising from mutated mammary epithelial stem cells (Callahan and Smith, 2000; Kordon and Smith, 1998). The frequency and latency of tumor formation varies with the strain of mouse infected and the virus. In highly susceptible mouse strains

such as the C3H, 100% of offspring can develop tumors within 7-10 months of age (Callahan and Smith, 2000; Luther and Acha-Orbea, 1997). At least one pregnancy appears to be necessary for tumor development, while tumor progression may or may not be pregnancy dependent, again depending on the strain of mouse and virus as well as the number of pregnancies which have occurred (Callahan and Smith, 2000).

The first gene to be identified as a proto-oncogene activated by MMTV was *Wnt*-1, which is activated by insertional mutagenesis in approximately 75% of infected C3H mice (Nusse and Varmus, 1982). Wnt-1 is a member of a family of at least 12 genes related to the Drosophila *Wingless* (*Wg*) gene (Nusse, 1991; Nusse, 1997). Two other members of the *Wnt* family have been observed to be activated by MMTV insertion: *Wnt*-3 (Roelink et al., 1990) and *Wnt*-10b (Lee et al., 1995). In addition to the *Wnt* family members of the fibroblast growth factor family: *Fgf*-3 (Dickson et al., 1984), *Fgf*-4 (Peters et al., 1989) and *Fgf*-8 (MacArthur et al., 1995); the Notch family: *NOTCH*-1 (Dievart et al., 1999) and *NOTCH*-4 (Gallahan and Callahan, 1997; Uyttendaele et al., 1996); and a gene encoding the p48 component of the translation initiation factor-3 (eIF-3p48) (Marchetti et al., 1995) have been shown to be deregulated by MMTV in mammary tumors.

Rare MMTV induced tumors are also observed to occur in tissues other than the mammary gland, most frequently in T-cells. Of the MMTV proviruses from these tumors that have been examined, all have genetic rearrangements in their LTR, involving deletions of 350 to 500 nucleotides in the U3 region (Dudley and Risser, 1984; Hsu et al., 1988; Michalides and Wagenaar, 1986; Michalides et al., 1982; Theunissen et al., 1989; Yanagawa et al., 1990). Moreover, naturally occurring MMTV proviruses with

rearranged LTRs in this region induce T-cell lymphoma rather than mammary carcinoma (Yanagawa et al., 1993). Furthermore type B leukemogenic virus (TBLV) is a MMTV variant with similar LTR alterations, which induces T-cell lymphoma but not mammary carcinoma (Ball et al., 1985; Ball et al., 1988). Several studies have demonstrated that this deleted region contains a negative transcriptional regulatory element (Hsu et al., 1988; Lee et al., 1991; Mink et al., 1990; Morley et al., 1987; Yanagawa et al., 1990) in addition to the 3' end of the SAg gene (Luther and Acha-Orbea, 1997). To date two genes which are common integration sites for genetically rearranged MMTV have been identified: the *Notch1* gene which was rearranged in 30% of MMTV induced T-cell lymphomas in BALB/c mice (Yanagawa et al., 2000) and the c-myc gene which was rearranged in 7% of similar tumors (Rajan et al., 2000).

(iv) Transcriptional regulation of MMTV

a) The hormone responsive region

Transcription of the MMTV genes is controlled by a promoter located in the LTR (see Figure 1, panel B). This promoter is complex and contains binding sites for multiple transcription factors. The primary regulatory elements occur in the promoter proximal region, within the first 200 bp 5' of the transcription start site. However several distally located elements also contribute to transcriptional control. MMTV transcription is strongly induced by steroid hormones, including glucocorticoids, progestins, androgens and mineralocorticoids but not estrogen (Archer et al., 1994; Cato et al., 1987; Cato et al., 1986; Cato and Weinmann, 1988). Steroid induction occurs primarily through the hormone responsive region, found in the nucleosome B region of the LTR between –184

to –78 relative to the transcription start site. This element contains a complex cluster of four hormone response elements (HREs) which are binding sites for steroid receptors (Payvar et al., 1983) (see Figure 1, panel 3). Of these four, the most 5' HRE, located between -184 and -170, has the highest affinity for steroid receptors (Scheidereit et al., 1983). The consensus DNA-binding sequence for steroid receptors (not including the estorgen receptor) is a palindrome of the sequence AGAACA separated by 3 base pairs. The 5'HRE is an imperfect palindrome in which the 5' half site is the non-consensus sequence GTTACA. The second HRE (-127 to -114) is also an imperfect palindrome in which the 5' half site contains the sequence GTATCA. It is bound at a lower affinity due to a sub-optimal two bp spacing between the palindrome half-sites (Scheidereit et al., 1983). The third (-98 to -93) and fourth (-83 to -78) HREs are both consensus half-palindromes (Scheidereit et al., 1983). In isolation half-palindromes are bound with low affinity, however in the context of MMTV where they are in proximity to other receptor binding sites the affinity is much higher and these sites are required for the full transcriptional response to steroid hormones (Chalepakis et al., 1988). In addition to the 4 promoter proximal HRE's, two additional sites recognized by the glucocorticoid receptor have recently been identified in the nucleosome C region between -299 and -274 (Fletcher et al., 2000). These appeared to act synergistically with the nucleosome B HRE's as their mutation resulted in a 50% loss of transcriptional activity but they were unable to activate transcription alone when the nucleosome B HRE's were mutated (Fletcher et al., 2000).

b) Role of chromatin structure in MMTV transcription

In all eukaryotic cells DNA is packaged into a protein-DNA structure, chromatin. Transcription of MMTV is critically regulated by the chromatin organization of the LTR. The basic subunit of chromatin is the nucleosome core particle, which consists of an octamer of core histone proteins, containing a tetramer of histone H3 and H4 and two dimers of histones H2A and H2B (Luger et al., 1997). Around the histone octamer are wrapped 146 bp of DNA in 1.75 superhelical turns (Luger et al., 1997). The packaging into chromatin results in a compaction which, when occurring in transcriptional regulatory regions, renders the affected region transcriptionally silent (Wong et al., 1995). In compacted chromatin the majority of transcription factors are unable to bind to their target sequences and first require remodeling of the chromatin (Beato and Eisfeld, 1997; Wallrath et al., 1994; Wu, 1997). Transcription factors that are capable of remodeling chromatin and counteracting this repressive effect are therefore critical in regulating gene activity (Kingston and Narlikar, 1999; Kornberg, 1999). A number of promoter and enhancer regions have been found to have specifically positioned nucleosomes that are located over defined regions of DNA (Beato and Eisfeld, 1997; Wolffe, 1994). This specific positioning is defined by two parameters: translational positioning which identifies the region of a given DNA sequence that is wrapped around the histone octamer and rotational positioning which refers to the angular orientation of individual base pairs on the nucleosome surface (Beato and Eisfeld, 1997).

Specific nucleosome positioning and transcriptional silencing by chromatin is exemplified in the MMTV LTR. When integrated into cellular chromosomes the MMTV LTR adopts a specific chromatin organization consisting of six positioned nucleosome

families, which have been labeled A-F in the 3' to 5' direction (Fragoso et al., 1995; Richard-Foy and Hager, 1987). Using high-resolution mapping the position of the two most 3' nucleosomes, A and B, have been shown to have variation in their translational positioning (Fragoso et al., 1995). The majority of the positions, or frames, are within a 50 to 60 bp region centered over the positions identified by low-resolution mapping (Fragoso et al., 1995). The 5' end of nucleosome B is located between –235 to –187 while the 3' end is between –86 and –36. The 5' end of nucleosome A is between –22 and +42 while the 3' end is between +121 and +186 (Fragoso et al., 1995). In addition the 5' end of nucleosome B extends at low frequency toward nucleosome C while at the 3' end there is a sharp discontinuity suggesting that there is a boundary constraint in the linker region between nucleosomes A and B (Fragoso et al., 1995).

The organization of positioned nucleosomes plays a key role in regulating the transcriptional responses from this promoter. At least three factors acting on the MMTV promoter are restricted from binding their sites in the "closed" chromatin conformation and require remodeling by GR in order to activate transcription. Adjacent to the HRE in nucleosome B is a binding site for the transcription factor NF-1 located from -76 to -63 that partially overlaps with the most 3' HRE (Buetti and Kuhnel, 1986; Cato et al., 1988; Cordingley and Hager, 1988; Cordingley et al., 1987; Nowock et al., 1985). In the linker region between nucleosome A and B, located between -57 to –38 (Bruggemeier et al., 1991; Buetti, 1994; Meulia and Diggelmann, 1990), are two octamer motifs bound by the octamer transcription factors Oct-1 and Oct-2 (Falkner and Zachau, 1984; Verrijzer et al., 1992). The TATA box, located between -27 to -20 and bound by TFIID, which includes the TATA-binding protein, is also located in the linker region (Nakajima et al., 1988;

Toohey et al., 1990). When the LTR is integrated into cells that haven't been exposed to hormone it adopts a "closed" conformation and transcription factors are unable to bind to it (Archer et al., 1992; Cordingley et al., 1987; Truss et al., 1995). Upon treatment of these cells with glucocorticoids, GR binds to the HREs and initiates chromatin remodeling resulting in a region which becomes hypersensitive to DNase I, methidiumpropyl-EDTA·iron(II) and restriction enzymes (Archer et al., 1991; Boronat et al., 1997; Richard-Foy and Hager, 1987; Richard-Foy et al., 1987; Truss et al., 1995). This remodeling leads to the binding of NF-1, octamer transcription factors and the TATA-binding protein (Archer et al., 1992; Cordingley et al., 1987; Lee and Archer, 1994). This is concomitant with transcriptional activation from the reorganized promoter (Archer et al., 1992; Cordingley et al., 1987; Lee and Archer, 1994; Truss et al., 1995).

GR mediated remodeling appears to be a two-step process in which GR first binds to the HREs then recruits a remodeling factor and dissociates from the DNA in an ATP dependent manner (Fletcher et al., 2000). The chromatin remodeling factor is unknown however increasing evidence points to members of the SWI-SNF complex. In mammalian cells remodeling has been shown to require BRG1 and hBrm, components of the mammalian SWI-SNF complex (Fryer and Archer, 1998; Muchardt and Yaniv, 1993). Also a drosophila ISWI-containing complex can facilitate a progesterone receptor dependent change in topology of MMTV minichromosomes (Di Croce et al., 1999). Finally GR enhanced the ability of SWI-SNF complexes to remodel GRE containing nucleosomes and to facilitate *in vitro* NF-1 binding (Ostlund Farrants et al., 1997).

The GR induced nucleosome remodeling is transient in nature, reaching maximal induction after 1 h and becoming refractory to GR induction after 24 h (Archer et al.,

1994; Lee and Archer, 1994). This refractory state requires continued exposure to glucocorticoids and is paralleled by a global dephosphorylation of histone H1 (Lee and Archer, 1998). Blocking histone H1 phosphorylation results in the promoter remaining refractory while dephosphorylation of histone H1 on the MMTV LTR assembled as chromatin results in a loss of ability to respond to glucocorticoid (Lee and Archer, 1998).

The nature of the chromatin remodeling is unclear, however the following observations suggest that the LTR DNA remains associated with nucleosome proteins: first, nucleosomal ladders are observed on glucocorticoid treated templates which are identical to those observed on uninduced templates (Richard-Foy and Hager, 1987; Truss et al., 1995). Moreover, glucocorticoid induction does not alter the position or occupancy of the multiple frames of nucleosome B (Fragoso et al., 1995). Finally, there is no observed decrease in the amount of core histone H2B associated with nucleosome B after induction, although the level of linker histone H1 does decrease (Bresnick et al., 1992). Interestingly the remodeled, or hypersensitive, region extends from –109 to –295, well past the boundary of nucleosome B and into nucleosome C, this region does not vary with the frame of the uninduced nucleosome B (Fragoso et al., 1998). This distal remodeling requires the nucleosome C HREs (Fletcher et al., 2000).

NF-1 and GR have long been known to activate MMTV transcription synergistically on organized chromatin templates (Buetti and Kuhnel, 1986; Cordingley and Hager, 1988; Cordingley et al., 1987). On transiently transfected templates however, NF-1 and GR can not co-occupy their DNA binding sites owing to stearic interference resulting from the partial overlap of the sites (Pina et al., 1990). On these templates NF-1 inhibits the transcriptional activation by glucocorticoids (Archer et al., 1992). On

MMTV LTR templates incorporated into chromatin and in a "closed" chromatin conformation, the NF-1 binding site is rotationally positioned such that the core histone octamer sterically prevents binding of NF-1 (see Figure 1, panel C) (Archer et al., 1991; Archer et al., 1992; Bruggemeier et al., 1991; Ostlund Farrants et al., 1997; Pina et al., 1990). Treatment with glucocorticoids and subsequent remodeling by GR allows NF-1 to access its site (Archer et al., 1991; Archer et al., 1992; Bruggemeier et al., 1991; Ostlund Farrants et al., 1997; Pina et al., 1990). In this case the winding of the DNA around the nucleosome eliminates the steric interference between GR and NF-1 and allows both factors to access their binding sites (Truss et al., 1995).

c) Synergistic transactivation of MMTV by GR and the octamer transcription factors

Transcriptional synergism between the octamer transcription factors and GR occurs by a different mechanism than with NF-1. The octamer motifs located in the linker region between nucleosome A and B are recognized by octamer transcription factors Oct-1 and Oct-2 (Falkner and Zachau, 1984; Verrijzer et al., 1992) however these sites deviate from the consensus octamer motifs and are therefore bound with relatively low affinity (Bruggemeier et al., 1991). Binding to the MMTV LTR octamer motifs requires the presence of GR or PR (Bruggemeier et al., 1991; Wieland et al., 1991). The cooperativity for DNA binding between Oct-1 or Oct-2 and GR has been observed both *in vitro* and *in vivo* (Bruggemeier et al., 1991; Truss et al., 1995; Wieland et al., 1991). Both Oct-1 and Oct-2 also activate transcription cooperatively with GR and PR (Bruggemeier et al., 1991; Wieland et al., 1991) but in the absence of steroid they have little transcriptional effect (Cato et al., 1986; Parks et al., 1976). The cooperativity between GR and Oct-1 occurs when the LTR is arranged in organized nucleosomes

despite the fact that the octamer motifs are located in the linker region between nucleosomes A and B and are expected to be accessible for DNA binding (Truss et al., 1995). Octamer transcription factors bind DNA through their POU domains (Wegner et al., 1993) which are bipartite domains consisting of a homeodomain and a POU specific helix-turn-helix motif that act cooperatively in binding their sequence-specific binding sites (Klemm et al., 1994). The cooperativity for DNA binding has been shown to be mediated by a direct protein-protein interaction between the DBD of GR and the homeodomain of Oct-1 or Oct-2 (Prefontaine et al., 1998). Octamer factor binding to GR results in a strong recruitment of Oct-1 or -2 to the octamer motifs and this correlated to an increase in MMTV transcription (Prefontaine et al., 1998). This interaction was found to occur in solution but did not occur when GR was bound to a GRE (Prefontaine et al., 1998). The *in vivo* interaction with Oct-2 was conserved among other members of the steroid receptor family including PR and AR but not MR or other members of the nuclear receptor family (Prefontaine et al., 1999). This correlated with the inability of MR to synergistically activate transcription with Oct-2 despite its having a similar ability to activate MMTV transcription as GR (Prefontaine et al., 1999). That GR-activated transcription was reduced in the presence of mutated octamer motifs suggests that the organization of the MMTV promoter limits the ability of glucocorticoids to activate transcription in the absence of Oct-2 (Prefontaine et al., 1999).

d) Other regulatory elements

In addition to the proximal promoter region the MMTV LTR carries a transcriptional enhancer located between –1090 to –900 (Gouilleux et al., 1991; Mink et al., 1990; Yanagawa et al., 1991). This enhancer activates transcription in a mammary

specific manner (Mink et al., 1992; Yanagawa et al., 1991) and is likely to be at least a partial determinant of the mammary gland specific expression of MMTV. This enhancer has also been postulated to be involved in the activation of proto-oncogenes following viral insertion (Mink et al., 1990). Binding by two factors, an NF-1 and an uncharacterized factor called mammary cell-activating factor (MAF) act in concert to promote transcription in mammary cells (Mink et al., 1992). Neither site in isolation is capable of mediating the mammary specific effect. A third factor which recognizes an ACAAG core motif can enhance the activities of the other two elements (Mink et al., 1992). Prolactin in concert with either EGF or TGFα has been shown to induce transcriptional activity of this enhancer (Haraguchi et al., 1997). This treatment also results in increased binding of nuclear factors to their DNA biding sites (Haraguchi et al., 1997).

A number of negative regulatory elements (NREs) have also been described in the MMTV LTR. One such element has been identified between the two 5' and two 3' HREs (Hartig et al., 1993; Langer and Ostrowski, 1988; Tanaka et al., 1991) however in genomic footprinting experiments no factor was observed binding to this site (Truss et al., 1995). An NRE located between –364 and –428 has also been identified (Mink et al., 1990; Morley et al., 1987). This sequence has been implicated in the tissue specificity of MMTV transcription (Ross et al., 1990). Also, when cloned in front of the thymidine kinase promoter it repressed transcription in a cell-type specific manner (Mink et al., 1990). Repression occurred irrespective of the orientation of the element relative to the promoter (Mink et al., 1990). Mutational analysis of this NRE suggested that it consisted of two regulatory regions since point mutations or small deletions within these regions

are deleterious to the repressive effect while similar mutations in the central region have little effect (Lee et al., 1991). The 3' mutation sensitive region extended from –394 to –365 and contains an extended polypurine/polypyrimidine sequence. This region was designated NRE1, it has been extensively characterized in our laboratory and will be described in detail in the following section. The 5' mutation sensitive region has not been characterized as extensively but recently an uncharacterized factor termed MMTV NRE-binding protein 1 (MNBP-1) from fractionated human HeLa and mouse Ltk$^-$ cells was shown to bind to an imperfect palindrome in this region (Kang and Peterson, 1999). The effect of binding by this factor on transcription has not yet been determined. A third NRE located between –560 and –631 has also been described, that can also mediate repression when cloned in front of a thymidine kinase promoter (Mink et al., 1990). This region repressed transcription in all cell lines examined and was postulated to be a general NRE (Mink et al., 1990). Unlike the –428 to –364 NRE this element only repressed transcription in its native orientation (Mink et al., 1990). Finally, the region between –286 and –268 has also been shown to repress basal MMTV transcription (Bramblett et al., 1995). This element is bound by the nuclear matrix attachment protein special AT-rich sequence-binding protein 1 (STAB-1) (Liu et al., 1997).

(v) Characterization of NRE1

Our interest in the negative regulatory element in the –428 to –364 region stemmed from the observation that in all cases of MMTV induced T-cell lymphoma that had been examined, all had deletions of the viral LTR including this region (Dudley and Risser, 1984; Hsu et al., 1988; Michalides and Wagenaar, 1986; Michalides et al., 1982; Theunissen et al., 1989; Yanagawa et al., 1993; Yanagawa et al., 1990). This suggested

that deregulation of the virus by loss of this NRE leads to an increased expression in T lymphocytes that ultimately results in cellular transformation. Sequence analysis of the GR strain MMTV LTR in this region identified a short element highly similar to the binding site for PRF, a B cell specific growth inhibitory DNA binding activity, which acts on the c-myc promoter between –277 and –290 (Kakkis and Calame, 1987; Kakkis et al., 1988; Kakkis et al., 1989). This element is polypurine/polypyrimidine and in the GR MMTV contains an overlapping direct repeat of the sequence GAGAAAGA with a 2 bp overlap. This element also corresponded to the 3' mutation sensitive domain identified in the –428 to –364 region (Lee et al., 1991).

a) NRE1 binding by factors in crude nuclear extracts

A 23 bp double-stranded oligonucleotide (MTV, see Table 1) containing this element (–398 to –375) was bound by nuclear factors of similar mobility from both Jurkat human T-cells and CAC-E1A mouse mammary epithelial cells in EMSA assays (Giffin et al., 1994). These cells also contained nuclear factors that bound both the polypyrimidine upper-strand and the polypurine lower-strand in EMSA. Both of these single-stranded binding activities were of identical mobility to the double-stranded binding activity (Giffin et al., 1994). These binding activities were found in a variety of cell lines, however the relative abundance of each varied between cell type, for example the double-stranded binding activities were underrepresented in immature lymphoid cells (Giffin et al., 1994). The single-strand binding activities could be distinguished from the double-strand binding activity based on their sequence preference. When an oligonucleotide lacking one copy of the direct repeat (MT, see Table 1) was used in EMSA, binding to the single-strands was severely compromised while the double-stranded binding was

comparable to that with the full-length element (Giffin et al., 1994). Binding to this element was confirmed by DNase I footprinting, which showed strong protection by factors in Jurkat nuclear extracts over the polypurine/polypyrimidine region in double-stranded DNA. On both upper and lower single-stranded DNA, this element was also strongly protected by CAC-E1A nuclear factors as determined by $KmNO_4$ footprinting (Giffin et al., 1994) and by Jurkat nuclear factors in single-stranded DNaseI footprinting assays (Torrance et al., 1998).

A more detailed examination of the binding to NRE1 revealed that binding induced DNaseI hypersensitive sites upstream of NRE1 in a Mg^{2+} dependent manner (Giffin and Hache, 1995). This appeared to be due to a topological change in the DNA structure spanning this upstream region induced by factor binding to NRE1. The hypersensitivity was not due to direct binding in the upstream region since no factor was observed binding to the upstream sequences in EMSA under identical conditions (Giffin and Hache, 1995). Moreover, NRE1 could induce similar Mg^{2+} dependent hypersensitivity in a heterologous DNA fragment. The upstream DNaseI hypersensitive region was also found to be hypersensitive to single-stranded DNA cleavage agents on both strands, also in a Mg^{2+} dependent manner, following binding to NRE1 (Giffin and Hache, 1995). This indicated that a significant structural change in the DNA was being induced.

Using UV cross-linking analysis three factors were found to directly contact NRE1. A DNA-cross-linked factor, migrating at 80 kD, cross-linked to the upper strand of double-stranded DNA but did not contact the lower strand (Giffin et al., 1994). This factor also cross-linked the single-stranded upper-strand. A second DNA-cross-linked

factor, migrating at 95 kD, contacted only the single upper-strand while a third DNA-cross-linked factor, migrating at 50 kD, contacted only to the single lower-strand (Giffin et al., 1994). These factors appeared to bind the different forms of NRE1 as a heteromeric complex since all three factors fractionated at 8S on sucrose gradients and in EMSA the factors binding to each form of NRE1 migrated at virtually identical mobilities (Giffin et al., 1994). DNA contact by these factors was influenced by Mg^{2+} and ATP. On double-stranded DNA, in the presence of Mg^{2+} or ATP, both the 80 and 95 kD factors cross-linked to the upper-strand while the 50 kD factor cross-linked to the lower strand (Giffin and Hache, 1995). Thus this cross-linking pattern on double-stranded DNA resembled that of the single-strands in the absence of Mg^{2+} or ATP suggesting an opening of the DNA was occurring.

b) NRE1 represses glucocorticoid induced transcription of MMTV

To determine if this binding site alone could act as a negative regulatory element, transient transfections were performed using reporter genes in which multiple copies of the MTV or MT oligonucleotides were inserted upstream of the hormone responsive region of the MMTV LTR (at –237). In both Jurkat and CAC-E1A cells four copies of the MTV strongly repressed the induction of transcription of MMTV by glucocorticoids (Giffin et al., 1994). In CAC-EIA cells 4 copies of MTV mediated a slight decrease in basal transcription while in Jurkat cells there was no effect on basal transcription (Giffin et al., 1994). By contrast in NMuMG mammary epithelial cells both basal and glucocorticoid induced transcription were strongly repressed by four copies of NRE1. However the fold-induction by glucocorticoids was unaffected. The region of the MMTV LTR from –398 to –375 was therefore identified as a negative regulatory element

that strongly repressed the induction of MMTV transcription by glucocorticoids and was designated NRE1. The single-stranded binding activities appeared to be important for the repression of glucocorticoid activation since nine copies of the MT element inserted upstream of the hormone responsive region had little effect on glucocorticoid induction (Giffin et al., 1994). In CAC-EIA cells nine copies of MT did repress the overall level of transcription however.

c) Characterization of NRE1 binding by the Ku autoantigen

The double-stranded NRE1 binding protein was purified using a DNA affinity approach and identified as the Ku autoantigen by microsequence analysis (Giffin et al., 1996). This was confirmed by EMSA in which purified Ku bound to NRE1 and migrated with the same mobility as the binding factors in Jurkat nuclear extracts. Moreover addition of an anti-Ku antibody to the binding reactions resulted in an identical decrease in mobility of DNA complexes with purified Ku and factors in nuclear extracts (Giffin et al., 1996). Purified Ku also protected the upper- and lower-strands of double-stranded NRE1 in DNaseI footprinting assays (Giffin et al., 1996).

Ku is a heterodimeric protein with subunits of 70 and 82 kD (see section 2 for a detailed discussion of Ku) (Liu and Lee, 1991; May et al., 1991). The Ku subunits corresponded approximately in size to the 80 and 95 kD factors observed in the UV cross-linking studies, the larger size of the cross-linked factors being attributable to the covalently linked DNA (Giffin et al., 1994). Ku had been previously shown to bind to DNA ends without sequence preference (Blier et al., 1993; de Vries et al., 1989; Falzon et al., 1993) and to translocate along the DNA following binding (de Vries et al., 1989; Paillard and Strauss, 1991). To eliminate the possibility that Ku bound to DNA ends then

migrated to NRE1, EMSA were performed using 223 bp DNA microcircles containing the NRE1 sequence. Ku was able to bind specifically to these circles but could not bind to identical circles lacking the NRE1 (Giffin et al., 1996). Binding to the circles could be competed by the MTV oligonucleotide and a closed circular plasmid containing the NRE1 sequence, but not by the closed-circular parent plasmid lacking the sequence (Giffin et al., 1996). Most importantly, in footprinting experiments purified Ku was also able to protect the NRE1 sequence on both strands of a closed-circular plasmid containing the MMTV LTR (Giffin et al., 1996).

Once bound to NRE1, Ku is able to translocate on the DNA in a Mg^{2+} dependent manner. In EMSA with purified Ku and NRE1-containing microcircles, addition of Mg^{2+} resulted in the appearance of lower-mobility complexes indicative of multiple copies of Ku bound to the circle (Giffin et al., 1996). Purified Ku also induced DNaseI hypersensitive sites flanking the NRE1 element suggestive of Ku translocation (Giffin et al., 1996). Ku also induced hypersensitivity to $KmNO_4$ on both strands of double-stranded closed circular MMTV LTR containing plasmids (Giffin et al., 1999). This hypersensitivity correlated closely with that observed using crude extracts (Giffin and Hache, 1995) and was dependent on the presence of Mg^{2+} (Giffin et al., 1999).

The upper NRE1 binding activity was purified and found to contain three components, two of which were identified as the 70 and 82 kD subunits of the Ku autoantigen, while the third remained unidentified (Torrance et al., 1998). That Ku was one of the upper-stranded binding activities was demonstrated by EMSA in which anti-Ku antibodies supershifted the factors bound to upper-NRE1 (Torrance et al., 1998). Footprinting of each of the single-strands of the MMTV LTR with purified Ku

demonstrated that it was able to reproduce the protection pattern observed with crude extracts (Torrance et al., 1998). Finally in UV cross-linking experiments both subunits of purified Ku were shown to contact the single upper-strand, while only the 70 kD subunit contacted the upper-strand when using double-stranded DNA. Addition of Mg^{2+} and ATP to the double-strand cross-linking reaction induced both subunits to contact the upper-strand (Torrance et al., 1998). These results paralleled exactly the results observed with crude extracts.

d) Identification of three classes of sequence-specific Ku binding sites

A number of other sequence-specific binding sites for Ku had previously been reported (DiCroce and Krontiris, 1995; Falzon et al., 1993; Kim et al., 1995; Knuth et al., 1990; Liu and Lee, 1991; May et al., 1991; Messier et al., 1993; Okumura et al., 1994). These fall into two classes (Giffin et al., 1997). The first class had high sequence identity with NRE1 and an overall polypurine/polypyrimidine character. These included the PRF element in the murine c-myc gene (Kakkis and Calame, 1987; Kakkis et al., 1988; Kakkis et al., 1989) and an element in the LTR of HTLV (Okumura et al., 1994). Alignment of NRE1 with the corresponding region of the LTR of the C3H strain of MMTV revealed a similar element that has also been implicated in repression of viral transcription (Lee et al., 1991; Lefebvre et al., 1991; Morley et al., 1987). A second class of binding sites had no apparent homology to NRE1, this included the octamer motif (May et al., 1991), a heat shock response element (Kim et al., 1995), an element from the U1 small nuclear RNA promoter (Knuth et al., 1990), and the EBP80 binding site from the *rc-mos* intracisternal A particle (IAP) LTR (Falzon et al., 1993; Falzon and Kuff, 1989; Falzon and Kuff, 1990).

To assess whether these were direct sequence-specific binding sites we took advantage of the following observation: in EMSA with NRE1-containing microcircles an excess of DNA ends was unable to compete for Ku binding to NRE1 (Giffin et al., 1996). Likewise in footprinting experiments on linear MMTV LTR, addition of excess DNA ends competed protection of the LTR ends by Ku but did not compete the protection over NRE1 (Giffin et al., 1996). This indicated that competition assays could distinguish between sequence-specific binding and end binding. EMSA were therefore performed in which oligonucleotides containing the proposed binding sites were used as competitors. Ku binding to the NRE1-containing microcircles could be competed by the NRE1-like binding sites however the second class of binding sites were unable to compete (Giffin et al., 1997). This suggested that the first class of binding sites were direct sequence-specific binding sites while the second class were indirect, possibly sites where Ku paused on the DNA. Furthermore, Ku was able to bind specifically to a microcircle containing the C3H NRE1 however it could not bind to microcircles containing the octamer motif, the heat shock element or the elements from the IAP LTR or the U1 promoter. This confirmed that Ku could bind directly to at least one other member of the NRE1-like class of binding sites but not the class two binding sites.

The sequence specificity of single-stranded Ku binding closely parallels that of double-stranded binding as Ku could recognize the polypurine rich strands of the NRE1-like class of binding sites including the C3H NRE1 element, the c-myc PRF element and the element from the HTLV U5 LTR (Torrance et al., 1998). In contrast Ku did not bind to single-strands from the second class of binding sites including the octamer motif and the heat shock element (Torrance et al., 1998).

The factor which binds to the truncated NRE1 element lacking one copy of the direct repeat (MT, see Table 1) has also been identified as the Ku autoantigen (Giffin et al., 1999). Differences in the manner in which Ku interacts with this sequence as compared to the full-length NRE1 indicate it represents a third class of sequence-specific binding site. Purified Ku is able to bind directly to the MT element incorporated into DNA microcircles and this reflects exactly the binding to MT observed using crude Jurkat nuclear extracts (Giffin et al., 1999). Unlike the full-length NRE1 however, Ku recognizes only the double-strand of MT and not the upper-strand (Giffin et al., 1999). Also distinguishing NRE1-binding from MT-binding is the fact that Ku appears to bind statically to MT and is unable to translocate from this element in a Mg^{2+} dependent manner (Giffin et al., 1999). Mg^{2+} and ATP are also unable to induce UV cross-linking of the 82 kD subunit of Ku to the upper-strand of the MT element as occurs on the full-length NRE1 (Giffin et al., 1999). Finally Ku bound to the MT element appears conformationally different from the NRE1 bound form. In EMSA using high percentage acrylamide gels MT-bound Ku migrated with a much lower mobility than Ku bound to double- or upper-stranded NRE1 or to DNA ends (Giffin et al., 1999). Treatment of these bound complexes with proteases suggested that the Ku bound to all of these forms of DNA had a core binding component that was resistant to proteases and that had identical mobilities in EMSA (Giffin et al., 1999). This core component appeared to have a significantly less stable interaction with DNA than the intact Ku (Giffin et al., 1999).

The features which distinguish NRE1-binding from MT-binding appear critical for transcriptional regulation by Ku. In MMTV LTR reporter constructs site-directed

mutagenesis was used to remove one copy of the direct repeat in the NRE1 element making it identical to the MT sequence. The transcriptional activity of this modified promoter in response to glucocorticoids was similar to that of a promoter with the entire NRE1 deleted (Giffin et al., 1999). The MT element was therefore unable to mediate repression of the glucocorticoid-induced transcription of MMTV. This was consistent with the observation that a multimerized MT element inserted upstream of the hormone responsive region of the MMTV LTR did not repress glucocorticoid-induced transcription (Giffin et al., 1994).

e) Characterization of DNA-PK activity on NRE1

The Ku heterodimer forms the DNA binding subunit of DNA-PK, a serine-threonine protein kinase. Ku recruits the DNA-PK catalytic subunit (DNA-PK_{cs}) to DNA and acts as an allosteric activator (Gottlieb and Jackson, 1993). When bound to DNA, DNA-PK is able to phosphorylate a number of protein substrates *in vitro* including several transcription factors (Anderson, 1993; Anderson and Lees-Miller, 1992). Moreover, both Ku subunits and DNA-PK_{cs} can be autophosphorylated by the DNA-PK holoenzyme (Lees-Miller et al., 1990) (see section 2 for a detailed discussion of DNA-PK). This suggested that Ku could recruit DNA-PK_{cs} to the NRE1 and that the DNA-PK kinase activity might be important for the transcriptional effects of NRE1. Indeed, in EMSA using DNA microcircles we observed that Ku could recruit DNA-PK_{cs} to the DNA if the microcircles contained NRE1 or MT (Giffin et al., 1999). This also occurred on non-specific DNA ends but did not occur on the upper-stranded NRE1 (Giffin et al., 1999).

The recruitment of DNA-PK_{cs} to NRE1 by Ku activated the DNA-PK kinase activity. This was shown using MMTV LTR-containing plasmids on which purified DNA-PK was able to phosphorylate both baculovirus expressed GR and a bacterially expressed Oct-1 *in vitro* (Giffin et al., 1996). Both of these factors bind to the MMTV LTR and are involved in the MMTV transcriptional response to glucocorticoids. This phosphorylation was dependent on the ability of Ku to bind to these plasmids as it only occurred on plasmids containing NRE1 or on linear plasmids with exposed DNA ends (Giffin et al., 1996). The requirement for co-localization of DNA-PK and its substrate was examined using a bacterially expressed DBD of GR (amino acids 407-568) fused to GST (GST-GR_{X568}). In *in vitro* kinase assays this protein was phosphorylated by DNA-PK on plasmids containing NRE1 or on linearized plasmids. Digestion of the phosphorylated GST-GR_{X568} with thrombin, which cleaves this protein between the GST domain and the GR DBD showed that the phosphorylation occurred in the GR DBD and not on the GST domain (Giffin et al., 1997). Further, by using a phosphorylation site mapping strategy similar to that outlined in Figure 14, as described in the Results section, it was determined that the DNA-PK phosphorylated the GST-GR_{X568} on a single site corresponding to serine 527 on the full-length receptor. This phosphorylation site was consistent with the previously reported consensus site for DNA-PK, consisting of serine followed by glutamine (Anderson and Lees-Miller, 1992). In GR this serine is located in the hinge region, adjacent to the DBD.

Phosphorylation required that GST-GR_{X568} be co-localized to the DNA with DNA-PK since a single amino acid substitution that eliminates GST-GR_{X568} DNA binding also eliminated phosphorylation (Giffin et al., 1997). Moreover, phosphorylation

of GST-GR_{X568} could be competed by addition of an excess of a closed-circular plasmid containing the MMTV hormone responsive region but not NRE1 (Giffin et al., 1997). The DNA binding requirements for phosphorylation paralleled the DNA binding properties of Ku. For example, phosphorylation of GST-GR_{X568} from DNA ends on a linear plasmid lacking NRE1 could be efficiently competed by a closed-circular plasmid that contained NRE1 (Giffin et al., 1997). By contrast phosphorylation from NRE1 on closed-circular plasmids was not efficiently competed by a linearized plasmid (Giffin et al., 1997). These results reflected the stronger binding of Ku to NRE1 compared to DNA ends.

The kinase activity of DNA-PK is not determined solely by the ability of Ku to bind DNA, instead it appears that the conformation of Ku on the DNA also influences its ability to recruit the DNA-PK_{CS}. For example the GST-GR_{X568} was not phosphorylated by DNA-PK *in vitro* on a closed-circular MMTV LTR-containing plasmid in which the NRE1 was substituted for the MT element (Giffin et al., 1999). Therefore although Ku bound to MT was observed to recruit DNA-PK_{CS} to the DNA in EMSA (Giffin et al., 1999) it did not support kinase activity. This could be explained by the differential contact of the Ku subunits to the MT element as compared to the NRE1 and the differential conformations of MT-bound Ku and NRE1-bound Ku (Giffin et al., 1999).

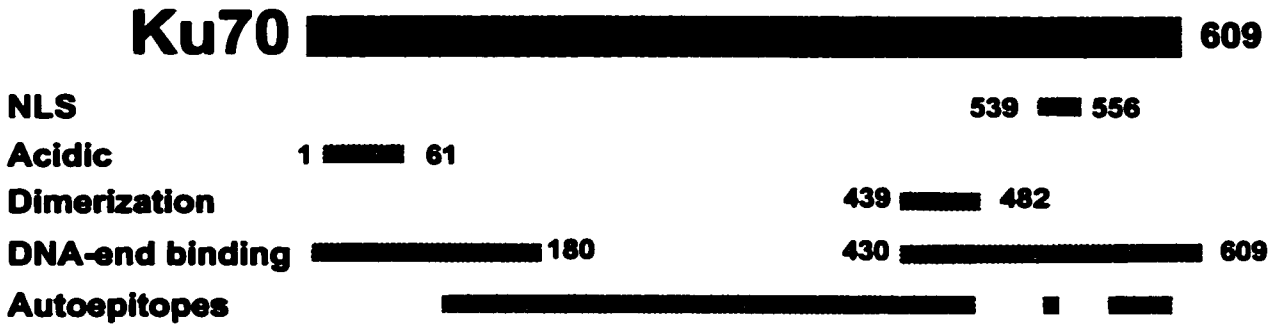
To examine the DNA-PK kinase activity on the NRE1 upper-strand, a synthetic peptide containing the sequence of the p53 DNA-PK phosphorylation site was used as a substrate. This peptide is phosphorylated by DNA-PK in trans and is widely used in measuring DNA-PK activity (Lees-Miller et al., 1992). Consistent with previous results this peptide was strongly phosphorylated by DNA-PK on DNA ends. However, if the

upper-stranded MTV oligonucleotide was used as a template, phosphorylation of this peptide did not occur (Giffin et al., 1999). This reflects the fact that DNA-PK_{CS} does not form a complex with Ku on single-stranded NRE1 as observed in EMSA (Giffin et al., 1999).

2. The DNA-dependent protein kinase and the Ku autoantigen

The DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase that is activated upon association with DNA (Gottlieb and Jackson, 1993; Smith and Jackson, 1999). The DNA-PK holoenzyme is composed of a ~470 kD catalytic subunit, termed DNA-PK_{CS}, and a regulatory factor termed Ku (Dvir et al., 1992; Gottlieb and Jackson, 1993). DNA-PK_{CS} is a member of the ATM family of proteins with homology to Phosphatidylinositol 3 kinase over its C-terminal ~500 residues (Hartley et al., 1995). Despite this homology, DNA-PK appears to have strictly protein kinase activity without any of the lipid kinase activity associated with phosphatidylinositol 3 kinase (Hartley et al., 1995; Smith et al., 1999). Other than this relatively small region the remainder of DNA-PK_{CS} appears unrelated to other known proteins (Hartley et al., 1995).

Ku is a heterodimeric protein originally identified as an autoantigen recognized by sera from patients with autoimmune diseases (Francoeur et al., 1986; Mimori et al., 1981; Mimori et al., 1986; Reeves, 1985). The Ku heterodimer is composed of two tightly associated subunits of 70 and 82 kD (Ku70 and Ku80 respectively) (Figure 2). Ku is abundant (4 X 10^5 copies per HeLa cell nucleus) and found in most human cells (Francoeur et al., 1986; Mimori et al., 1981). Ku homologues have been identified in organisms from yeast to humans, and potentially plants (Tuteja and Tuteja, 2000). The


Ku70
609
NLS
539
556
Acidic
1
61
Dimerization
439
482
DNA-end binding
180
430
609
Autoepitopes

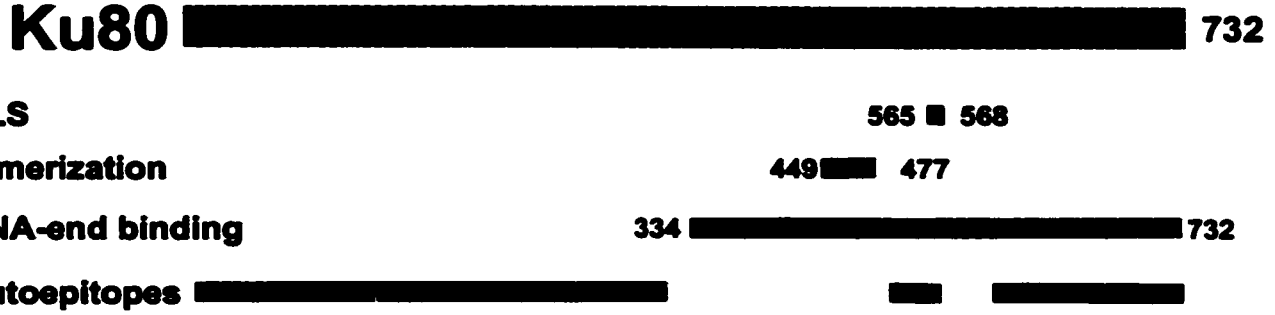

Ku80
732
NLS
565
568
Dimerization
449
477
DNA-end binding
334
732
Autoepitopes

Figure 2. Schematic representation of the domains of Ku70 and Ku80.

The Ku70 and Ku 80 subunits are represented schematically. Beneath the full length subunits the positions of known domains are indicated. Domains which are indicated are: NLS - nuclear localization signal. Acidic - acidic region of Ku70. Dimerization - domains required for heterodimerization of the subunits. DNA-end binding - domains necessary for DNA-end binding. Autoepitopes - epitopes recognized by antibodies in patients with autoimmune disease.

Ku/DNA-PK_{CS} complex has been shown to be crucial for DSB repair and V(D)J recombination (Smith and Jackson, 1999). Additionally, the holoenzyme or its components have been implicated in transcription, telomere maintenance, cell cycle regulation and replication (Tuteja and Tuteja, 2000).

(i) Activities of the Ku heterodimer

a) Binding to DNA ends or structures

Early studies on Ku discovered that it was able to bind to DNA ends independently of the sequence (Falzon et al., 1993; Mimori and Hardin, 1986; Paillard and Strauss, 1991). The structure at the end also appears unimportant as it can recognize 5' and 3' overhangs, hairpin ends and ends produced by ionizing radiation or cisplatin damage (Griffith et al., 1992; Mimori et al., 1981; Pang et al., 1997; Turchi and Henkels, 1996). Blunt and overhanging ends are recognized with approximately equal affinity (Griffith et al., 1992). Ku is unable to bind to closed circular DNA and only weakly to single-stranded DNA (Griffith et al., 1992; Mimori and Hardin, 1986; Tuteja et al., 1994), with the exception of NRE1 containing circles or single-strands (Giffin et al., 1997; Giffin et al., 1996; Torrance et al., 1998). Ku also appears to bind to virtually any type of single- to double-strand transition including closed hairpins and gapped and nicked molecules (Blier et al., 1993; Falzon et al., 1993; Tuteja et al., 1994). Moreover, Ku is able to translocate from one DNA molecule to another if the two DNA molecules have complementary ends (Bliss and Lane, 1997).

. Despite several attempts to identify the DBDs within the two Ku subunits, their localization has remained controversial (Figure 2). It appears that both subunits are

required for DNA end binding (Griffith et al., 1992; Ochem et al., 1997; Ono et al., 1994; Wu and Lieber, 1996) however in southwestern (Chou et al., 1992) and immunoprecipitation assays Ku70 appears able to bind in isolation (Wang et al., 1994a; Wang et al., 1994b). Using *in vitro* translated Ku it was demonstrated that the C-terminal 40 kD of Ku70 and the C-teminal 45 kD of Ku80 are necessary for binding in EMSA (Wu and Lieber, 1996). However Osipovich et al. have shown that the region between 210 to 531 of Ku80 forms a DNA end binding core (Osipovich et al., 1999). Amino acids 536 to 609 of Ku70 appear to be necessary and sufficient for binding to DNA ends in a southwestern assay (Chou et al., 1992).

Ku binds to double-stranded DNA termini, and once bound is able to traverse along the DNA without an energy requirement (Blier et al., 1993; de Vries et al., 1989; Paillard and Strauss, 1991). *In vitro* many Ku heterodimers can bind a DNA molecule, with each recognizing the end then translocating internally, appearing like beads on a string (de Vries et al., 1989). Each dimer contacts 13-21 bp and successive molecules bind at 25-30 bp intervals (de Vries et al., 1989).

b) Binding to specific sequences

Several sequence specific binding sites for Ku have been reported. Putative Ku binding sites have been found in transcriptional regulatory regions of genes encoding the transferrin receptor (Osipovich et al., 1999), U1 snRNA (Knuth et al., 1990), *grp78* (Alexandre et al., 1991), T-cell receptor β chain (Messier et al., 1993), collagen IV (Genersch et al., 1995), parathyroid hormone (Chung and Miller, 1988) and c-myc (Giffin et al., 1997). Binding has been reported to sequences in HTLV-1 (Okumura et al., 1996; Okumura et al., 1994) and MMTV (Giffin et al., 1996). Binding of Ku or Ku like

proteins has been reported to the immunoglobulin octamer motif (May et al., 1991), the AP-1 binding site (Quinn and Farina, 1991) and a heat shock element (Kim et al., 1995). Finally putative sites have also been observed in a replication origin (Toth et al., 1993) and in a BCL2 major breakpoint (DiCroce and Krontiris, 1995).

As was discussed in detail in Section I.(v).d) these binding sites fall into two classes, those carrying extensive polypurine/polypyrimidine sequences which form direct binding sites for Ku and those without polypurine/polypyrimidine sequences which are not bound directly (Giffin et al., 1997). The MT element comprises a third class which is bound directly but displays different characteristics than NRE1 as discussed in Section I.(v).d).

c) ATPase and helicase activities

Ku has been reported to have associated DNA-dependent ATPase and ATP-dependent helicase activities (Cao et al., 1994; Ochem et al., 1997; Tuteja et al., 1997; Tuteja et al., 1994). The helicase activity was reported to progress in the 3' to 5' direction (Tuteja et al., 1994). This activity required a single- to double-strand transition but did not act from nicks (Tuteja et al., 1994). Finally, activity was strongest on duplex DNA with forked ends (Tuteja et al., 1994). This activity would help to explain the suggestion that DNA-PK activity requires contact with both double- and single-stranded DNA (Leuther et al., 1999; Suwa et al., 1994). It could also partially explain the hypersensitivity to nucleases induced in the MMTV promoter upon Ku binding to NRE1 (Giffin et al., 1999; Giffin and Hache, 1995) although this does not involve complete unwinding of the DNA. Despite this, these activities remain controversial and it has been

suggested that they may have been due to co-purifying or co-immunoprecipitating factors (Dynan and Yoo, 1998).

d) Stimulation of mammalian DNA ligases

Atomic force microscopy studies have observed both DNA end bound and internal DNA-PK complexes (Cary et al., 1997). These studies also demonstrated that Ku could juxtapose two DNA ends, likely through interactions between two heterodimers (Cary et al., 1997). This correlates with the ability of Ku to stimulate DNA end joining by human DNA ligases I, II and IV (Ramsden and Gellert, 1998). This stimulation of ligation however appears specific for mammalian ligases since Ku inhibits the function of prokaryotic ligases probably by blocking access of the ligase to the ends (Griffith et al., 1992).

(ii) The kinase activity of DNA-PK

DNA-PK was originally identified as a kinase that could phosphorylate a number of proteins in a DNA dependent manner. Targets of DNA-PK include hsp90, SV40 Large TAg, p53, Ku, RNA pol II, serum response factor, RP-A, topoisomerases and transcription factors such as c-jun, c-fos, Oct-1, SP-1, c-myc, NF-1 and TFIID (Anderson and Lees-Miller, 1992; Carter et al., 1990; Lees-Miller et al., 1990). DNA-PK appears to have relatively low substrate specificity, recognizing either serine or threonine followed by glutamine (S/T-Q) (Anderson and Lees-Miller, 1992).

Kinase activity is most efficient when the substrate molecule is bound to the same DNA molecule as DNA-PK itself, indicating that juxtaposition of DNA-PK with its target is part of the activating effect of DNA (Gottlieb and Jackson, 1993). However,

DNA also activates phosphorylation of non-DNA-binding proteins indicating that an activating conformational change is induced upon DNA binding. Ku was shown to co-purify with DNA-PK_{CS} and was later shown to be the component of DNA-PK that confers DNA binding (Dvir et al., 1992; Dvir et al., 1993; Gottlieb and Jackson, 1993; Lees-Miller et al., 1990; Morozov et al., 1994). Ku therefore is also an allosteric activator of DNA-PK_{CS} (Yaneva et al., 1997). DNA can activate DNA-PK_{CS} in the absence of Ku, although Ku increases the degree of activation and appears to stabilize the DNA interaction (Hammarsten and Chu, 1998; West et al., 1998; Yaneva et al., 1997). These data suggest a model whereby DNA-PK_{CS} is recruited to DNA by Ku whereupon it makes DNA contacts which confer activating conformational changes (Smith and Jackson, 1999). The structure of DNA-PK_{CS} has been resolved at a resolution of ~20 and 22 Å (Chiu et al., 1998; Leuther et al., 1999) and interestingly, biochemical analysis based on these structures suggests that kinase activation requires interaction with both double- and single-stranded DNA (Leuther et al., 1999; Suwa et al., 1994).

In addition to Ku and DNA, other factors have been proposed to regulate DNA-PK activity. First, DNA-PK has been shown to be auto-regulatory. DNA-PK_{CS} and both of the Ku subunits are phosphorylated by the holoenzyme, this 'autophosphorylation' has been demonstrated to induce dissociation from Ku and to inhibit the kinase activity (Chan and Lees-Miller, 1996; Lees-Miller et al., 1990). Phosphorylation by the c-Abl kinase has also been shown to dissociate the DNA-PK_{CS} from Ku *in vitro* (Jin et al., 1997; Kharbanda et al., 1997). Secondly, interactions of other proteins with DNA-PK have been observed *in vitro* and suggested to regulate the kinase. A putative leucine zipper has been reported to be required for interaction with the high affinity DNA binding protein

C1D (Yavuzer et al., 1998). C1D bound to DNA can stimulate kinase activity in the absence of DNA ends, possibly by perturbing the structure of the DNA double helix (Yavuzer et al., 1998). HMG proteins 1 and 2 also activate DNA-PK *in vitro*, suggesting an influence of chromatin context (Watanabe et al., 1994; Yumoto et al., 1998). Finally HSF1 stimulates DNA-PK *in vitro* likely through interactions with Ku and DNA-PK_{CS} suggesting stabilization of the kinase on the DNA (Huang et al., 1997; Peterson et al., 1995). A third mechanism by which DNA-PK is regulated is by proteolysis following apoptosis. Caspase-3 or a related protease cleaves DNA-PK_{CS} during apoptosis. This is likely to prevent activation of the non-homologous DNA end-joining pathway in response to the degraded genomic DNA generated during apoptosis (Han et al., 1996; Le Romancer et al., 1996; Song et al., 1996).

(iii) The role of DNA-PK in DSB repair and V(D)J recombination

The involvement of DNA-PK in DSB repair was established through analyses of mutant rodent cell lines that were hypersensitive to ionizing radiation and were defective in the repair of chromosomal DNA DSBs and the V(D)J recombination pathway (Zdzienicka, 1995). These cells were grouped into three distinct complementation groups, IR4, IR5 and IR7, with their complementing genes called XRCC 4, 5 and 7 (for X-ray cross-complementing). Through a series of studies it was demonstrated first that the IR5 cells lacked a Ku-like end binding activity, and that the defect was complemented by the Ku80 gene (Rathmell and Chu, 1994a; Smider et al., 1994; Taccioli et al., 1994). This discovery prompted studies that demonstrated that IR7 cells lacked DNA-PK activity and that the defect was complemented by the DNA-PK_{CS} gene (Blunt et al., 1995; Kirchgessner et al., 1995). Later it was established that IR5 and IR7 cells harbor

inactivating mutations in the Ku80 and DNA-PK_{CS} gene (Araki et al., 1997; Blunt et al., 1996; Danska et al., 1994; Errami et al., 1996). Later, targeted disruption of the Ku70 gene demonstrated it had similar defects, and such cells were designated IR6 (Gu et al., 1997). The gene encoding XRCC4 was identified as a novel protein that interacts with and stimulates DNA ligase IV (Leber et al., 1998).

In mammalian cells DSB repair occurs primarily through the NHEJ pathway. It is this pathway in which Ku and DNA-PK_{CS} participate. DSB repair in this pathway does not require extensive homologies between the recombining DNA molecules nor does it require an undamaged DNA partner, differentiating it from the homologous recombination pathway (Chu, 1997; Critchlow and Jackson, 1998). How NHEJ occurs and what roles the DNA-PK components play is poorly understood although several possibilities have been proposed. First, the DNA end binding properties of Ku suggest that it directly binds DSBs in vivo and protects the end from nucleolytic degradation (de Vries et al., 1989; Gottlieb and Jackson, 1993; Mimori and Hardin, 1986). Alternatively the DNA-PK holoenzyme could recruit other NHEJ factors or modify their activity through phosphorylation. For example XRCC4 is phosphorylated by DNA-PK *in vitro* and XRCC4/ligase IV complexes interact with DNA-PK (Leber et al., 1998). Another possibility is that Ku/ DNA-PK_{CS} promote ligation by tethering two DNA ends, corresponding to its ability to juxtapose double-stranded ends *in vitro* and to promote ligation (Cary et al., 1997; Ramsden and Gellert, 1998). Ku/DNA-PK_{CS} could dissociate repair factors from the DNA following repair or remove other factors that might block repair (Zhu et al., 1996). Finally, yeast Ku has been shown to interact with Sir4p, a component of heterochromatin and this prompted the suggestion than Ku could recruit

the Sir complex to DSBs which could facilitate chromatin condensation, and block access to nucleases or promote juxtaposition of the DNA termini (Tsukamoto et al., 1997).

Differences in the functions of the DNA-PK components are suggested by observations of the V(D)J recombination pathway. V(D)J recombination is the process by which immunoglobulin and TCR genes are rearranged, leading to the formation of coding joints, which encode the highly variable-antigen binding regions of the antigen receptors and nonfunctional signal joints (Gellert, 1997). The first step in this process is the cleavage of the DNA between signal and coding sequences by the RAG1 and 2 proteins, generating blunt ended signal termini and covalently-closed hairpin coding termini (Ramsden et al., 1997). Religation of the ends follows, which for signal termini involves simple end joining whereas for the coding termini the hairpins must first reopen before ligation can ensue (Ramsden et al., 1997). These steps are affected differentially in *scid* mice, which are members of IR7 and have a defect in the DNA-PK_{CS} gene that truncates the final 83 amino acids of the protein (Araki et al., 1997; Blunt et al., 1996; Danska et al., 1994). In these mice, after the formation of the DSBs, formation of coding joints is highly impaired, while signal joints still form but with reduced efficiency (Bogue et al., 1998). A similar effect is observed in DNA-PK_{CS} knockout mice (Jhappan et al., 1997; Taccioli et al., 1998). By contrast, in Ku80 or Ku70 knockout mice both coding ends and signal ends accumulate (Zhu et al., 1996) however in $Ku70^{-/-}$ mice residual signal joints are observed (Gu et al., 1997). These observations suggest that there are mechanistic differences between coding and signal joint formation. It appears that DNA-PK_{CS} plays a more critical role in resolving the hairpin ends of the coding joints. Interestingly DNA-PK can bind hairpin ends but is not active on them (Smider et al.,

1998). It could potentially act by recruiting factors involved in resolving the hairpin such as RAG1/2 (Zhu et al., 1996).

(iv) Transcription

The role of Ku/ DNA-PK_{CS} in transcriptional regulation of the glucocorticoid-induced transcription of MMTV was introduced in Section I.(v).d). As has already been described, Ku has been reported to bind to a number of specific sequences, several of these have been suggested to have transcriptional regulatory implications. Ku has been proposed to regulate a number of other genes, examples of which include: negative regulation of the human parathyroid hormone gene (Chung et al., 1996); repression of HTLV-1 basal transcription (Okumura et al., 1994); silencing of the metallothionein-I-Promoter due to hypermethylation in response to Ku overexpression (Majumder et al., 1999); and finally the inhibition of RNA pol I-directed transcription by Ku alone or, more strongly, by the DNA-PK holoenzyme (Kuhn et al., 1995; Kuhn et al., 1993).

Moreover, Ku-like proteins have been implicated in transcription of several genes including: activation of the U1 gene (Knuth et al., 1990); repression of heat shock genes (Li et al., 1995); regulation of the human collagen IV gene (Genersch et al., 1995); and activation of the Drosophila yolk protein-1 gene (Jacoby and Wensink, 1994).

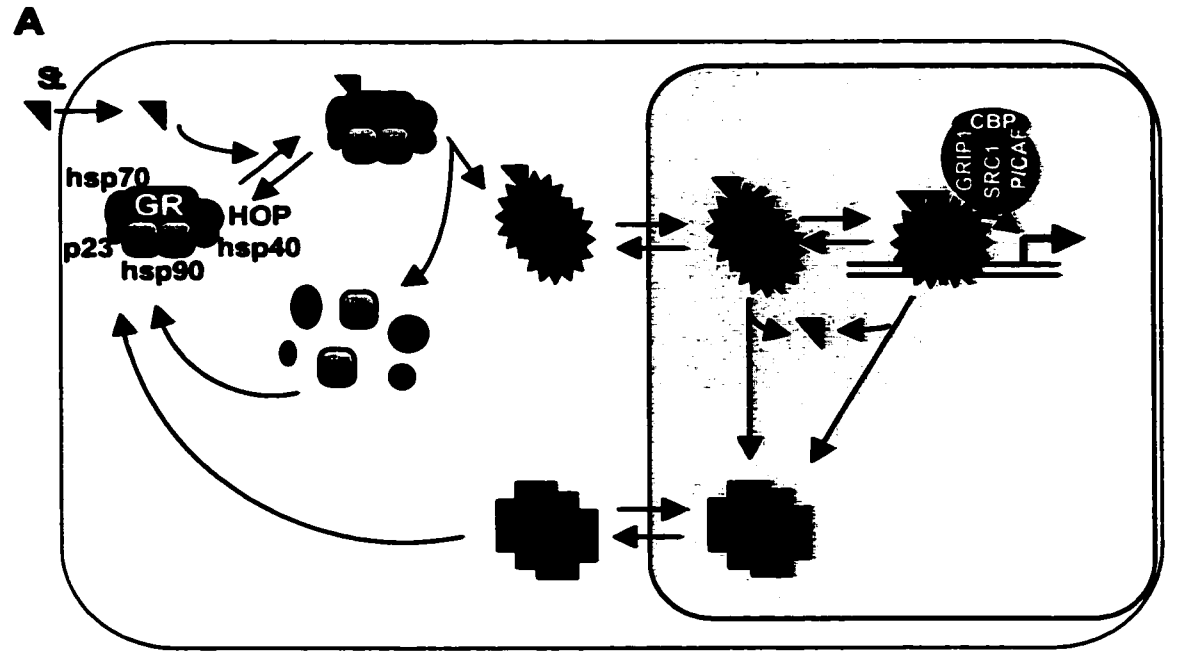
3. The glucocorticoid receptor

GR is member of the nuclear receptor superfamily, which is comprised of over 300 gene regulatory, DNA binding proteins (Laudet, 1999; Sluder et al., 1999). GR belongs to a subset of this family comprised of the steroid hormone receptors which are

ligand-activated transcription factors. The steroid receptors represent the most recent evolutionary grouping of the nuclear receptors, being found to date only in vertebrates (Whitfield et al., 1999). The members of this group, which contain the receptors for the adrenal steroids (glucocorticoids, mineralocorticoids) and the sex steroids (progestins, androgens and estrogens), are highly similar not only in their primary sequence but also in their overall domain structure (Whitfield et al., 1999).

(i) Glucocorticoid hormone action

GR mediates the effects of glucocorticoid hormones in vertebrates (Whitfield et al., 1999). Figure 3, panel A, shows a summary of the classical pathway by which glucocorticoids act. In the absence of hormone GR is localized in the cytoplasm in a large heterocomplex that sediments at approximately 8S on sucrose gradients (Pratt and Toft, 1997). This complex contains a large number of proteins including heat shock proteins (hsps 90,70 and 40), high molecular weight immunophilins (p56/59 and CyP-40) and other non receptor proteins (p23 and p50) (Pratt, 1993; Pratt et al., 1996; Pratt and Toft, 1997; Smith, 1993). This complex is required for ligand binding activity by GR (Bresnick et al., 1989; Hutchison et al., 1992). A large amount of evidence supports the hypothesis that this complex opens a hydrophobic pocket in the LBD to allow access by steroids (Giannoukos et al., 1999; Hutchison et al., 1992; Pratt, 1993; Smith, 1993; Stancato et al., 1996; Xu et al., 1998). Recently purification of components of the complex has shown that five proteins are required for its optimal assembly: hsp90, hsp70, Hop, hsp40 and p23 (Morishima et al., 2000a). While only hsp90 and hsp70 are absolutely required for opening the steroid binding pocket of GR, the other proteins act as co-chaperones increasing the efficiency (Morishima et al., 2000a). Other members of the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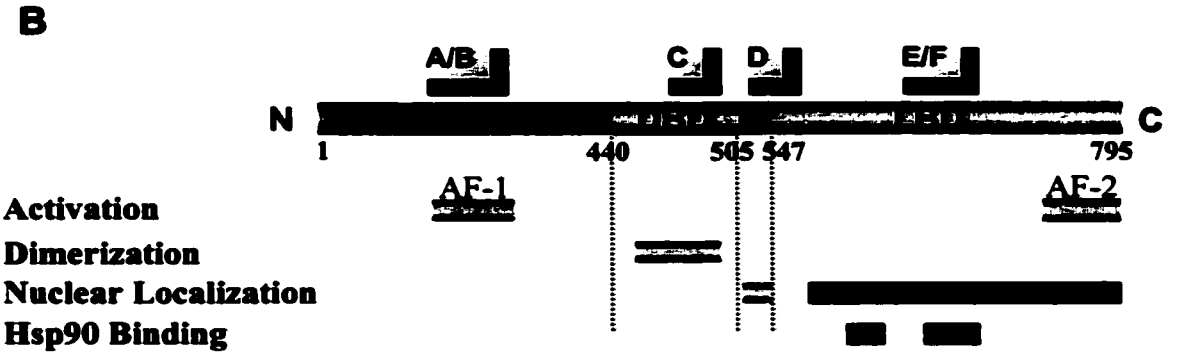
hsp70
GR
HOP
p23
hsp40
hsp90
CBP
GRIP1
SRC1
P/CAF


B
A/B
C
D
E/F
N
C
1
440
505
547
795
AF-1
AF-2
Activation
Dimerization
Nuclear Localization
Hsp90 Binding

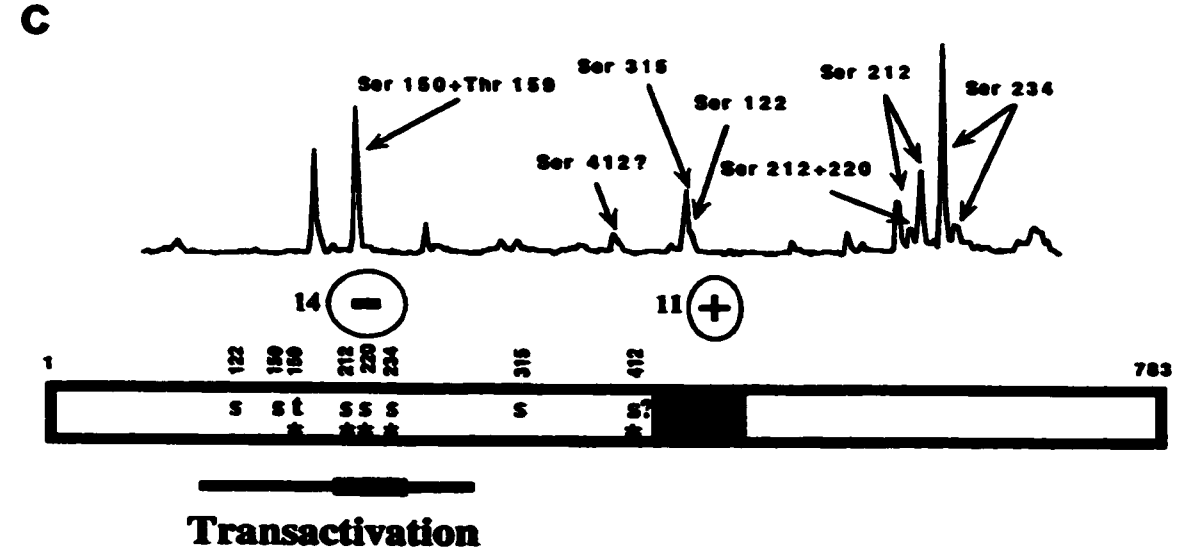

C
Ser 150+Thr 159
Ser 315
Ser 122
Ser 212
Ser 234
Ser 412?
Ser 212+220
14
11
1
783
Transactivation

Figure 3. Glucocorticoid action, GR structure and phosphorylation.

Panel A: Glucocorticoid hormone acion.Glucocorticoids enter the cell through diffusion. They bind to the GR 8S complex and promote the dissociation of the complex and the activation of GR. Transformed GR (4S) translocates to the nucleus and binds the GREs to regulate target gene expression. Disassociation of the steroid results in the disassociation from the DNA and eventual recycling into the 8S complex and cytoplasmic localization.
Panel B: The domain organization of rat GR. The domains (A to F) are shown and the relative positions of the DNA binding domain (DBD), ligand binding domain (LBD), transcriptional activation domains, dimerization domains, nuclear localization signals and hsp90 binding sites.
Panel C: Phosphorylation sites on mouse GR. The seven sites are indicated at the bottom in the domain structure. Sites indicated with a * are proline directed sites. Serine 412 is tentatively identified. Regions of excess negative and positive charge are identified. At the top is an HPLC profile showing the assignment of peaks to peptides containing the phosphorylation sites. The horizontal axis is the fraction eluted from the HPLC while the vertical axis represents the level of ^{32}P in each fraction (taken from Bodwell, 1998)

steroid receptor family associate into similar complexes however only GR and MR appear to require this for ligand binding (Alnemri and Litwack, 1993; Caamano et al., 1993; Eul et al., 1989; Seielstad et al., 1995; Smith, 1993; Young et al., 1990). This suggests the complex may play other roles such as inhibiting DNA binding, dimerization, transactivation or, for GR and MR at least, nuclear translocation (Bresnick et al., 1989; Picard et al., 1990; Pratt, 1993; Pratt et al., 1996; Pratt and Toft, 1997).

Upon hormone binding, GR dissociates from the heterocomplex, transforming into a conformation that sediments at 4S on sucrose gradients (Pratt and Toft, 1997). Through this transformation GR acquires the ability to dimerize, bind DNA and activate transcription (Pratt and Toft, 1997). Immediately following hormone binding, GR translocates to the nucleus where it binds as a homodimer to its DNA recognition sequence (or GRE) in target genes and alters gene expression. GR is widely regarded as a transcriptional activator due to its ability to promote the transcription of a wide variety of genes such as MMTV. The mechanisms involved in transactivation will be discussed in greater detail in section 3 (iii). A number of genes are also repressed by GR, this repression typically occurs via direct protein-protein interactions or by competitions for DNA binding sites. Several genes activated by the transcription factor AP-1, such as the collagenase gene, are repressed by GR in the absence of a GRE (Heck et al., 1994; Konig et al., 1992). This occurs by a direct protein-protein interaction between the DBD of GR and AP-1 which inhibits the ability of AP-1 to activate transcription (Heck et al., 1994; Konig et al., 1992). A similar example occurs with proinflammatory genes that are activated by NF-κB (Mukaida et al., 1992; Ray and Prefontaine, 1994). GR appears to act by binding NF-κB directly and interfering with a necessary phosphorylation event on

RNA polymerase II (Nissen and Yamamoto, 2000). Alternatively, cyclic-AMP mediated activation of the gene for the α-subunit of the glycoprotein hormones by CREB is repressed by GR through a mechanism proposed to involve competition for the DNA binding sites (Akerblom et al., 1988).

Steroid binding is transient ($t_{1/2}$ = 5-10 min) (Munck and Holbrook, 1984), and the loss of hormone from the receptor results in dissociation from the DNA and reassembly into an 8S complex (Pratt and Toft, 1997). This process involves two ATP-dependent steps, the first being the binding of hsp70 and the second the binding of hsp90 (Morishima et al., 2000b). GR also relocalizes to the cytoplasm although this occurs relatively slowly requiring 12 to 24 h (Hache et al., 1999; Qi et al., 1989; Sackey et al., 1996).

(ii) Structural and functional domains of GR

Like other members of the nuclear receptor superfamily, GR is modular in structure with domains that largely retain their activities in isolation from the full protein (Evans, 1988). The functional domains of GR are organized into six homologous domains designated A-F (see Figure 3, panel B). Many of the domains contain several overlapping functions. In the discussions of the domains below all amino acid positions refer to the positions in the rat GR.

a) The N-terminal or A/B domain

The most important function ascribed to the N-terminal, or A/B domain, (amino acids 1-439) is transcriptional activation. This activity, referred to as activation function 1 (AF-1), (Bocquel et al., 1989; Hollenberg and Evans, 1988; Tasset et al., 1990) is

hormone independent and has been mapped to amino acids 98-292 of the rat GR by deletion experiments (Hollenberg and Evans, 1988). Transcriptional activation by AF-1 appears to occur by recruitment of target factors to the regulated promoters. Hydrophobic residues in this region have been found to be critical for both *in vivo* gene activation (Almlof et al., 1997) and *in vitro* interactions with target factors (Almlof et al., 1998). The AF-1 domain has been shown to mediate interactions with TBP (Ford et al., 1997), CBP (Almlof et al., 1998) and Ada2 (Henriksson et al., 1997). Otherwise, this domain is not directly involved in DNA binding, hormone binding, receptor activation or dimerization and hence has been referred to as a modulatory domain. One additional property ascribed to this region is the ability to decrease non-specific DNA binding (Danielsen et al., 1987). This has been postulated to be due to the excess negative charge in this region.

b) The DNA binding or C domain

Among the nuclear receptor family the DBD is the most highly conserved domain (Truss and Beato, 1993). The DBD (amino acids 440-504) mediates both specific and non-specific DNA binding and is characterized by two Zn^{2+} fingers each formed by the tetrahedral coordination of four cysteine residues to a Zn^{2+} atom (Freedman et al., 1988; Hard et al., 1990a; Hard et al., 1990b). GR binds to DNA as a homodimer recognizing bipartite DNA sequences, or glucocorticoid response elements (GRE). The consensus GRE consists of a palindrome of the sequence 5'-AGAACA-3' separated by three nucleotides (Beato, 1989; Beato et al., 1989; Green et al., 1988; Gronemeyer, 1991; Truss and Beato, 1993). The GR monomers within the homodimer interact to cooperatively bind DNA. The two Zn^{2+} fingers play distinct roles in GRE recognition, the N-terminal

finger makes nucleotide specific contacts with the DNA providing the DNA binding specificity (Luisi et al., 1991). The specificity is determined by the P-box, a cluster of amino acids at the C-terminal end of this finger which for GR as well as the other steroid receptors (but not the ERs) is highly divergent from that of the other nuclear receptors. The C-terminal finger mediates the cooperativity of binding between the two GR monomers by forming amino acid contacts through an interface known as the D-box (Dahlman-Wright et al., 1992; Danielian et al., 1992; Luisi et al., 1991; Umesono and Evans, 1989). Hence the D-box mediates the second major function of this domain, DNA-dependent dimerization. In the absence of DNA binding dimerization does not occur through this interface even at high protein concentrations (Hard et al., 1990b).

This domain also mediates several protein-protein interactions between GR and other transcription factors. Proteins observed to interact with this domain include Oct-1 and 2 (Prefontaine et al., 1998), Stat 5 (Stocklin et al., 1996), Nurr 77 (Philips et al., 1997), NFκB (Ray and Prefontaine, 1994), NF-IL6 (Nishio et al., 1993) and AP-1 (Heck et al., 1994). Additionally this domain has been shown to harbor a transcriptional activation domain, in which mutations can affect transactivation potential without affecting DNA binding (Hollenberg et al., 1987; Miesfeld et al., 1987; Schena et al., 1989). Finally, a NLS mapped to the hinge region partially overlaps with the DBD (Picard and Yamamoto, 1987; Tang et al., 1997).

c) The hinge or D domain

The D domain (amino acids 505-546) separates the DBD from the C-terminal ligand binding domain is called the hinge region as it has been thought of as a flexible link between the two domains. The hinge region contains a nuclear localization signal,

NL1, that is similar to the SV40 T antigen NLS (Hollenberg et al., 1987; Miesfeld et al., 1987). NL1 is a tripartite NLS with a core sequence adjacent to the DBD that is required for nuclear import. The NLS activity of this core sequence is enhanced by two clusters of basic amino acids in the C-terminus of the DBD (Tang et al., 1997; Ylikomi et al., 1992).

d) The ligand binding or E/F domain

The first function assigned to the GR LBD (amino acids 547-795) was the ability to bind glucocorticoids. This binding is thought of as a molecular switch that transforms the receptor from an inactive state to a transcriptionally active form (Truss and Beato, 1993). The LBD is now known to be a multifunctional domain that participates in several other receptor functions.

The LBD is the region of the receptor that interacts directly with hsp90 in the 8S receptor complex (Dalman et al., 1991; Howard et al., 1990; Pratt et al., 1988; Xu et al., 1998). A specific hsp90 binding site has not been identified, however it is thought to make multiple protein contacts in the LBD with the sequences from 537-673 being sufficient for this interaction. A further function assigned to the LBD is nuclear localization (Picard and Yamamoto, 1987). This activity called NL2 is dependent on binding to ligand (Picard and Yamamoto, 1987; Savory et al., 1999). It has not been assigned to specific amino acids in the LBD, however unlike NL1 it does not appear to be a typical NLS consisting of clusters of basic amino acids. Further differentiating NL2 from NL1 is the fact that NL2 appears to participate in a nuclear import pathway independent of pendulin/importin α (Savory et al., 1999). A final function localized in the LBD is a transcriptional activation function designated AF-2, a ligand-dependent transactivation domain (Bocquel et al., 1989; Danielian et al., 1992; Hollenberg and

Evans, 1988; Tasset et al., 1990; Webster et al., 1988). Within AF-2 is an autonomous activating domain, termed AF-2 AD which maps to the extreme C-terminus of the LBD and is highly conserved in the nuclear receptor superfamily (Barettino et al., 1994; Durand et al., 1994; Leng et al., 1995). AF-2 AD is required for ligand dependent transactivation and for interaction between the LBD and the coactivator molecules such as GRIP-1, SRC-1 and CBP/p300 (Hong et al., 1996; Onate et al., 1995).

(iii) Transcriptional regulation by the glucocorticoid receptor

Transcriptional activation by the glucocorticoid receptor is mediated primarily by its two transactivation domains, AF-1 and AF-2 which act in a constitutive and ligand inducible manner respectively. The AF domains form interactions with coactivator proteins that mediate the downstream transcriptional effects of GR (Collingwood et al., 1999; Freedman, 1999). There are four major mechanisms by which GR regulates transcription, these include: chromatin remodeling, TBP/TATA targeting, pre-initiation complex stabilization and interactions with other transcription factors (Collingwood et al., 1999; Freedman, 1999; Whitfield et al., 1999).

It is thought that one of the major functions of coactivators is to relieve the repressive effects of chromatin through chromatin remodeling (Collingwood et al., 1999; Freedman, 1999). There are two major classes of chromatin modifying coactivators: the histone acetyltransferase (HAT) proteins and the SWI/SNF proteins. HAT coactivators were identified based on their interaction with the LBD of nuclear receptors (Torchia et al., 1998). These factors acetylate the N-terminal tails of histone genes destabilizing the chromatin (Collingwood et al., 1999; Freedman, 1999; Lemon and Freedman, 1999).

It is also becoming increasingly apparent that acetylation of non-histone proteins plays an important role in transcriptional regulation. For example, acetylation of p53 and GATA1 by p300 increases their sequence-specific DNA binding and transcriptional activity (Gu and Roeder, 1997; Boyes et al., 1998). Acetylation of the orphan nuclear receptor HNF-4 by CBP is necessary for proper nuclear retention and increases DNA binding activity (Soutoglou, 2000). By contrast, acetylation of HMG I(Y) by CBP dissrupts its DNA binding resulting in a dissruption of the enhanceosome and decreased IFN-B transcription (Munshi et al., 1998).

The p160 family is the best characterized family of HAT factors. GR interacts with two members of this family in a ligand dependent manner: SRC-1/NcoA-1 (Kamei et al., 1996; Onate et al., 1995) and GRIP-1/TIF2/NcoA-2 (Hong et al., 1996; Torchia et al., 1997; Voegel et al., 1996). A distinctive structural feature of the p160 coactivators is the presence of multiple LXXLL motifs which comprise determinants for direct interactions with nuclear receptor AF-2s (Xu et al., 1999). Biochemical and crystallographic data have revealed that an α-helix in the receptor AF-2 core becomes reorientated upon ligand binding allowing direct contacts with the LXXLL motifs (Xu et al., 1999). These coactivators recruit other HAT factors including CBP and p300 as well as the p300/CBP associated factor PCAF (Hanstein et al., 1996; Kamei et al., 1996; Voegel et al., 1998; Yao et al., 1996). These interactions indicate that very large HAT factories are assembled in the vicinity of ligand-bound receptor (Wade et al., 1997). Indeed different HAT factors have been observed in distinct complexes in the cell (McKenna et al., 1998).

A further mechanism of transcriptional regulation by members of the nuclear receptor family is recruitment of corepressors. The corepressors SMRT and N-CoR interact with the LBDs of unliganded TR, RAR and other nuclear receptors, mediating repression by DNA-bound, unliganded receptors (Chen and Evans, 1995; Chen et al., 1996; Horlein et al., 1995; Kurokawa et al., 1995; Sande and Privalsky, 1996; Lee et al., 1995). Both of these corepressors are components of large protein complexes that include the deacetylases HDAC1 and Sin3A (Taunton et al., 1996; Ayer et al., 1996; Schreiber-Agus et al., 1995), which can deacetylate histones, resulting in a chromatin state that is refractory to transcription (Grunstein, 1997; Wolffe et al., 1997). To date neither SMRT nor N-CoR have been observed binding to unliganded GR or other members of the steroid receptor family. However, antagonist bound ER and PR have both been observed to bind to these correpressors, presumably maintaining the receptors in a transcriptionally inactive state (Jackson et al., 1997; Zhang et al., 1998). Moreover, GR has been shown to recruit HDAC-2, mediating a repression of GM-CSF expression (Ito et al., 2000). These obeservations suggest that transcriptional regulation by GR may also be influenced by corepressors/histone deacetylase factors.

In contrast to the HAT/HDAC factors are the SWI/SNF family of proteins that have intrinsic ATPase activity and remodel nucleosomes by uncoupling ionic interactions between histones and DNA (Carlson and Laurent, 1994). These were first characterized in yeast as transcriptional regulatory proteins (Neigeborn and Carlson, 1984; Stern et al., 1984). The mammalian counterparts to the yeast proteins hbrm and BRG-1 are found in distinct complexes with BRG-1 associated factors (BAFs) (Wang et al., 1996). Evidence for the involvement of these factors in GR activated transcription first came from yeast,

where glucocorticoid-induced transcription was lost in Swi mutant yeast strains (Yoshinaga et al., 1992). Also the SWI/SNF factor SWP73 could potentiate GR activated transcription (Cairns et al., 1996). In mammalian cells hbrm cooperates with GR (Muchardt and Yaniv, 1993) and BRG-1 has been shown to be a GR target (Fryer and Archer, 1998). Moreover GR enhances the ability of a mammalian SWI/SNF complex to remodel MMTV LTR chromatin and facilitate NF-1 binding *in vitro* (Ostlund Farrants et al., 1997).

Transcriptional activation by GR can also occur by directly influencing components of the general transcription machinery to increase transcription rates. For example the AF-1 region of GR has been shown to interact directly with TBP *in vitro* suggesting the possibility that GR promotes targeting of TBP to the TATA box (Ford et al., 1997). Moreover, PCAF has been shown to associate with several TAFs which are components of TFIID and that interact with TBP (Ogryzko et al., 1998) indicating that GR coactivators can also potentially target TBP to the TATA box. It has also been demonstrated that CBP/p300 interact with RNA pol II (Kee et al., 1996; Nakajima et al., 1997) and TFIIB (Kwok et al., 1994; O'Connor et al., 1999) suggesting the possibility that GR, through its coactivators, can stabilize the pre-initiation complex.

GR has also been demonstrated to interact with several other transcription factors resulting in a modulation of their activity. For example an interaction between the GR AF-2 domain and C/EBP β on the thymidine kinase promoter leads to a potentiation of C/EBP-β activated transcription (Boruk, 1998). By contrast, a tethering interaction between the LBD of GR with AP-1 has been shown to repress the expression of collegenase type-I and collegenase-3 (Jonat et al., 1990; Schüle et al., 1990; Yang Yen et

al., 1990; Konig et al., 1992). Likewise, GR inhibits expression of a number of proinflammatory genes, one possible mechanism being via a tethering interaction with NF-κB (Ray and Prefontaine, 1994; Caldenhoven et al., 1995; Nissen and Yamamoto, 2000). Recent genetic evidence has suggested the importance of these tethering interactions. $GR^{-/-}$ knockout mice die shortly after birth and display several severe abnormalities, indicating that GR is essential for viability (Cole et al., 1995). However a targeted point mutation of the GR gene within the D-loop of the DBD which disrupts homo-dimerization and DNA binding resulted in viable mice, sugesting that the activites of GR which are required for viability are independent of DNA-binding (Reichardt et al., 1998).

(iv) Phosphorylation of the glucocorticoid receptor

Like all members of the steroid-homone receptor family GR is a phosphoprotein which undergoes hormone-dependent hyperphosphorylation (Orti et al., 1992; Weigel, 1996). Prior to stimulation by glucocorticoids the level of GR phosphorylation is relatively low, for example in mouse thyoma WEHI-7 cells there is an average of 2.6 phosphates per receptor (Mendel et al., 1987). Glucocorticoid-stimulation results in a 2-4 fold increase in phosphate incorporation in GR (Hock et al., 1989; Hoeck and Groner, 1990; Hoeck et al., 1989; Orti et al., 1989). Hyperphosphorylation is observed within 5 min following hormone treatment and persists for 20 h (Orti et al., 1993; Orti et al., 1989). The half-time for receptor dephosphorylation is 90 to 120 min indicating that hyperphosphorylation is due to increased phosphorylation and not decreased dephosphorylation (Orti et al., 1993). The antagonist RU486 does not induce hyperphosphorylation and blocks the glucocorticoid-induced phosphorylation indicating

that this effect is agonist specific and requires GR activation (Hock et al., 1989; Hoeck and Groner, 1990; Hoeck et al., 1989; Orti et al., 1993; Orti et al., 1989).

a) Sites of Phosphorylation

Seven phosphorylation sites in the mouse GR were identified by microsequencing of phosphotryptic peptides separated by HPLC (Bodwell et al., 1991). These sites include serines 122, 150, 212, 220, 234 and 315 and threonine 159. These sites were present in the endogenous GR of mouse WEHI-7 cells and in mouse GR over-expressed in CHO cells suggesting that they are independent of cell-type. An eighth site has been speculated to occur at serine 412 although if it is a legitimate site phosphorylation is only weakly detected following hormone treatment (Bodwell et al., 1995; Bodwell et al., 1998). All the sites are located in the N-terminal domain, located within or in close proximity to the AF-1 region (see Figure 3, panel 3). Serines 212, 220 and 234 are found within the core of AF-1 (Bocquel et al., 1989; Hollenberg and Evans, 1988; Tasset et al., 1990). This region is also highly acidic with 14 excess negative charges provided by aspartic or glutamic acids, not counting contributions by phosphorylations, and has been shown to reduce non-specific DNA binding (Danielsen et al., 1987). These three phosphorylated serines have recently been shown to be conserved sites in the rat GR (corresponding to serines 224, 232 and 246), along with threonine 171 (corresponding to threonine 159) (Krstic et al., 1997). In mouse GR all sites except serine 150 and threonine 159 were found to be hyperphosphorylated in response to glucocorticoid treatment, serine 220 being the most strongly so (Bodwell et al., 1995). No novel phosphorylation sites were observed following hormone treatment (with the possible exception of serine 412) (Bodwell et al., 1995; Bodwell et al., 1998). Likewise in rat GR

threonine 171 was not hyperphosphorylated while serines 224 and 232 were. In rat GR however serine 246 (serine 234 in mouse) was not hyperphosphorylated (Krstic et al., 1997).

In mouse GR, in the basal state, there are only 2.6 mol of phosphate per mol of GR even though there are seven phosphorylation sites (Mendel et al., 1987). Moreover, in steady state labeling experiments, there were significant differences in the extent of phosphorylation at each site (Bodwell et al., 1991). These results suggest that there are subpopulations of GR having different combinations of phosphorylated and non-phosphorylated residues. Hormone-induced hyperphosphorylation must involve phosphorylation of GRs with vacant sites. Consequently this could alter the distribution of the GR subpopulations.

Several of the phosphorylation sites are consensus sequences for known kinases. The four sites conserved between mouse and rat GR are followed by a proline, forming the so-called proline-directed motif, which can be targeted by multiple MAPKs and CDKs (Bodwell et al., 1998). Additionally, serine 122 of mouse GR is in a casein kinase II consensus motif however it is not yet known if GR is actually a substrate for this kinase (Bodwell et al., 1991).

b) Effects of phosphorylation on GR function

Several studies have attempted to determine the effects of phosphorylation on GR activity in mammalian cells however to date no striking effects have been described. A central theme that has emerged is that the effects are subtle and may only occur in specific contexts (Bodwell et al., 1998). In an initial study, mutation of the mouse GR phosphorylation sites to alanines or aspartic acids had little effect on GR transcriptional

activity on a MMTV reporter in COS-1 cells (Mason and Housley, 1993). Mutation of all seven sites resulted in only a minor decrease in transcriptional activity. Similar mutants in human GR were also found to have essentially unaltered transcriptional activity on a reporter containing two copies of the GRE linked upstream of the thymidine kinase promoter (Almlof et al., 1995). Moreover, mutation of mouse GR phosphorylation sites had no effect on sub-cellular localization (Jewell et al., 1995).

Recent work expanding on these earlier studies has demonstrated some effects of mutating the mouse GR phosphorylation sites. The most dramatic effect was observed in the transcriptional activity of phosphorylation-site mutants on a simple glucocorticoid-responsive promoter in COS-1 cells. Mutation of any of the three serines in the transactivation core region (serines 212, 220 or 234) of mouse GR reduced the glucocorticoid-induced transcription of a reporter containing two GREs linked upstream of the adenovirus E1B TATA box (Webster et al., 1997). Substituting serine 212 or 220 for alanine resulted in a 3.5-fold decrease in the induction by Dex on this reporter, while substitution of serine 234 resulted in only a 2-fold decrease. Mutants containing combinations of these substitutions or additional substitutions at the other sites did not cause any further decrease beyond that observed with serine 212 or 220 (Webster et al., 1997). These observations suggest that phosphorylation of mouse GR can affect the transcriptional activity of the receptor in a promoter specific manner.

Glucocorticoids down-regulate the expression of their own receptor at the level of transcription (Burnstein et al., 1994) and the determinants for this down-regulation are found in the GR coding region (Burnstein et al., 1990). In COS-1 cells transiently transfected with mouse GR, after 24 hours of Dex treatment, GR expression decreased to

approximately 40% of that seen in untreated cells as indicated by RNA and protein levels (Webster et al., 1997). Substitution of serines 212, 220 or 234 for alanine resulted in a less-dramatic down-regulation to approximately 65% of the level in untreated cells (Webster et al., 1997). As the number of mutated phosphorylation site was increased the degree of down-regulation decreased. In a mutant containing alanine substitutions of all eight phosphorylation sites (including the speculative site at serine 412) the level of receptor RNA was equivalent to that in untreated cells while the protein level was 50% higher (Webster et al., 1997). This suggests that multiple phosphorylations of GR are required for the auto-down-regulation of the receptor. Furthermore, the stability of mouse GR was shown to increase following substitution of the eight phosphorylation sites with alanine. The half-life of this mutant receptor was found to be two-fold higher than wild type GR (Webster et al., 1997). Moreover, a Dex-dependent decrease in the half-life, that was observed with wild type GR, was not observed with this mutant (Webster et al., 1997). This suggests that phosphorylation of mouse GR results in decreased receptor stability and that this is enhanced following glucocorticoid treatment.

c) Cell cycle dependence of GR phosphorylation and activity

Early studies demonstrated that GR activity varies thoughout the cell-cycle. A number of glucocorticoid responsive genes in a variety of cell-types have been shown to be sensitive to glucocorticoids in late G1 and S phases but resistant in G2, M and early G1 (Fanger et al., 1986; Griffin and Ber, 1969; Hsu et al., 1992; Martin et al., 1969). Evidence that phosphorylation played a role in this cell cycle dependence came from the observations that GR is more negatively charged in G2 and early G1 than in late G1 and S (Currie and Cidlowski, 1982; Fanger et al., 1986) which could be explained by

increased phosphorylation. Moreover, alterations in two-dimensional phosphopeptide maps of GRs from cells arrested in different stages of the cell cycle were observed (Hsu et al., 1992). Finally, as previously mentioned, all of the rat GR and most of the mouse GR phosphorylation sites are in consensus motifs for cyclin-dependent kinases (Bodwell et al., 1991; Hu et al., 1994).

A more detailed examination of mouse GR phosphorylation through the cell cycle was carried out in CHO cells stably overexpressing mouse GR. In the absence of hormone the receptor had a 3-fold higher level of phosphate in G2/M than in S (Hu et al., 1994). Hormone treatment resulted in hyperphosphorylation in S phase but not in G2/M, although the level of phosphorylation in G2/M was still higher than in S due to the increased level observed in the basal state (Hu et al., 1994). The lack of hyperphosphorylation in G2/M was not due to saturation of the phosphorylation sites in this phase (Hu et al., 1994; Hu et al., 1997). In a mouse GR mutant in which all phosphorylation sites except serine 122 had been mutated to alanine, hyperphosphorylation of serine 122 was observed in both the S and G2/M phases (Hu et al., 1997). This result suggests that the differences in hyperphosphorylation between the S and G2/M phases is not due to differential expression of kinases in these phases since a kinase for at least serine 122 is present in both phases (Hu et al., 1997). In addition if the same sites were mutated to glutamic acid, hyperphosphorylation of serine 122 did not occur in either phase (Hu et al., 1997). This suggests that the hyperphosphorylation observed in S phase was dependent on the lower overall negative charge in the mouse GR N-terminus in the basal state, while the high negative charge in this region in G2/M prior to hormone treatment was refractory to hyperphosphorylation.

d) Phosphorylation of GR by MAPKs and CDKs

Consistent with the observation that the four phosphorylation sites in rat GR occur in consensus sites for MAPKs and CDKs, it was observed that GR can be a substrate for these kinases. The MAPK ERK as well as several combinations of cyclins and CDKs can phosphorylate a bacterially expressed N-terminal peptide from rat GR *in vitro* (Krstic et al., 1997). Serines 224 and 232 appear to be specific sites for CDKs while serine 246 and threonine 171 are phosphorylated by ERK (Krstic et al., 1997; Rogatsky et al., 1998). Moreover, mutations of CDK subunits repress GR dependent transcriptional activation of a simple promoter with a single GRE in yeast cells. By contrast, in yeast harboring MAPK mutations, GR transcriptional activation is enhanced (Krstic et al., 1997).

Further characterization of MAPK phosphorylation of GR demonstrated that the MAPK JNK could also phosphorylate the same GR peptide while the p38 MAPK could not (Rogatsky et al., 1998). Like ERK, JNK was specific for serine 246 and threonine 171, with serine 246 being the major site. In human osteosarcoma SAOS2 cells stably expressing rat GR, activation of JNK, and to a lesser degree ERK, by serum stimulation of quiescent cells resulted in a strong induction of phosphorylation of serine 246 (Rogatsky et al., 1998). Phosphorylation of serines 224 and 232 by cyclin/CDK complexes was also observed consistent with the activation of members of this family of kinases upon reentry into the cell cycle following serum stimulation (Rogatsky et al., 1998). Phosphorylation of threonine 171 was observed in serum deprived cells in which MAPKs are largely inactive suggesting other kinases are responsible for phosphorylation of this residue. It has been suggested that glycogen synthase kinase-3 may target this site (Rogatsky et al., 1998).

The effect of ERK and JNK on GR transcriptional activity were assessed using transient transfections with a simple glucocorticoid-responsive reporter gene. (Webster et al., 1997). Selectively activating either ERK or JNK pathways resulted in a 4-fold decrease in the glucocorticoid-induced transcription on this reporter (Rogatsky et al., 1998). Mutation of serine 246 to alanine partially relieved the JNK mediated repression but had no effect on the ERK mediated repression (Rogatsky et al., 1998). Consistent with this JNK activation in HeLa cells leads to a 5-fold increase in phosphate incorporation of a GR mutant lacking serines 232 and 224 *in vivo* (Rogatsky et al., 1998). By contrast, activation of ERK resulted in no appreciable increase in GR phosphorylation (Rogatsky et al., 1998). These results suggest that direct phosphorylation of serine 246 by JNK decreases the transcriptional response of GR to glucocorticoids on a simple promoter. However, since mutation of serine 246 only partially alleviated the repressive effect of JNK it appears other mechanisms may also be involved. The ERK-mediated repression of glucocorticoid-induction on the other hand likely occurs through a mechanism independent of serine 246 phosphorylation.

In conclusion several effects of phosphorylation of GR have been described. These effects are generally modest in nature and occur only in specific contexts. The best described example of an effect on GR activity is the repression of glucocorticoid induced transcription as a result of the phosphorylation of serine 246 of the rat GR by JNK although, as mentioned, this only partially accounts for the JNK mediated repression. It is likely that roles of phosphorylation that have yet to be identified may occur in only specific contexts such as on specific promoters or in specific cell types. The variation in the degree of hyperphosphorylation throughout the cell cycle suggests that effects of

phosphorylation may also be cell-type specific. Moreover, the likelihood of the existence of unidentified phosphorylation sites suggests that phosphorylation at these sites will play a role in regulating GR activity, possibly in concert with the already identified sites.

4. Phosphorylation as a mechanism for transcriptional regulation

Phosphorylation plays a key role in the regulation of the function of many transcription factors (Hill and Treisman, 1995). Frequently, transcription factors are multiply phosphorylated and may contain phosphorylations that enhance activity, as well as others that reduce activity (Karin, 1992). Moreover, the phosphorylation and dephosphorylation reactions are often carried out by multiple enzymes implying that different signaling pathways can regulate transcription factor activity (Hill and Treisman, 1995; Hunter and Karin, 1992). To carry out their functions, transcription factors must be located in the nucleus, bind to DNA and interact with other factors such as coactivators or the basal transcription machinery (Hill and Treisman, 1995; Hunter and Karin, 1992). Phosphorylations which regulate the activity of transcription factors may therefore affect one or more of these processes or affect the stability of the transcription factor.

(i) Control of sub-cellular localization by phosphorylation

Transcription factors require access to their DNA target sequences for their function. The nuclear envelope provides a barrier through which transcription factors must pass in order to access these targets. Several examples have been described where passage across the nuclear envelope is regulated by phosphorylation (Jans and Hubner, 1996). Typically the transcription factor is sequestered within the cytoplasm and a change

in phosphorylation status, of either itself or a cytoplasmic anchor protein, allows translocation to the nucleus (Hunter and Karin, 1992; Jans and Hubner, 1996). The SV40 T-Ag provides an example of a transcription factor whose nuclear import is regulated by phosphorylation. T-Ag nuclear import is mediated by its CcN motif which is comprised of a NLS together with phosphorylation sites for CKII, the cyclin-dependent kinase cdc2 and DNA-PK (Jans et al., 1991; Xiao et al., 1997). T-Ag nuclear import strictly requires the intact NLS (Rihs et al., 1991; Rihs and Peters, 1989) however it is regulated by the phosphorylation status of the neighboring sites. Phosphorylation of the CKII site accelerates the rate of nuclear import about 50-fold (Jans et al., 1991; Rihs et al., 1991). Cdc2 phosphorylation in contrast reduces maximal nuclear accumulation by approximately 70% (Jans et al., 1991). However this kinase is unlikely to be physiologically important due to its exclusive nuclear localization (Xiao et al., 1997). The DNA-PK site is required for efficient nuclear accumulation and the introduction of a negative charge at this site, by site-directed mutagenesis of the wild type serine to aspartic acid, results in a faster nuclear import rate and a higher affinity for the importin 58/97 subunits (Xiao et al., 1997). This mutation also results in more efficient phosphorylation by CKII (Xiao et al., 1997) which is known to also increase the rate of T-Ag nuclear import and affinity for importin 58/97 (Hubner et al., 1997). However, it remains unclear whether the predominately nuclear DNA-PK can sufficiently phosphorylate the cytoplasmic T-Ag *in vivo* to effect its nuclear import.

In contrast to T-Ag whose nuclear import is enhanced by phosphorylation, the SWI5 transcription factor in *Saccharomyces cerevisiae* remains cytoplasmic while phosphorylated and must be dephosphorylated for nuclear import. SWI5 remains

cytoplasmic throughout the cell cycle but enters the nucleus and activates transcription during the G1 phase (Nasmyth et al., 1990). Two serines adjacent to the SWI5 NLS are phosphorylated by cdc28 resulting in sequestration within the cytoplasm (Jans et al., 1995; Moll et al., 1991). During G1 SWI5 is dephosphorylated, likely by the cdc14 phosphatase, undergoes nuclear import and is able to activate transcription (Jans et al., 1995; Moll et al., 1991; Nasmyth et al., 1990; Visintin et al., 1998).

Phosphorylation can also indirectly regulate the nuclear import of transcription factors. An example of this and of phosphorylation affecting the stability of transcription factors, is provided by the Rel/NF-κB family of transcription factors. This family activates transcription in response to a variety of stimuli including phorbol esters, proinflammatory cytokines, tumor necrosis factor, interleukin 1, bacterial lipopolysacharide, ds RNA, viruses and the Tax protein of HTLV-1 (Baldwin, 1996; Grossmann et al., 1999). The NF-κB family members form heterodimers via interactions between their Rel homology domains (RHD), conserved domains which contain the DBD and the NLS (Baeuerle and Henkel, 1994; Parry and Mackman, 1994; Ryseck et al., 1995; Siebenlist et al., 1994). In the absence of stimuli, the NF-κB heterodimers are cytoplasmic and complexed with members of the inhibitors of NF-κB (IκB) family (Baeuerle and Henkel, 1994; Davis et al., 1991; Haskill et al., 1991; Siebenlist et al., 1994). The IκBs contain a series of six or more ankyrin repeats which mediate binding to the RHD of NF-κB (Beg and Baldwin, 1993; Grossmann et al., 1999). By binding to the RHD the IκBs mask the NLS and cause cytoplasmic retention (Beg and Baldwin, 1993). Following the appropriate stimulation of the cell, the IκBs are phosphorylated at two specific serine residues (Brown et al., 1995; Chen et al., 1995; DiDonato et al., 1996) by

the IκB kinase (IKK) (DiDonato et al., 1997; Karin, 1999; Mercurio et al., 1997). This phosphorylation triggers polyubiquitination of the IκBs, leading to their rapid degradation by the 26 S-proteasome (Alkalay et al., 1995; DiDonato et al., 1995). Following degradation of IκB the NLS of the NF-κB dimer is exposed allowing NF-κB to enter the nucleus and activate transcription (Baldwin, 1996; Grossmann et al., 1999). Thus both sub-cellular localization and protein stability are controlled by phosphorylation in this example.

(ii) Control of DNA binding by phosphorylation

The DNA binding activity of transcription factors is a second mechanism by which phosphorylation can regulate their function. Interactions between the transcription factor DBDs and the DNA binding site normally involve electrostatic interactions between positively charged residues within the DBD and the negatively charged phosphate backbone of the DNA. Phosphorylation within or near the DBD introduces a negative charge that can directly interfere with these interactions causing a decrease in affinity for the DNA binding site (Hill and Treisman, 1995; Hunter and Karin, 1992). For example, the transcription factor c-jun, a member of the AP-1 complex, is phosphorylated on three sites adjacent to its DBD. While phosphorylated it has a relatively low affinity for DNA, however after stimulation of cells with TPA, growth factors or viral oncogenes, c-jun becomes dephosphorylated and DNA binding activity increases (Binetruy et al., 1991; Boyle et al., 1991; Lin et al., 1992; Smeal et al., 1992; Smeal et al., 1991). Alterations in DNA binding affinity can also occur via phosphorylations in domains distant from the DBD that influence DNA binding through conformational changes. This is thought to occur with SRF (Janknecht et al., 1992;

Rivera et al., 1993) and Elk-1 (Gille et al., 1992), both of which undergo an increase in DNA binding affinity in response to phosphorylation.

Many transcription factors bind DNA as oligomers, and phosphorylation can therefore regulate binding by affecting oligomerization, as well as direct protein-DNA interactions. Phosphorylation sites on the human ER, for example, have been shown to inhibit dimerization of the receptor and consequently DNA binding (Arnold et al., 1995a; Arnold et al., 1995b). By contrast, dimerization and DNA binding of STAT factor are induced by phosphorylation (Shuai et al., 1994). Another case exists for TRα and its oncogenic derivative v-ERB A which are phosphorylated by PKA on serines 16 and 17 (Goldberg et al., 1988). This phosphorylation appears to enhance the oncogenic activity of v-ERB A (Glineur et al., 1990) and the transcriptional activity of TRα (Jones et al., 1994). Phosphorylation at these sites has been shown to decrease the DNA-binding activity of TRα and v-ERB A monomers while having no effect on the binding of homodimers or heterodimers with RXR (Tzagarakis-Foster and Privalsky, 1998).

(iii) Effects of phosphorylation on transactivation

Several transcription factors have transcriptional activation domains that can be regulated by phosphorylation. Generally, phosphorylation has a positive effect on transcription and is thought to facilitate interactions with coactivator proteins or the basal transcription machinery (Hill and Treisman, 1995). An example is the CREB transcription factor that is phosphorylated on serine 133 by PKA, calmodulin dependent kinases or a nerve growth factor dependent kinase (Ginty et al., 1994; Gonzalez and Montminy, 1989; Sheng et al., 1991). Phosphorylation of this residue allows CREB to associate with CBP, a coactivator protein (Arias et al., 1994; Chrivia et al., 1993; Kwok

et al., 1994; Parker et al., 1996; Radhakrishnan et al., 1997), and this interaction leads to activation of transcription of CREB responsive genes (Cardinaux et al., 2000).

Phosphorylation-dependent recruitment of cofactors has also been described for several members of the nuclear receptor family. ERβ, like several other members of the steroid receptor family, can be activated by nonsteroidal agents (Weigel and Zhang, 1998). For example the Ras pathway can promote activation of ERβ and this effect is mediated by serine phosphorylation in the AF-1 region (Tremblay et al., 1997). The AF-1 region recruits the coactivator SRC-1 which enhances steroid-independent transcription (Tremblay et al., 1999). Two serines (106 and 124) are phosphorylated by MAPK and phosphorylation of these sites enhances the recruitment of the coactivator SRC-1 (Tremblay et al., 1999). Mutation of these sites blocked the enhancement of transcription by SRC-1 (Tremblay et al., 1999). Interestingly these serine residues are conserved in GR and PR (Krstic et al., 1997; Zhang et al., 1997). Similarly the orphan nuclear receptor SF-1 is also phosphorylated by MAPK in the AF-1 region leading to increased transcriptional activity (Hammer et al., 1999). This phosphorylation appears to mediate recruitment of both the coactivator GRIP-1 and the corepressor SMRT implying that phosphorylation may reduce or increase SF-1 activity in different contexts (Hammer et al., 1999). A third member of the nuclear receptor family, PPARγ, phosphorylation of serine 112 in the AF-1 region appears to modulate interdomain communication between the AF-1 domain and the C-terminal LBD (Shao et al., 1998). This enhances the ligand dependent recruitment of SRC-1 (Shao et al., 1998).

5. Working Hypothesis

In the MMTV LTR the NRE1 element mediates repression of glucocorticoid-induced transcription. This element is bound by nuclear proteins including the Ku autoantigen and DNA-PK, which participate in the repression. Inhibition of MMTV transcriptional activators via phosphorylation by DNA-PK is one mechanism by which this repression may occur. As the NRE1-mediated repression is specific for glucocorticoid-induced transcription, the most likely target for DNA-PK phosphorylation is GR. If this is true, it would be expected that mutation of DNA-PK phosphorylation sites within GR would abrogate the repression of glucocorticoid-induced transcription of MMTV.

6. Project Goals

The general goal of this project was to further characterize the role of NRE1 in the repression of glucocorticoid-induced transcription of the MMTV promoter. Specifically the first goal was to continue the characterization of nuclear factor binding to NRE1 in crude nuclear extracts. I first determined the kinetics of NRE1 binding by these factors. I also investigated the sequence specificity of this binding by examining a cross-recognition of the octamer motif by these factors. After the identification of Ku as the double-strand and upper-strand NRE1 binding factor, I determined the affinity to which Ku bound both double-stranded NRE1 and upper-stranded NRE1. I also determined the kinetics of binding to upper-stranded NRE1.

A second goal was to establish the requirement of Ku and DNA-PK in the repression of glucocorticoid-induced MMTV transcription. This was accomplished using

transient transfection assays with MMTV reporters either containing or lacking NRE1 in mutant cell lines which did not express either Ku or DNA-PK.

The third goal was to identify the DNA-PK phosphorylation sites in the full-length glucocorticoid receptor. This was accomplished using a strategy in which an epitope tagged GR was kinased *in vitro* and the phosphorylation sites were identified through a combination of protease digestion and Edman degradation.

The final goal of this project was to determine the role of phosphorylation at the mapped sites. I accomplished this by using site-directed mutagenesis to create GR mutants that could not be phosphorylated. I then determined the effect of these mutations on the repression of glucocorticoid induction of MMTV transcription by performing transient transfection assays with MMTV reporters either containing or lacking NRE1.

II. MATERIALS AND METHODS

1. Enzymes, antibodies and chemicals

(i) Enzymes

All DNA modifying enzymes and restriction enzymes were obtained from New England Biolabs (Mississauga, ON, Canada). TPCK -treated trypsin was obtained from Worthington Biochemicals Ltd. (Freehold, NJ). Specific proteases AspN and GluC were obtained from Boeringher Manheim (Laval, QC, Canada).

(ii) Antibodies

a) Purchased antibodies

Anti-Oct-1 antibody YL15 was provided by W. Herr (Lai and Herr, 1992). Anti-GR monoclonal antibody BuGR2 (Gametchu and Harrison, 1984) was purchased from Affinity Bio Reagents. Anti-GAL4-DBD monoclonal antibody RK5C1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sheep anti-mouse IgG conjugated to HRP (SAM-HRP) was from Amersham (Baie d'Urfé, PQ, Canada).

b) Preparation of 9E10 antibody from hybridoma supernatant

Cultures of 9E10 hybridoma cells were expanded to 2 L and grown until just confluent. Cells were removed from the media by centrifugation at 2000 g for 5 min. The supernatant was collected and re-inoculated with approximately 5% of the cell pellet. The cells were then allowed to grow to saturation and death. As saturation was approached, glucose and HEPES were added to 1% and 25 mM respectively. The cells

and debris were removed by centrifugation at 3000 g for 30 min at RT. The supernatant was collected and 0.5 volumes of saturated ammonium sulfate pH 7.0 was slowly added. This solution was incubated overnight at 4 °C then centrifuged at 3000 g for 30 min. The supernatant was collected and an additional 0.5 volumes of saturated ammonium sulfate pH 7.0 was slowly added. This was incubated and centrifuged as before. The pellet was collected and resuspended in 0.1 volumes of the starting volume of PBS (137 mM KCl, 2.7 mM NaCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4). This was dialyzed against 2 L of PBS overnight at 4 °C with 3 changes of dialysis buffer. The antibody was then stored at -80 °C in 1 ml aliquots.

(iii) Chemicals

The synthetic GR ligand 1,4-Pregnadien-9α-fluoro-16α-methyl-11β,17,21-triol-3,20-dione (dexamethasone, Dex) was obtained from Steraloids Inc. (Wilton, NH, USA). All tissue culture media and reagents were obtained from Life Technologies except FBS which was obtained from either Life Technologies or Hyclone. All chemicals for electrophoresis were obtained from BDH (PQ, Canada). Protein A Sepharose CL-4B beads were obtained from Sigma. Glutathione Sepharose beads were purchased from Pharmacia (Baie d'Urfé, PQ, Canada). ^{14}C-chloramphenicol (0.05 mCi/ml) was obtained from Amersham (Baie d'Urfé, PQ, Canada).

2. Plasmids

(i) MMTV reporter constructs

The MMTV reporter plasmids pHC17 and pHC364 were graciously provided by Dr. A. Cato and were previously described (Mink et al., 1990). pHC17 contains the segment of the MMTV LTR spanning -421 to +125 bp relative to the start of transcription while pHC364 contains sequences from -364 to +125 bp. In both plasmids the MMTV LTR segment is upstream of and drives expression of the CAT gene.

(ii) Glucocorticoid receptor expression constructs

Full-length wild type rat GR was expressed from the p6RGR plasmid, graciously provided by Dr. Keith Yamamoto (Pearce and Yamamoto, 1993). This plasmid is derived from pSP65 (Promega, Madison, WI, USA) and contains a 500 bp fragment of the RSV LTR which drives expression of full length rat GR.

The mammalian full-length GR expression vector pTL2-rGRwt was provided by Joanne Savory and its construction was previously described (Savory et al., 1999). This vector contains the full rat GR gene from p6RGR sub-cloned into pTL2 (Green et al., 1988). The pTL2 vector is a derivative of pSG5, which in addition to mammalian expression, also allows bacterial expression driven by a T7 promoter and single stranded DNA synthesis from an M13 origin of replication.

pTL2-rGR$_{S527A}$, pTL2-rGR$_{S527D}$ and pTL2-rGR$_{S527E}$ containing mutations of serine 527 of rat GR to alanine, aspartic acid and glutamic acid respectively were constructed by PCR amplifying an 833 bp PstI/BamHI fragment of pTL2-rGRwt using 5' primers containing substitutions in the serine 527 codon. Each of the 5' primers contained

the PstI site adjacent to amino acid position 525 of rat GR. The sequences were as follows: S527A (5'-GCCACTGCAGGAGTCGCACAAGAC-3'), S527D (5'-GCCACTGCAGGAGTCGACCAAGAC-3'), S527E (5'-GCCACTGCAGGAGTCGAACAAGAC-3'). The mutated codon corresponding to position 527 of rat GR is in bold face. The 3' primers in each case were identical and contained the BamHI site adjacent to the amino acid position 795. The sequence was as follows: (5'-GCCACTGCAGGAGTCACACAAGAC-3'). Following PCR amplification the products were doubly cleaved with PstI/BamHI and isolated on 1.0% agarose gels. The digested products were then sub-cloned into the corresponding sites of pTL2-rGRwt. The presence of the appropriate mutations and accuracy of the PCR reaction was confirmed by sequencing. pTL2-rGR$_{S527A}$ and pTL2-rGR$_{S527E}$ were constructed by Claudia Bayer.

Rat GR fused to an N-terminal 6 X c-myc epitope tag was expressed from pTL2-mycGR. The pTL2-mycGR vector was obtained from Gratien Préfontaine and construction was as previously described (Prefontaine et al., 1998). The first 21 amino acids of the rat GR were deleted to accommodate the sub-cloning. As with pTL2-rGRwt vector, mammalian expression is driven by the SV40 early promoter.

To express the serine 527 mutants with an N-terminal 6 X c-myc epitope tag, each of pTL2-rGR$_{S527A}$, pTL2-rGR$_{S527D}$ and pTL2-rGR$_{S527E}$ were grown in *Escherichia coli* strain RB404. These were then doubly digested with MscI/BamHI and the resulting 2335 bp fragments were subcloned into the SmaI/BamHI fragment of pTL2-myc yielding the vectors pTL2-mycGR$_{S527A}$, pTL2-mycGR$_{S527D}$ and pTL2-mycGR$_{S527E}$ respectively. The

presence of the appropriate mutations was confirmed by sequencing. pTL2-mycGR$_{S527A}$ and pTL2-mycGR$_{S527E}$ were constructed by Claudia Bayer.

The pGEX-2T-X568 vector was constructed and provided by Maya Traykova-Andonova (Giffin et al., 1997). This plasmid expressed the rat GR DNA binding domain (amino acids 407-568) fused to an N-terminal GST domain (GST-GR$_{X568}$). Expression was driven by the T7 promoter.

(iii) Ku expression constructs

For expression of Ku 80 in mammalian cells, the pBJ6-Ku80 vector was used. This vector was constructed and provided by Gilbert Chu (Smider et al., 1994). This expression plasmid expresses high levels of human Ku 80, driven by the SRα promoter. The SRα promoter consists of the SV40 early promoter fused to a segment of the LTR of HTLV-1 (Takebe et al., 1988).

(iv) Other constructs

In developing stable cell lines, transfected cells were rendered resistant to the antibiotic G418 (Life Technologies), an aminoglycoside antibiotic related to Gentamicin (Jimenez and Davies, 1980), by cotransfection of the vector pSV2NEO^r (Southern and Berg, 1982). This vector expresses the enzyme aminoglycoside phosphotransferase 3' (Southern and Berg, 1982), which renders resistance to aminoglycoside antibiotics. Expression is under control of the SV40 early promoter.

In transient transfection experiments the pRSVβgal (Edlund et al., 1985) vector was cotransfected as an internal control to allow the transfection efficiency to be monitored. This vector contains the lac Z gene under the control of the RSV promoter.

To generate NRE1-containing micro-circles the plasmid pNRE1 was first generated containing a single copy of the MTV oligonucleotide (see Table I) ligated into the SmaI site of pBluescript (Stratagene).

3. Recombinant proteins

(i) Baculoviral Ku

Expression and purification of Ku from insect cells was essentially by the protocol of Ono et al. (Ono et al., 1994). Recombinant baculoviruses expressing human Ku 80 (VBB2-Kup80) or Ku 70 fused at the C terminus to a hexahistadine tag (VBB2-Kup70tH6) were obtained from Donald Capra (Ono et al., 1994). These recombinant viruses were co-infected into Sf9 cells. Three days post-infection cells were harvested and lysed by sonication in 40 mM HEPES, pH 7.9, 1 mM EDTA, 2 mM DTT, 0.1% NP-40, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 1 mM PMSF. Ku heterodimers were then purified using a Ni 21 affinity resin (His-Bind Resin, Novagen).

(ii) Bacterial GST-GR_{X568}

GST-GR was prepared using the protocol of Lai et. al (Lai et al., 1992) as modified by Giffin et. al (Giffin et al., 1997). *E. coli* BL21 cells transformed with pGEX-2T-X568 were grown at 37°C overnight, followed by induction with 1 mM isopropyl-1-thio-b-D-galactopyranoside for 16 h at 23°C. Induced bacteria were harvested and resuspended in lysis buffer (25 mM HEPES, pH 7.9, 100 mM KCl, 20% glycerol, 2 mM EDTA, 0.2 mM PMSF) supplemented with 100 mg/ml lysozyme and incubated for 10 min at 4°C. Cells and DNA were sheared by passage through a series of 18-, 20-, and 25-gauge needles, followed by sonication 10 times for 40 s in the presence

of 0.1% NP-40 at 4°C. Lysates were centrifuged 10 min at 10,000 x g, and the supernatants were incubated with 3 ml of glutathione-Sepharose 4B beads (Pharmacia) for 90 min. The beads were subsequently washed five times with 10 bed volumes of lysis buffer and three times with 20 bed volumes of 60% lysis buffer containing 0.1% NP-40. GST-GR_{X568} beads were stored at 4 °C with 1 mM PMSF and 0.02% NaN_3 .

4. Plasmid preparation

Plasmid DNAs were transformed by either a $CaCl_2$ heat shock method (Chung and Miller, 1988) or by electroporation, with an E. coli Pulser (Bio Rad, Mississauga, ON, Canada) as per the manufacturers protocols, into competent *E. coli* DH5α strain and plated on LB plates (1.5% w/v agar) containing 150 μg/ml ampicillin. Colonies were allowed to grow overnight at 37°C and single colonies were used to inoculate 5 ml overnight cultures in LB/ampicillin. These cultures were used to inoculate 1 L cultures in LB/ampicillin which were grown at 37°C to an OD (650 nm) of 0.6 after which 0.2 g/L of chloramphenicol was added and cultures were returned to 37°C overnight. Plasmid DNA was prepared using the alkaline-lysis maxi-preparation procedure followed by isolation on 2 sequential $CsCl_2$ gradients.

5. Electrophoretic mobility shift assays (EMSA)

DNA binding assays were performed using a modification of the original EMSA protocol (Fried and Crothers, 1981; Garner and Revzin, 1981). Binding activity in either crude nuclear extracts (1 μg) or in purified recombinant baculoviral Ku (1 μl) was examined. Cells were harvested and nuclear extracts were prepared by the method of Andrews and Fallon (Andrews and Faller, 1991). For standard binding reactions 0.5 ng

^{32}P-kinased specific oligonucleotide was used as probe. Binding reactions were carried out in 20 µl reaction volumes in binding buffer (12 mM HEPES pH 7.9, 12% glycerol, 60 mM KCl, 0.12 mM EDTA, 1 µg BSA and 1 µg of double or single-strand sheared calf thymus DNA). 25 - 200 ng of specific competitor DNA's were also included when indicated. Binding reactions were performed for 30 min at 20°C. Samples were loaded onto 0.8 mm thick, 4%, polyacrylamide gels (acrylamide : bisacrylamide = 40:1) in 0.5 x TBE and electrophoresed for 225 V/h. After electrophoresis gels were dried and exposed to autoradiography film (Kodak, Rochester, NY).

The oligonucleotides used as radiolabeled probes and as specific competitors were: MTV (upper-strand; 5'-AACTGAGAAAGAGAAAGACGACA-3'), MT (upper-strand; 5'-AACTGAGAAAGACGACA-3'), OCT (upper-strand; 5'-AGCTTGCTTATGCAAATAAGGTG-3') and mtOCT (upper-strand; 5'-AGCTTGCTTCGGCAAATAAGGTG-3'). (Also see Table I). Oligonucleotides were labeled using T4-polynucleotide kinase and γ^{32}P-dATP. Free ^{32}P-dATP was removed by passage through G-50 Sephadex (Pharmacia), spin columns. The oligonucleotides were then purified by extracting in phenol/chloroform followed by ethanol precipitation. Double-stranded probes were prepared by combining equimolar amounts of the labeled oligonucleotide with the corresponding complementary strand, heating the mixture to 90°C and allowing it to slowly cool to room temperature.

6. Analysis of kinetics and affinity of DNA binding

(i) On-rates

To measure the rate of association of DNA binding factors with a specific DNA binding site, kinetic studies were performed using EMSA. Standard binding reactions were performed in which the crude nuclear extract or purified baculoviral recombinant Ku was added to the ^{32}P-labelled oligonucleotide for times ranging from 1 to 240 min. Binding reactions were synchronized to conclude at the same time, and were resolved by EMSA. For experiments with crude nuclear extracts, relative levels of binding were quantified using densitometric analysis of exposed films, performed on an LKB Ultroscan XL (Pharmacia). In experiments with purified recombinant Ku, relative binding was quantified by phosphorimage analysis using a Bio-Rad GS-525 Molecular Imager system. Half-times of binding ($t_{1/2}$) were calculated by plotting the binding versus time and extrapolating the time at which 50% of maximal binding occurred.

(ii) Off-rates

To measure the rate of dissociation of DNA binding factors from a specific DNA binding site, kinetic studies were performed using EMSA. Standard binding reactions were performed in which the crude nuclear extract or purified baculoviral recombinant Ku was added to the ^{32}P-labelled oligonucleotide probe and binding was allowed to equilibrate at room temperature for 30 min. Dissociation of the DNA binding factors from the DNA was monitored by addition of an excess of unlabelled oligonucleotide, identical to the labelled probe, which acted as a specific competitor and prevented appreciable reassociation of the binding factors with the probe. 200 ng (400 fold excess)

or 1 μg (2000 fold excess) of an unlabelled oligonucleotide competitor was then added for times ranging from 0 to 24 h. Competitions were synchronized to conclude at the same time, and were resolved by EMSA. For experiments with crude nuclear extracts, relative levels of binding were quantified using densitometric analysis of exposed films, performed on an LKB Ultroscan XL (Pharmacia). In experiments with purified recombinant Ku, relative binding was quantified by phosphorimage analysis using a Bio-Rad GS-525 Molecular Imager system. Half-times of dissociation ($t_{1/2}$) were calculated by plotting the level of binding versus time and extrapolating the time at which 50% of binding had dissociated.

(iii) Affinity of DNA Binding

EMSA was used to determine the affinity of Ku binding to the double-strand and upper-strand of NRE1. A constant amount of recombinant Ku expressed in Baculovirus (1 μl) was bound to an increasing amount of radiolabeled DNA probe.

To measure the affinity of binding to double-stranded NRE1, a micro-circular probe was used to eliminate binding of Ku to DNA ends. To generate the micro-circles a 223 bp PvuII/XhoI fragment from pNRE1 (containing a single copy of the NRE1) was blunt-ended by filling in the ends in the presence of α^{32}P-ATP (6000 Ci/mmol,Dupont). The blunt-ended fragment was ligated and the closed circular form was gel purified. The closed circular conformation was verified by demonstrating resistance of the micro-circles to exonuclease III, Bal31 and S1 nuclease. Labeling efficiency was 100%, as formation of covalently closed microcircles required incorporation of α^{32}P-ATP during fill in of the XhoI site prior to microcircle ligation. This allowed direct quantitation of the micro-circle by scintillation counting. The following amounts of microcircle were

used in the EMSA: 2.7, 5.4, 10.8, 21.7 and 43.5 pmol. Following EMSA, bound and free DNA were quantified by phosphorimager analysis. To determine the concentration of bound DNA, a standard curve was created using the total (bound + free) adjusted volume (counts x mm^2) from the phosphorimager versus concentration of total DNA. Scatchard analysis (Scatchard, 1949) was performed by plotting the ration of bound/free versus the concentration of bound DNA. The slope of the curve was determined by linear regression and from this affinity was determined using the following equation:

$$K_d = \frac{-1}{\text{slope}}$$

To determine the affinity of binding to the single upper-strand EMSA were performed using the upMTV oligonucleotide (see Table 1) labeled with γ^{32}P-ATP and T4 polynucleotide kinase. The concentration of upMTV oligonucleotide was determined by spectrophotometry. The following amounts of radiolabeled upMTV were used in the EMSA: 7, 14, 35, 70, 140, 280 fmol. The affinity was determined in the same manner as described above for the closed micro-circular DNA.

7. SDS-Polyacrylamide gel electrophoresis

SDS-PAGE was performed essentially as previously described (Laemmli, 1970). Protein samples were dissolved in sample buffer (62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.125% (w/v) bromophenol blue) and denatured by boiling for 5 min at 95°C. Samples were loaded on SDS-PAGE gels which consisted of a stacking gel (4% (w/v) 36.5:1 acrylamide:N,N'-methylenebisacrylamide, 62.5 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate, 0.1% (v/v) TEMED) and a separating gel of (6-12% (w/v) 36.5:1

acrylamide:N,N'-methylenebisacrylamide, 375 mM Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate, 0.05% (v/v) TEMED). For analytical gels a mini-protein gel apparatus (Bio Rad) with a gel thickness of 0.75 mm was used. For preparative gels a Protean gel apparatus was used (Bio Rad) with a gel thickness of 1.5 mm. Gels were run at 100 volts for 60-75 min for analytical gels or 180-200 min for preparative gels in electrophoresis buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% (w/v) SDS). Gels used for separation of radiolabeled proteins were analyzed by autoradiography or phosphoimaging while gels used for non-radiolabeled proteins were examined by silver or amido black staining or by western or southwestern blotting.

8. Staining of SDS-PAGE gels

(i) Silver Staining

For samples with low levels of proteins (<500 ng/protein band) SDS-PAGE gels were stained using the Silver Stain Plus kit from Bio Rad as per the manufacturers instructions.

(ii) Amido Black Staining

For samples with higher levels of protein (>500 ng/protein band) SDS-PAGE gels were stained with amido black. Gels were immersed in amido black staining solution (0.1% amido black, 40% methanol, 10% acetic acid) for 30 min then destained in several changes of 40% methanol, 10% acetic acid.

9. Western Blotting

Western blotting was performed as described (Burnette, 1981; Towbin et al., 1979). After separation by SDS-PAGE, proteins were electrophoretically transferred from the gel to a PVDF membrane (Immobilon P, Millipore Corp., Bedford, MA) in transfer buffer containing 25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol. Prior to use, the PVDF membrane was prepared by soaking in methanol for 15 sec, H_2O for 2 min and transfer buffer for 5 min. Transfers were carried for 60-120 min with cooling at 100 V using a BioRad transblot apparatus. After transfer the membrane was rinsed in PBS-T (PBS with 0.05% Tween-20) for 15 min at room temperature, then blocked with 10% (w/v) skim milk in PBS-T. The membrane was then rinsed with PBS-T and incubated with primary antibody overnight at 4°C. The primary antibodies BuGR2 and 9E10 were diluted 1:2000 and 1:500 respectively in PBS-T for incubation. The membrane was then washed once for 5 min then twice for 20 min with PBS-T at room temperature with gentle shaking. It was then incubated for 1 h SAM-HRP secondary antibody, diluted 1:50000 in PBS-T. Washing was then repeated as for the primary antibody, then antibody-labeled proteins were detected by enhanced chemiluminescence using the Amersham ECL kit (Amersham Life Sciences Inc., Oakville, ON, Canada) as per the manufacturers instructions.

10. Southwestern blotting

Detection of NRE1 binding proteins by southwestern blotting (Bowen et al., 1980; Miskimins et al., 1985) was performed as follows: 100 µg of Jurkat nuclear extract was separated on 8.5% SDS-PAGE gels. Jurkat cells were harvested and nuclear extracts

were prepared by the method of Andrews and Fallon (Andrews and Faller, 1991). The gels were washed overnight at room temperature with 3 changes of renaturation buffer (4 M Urea, 10 mM Tris-HCl pH 7.4, 50 mM NaCl, 2 mM EDTA and 0.1 mM DTT). Proteins were transferred to PVDF membranes by electroblotting in a transfer buffer containing 192 mM glycine and 88 mM Tris-HCl pH 8.3. Blots were incubated in blocking solution (5% milk, 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM β-Mercaptoethanol) for 45 min at room temperature, then for an additional 45 min in blocking solution containing a 500 fold excess (relative to the radiolabeled oligonucleotide probe) of highly sheared salmon sperm DNA. The blots were then cut into individual lanes and incubated for 3.5 h in blocking solution containing salmon sperm DNA and 100 ng (1-1.5 x 10^6 cpm) of radiolabeled oligonucleotide probe. Following hybridization blots were washed 3 times in blocking solution for 15 min each then rinsed in 10 mM Tris-HCl pH 8.0, 2 mM MgCl and 1 mM β-Mercaptoethanol. Blots were then exposed to film for 24 h.

Oligonucleotides used for radiolabeled probes were: MTV, MT, OCT (see Table 1). In some cases these probes were multimerized by using T4 DNA ligase. As a non-specific probe, a 35 bp fragment from the polylinker of pGEM was excised from the plasmid and labeled as above.

11. Tissue culture

Jurkat human T-cell lymphoma cells and 9E10 hybridoma cells were cultured in RPMI 1640 supplemented with 10% FBS. V79 and V15B hamster fibroblasts, CB17 and Sf7 mouse fibroblasts and HeLa human cervical carcinoma fibroblasts were cultured in DMEM supplemented with 10% FBS. All stable cell lines derived from Sf7 cells were

cultured in DMEM supplemented with 10% FBS and 500 μg/ml G418. All cell lines were cultured in a tissue culture incubator at 37°C in a humified atmosphere of 5% CO_2.

12. Transfection of tissue culture cells

(i) Transient transfections

a) DEAE-Dextran Transfection

One method used to transfer DNA into mammalian cells was the DEAE-dextran method (Lopata et al., 1984). Cells to be transfected were seeded in 100 mm dishes and grown to 70% confluence. Plasmid DNAs to be transfected were diluted in DMEM supplemented with 2.5% FBS and 100 μM chloroquine. The plasmid DNAs used were: pHC364 or pHC17 (4 μg/plate), p6RGR (2 μg/plate) and pRSVβgal (2 μg/plate). DEAE-dextran was added to a final concentration of 100 μg/ml. Media in the tissue culture dishes was aspirated and replaced with the DNA/DEAE-dextran containing media. Plates were incubated at 37°C for 4 h. Plates were then aspirated and incubated with 10% DMSO in PBS for 2 min. They were then washed with PBS and returned to DMEM with 10% FBS and incubated for 48 h at 37°C prior to harvesting to assay CAT and β-gal activity as described in section 13 and 14.

b) Lipofectamine Transfection

Transfection with lipofectamine polycationic lipid transfection agent (Life Technologies) was performed according to the manufacturers protocols as follows: Cells were seeded in 60 mm dishes and grown to 70% confluence. Plasmids used were pHC364 or pHC17 (1 μg/plate), p6RGR (200 ng/plate) and pRSVβgal (500 ng/plate).

These were diluted in 300 µl Optimem (Life Technologies). 20 µl lipofectamine reagent was diluted in 300 µl Optimem, combined with the DNA solution and incubated at room temperature for 30 min then diluted to a 2 ml final volume. The tissue culture dishes were washed 2 times with PBS then incubated with the 2 ml of diluted DNA/lipofectamine solution. The plates were incubated for 5 h at 37°C then 2 ml of Optimem containing 20% FBS was added. The following day the Optimem solution was replaced with normal growth media (DMEM + 10% FBS) either containing 10^{-6} M Dex. The plates were then incubated for 48 h at 37°C prior to harvesting to assay CAT and β-gal activity as described in section 13 and 14.

c) Exgen 500 Transfection

Transient transfection using the Exgen 500 cationic transfection reagent (MBI Fermentas) (Boussif et al., 1995; Ferrari et al., 1997) was used according to the manufacturers protocols as follows: Cells were seeded in 60 mm dishes and grown to 70% confluence. Plasmids used were pHC364 or pHC17 (50 ng/plate), p6RGR (250 ng/plate) and pRSVβgal (50 ng/plate). These were diluted in 50 µl of 150 mM NaCl. 2.8 of Exgen 500 reagent (8 µl/µg DNA) was diluted in 50 µl of 150 mM NaCl and combined with the 50 µl DNA solution. The mixture was incubated at room temperature for 10 min then 200 µl DMEM was added. The tissue culture dishes were washed 2 times with PBS, 900 µl of DMEM (serum and antibiotic free) was added, then the 300 µl DNA/Exgen 500 solution was added. The plates were incubated for 3 h at 37°C then 2 ml of DMEM containing 20% FBS was added. The following day the transfection media was replaced with normal growth media (DMEM + 10% FBS) either containing or

lacking Dex. The plates were then incubated for 24 h at 37°C prior to harvesting to assay CAT and β-gal activity as described in section 13 and 14.

(ii) Stable transfections

To stably express mycGR or mycGR$_{S527A}$ the plasmids pSV2NEO^r (1 μg) and pTL2-mycGR or pTL2-mycGR$_{S527A}$ (7 μg) were co-transfected into approximately 10^7 Sf7 cells (100 mm dish) using the lipofectamine protocol described above. 48 h post-transfection the growth media (DMEM + 10% FBS) was replaced with selection media consisting of DMEM +10% FBS + 500 μg/ml G418 (Life Technologies). Cell death occurred over the next 2 weeks during which time the selection media was replaced daily. G418 resistant colonies were allowed to grow to sufficient size (approximately 1 mm). To pick colonies, the plates were washed with PBS and a sterile paper disc soaked in trypsin was laid over the colony. After 60 s the disc was transferred to a well of a 24 well tissue culture plate. The paper discs were removed after 2 days. Twenty colonies were picked and the surviving clonal lines were expanded to 60 mm dishes. Nine clones were selected randomly and screened for expression of mycGR by western analysis of whole cell extracts. The clone expressing the highest level of mycGR was then selected for further use.

13. CAT assays

(i) TLC based CAT assay

To prepare cytosolic extracts for CAT assays transfected tissue culture plates were washed twice with PBS and the cells were collected by scraping and kept on ice.

The cells were pelleted by centrifugation at 8000 rpm then resuspended in 100 µl FT buffer (0.25 M sucrose, 10 mM Tris-HCl pH 7.4, 10 mM EDTA). The cells were lysed by 4 cycles of freezing in liquid N_2 and thawing at 37°C. Nuclei and cellular debris were removed by centrifugation at 13 000 rpm.

CAT reactions were carried out by by the method of Gorman (Gorman et al., 1982) as follows: 10-50 µl of cellular extract were incubated in 0.25 M Tris-HCl (pH 7.8), 16 µM ^{14}C-chloramphenicol (0.2 µCi), 1.4 mg/ml acetyl coenzyme A for 4 h at 37°C. Chloramphenicol derivatives were extracted with 800 µl of ethyl acetate. 750 µl of the ethyl acetate phase was dried and resuspended in 10µl ethyl acetate which was then spotted onto TLC plates. Plates were 20 cm X 20 cm, and samples were applied 2 cm above the base at 1.5 cm intervals. Chromatography was carried out in a sealed chamber containing 100 ml of chloroform:methanol (95:1) until solvent was 4 cm from the top of the TLC plate. Either phosphoimage analysis or autoradiography were used to visualize the acetylation of the chloramphenicol. In the case of autoradiography, acetylation was quantified by excising the radiolabeled spots from the TLC plate and measuring radioactivity by liquid scintillation counting. CAT activity was calculated as the percent conversion of the chloramphenicol (percentage of radioactivity in the higher mobility acetylated forms relative to the total radioactivity) and was corrected for transfection efficiency by dividing by the β-Gal activity.

(ii) Phase-extraction CAT assay

Phase-extraction CAT assays (Seed and Sheen, 1988) were performed as follows: cytosolic extracts and CAT reactions were carried out as for the TLC based assay except that n-butyryl coenzyme A was used instead of acetyl coenzyme A. After the reaction

butyrlated chloramphenicol derivatives were extracted with 300µl of mixed xylenes. The xylene phase was then back extracted twice with 100 µl of 0.25 M Tris-HCl (pH 7.8). A 200 µl aliquot of the xylene phase was then scintillation counted. CAT activity was determined as the total counts in the xylene phase and was corrected for transfection efficiency as above.

14. β-galactosidase assay

β-galactosidase activity was assayed by the method of Zuber and Losick (Zuber and Losick, 1983) as follows: cytoplasmic extracts were prepared as described for CAT assays. 10-40 µl of cell extract was mixed with Z buffer (60 mM Na_2PO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-Mercaptoethanol, pH 7.0) to 200 µl. 40 µl of ONPG reaction buffer (4 mg/ml ONPG in 100 mM Phosphate buffer pH 7.0) was added and reactions were incubated at 30°C. Reactions were timed and stopped when a faint yellow colour appeared by addition of 100 µl of 1 M Na_2CO_3, then the absorbance at 420 nM was determined. β-galactosidase activity was calculated as follows:

$$\beta\text{-gal U/ml} = \frac{A_{420}/0.0045}{\text{Reaction Time} \times \text{Volume of Cell Extract}}$$

15. Immunoprecipitation and Phosphorylation of mycGR

Sf7 cells stably expressing mycGR were treated with 1 µM Dex for 1 hr. Cells were harvested and nuclear extracts were prepared by the method of Andrews and Faller (Andrews and Faller, 1991), all buffers were supplemented with 1 µM Dex. The NaCl

concentration of the extracts was adjusted to 150 mM with hypotonic buffer, and anti c-myc antibody 9E10 was added at 1 µl per 100 µg of nuclear extract and incubated for 16 h at 4°C. 2.5 mg aliquots were incubated with 10 µl of a 1:1 slurry of protein A-sepharose beads in immunoprecipitation buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% NP-40) for 30 min at 4°C. Beads were washed twice with immunoprecipitation buffer supplemented with 1 µM Dex, then twice with kinase buffer (50 mM HEPES pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.2 mM EGTA) supplemented with 1 µM Dex.

mycGR bound to the protein A-sepharose beads was phosphorylated by DNA-PK essentially as previously described (Lees-Miller et al., 1992). Beads were incubated for 30 min at 30°C in 20 µl reaction volumes containing kinase buffer (50 mM HEPES pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.2 mM EGTA), 40 ng linearized pHC17 plasmid, 5 µCi γ^{32}P-ATP (6000 Ci/mmol,Dupont), 50 mM ATP and 0.5 units of DNA-PK (Promega, Madison, WI, USA). The pHC17 plasmid was linearized with HindIII and was purified through agarose gels prior to use in kinase assays. Following kinasing the beads were washed 3 times in kinase buffer to remove excess ^{32}P. The mycGR was eluted by boiling in SDS-PAGE sample buffer. ^{32}P incorporation was determined by autoradiography or phosphorimage analysis of 6% SDS PAGE gels.

16. Identification of DNA-PK phosphorylation sites on mycGR

The method of Zhang et al. (Zhang et al., 1994) was used to identify mycGR phosphorylation sites as follows: SDS PAGE gels containing mycGR were stained with amido black and the corresponding bands were excised from the gel. All chemical used

were HPLC grade. Gel slices were washed with 50% methanol for 1 h, H_2O for 30 min and twice with 50 mM NH_4HCO_3. The slices were then resuspended in 0.5 ml 50 mM NH_4HCO_3 and 20µl of TPCK-trypsin (1 mg/ml in 50 mM NH_4HCO_3) was added. These were incubated overnight at 37°C with 4 further additions of 20µl of TPCK-trypsin. Gel slices were then removed, the eluate was dried, and the trypsin-digested peptides were resuspended in alkaline gel sample buffer (0.125 M Tris-HCl, pH 6.8; 6 M urea). These were then separated on 40% poly-acrylamide alkaline gels (stacking gel: 40% acrylamide, 0.75 M Tris-HCl pH 8.8; separating gel: 3.3% acrylamide, 0.125 M Tris-HCl pH 6.8, 6 M Urea). Wet gels were exposed overnight and radiolabeled bands were excised. Alkaline gel slices were washed twice for 5 min in 50% methanol and twice for 5 min in H_2O then the peptides were eluted in 0.5 ml H_2O overnight at 4°C. Peptides were then dried and subjected to either manual Edman degradation or secondary protease digestion.

Manual Edman degradation was carried out using the protocol of Sullivan and Wong (Sullivan and Wong, 1991) as modified by Zhang et al. (Zhang et al., 1994). Peptides were linked at their carboxy terminus to PVDF membranes derivatized with aryl amine groups using the Sequelon-AA reagent kit (Millipore) according to the manufacturers directions. After linkage the discs were washed 4 times with 1 ml H_2O, 5 times with 0.5 ml TFA then 3 times with 1ml methanol. Next 0.5 ml coupling reagent (7:1:1:1 methanol: H_2O:triethylamine:phenyl isothionate) was added and discs were incubated at 50°C for 10 min. The reagent was removed and the disc was washed 5 times with 1 ml methanol. The disc was then vacuum dried for 5 min then 0.5 ml TFA was added and incubated at 50°C for 6 min. The TFA was collected and the disc was

extracted with 1 ml 4.25% phosphoric acid in TFA. The 4.25% phosphoric acid solution was recovered and added to the collected TFA then the radioactivity released in this solution was determined by scintillation counting. The disc was then washed 5 times with 1 ml methanol and the cycle was repeated starting with the addition of coupling reagent. Cycles were continued until a significant release of radioactivity was detected.

Digestion of alkaline gel-purified phosphopeptides with sequencing grade endoproteinase AspN or GluC (Roche) was performed according to the manufacturers recommendations. For digestion with AspN the dried peptides were resuspended in 200 µl sodium-phosphate buffer pH 8.0. 1 µl AspN (0.2 µg/µl) was added and the sample was incubated at 37°C for 12 h with 2 additions of AspN. For digestion with GluC the dried peptides were resuspended in 200 µl 25 mM NH_4HCO_3 pH 7.8. 1 µl GluC (1 µg/µl) was added and the sample was incubated at 25°C for 18 h with 3 additions of GluC. Digestion products were resolved on 40% alkaline polyacrylamide gels and subjected to manual Edman degradation as described above.

III. RESULTS

1. Analysis of binding of factors to NRE1

The MMTV LTR transmits one of the strongest transcriptional responses to steroid hormones described to date (Beato, 1989; Ringold et al., 1975; Ucker and Yamamoto, 1984). This makes it an ideal model system for studying transcription responses to glucocorticoids as well as other steroid hormones including mineralocorticoids, progestins, and androgens (Archer et al., 1994; Cato et al., 1987; Cato et al., 1986; Cato and Weinmann, 1988). The transcriptional regulatory region of the virus primarily responsible for steroid hormone responsiveness occurs proximally to the promoter within the region from -187 to -37 of the LTR and contains a complex HRE as well as binding sites for NF1 and octamer transcription factors (Archer et al., 1994; Archer et al., 1992; Beato, 1991; Buetti, 1994; Cordingley et al., 1987; Toohey et al., 1990).

In certain cell types response to glucocorticoids is also mediated by cis-acting elements. In particular a negative regulatory element within -398 to -376 of the LTR, called NRE1, had been shown to repress the response to glucocorticoids in human Jurkat T cells and mouse epithelial CAC-E1A cells (Giffin et al., 1994). At the outset of this study, three factors had been identified which bound to the NRE1 sequence. One of these factors, with an apparent size of 80 kDa when cross-linked to DNA, interacted specifically with both the double-stranded element as well as the single upper-strand (Giffin et al., 1994). A 95 kDa DNA cross-linked factor interacted only with the single upper stand, while a 50 kDa factor contacted only the lower-strand (Giffin et al., 1994).

The initial goal of this project was to characterize the DNA binding properties of each of these factors.

(i) Properties of NRE1-binding factors in crude nuclear extracts

Evidence suggested that the three factors formed a heteromeric complex that was able to recognize the double as well as each of the single-stranded forms of NRE1 (Giffin et al., 1994). The ability to bind the single-strands appeared to be important for the transcriptional repression since a truncated form of NRE1 which did not support single-stranded binding was also unable to repress the glucocorticoid response (Giffin et al., 1994). It therefore became important to understand the relationship between the different DNA binding activities of the complex. As a first step, the kinetics of binding to each of the three forms of NRE1, as well as the truncated form, was assessed.

a) Kinetics of binding to NRE1

A number of classical approaches have been used to measure the kinetics of association and disassociation of proteins with DNA including nitrocellulose filter binding assays (Riggs et al., 1970), the electrophoretic mobility shift assay (EMSA) (Fried and Crothers, 1981; Garner and Revzin, 1981) and footprinting (Brenowitz et al., 1986; Galas and Schmitz, 1978). More recently a variety of spectroscopic approaches have been developed (Lohman and Bujalowski, 1991) including fluorescence anisotropy (Ozers et al., 1997; Perez-Howard et al., 1995; Sevenich et al., 1998; Takahashi et al., 1990) and non-spectroscopic approaches including isothermal titration calorimetry and surface plasmon resonance biosensoring (Oda and Nakamura, 2000). EMSA was used in

this study as it provides several advantages over the other techniques, especially in analyzing the DNA-binding of factors in crude nuclear extracts. This assay involves the addition of protein to radioactively labeled DNA fragments followed by the separation of protein-DNA complexes from free DNA by electrophoresis through non-denaturing gels and visualization of the labeled DNA by autoradiography or phosporimagery (Lane et al., 1992). The advantages this technique provides are (1) its requirement for very small quantities of materials; (2) its ability to differentiate the simultaneous binding of several proteins or protein complexes; (3) the amount of detailed information it can provide including the specificity of the interaction, the kinetics and the affinity of the binding (Lane et al., 1992). Additionally EMSA is technically simple to perform, rapid and requires only basic laboratory equipment (Lane et al., 1992).

To compare on-rates for NRE1 binding EMSAs were performed in which ^{32}P-labeled NRE1 containing oligonucleotides were incubated with Jurkat nuclear extract for times varying from 1 minute to 4 hours prior to separation on 4% non-denaturing polyacrylamide gels. The reactions were initiated at intervals designed so they would finish at the same time, enabling them to be loaded on the gels concurrently. Binding was then visualized by autoradiography and quantified by densitometry.

The rates of association with four forms of the NRE1 were examined. These forms included the double-strand as well as each of the single-strands of the full NRE1 element. For these, the MTV oligonucleotides described in Table 1 were used, which contain LTR sequences between -398 and -375 centered over NRE1. The rate of binding was also measured to a truncated form of NRE1 that is recognized by the double-strand

Name	Description	Strand	Sequence
MTV	MMTV LTR -398 to -375	up	5'-AACT**GAGAAAGAGAAAGA**CGACA-3'
		lo	5'-TGTCG**TCTTTCTCTTTCTC**AGTT-3'
MT	MMTV LTR -398 to -375 1 copy of polyR repeat deleted	up	5'-AACT**GAGAAAGA**CGACA-3'
		lo	5'-TGTCG**TCTTTCTC**AGTT-3'
OCT	Histone H2B octamer motif	up	5'-AGCTTGCTT**ATGCAAAT**AAGGTG-3'
		lo	5'-CACCTT**ATTTGCAT**AAGCAAGCT-5'
mtOCT	Mutated histone H2B octamer motif	up	5'-AGCTTGCTT**cgGCAAAT**AAGGTG-3'
		lo	5'-CACCTT**ATTTGCcg**AAGCAAGCT-5'

Table 1. Sequences of oligonucleotides used in DNA binding studies.

The oligonucleotides used in the DNA binding studies are shown. MTV is a 23 mer containing the full NRE1 element from the MMTV LTR. MT is a 17 mer containing the NRE1 element from the MMTV LTR but having 1 copy of the poly-purine (polyR) direct repeat deleted. OCT is a 23 mer containing the consensus octamer motif from the histone H2B promoter. mtOCT is a 23 mer identical to OCT except for the substitution of 2 bases shown to eliminate the binding by octamer transcription factors.

binding factors but does not support single-stranded binding (Giffin et al., 1994). For this the MT oligonucleotide described in Table 1 was used which has a 6 nucleotide deletion within NRE1 that results in the loss of one copy of the GAGAAAGA overlapping direct repeat.

EMSA showing the time courses of binding to each of these forms of NRE1 are shown in Figure 4 (panel A to D). These results were quantified by densitometry and plotted as a function of time in panel E. Although only the bound complexes are shown in Figure 4, the binding-activities to all four forms of NRE1 had identical mobilities (see Figure 7, lanes 3 to 6 for a direct comparison of mobilities) as was previously observed (Giffin et al., 1994). Binding to all four forms appeared to follow a curve with two apparent phases. In the first phase, binding increased logarithmically. In the second phase binding was plateaued at its maximal value (panel E). The duration of the first phase, or the time required to reach equilibrium, varied between the different forms of NRE1 used. This phase lasted 10 min with upper-stranded MTV (panel B and E), and was longer with the lower-stranded MTV at approximately 20 min (panel C and E). This first phase of binding was longest with the double-stranded NRE1, lasting approximately 60 min (panel A and E). For binding to the MT oligonucleotide, containing the NRE1 half-site, the first phase lasted an intermediate time of 20 min (panel D). After this first phase of increasing binding, the curves for all four forms of NRE1 became essentially horizontal at the maximal level of binding, indicating equilibrium had been reached. This second phase lasted for the duration of the experiment for each of the binding sites (panels A to E).

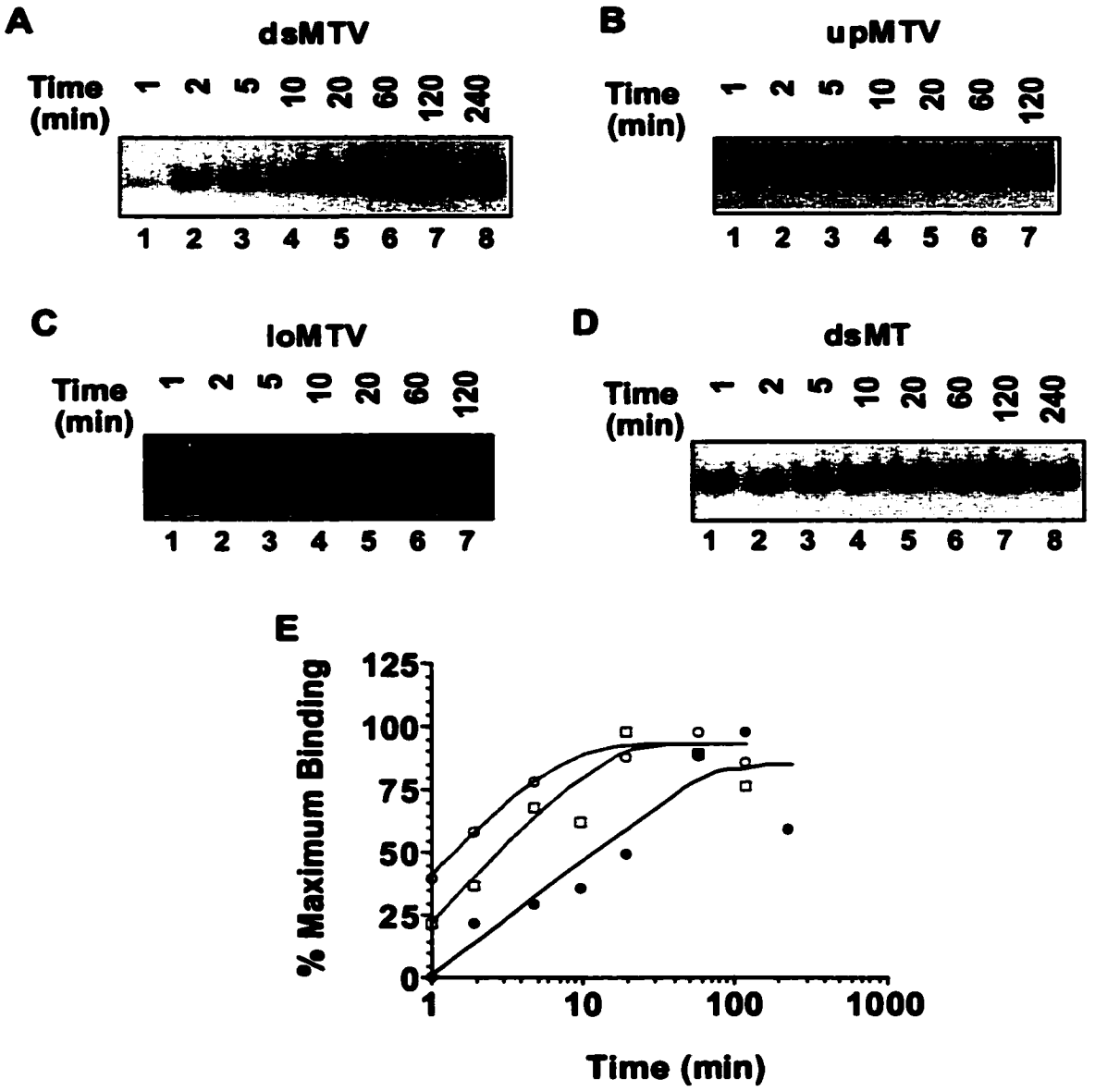

A
dsMTV
Time (min)
1 2 5 10 20 60 120 240
1 2 3 4 5 6 7 8
B
upMTV
Time (min)
1 2 5 10 20 60 120
1 2 3 4 5 6 7
C
loMTV
Time (min)
1 2 5 10 20 60 120
1 2 3 4 5 6 7
D
dsMT
Time (min)
1 2 5 10 20 60 120 240
1 2 3 4 5 6 7 8
E
% Maximum Binding
125
100
75
50
25
0
1
10
100
1000
Time (min)

Figure 4. On rates of specific factor binding in Jurkat crude nuclear extract to various forms of NRE1 containing oligonucleotides.

EMSA were performed with 1 μg Jurkat crude nuclear extract, incubated with 0.5 ng ^{32}P-labeled double-strand (ds) MTV (Panel A), upper-strand (up) MTV (Panel B) and lower-strand (lo) MTV (Panel C) for times up to 240 min. All incubations were timed to end concurrently. In all EMSA only the bound fraction is shown however the unbound fraction contained at least a 20-fold excess of probe. Experiments were repeated 3 times, a representative experiment is shown in each Panel. Panel E, for the shown gel shifts, binding was quantified by densitometric analysis of the autoradiographs. The amount of binding in each lane was expressed as a percentage of the maximum binding, and plotted against the time of the incubation. For clarity the curve for ds MT is not shown. Time was presented on a log scale to better separate the curves. ds MTV (●); up MTV (○); lo MTV (□).

The half-times of binding ($t_{1/2}$) were determined as a measure of the on-rates. These were measured by determining the time at which 50% of maximal binding occurred in the curves shown in panel E. The on-rates for binding to the three forms of NRE1 by factors in Jurkat nuclear extract were clearly distinguishable (Figure 4). Binding to upper-strand NRE1 occurred most rapidly, with a half time of 1.5 minutes (Figure 4, panel B and E). Lower-strand NRE1 binding was slightly slower, with an on-rate half time of 3 minutes (panel C and E). By contrast, the $t_{1/2}$ of binding to double-stranded NRE1 was substantially longer at 11 minutes (panel A and E). Notably, the on-rate for binding to double-stranded MT, at 4 minutes, was 3-fold faster than binding to the full-length element (panel D). This indicates that removal of one copy of the overlapping direct repeat in NRE1 lead to a more rapid formation of a stable protein-DNA complex on double-stranded DNA.

Off-rates of binding are the time in which a factor already bound to DNA dissociates from its binding site. To measure off-rates Jurkat nuclear extracts were pre-incubated with the ^{32}P-labeled oligonucleotides and binding was allowed to occur for 30 min. Subsequently a 400 fold excess of the identical unlabeled oligonucleotide was added and allowed to compete binding for times ranging from 1 min to 24 h. Again the competitor was added at times designed to allow the competitions to conclude at the same time. The reactions were then resolved on 4% non-denaturing gels, visualized by autoradiography and quantified by densitometry.

Off-rates were determined for the double-stranded and each of the single-stranded forms of MTV containing the full NRE1 element as well as the double-stranded form of

MT which contained the truncated NRE1 element with one copy of the overlapping direct repeat deleted (see Table 1 for the sequences).

For double and upper-stranded MTV and double-stranded MT, binding was observed to decrease over time following addition of a 400 fold excess of unlabeled competitor. Half-times of release ($t_{1/2}$) from the DNA were used as a measure of the off-rates. These were determined as the length of time required for the binding to reach 50% of maximal binding following addition of the competitor. The upper-stranded NRE1 binding activity in Jurkat nuclear extract released from the DNA with a $t_{1/2}$ of 30 minutes (Figure 5, panel B and F). Release from double-stranded full-length NRE1 was slower, with a $t_{1/2}$ of one hour (panel A), while the $t_{1/2}$ for release from the MT was even longer at 4 h (panel E and F). Our first attempt at competition of lower-strand binding activity with 200 ng of cold competitor showed no detectable release of the lower-strand binding factor (panel C). The experiment was repeated with the amount of competitor increased to 1 μg. In this case competition was barely detectable with a $t_{1/2}$ of at least 12 h (panel D and F).

For all forms of NRE1, the off-rates were unusually slow indicating that binding was very stable. This was somewhat surprising, as it was previously shown that a 200-fold excess of specific competitor DNA, when combined with the labeled DNA prior to addition of the nuclear extract, was sufficient to prevent binding to all four forms of NRE1 (Giffin et al., 1994).

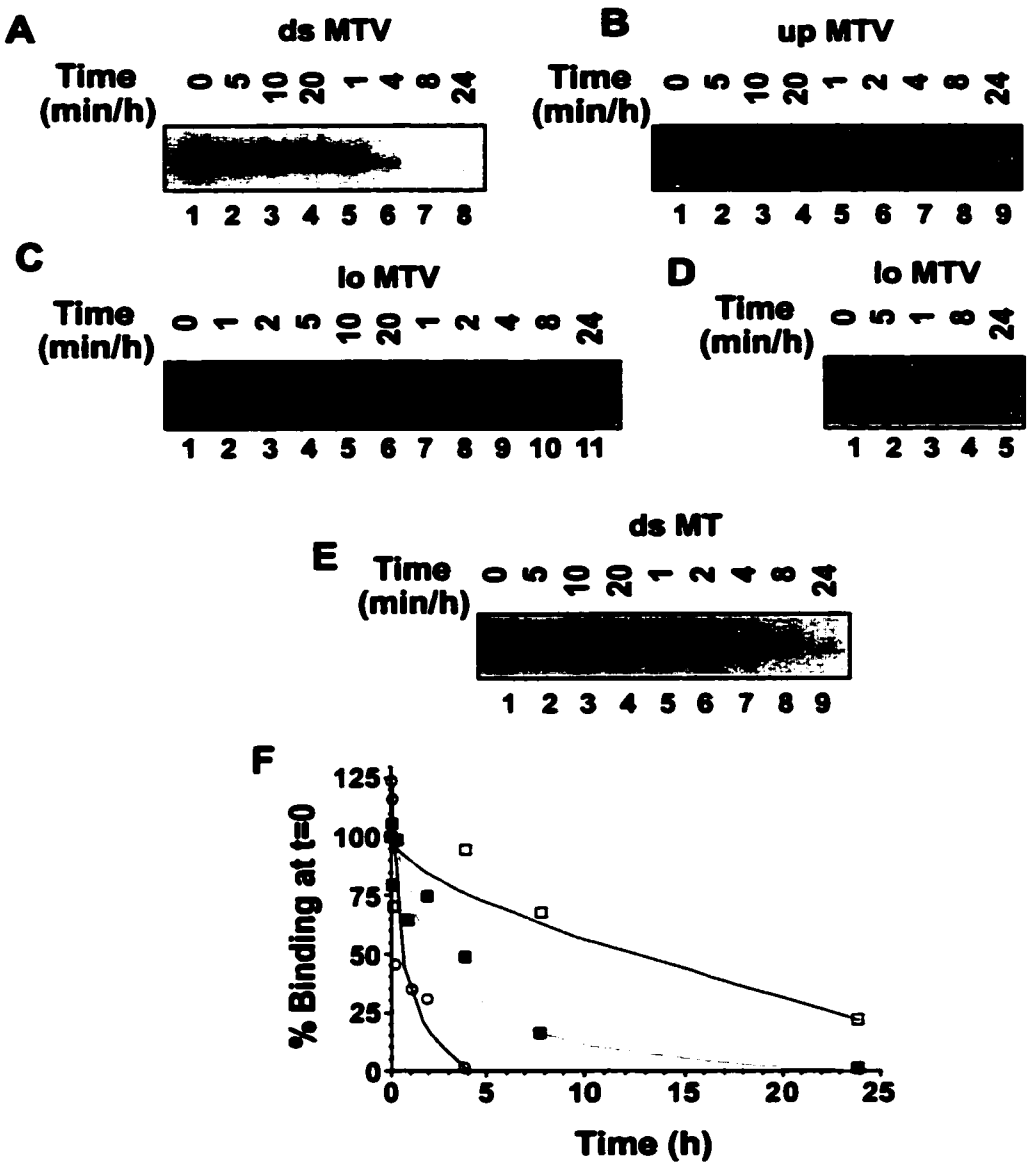

A
ds MTV
Time (min/h)
0 5 10 20 1 4 8 24
1 2 3 4 5 6 7 8
B
up MTV
Time (min/h)
0 5 10 20 1 2 4 8 24
1 2 3 4 5 6 7 8 9
C
lo MTV
Time (min/h)
0 1 2 5 10 20 1 2 4 8 24
1 2 3 4 5 6 7 8 9 10 11
D
lo MTV
Time (min/h)
0 5 1 8 24
1 2 3 4 5
E
ds MT
Time (min/h)
0 5 10 20 1 2 4 8 24
1 2 3 4 5 6 7 8 9
F
% Binding at t=0
125
100
75
50
25
0
0 5 10 15 20 25
Time (h)

Figure 5. Off rates of specific factor binding in Jurkat crude nuclear extract from various forms of NRE1 containing oligonucleotides.

EMSA were performed with 1 μg Jurkat crude nuclear extract added to 0.5 ng ^{32}P-labeled double-strand (ds) MTV (Panel A), upper-strand (up) MTV (Panel B), lower-strand (lo) MTV (Panel C, D) or double-strand (ds) MT (Panel E). Binding was allowed to proceed for 30 min. Subsequently an excess of the same unlabeled oligonucleotide was added and incubated for times up to 24 h. All incubations were timed to end concurrently. 200 ng (Panel A, B, C, E) or 1 μg (Panel D) of competitor was added. In all EMSA, only the bound fraction is shown however the unbound fraction contained at least a 20-fold excess of probe. Experiments were repeated 3 times, a representative experiments is shown in each Panel. Panel F, for the shown gel shifts, binding was quantified by densitometric analysis of the autoradiographs. The amount of binding for each incubation was expressed as a percentage of the binding in the absence of competitor, and plotted against the time of the competition. dsMT (■); upMTV (●); loMTV (□).

b) NRE1 binding factors from Jurkat nuclear extracts cross recognize an octamer motif

With the goal of developing a strategy to identify the NRE1 binding factors, southwestern blotting experiments were carried out. The southwestern blotting technique involves the visualization of proteins based on their DNA binding specificity (Bowen et al., 1980; Miskimins et al., 1985). In this procedure proteins are initially separated by SDS-PAGE then transferred to a membrane such as PVDF. The membranes are then probed with ^{32}P-labeled DNA elements containing the sequence of interest. DNA-binding proteins on the membrane can then be visualized by autoradiography. This procedure has been modified to allow cloning of sequence-specific DNA binding proteins (Singh et al., 1988; Staudt et al., 1988). In this technique filter replicas of cDNA expression libraries, typically in the λgt11 phage expression vector, are probed with ^{32}P-labeled recognition site DNA. The cDNAs encoding a number of transcription factors have been isolated using this technique including MBP-1 (Singh et al., 1988), Oct-1 (Sturm et al., 1988), Oct-2 (Muller et al., 1988; Staudt et al., 1988), CREB (Hoeffler et al., 1988) and Pit-1 (Ingraham et al., 1988).

As an initial step towards identifying the NRE1 binding factors using this approach, I performed southwestern blotting on SDS-PAGE separated proteins. Jurkat crude nuclear extracts were separated through SDS-PAGE gels, these were electroblotted onto PVDF membranes and the membranes were probed with the ^{32}P-labeled oligonucleotides described in Table 1, either as concatamers or as single copies. The results of this are shown in Figure 6. Four major factors from Jurkat nuclear extracts were observed to bind the double-stranded MTV probe containing the full NRE1 element

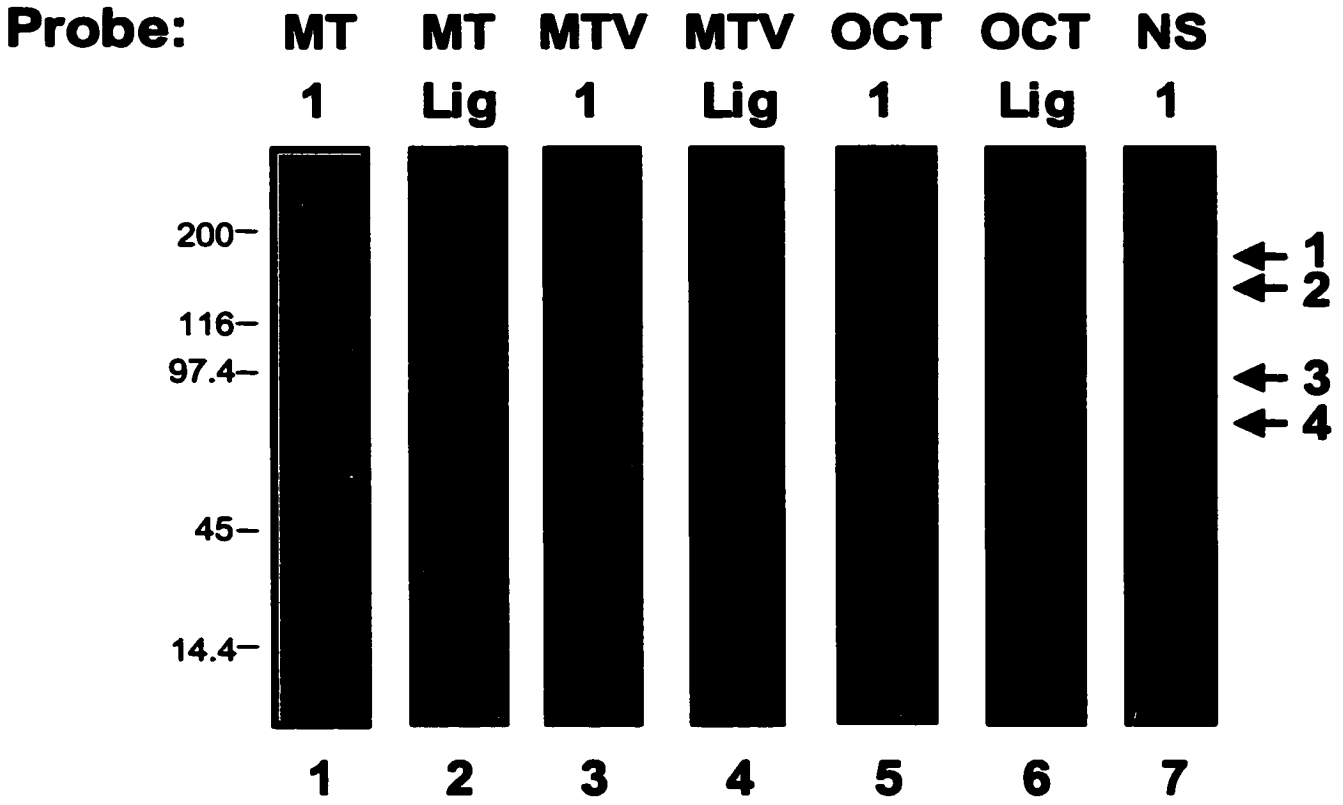

Probe:
MT 1
MT Lig
MTV 1
MTV Lig
OCT 1
OCT Lig
NS 1
200
116
97.4
45
14.4
1
2
3
4
1
2
3
4
5
6
7

Figure 6. Nuclear factors from Jurkat extracts cross recognize NRE1 and octamer motifs.

100 μg of Jurkat nuclear extract was separated by SDS-PAGE in multiple wells. The gels were soaked in renaturation buffer (4% urea) and transferred to PVDF membrane. Individual lanes were cut from the membrane and probed with double-stranded radiolabeled oligonucleotides. The MT, MTV and OCT probes are described in Methods and Materials. NS is a non-specific probe consisting of a 35 bp polylinker fragment from pGEM. The probes were either single copy (1) or multimerized with DNA ligase (lig). This experiment was repeated 3 times, with a representative experiment shown.

(Figure 6, lanes 3 and 4). These factors were of approximately 185, 145, 88 and 67 kD (labeled 1-4 respectively). There were no significant differences dependent on the concatamerization of the MTV probe. By contrast, only factor 4 was able to bind to a multimerized form of the truncated MT element (lane 2) while it did not recognize a single copy of this element (lane 1). This suggested that this factor had different requirements for sequence-specific binding than the other factors. Furthermore, given that the factors binding to MTV and MT appeared to be identical based on their mobilities in EMSA gels (see Figure 7, lanes 3 to 6, discussed below and (Giffin et al., 1994)) and that MT competed for binding to MTV (Giffin et al., 1994), this suggested that the 67 kD factor observed binding to both MTV and MT in southwestern blots may have been a specific NRE1 binding factor.

In lanes 5 and 6 a DNA probe containing the octamer motif, the optimal binding site for octamer transcription factors, was used as a positive control. Suprisingly, this element was bound by the same pattern as factors that recognized MTV. The binding appeared to be specific since the hybridizations were carried out in the presence of a 500-fold excess of competitor DNA and a non-specific labeled oligonucleotide was not bound (lane 7). This suggested that NRE1 binding factors could also recognize an octamer motif.

To more intensively examine the ability of Jurkat nuclear factors to cross recognize NRE1 and octamer motifs, I performed EMSA using NRE1 and octamer motif-containing oligonucleotides and specific competitors. As previously observed (Giffin et al., 1994) binding only occurred on the double-stranded MT element, while no binding occurred to either of the single-strands (Figure 7; panel A, compare lanes 1 and 2 with

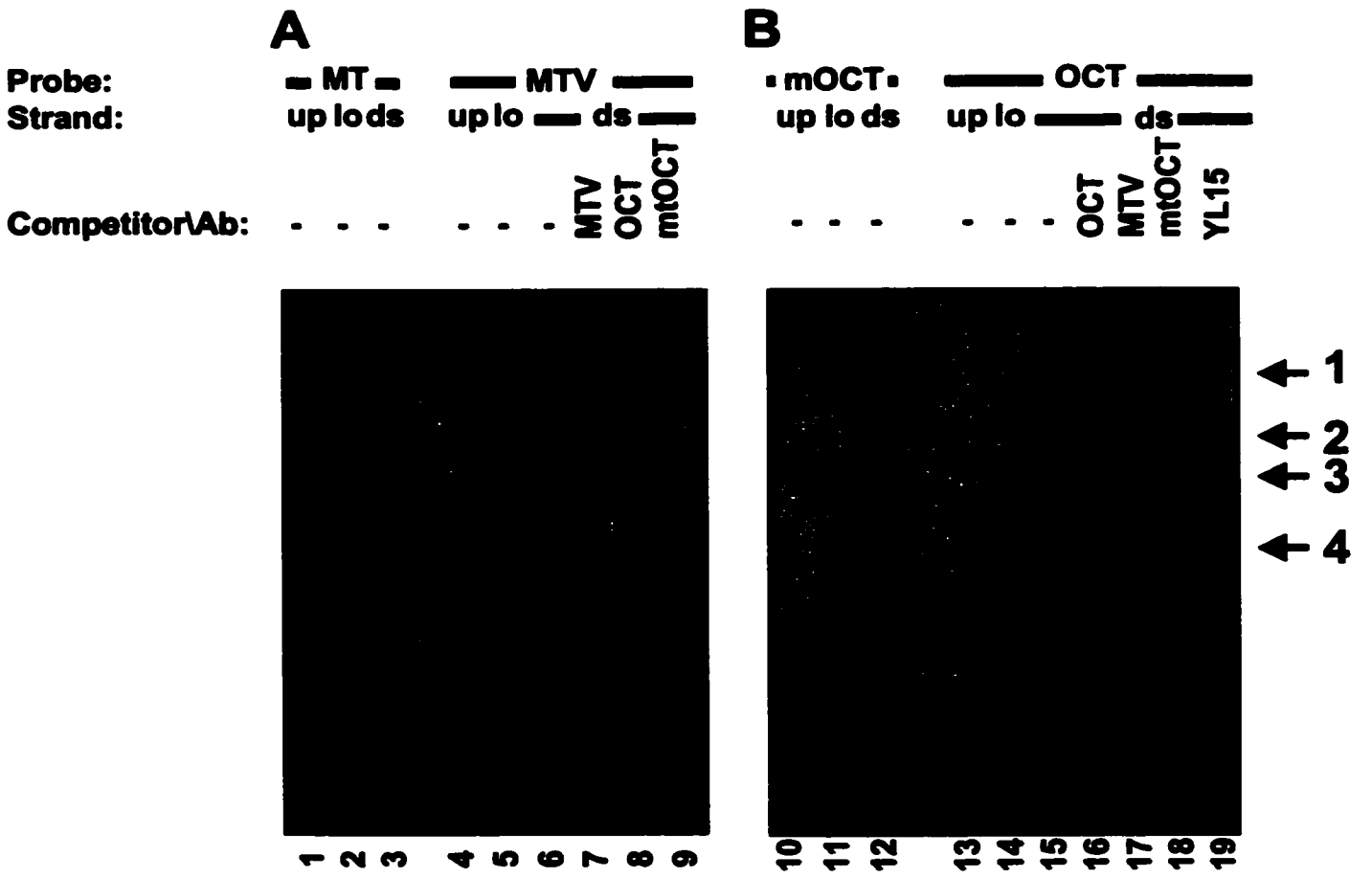

A
B
Probe:
MT
MTV
mOCT
OCT
Strand:
up lo ds
up lo ds
up lo ds
up lo ds
Competitor\Ab:
MTV
OCT
mtOCT
OCT
MTV
mtOCT
YL15
1
2
3
4
1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16 17 18 19

Figure 7. NRE1 competes for specific factor binding to octamer motifs.

Panel A: EMSA were performed with Jurkat nuclear extracts binding to 0.5 ng of radiolabeled MT (lanes 1-3) or MTV (lanes 4-9). Probes were upper-strand (lanes 1, 4), lower-strand (lanes 2, 5) or double-strand (lanes 3, 6-9). A 200-fold excess of unlabeled competitor oligonucleotide was added prior to addition of the nuclear extract. The double-stranded competitors used were MTV (lane 7), OCT (lane 8) and mtOCT (lane 9). Panel B: EMSA was repeated using 0.5 ng of a mutant octamer motif which does not support OCT-1 binding, mtOCT (lanes 10-12) or the wild type octamer motif, OCT (lanes 13-20). Probes were upper-strand (lanes 10, 13), lower-strand (lanes 11,14) or double-strand (lanes 12, 15-20). The four complexes observed were labeled 1-4 as indicated on the right side of the Panel. A 200-fold excess of competitor oligonucleotide was added prior to addition of the nuclear extract. The double-stranded competitors used were OCT (lane 16), MTV (lane 17) and mtOCT (lane 18). MTV exclusively competes binding of factor 3 (lane 17). YL15, an OCT-1 specific antibody, was incubated with the nuclear extract prior to addition of probe DNA, inhibiting formation of complex 1 (lane 19). All oligonucleotides are described in detail in Table 1 and Material and Methods. These experiments were repeated twice, with a representative experiment shown.

lane 3). A Jurkat nuclear factor binding to the full double-stranded MTV element was also observed, while binding to each of the upper or lower-strands was weaker (compare lanes 4 and 5 with lane 6) as previously observed (Giffin et al., 1994). Addition of a 200-fold excess of unlabelled MTV to the labeled probe prior to the addition of nuclear extract resulted in a specific competition of binding (lane 7). Similarly, addition of an unlabelled wild type octamer motif also competed binding to NRE1 (lane 8) indicating that this factor cross-recognized an octamer motif. Surprisingly, a mutated octamer motif, mtOCT, which does not support OCT-1 binding (Cleary et al., 1993), also competed NRE1 binding (lane 9) suggesting that the sequences within the octamer motif recognized by the NRE1 binding factor are different from those necessary for binding by OCT-1. In all cases the competition by the added oligonucleotides was considered specific since all reactions contained 1 μg of non-specific sheared calf-thymus DNA.

To confirm this cross recognition, EMSA were performed using labeled octamer motifs as probes. Four factors from Jurkat nuclear extracts were observed to bind to the octamer motif (Figure 7; panel B, lane 15) and these were labeled 1 to 4 as shown in Figure 7 (right side of panel B). Factor 3 corresponded in mobility to the NRE1 double-stranded binding factor (compare panel B, lane 15 with panel A, lane 6). This factor also recognized the double-stranded mtOCT (panel B, lane 12) confirming that this element was also cross recognized even though binding by other factors was compromised. Neither the single upper- or lower-strands of the wild type octamer motif or the mutant octamer motif were recognized (lanes 13-14 and 10-11 respectively) indicating that although the NRE1 binding factor could recognize single-stranded NRE1 it could not recognize single-stranded octamer motifs. Binding by complexes 1, 2 and 3 appeared

specific since addition of a 200-fold excess of unlabelled octamer motif to the labeled probe significantly reduced their levels of binding (lane 16). Complex 4 was not significantly affected by this competition (lane 16) indicating it was not a specific octamer-binding factor. A similar addition of unlabelled MTV resulted in the exclusive competition of binding by factor 3 (lane 17) indicating that this factor solely recognized the NRE1 element, giving further evidence that it was identical to the factor observed binding to MTV (panel A, lane 6).

Preincubation of Jurkat nuclear extract with 1 μl of the anti-OCT-1 antibody YL15 prior to the addition of ^{32}P-labeled DNA did not significantly affect the binding of factor 3, showing that the NRE1 binding factor was not immunologically related to OCT-1 (lane 19). The YL15 antibody recognizes the OCT-1 homeodomain and prevents DNA binding of OCT-1 (Lai and Herr, 1992). In contrast with factor 3, the binding of factor 1 to the octamer motif was significantly impaired by preincubation with YL15 (lane 19) indicating that factor 1 is OCT-1, consistent with a previous study showing OCT-1 as the lowest mobility octamer-binding factor in Jurkat nuclear extracts (Bhargava et al., 1993).

These results taken together support the notion that the Jurkat nuclear NRE1 binding factors can cross recognize sequences within an octamer motif and that the sequence requirements are distinguishable from the requirements for binding by OCT-1.

(ii) Binding of purified Ku autoantigen to NRE1

During the course of these studies the factor binding to the double-strands of NRE1 was purified and identified as the Ku autoantigen by direct protein sequencing (Giffin et al., 1996). For this reason the development of the southwestern cloning strategy was abandoned. Ku is a heterodimer consisting of 82 kD and 70 kD subunits,

localized primarily in the nucleus. This was consistent with the results of the southwestern assay, as two of the observed Jurkat nuclear NRE1 binding proteins (Figure 6, factor 3 and 4) corresponded in size to the two Ku subunits. Further, U.V. cross-linking studies have shown that only the 70 kD subunit of Ku makes contact with the MT element (Giffin et al., 1999), consistent with the observation that only the 67 kD factor bound to the multimerized MT element. Moreover, Ku has been reported to bind octamer motifs (May et al., 1991), albeit with lower affinity than to NRE1 (Giffin et al., 1997), consistent with the southwestern assay and EMSA results (Figure 6 and 7).

That the Ku autoantigen was the double-stranded NRE1 binding activity was confirmed with Ku specific antibodies which supershifted the protein-DNA complex in EMSA (Giffin et al., 1996). Moreover, it was demonstrated that binding occurred specifically and directly to NRE1 using EMSA on closed circular DNA templates eliminating the possibility that Ku was binding to the ends of the NRE1 oligonucleotide (Giffin et al., 1996). Ku was also demonstrated to bind directly to the NRE1 element of MMTV by via DNaseI footprinting of relaxed closed circular plasmids containing the MMTV LTR (Giffin et al., 1996).

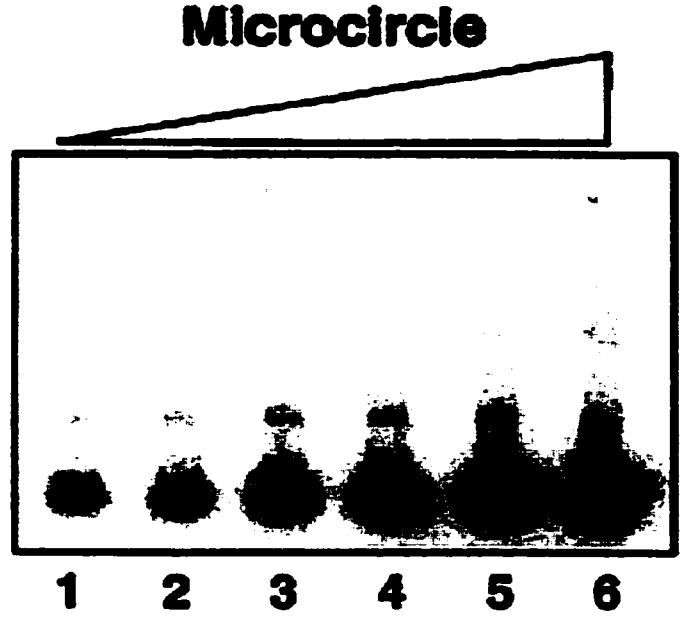
a) Purified Ku binds to NRE1 with high affinity

The following lines of evidence suggested that Ku was able to bind in a sequence-specific manner to the NRE1 element preferentially to DNA ends: In the EMSA experiments with crude Jurkat nuclear extracts (Figure 4, 5 and 7) all binding reactions were in the presence of 1 μg of highly sheared calf thymus DNA. This did not compete binding to NRE1. However addition of only 100 ng of unlabelled NRE1 oligonucleotide was able to completely compete this binding (Figure 5, panel A and Figure 7, lanes 7 and

17). In addition, in southwestern blotting the NRE1 binding factor was able to bind specifically to NRE1 (Figure 6) despite the presence of a 500-fold excess of DNA ends present in the hybridization reactions. This was also observed in EMSA and footprinting assays using purified Ku (Giffin et al., 1996). Additionally the purification of Ku as the NRE1 binding factor was anchored by a DNA affinity column that was run in the presence of 25 μg/ml of highly sheared calf thymus DNA (Giffin et al., 1996).

To directly ascertain the affinity of Ku for double-stranded NRE1, I performed a Scatchard analysis to determine the equilibrium binding constant (Scatchard, 1949). To accomplish this I carried out an EMSA using a constant amount of Ku binding to an increasing amount of NRE1-containing microcircle (Figure 8). This circle was prepared by ligation of a 223-bp DNA fragment from pNRE1 containing the NRE1 element. The single-copy closed-circular form was gel-purified and shown to be resistant to S1 nuclease (Vogt, 1980), Exonuclease III (Rogers and Weiss, 1980) and Bal31 (Gray et al., 1975) indicating that the microcircles were free of nicks, ends or other structural features. In this assay the recircularization protocol requires incorporation of a single ^{32}P label for formation of the covalently closed circular microcircle. Thus the labeling efficiency of the purified microcircles used in this assay was 100%, allowing exact determination of the quantity of DNA used. A representative EMSA is shown in Figure 8, panel A and a Scatchard analysis is shown in panel B. An equilibrium binding constant obtained by averaging three independent experiments yielded a K_d of 0.84 ± 0.24 nM for direct sequence-specific DNA binding of recombinant Ku to NRE1. This value is comparable with the values obtained for the sequence-specific DNA binding of many transcription

A


Microcircle
1
2
3
4
5
6

B

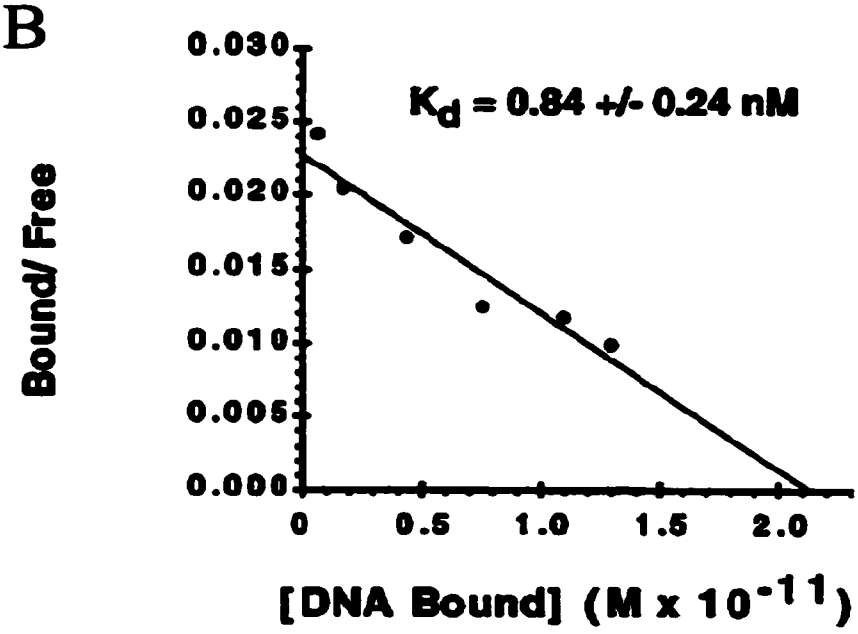

K_d = 0.84 +/- 0.24 nM
Bound/ Free
0.030
0.025
0.020
0.015
0.010
0.005
0.000
0
0.5
1.0
1.5
2.0
[DNA Bound] (M x 10^{-11})

Figure 8. Determination of the equilibrium binding constant of Ku to double-stranded NRE1.

Panel A: EMSA was performed with 2.7 to 43.5 pmol of ^{32}P-labeled NRE1-containing microcircle DNA and a constant 1 μl amount of recombinant purified Ku. The amounts of DNA probe added to each incubation were 2.7 pmol (lane 1), 5.4 pmol (lane 2), 10.8 pmol (lane 3), 21.7 pmol (lane 4), 43.5 pmol (lane 5) and 87 pmol (lane 6) as determined by scintillation counting. Panel B: bound and free DNAs in Panel A were quantified by phosphorimager and the K_d of NRE1 binding to Ku was determined by Scatchard analysis. One representative Scatchard plot is displayed together with the K_d (± S.E.) calculated from three independent repetitions of the assay.

factors and is 3-fold higher than that reported for Ku binding to a short non-specific oligonucleotide (K_d = 2.4 nM).

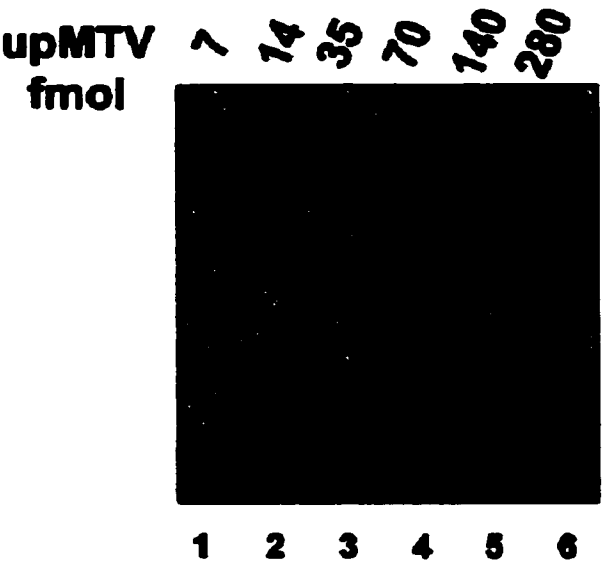
b) Purified Ku binds to upper NRE1 with intermediate affinity

Initial studies, performed in our laboratory, into the identity of the factors responsible for binding to the single-stranded forms of NRE1 identified Ku as also being one of two upper NRE1 binding factors (Torrance et al., 1998). Ku was purified from Jurkat nuclear extracts on a single-stranded upper NRE1 DNA affinity column (Torrance et al., 1998). It was confirmed that Ku was the upper-strand binding factor by EMSA experiments in which purified Ku bound to upper MTV was supershifted by a Ku specific antibody (Torrance et al., 1998). Purified Ku was also able to protect the NRE1 element in both DNaseI and $KMnO_4$ single-stranded footprinting assays (Torrance et al., 1998). In addition, recombinant Ku expressed and purified from baculovirus infected insect cells was able to specifically bind the upper and double-stranded NRE1 (Torrance et al., 1998).

As with double-strand binding, several lines of evidence described below suggested that Ku bound to the single upper-strand with a higher affinity than to DNA ends. First, in the single-stranded EMSA the presence of a 200-fold excess of non-specific single-stranded DNA ends did not compete with Ku binding to the upper NRE1 (Torrance et al., 1998). Likewise, in single-stranded footprinting the presence of a 50-fold excess of single-stranded DNA ends did not prevent Ku from protecting the NRE1 (Torrance et al., 1998). Finally, the single-stranded DNA affinity column used for purification was run in the presence of an excess of single-stranded competitor DNA (Torrance et al., 1998). These observations suggested that Ku bound to the upper-strand with higher affinity than to DNA ends.

To directly determine the affinity of Ku binding to the single, upper-strand, of NRE1 I again perform Scatchard analyses to determine the equilibrium binding constant (Scatchard, 1949). EMSA was performed by binding a constant amount of recombinant, purified Ku to an increasing amount of the upper MTV oligonucleotide (Figure 9). A representative EMSA is shown in Figure 9, panel A and a Scatchard analysis is shown in panel B. An equilibrium binding constant was obtained by averaging three independent experiments yielding a K_d of 3.5 ± 1.3 nM for sequence-specific single-stranded Ku binding. This result indicates that the affinity of recombinant Ku for upper-stranded NRE1 was approximately 4-fold lower than the 0.84 ± 0.24 nM K_d that I observed for the direct binding of recombinant Ku to double-stranded NRE1 under the same binding conditions. This lower value for the affinity of Ku for single-stranded NRE1 is consistent with the observation that the DNA ends in highly sheared calf thymus DNA begin to compete Ku binding to upper MTV at lower concentrations than they do double-stranded NRE1 binding (Torrance et al., 1998). This is also consistent with previous results using crude Jurkat nuclear extracts in which the double-stranded MTV oligonucleotide competed 3-5-fold more effectively for double-stranded NRE1 binding than did the upper MTV sequence (Giffin et al., 1994). However, competition of upper MTV binding by DNA ends still requires a greater than 100-fold molar excess of DNA ends (Torrance et al., 1998). Together these observations indicate that Ku binds to the upper-stranded NRE1 with an affinity intermediate to double-stranded NRE1 binding and DNA end-binding.

A


upMTV
fmol
7
14
35
70
140
280
1
2
3
4
5
6

B

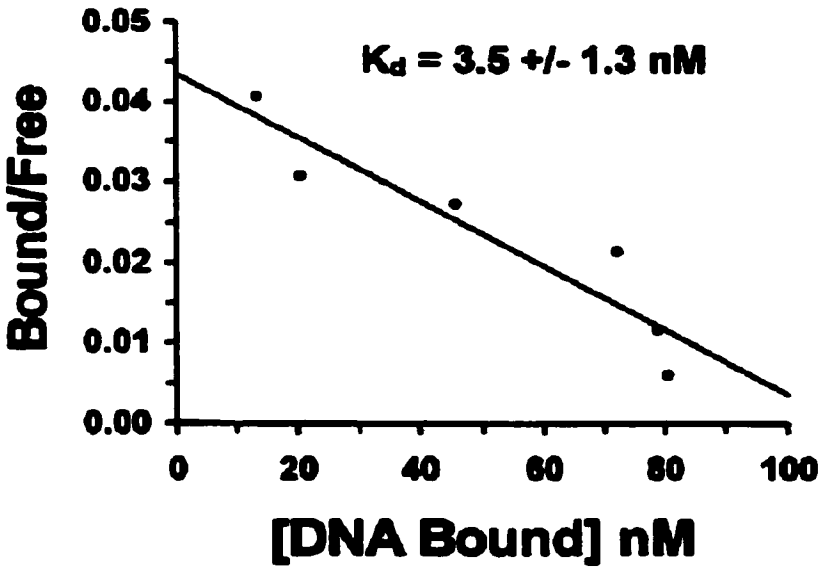

K_d = 3.5 +/- 1.3 nM
Bound/Free
0.05
0.04
0.03
0.02
0.01
0.00
0
20
40
60
80
100
[DNA Bound] nM

Figure 9. Determination of the equilibrium binding constant of Ku to the upper-strand of NRE1.

Panel A: EMSA was performed with 7 to 280 fmol of ^{32}P-labeled upper-stranded MTV oligonucleotide incubated with a constant 1 µl amount of recombinant purified Ku. The amounts of DNA probe added to each incubation were 7 fmol (lane 1), 14 fmol (lane 2), 35 fmol (lane 3), 70 fmol (lane 4), 140 fmol (lane 5) and 280 fmol (lane 6) as determined by scintillation counting. Panel B: Bound and free DNAs separated by EMSA in Panel A were quantified by phosphorimager and the K_d for recombinant Ku binding to the upper-strand of NRE1 was determined by Scatchard analysis. One representative Scatchard plot is displayed, together with the K_d (±S.E.) calculated from three independent repetitions of the assay.

A


1 2 5 10 20 60 min
1 2 3 4 5 6

B

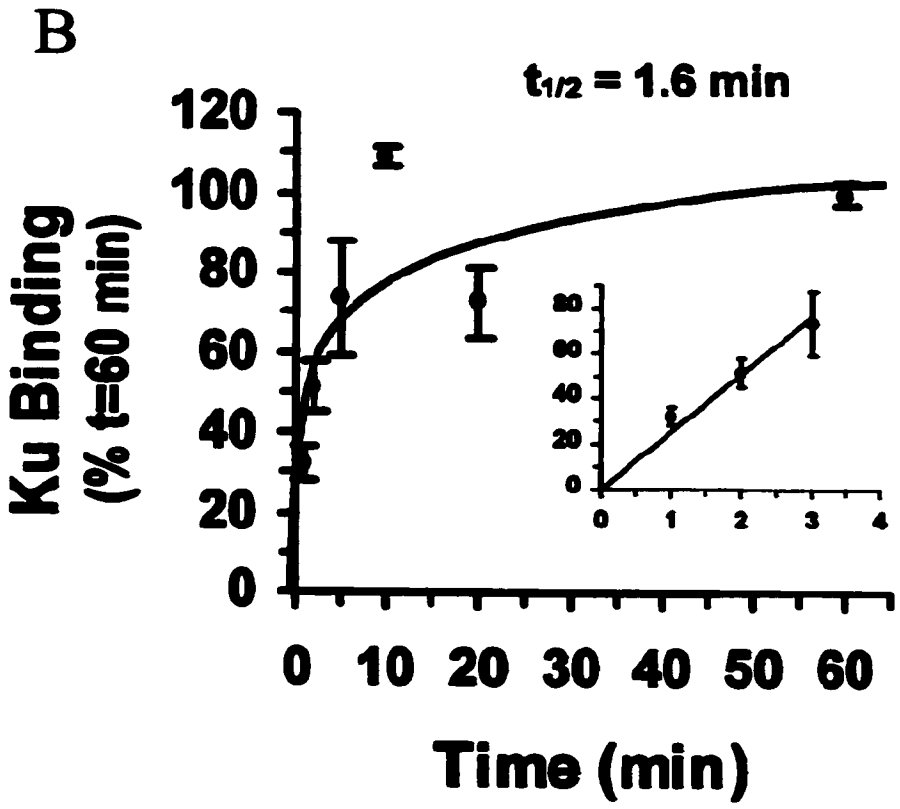

$t_{1/2}$ = 1.6 min
Ku Binding
(% t=60 min)
120
100
80
60
40
20
0
0 10 20 30 40 50 60
Time (min)
0 1 2 3 4

Figure 10. Analysis of the kinetics of Ku binding to the upper-strand of NRE1.

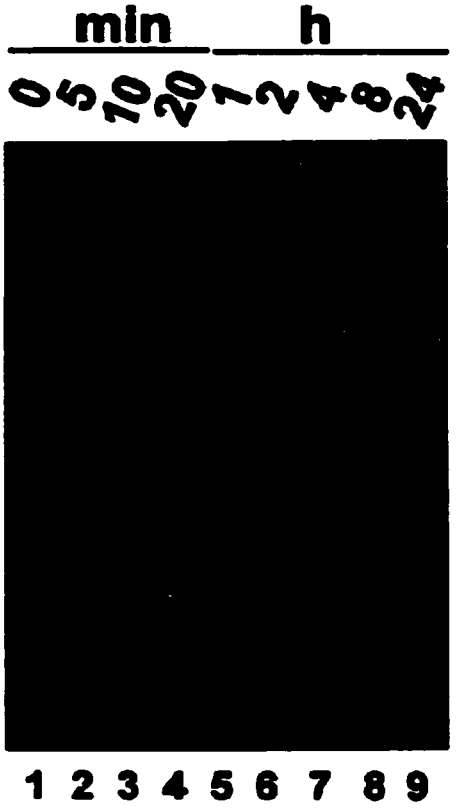
Panel A: the on-rate for recombinant Ku binding to the upper-strand of NRE1 was determined by EMSA. Recombinant purified Ku was incubated with ^{32}P-labeled upper MTV oligonucleotide for times increasing from 1 to 60 min (lanes 1-6). Reactions were timed to end concurrently. This experiment was repeated three times with one representative EMSA shown. Panel B: EMSA were quantified by phosphorimager and the percent of maximum Ku binding as a function of the time was plotted. The graph represents the means of three independent experiments and error bars indicate the standard error. The inset shows the initial rate of binding plotted on an expanded time scale.

c) Kinetic analysis of Ku binding to upper NRE1

To further analyze the single-stranded NRE1 binding properties of Ku, I examined the kinetics with which recombinant, purified Ku bound to the upper NRE1 oligonucleotide (Figure 10). An on-rate analysis was performed using EMSA in the same manner as described for crude extracts. A representative EMSA is shown in Figure 10 panel A, while the average of three separate experiments quantified by phosphorimager are shown in panel B. As with binding of activities in Jurkat crude nuclear extracts, the binding of purified Ku to NRE1 occurred in two phases (panel B). The first phase with increasing binding occurred over the first 10 minutes, after which equilibrium was reached and the curve became essentially horizontal. The $t_{1/2}$ of binding of Ku to upper MTV was 1.6 min. This is consistent with the rates observed with crude nuclear extract binding to upper MTV (Figure 4, panel B).

Off-rates were also determined, the results of which are shown in Figure 11. A representative EMSA is shown in Figure 11 panel A, while the average of three separate experiments quantified by phosphorimager are shown in panel B. The release of Ku from upper MTV following the equilibration of binding was unusually slow, with a $t_{1/2}$ of 68 min. This is 2-fold longer than was observed with crude nuclear extracts release from upper MTV however it confirms that binding to the upper-strand is extremely stable. These data suggest that Ku can rapidly access the single, upper-strand of NRE1. However, once binding has occurred, Ku may remain stably associated with the upper-strand of NRE1 for an extended period of time.

A


min
h
0 5 10 20 1 2 4 8 24
1 2 3 4 5 6 7 8 9

B

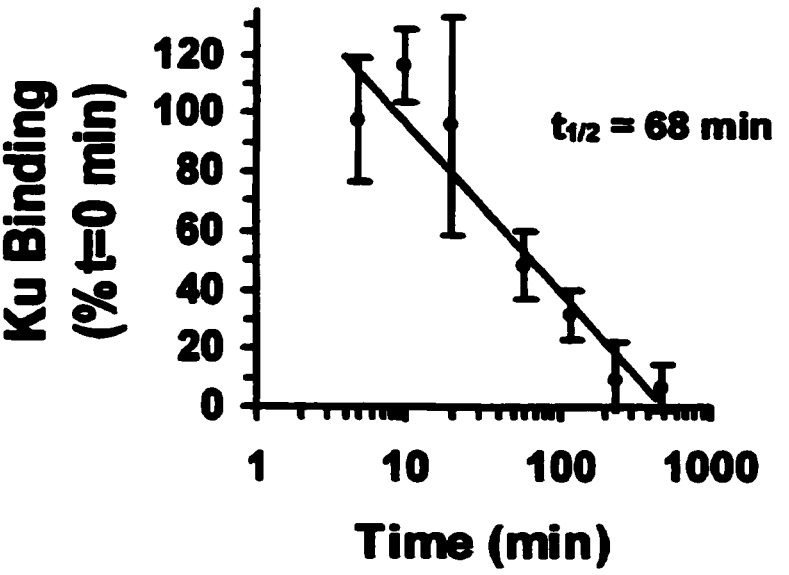

Ku Binding
(%t=0 min)
120
100
80
60
40
20
0
$t_{1/2}$ = 68 min
1
10
100
1000
Time (min)

Figure 11. Analysis of the kinetics of Ku release from the upper-strand of NRE1.

Panel A: the off-rate for recombinant purified Ku from the upper MTV oligonucleotide. Ku binding to 0.5 ng of ^{32}P-labeled upper MTV was allowed to equilibrate for 30 min and was analyzed by EMSA (lane 1). Alternatively, 200 ng of unlabeled upper MTV competitor was added to each incubation and EMSA was used to monitor the competition over a period ranging from 5 min to 24 h (lanes 2 to 9). Reactions were timed to end concurrently. EMSA were repeated three times and one representative trial is shown. Panel B: EMSA were quantified by phosphorimager and the percent Ku binding relative to the equilibrated binding of Ku was plotted as a function of time of competition. The graph represents the means of three independent experiments and error bars indicate the standard error. The time axis is plotted on a logarithmic scale for clarity.

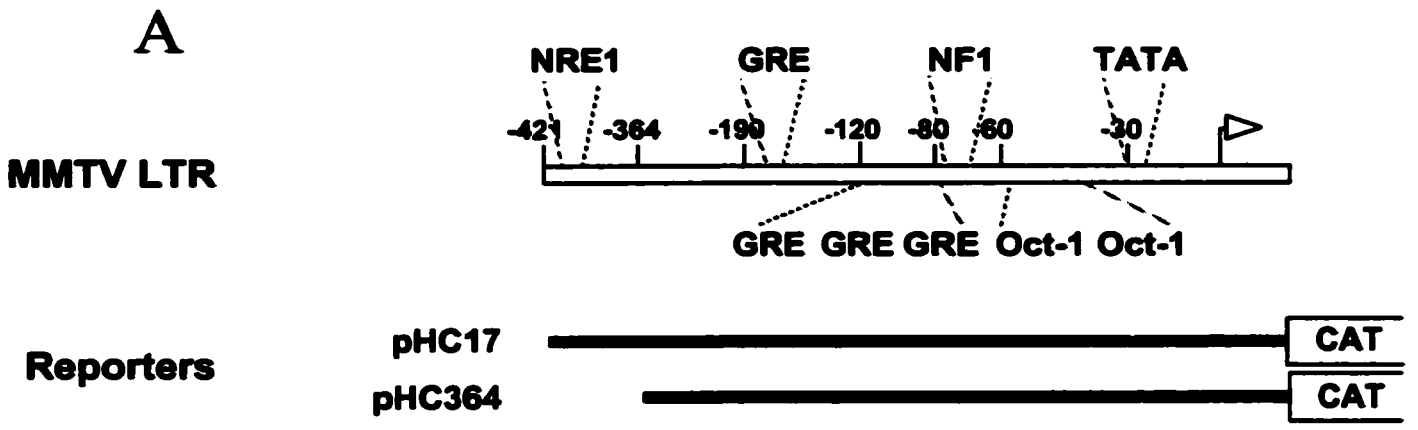
2. Functional analysis of the role of Ku/DNA-PK in NRE1 mediated repression of MMTV transcription

Following the demonstration that Ku was the major NRE1 binding factor it became important to determine whether it was responsible for the NRE1-mediated repression of glucocorticoid-induced transcription of MMTV. To this end we took advantage of a described cell line which carried an inactivating mutation in the Ku80 subunit. V15B cells, a derivative of V79 hamster fibroblasts, are sensitive to x-rays and deficient in non-homologous DNA break repair (Zdzienicka et al., 1988) and were also shown to lack a DNA end-binding activity immunologically related to the Ku autoantigen (Rathmell and Chu, 1994a; Rathmell and Chu, 1994b). The mutation causing these defects was localized to the Ku80 gene and it was demonstrated that reintroduction of Ku80 rescued the mutant phenotypes (Errami et al., 1996; Smider et al., 1994; Taccioli et al., 1994). The Ku70 gene is functional in V15B cells however the Ku70 protein is present at such low levels as to be barely detectable (Rathmell and Chu, 1994b; Smider et al., 1994; Taccioli et al., 1994). This is likely due to an instability of Ku70 in the absence of its heterodimerization partner.

To assess the role of Ku in NRE1 mediated repression of glucocorticoid induced transcription of MMTV, I carried out transient transfection assays. In these assays I used reporter constructs in which MMTV LTR segments drive expression of the CAT gene, allowing quantification of transcriptional activity by determination of CAT activity. The reporters used are described in Figure 12 panel A. pHC17 contains the MMTV sequence from -421 to +125 cloned upstream of the CAT gene and contains the NRE1 element while pHC364 contains the sequence from -364 to +125. Both reporters contain the

intact promoter proximal region including response elements for GR, NF1 and Oct-1. Upon transient transfection of these reporters into wild type V79 cells, it was observed that in the absence of NRE1 the synthetic glucocorticoid Dex induced transcription by approximately 100 fold (Figure 12; panel B, lane 1: compare + and - Dex). When NRE1 was present the glucocorticoid induction was repressed approximately 10 fold (lane 2). By contrast, in the V15B cells lacking Ku, the presence of NRE1 resulted in a slight increase in the level of transcription following glucocorticoid treatment (compare lanes 3 and 5). Introduction of exogenous Ku80 by co-transfection of a Ku80 cDNA expression plasmid rescued NRE1 mediated repression but had no effect in the absence of NRE1 (compare lanes 4 and 6). These results demonstrate that Ku is required for the glucocorticoid mediated repression of MMTV transcription.

Ku is the DNA binding subunit and an allosteric coactivator of DNA-PK (Gottlieb and Jackson, 1993). This close relationship between Ku and DNA-PK prompted us to investigate the role of DNA-PK in the repression of glucocorticoid induced transcription on MMTV. Experiments performed by others in our laboratory showed that DNA-PK was fully active on supercoiled and relaxed closed circular MMTV LTR plasmids containing NRE1 while deletion of NRE1 resulted in a loss of DNA-PK activity (Giffin et al., 1996). The demonstration that DNA-PK was active on NRE1 as well as DNA ends suggested that it might also be involved in the NRE1 mediated repression of MMTV transcription. To assess this I repeated the transient transfection experiments using the *scid* mouse fibroblast cell line Sf7 in which a mutation of the DNA-PK_{cs} gene results in a loss of DNA-PK kinase activity without affecting Ku (Blunt et al., 1995; Kirchgessner et al., 1995; Rathmell and Chu, 1994b). In wild type CB17 cells the presence of NRE1 in


A
NRE1
GRE
NF1
TATA
-421
-364
-190
-120
-80
-60
-30
MMTV LTR
GRE GRE GRE Oct-1 Oct-1
Reporters
pHC17
CAT
pHC364
CAT

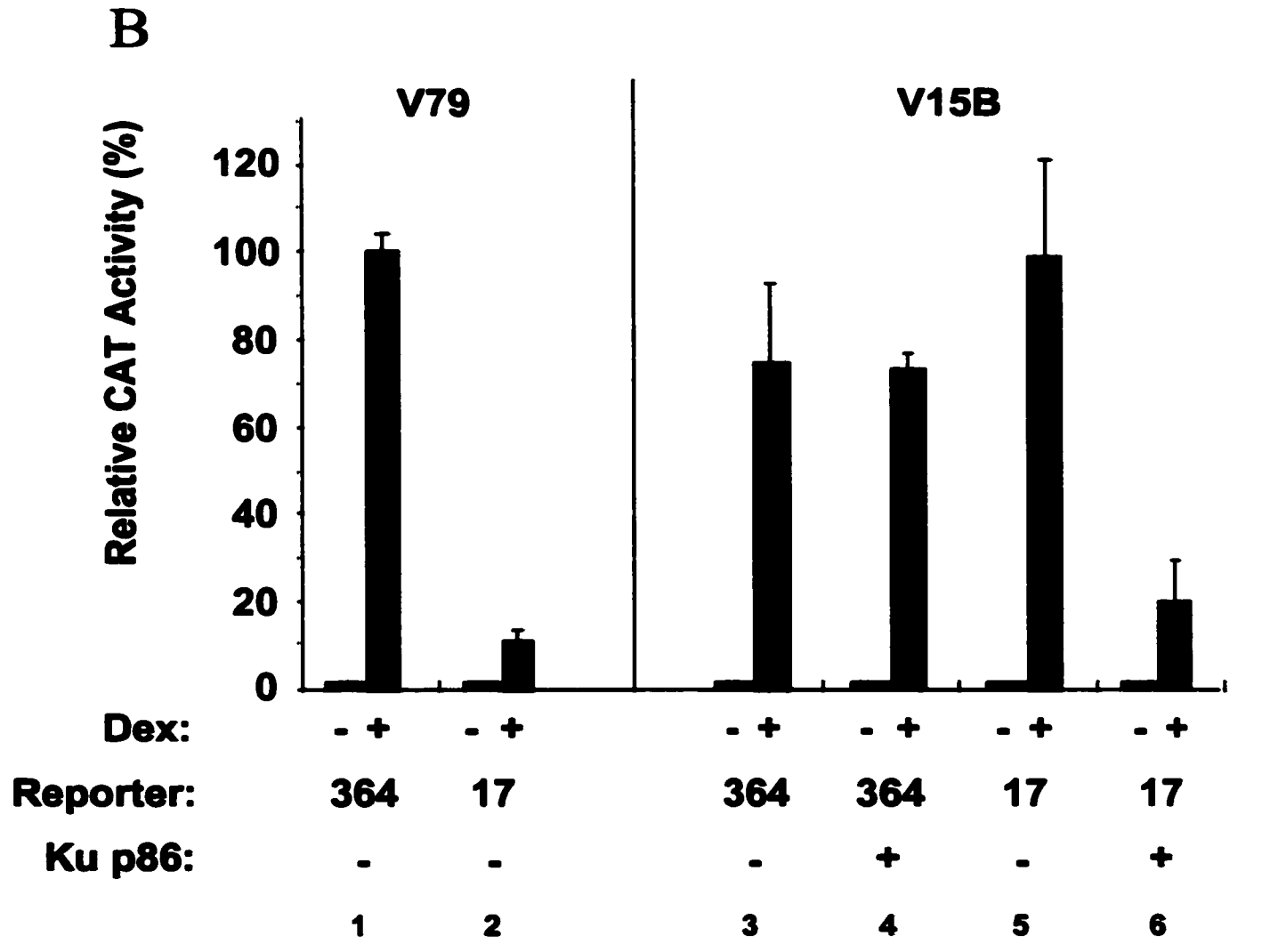

B
V79
V15B
Relative CAT Activity (%)
120
100
80
60
40
20
0
Dex: - + - + - + - + - + - +
Reporter: 364 17 364 364 17 17
Ku p86: - - - + - +
1 2 3 4 5 6

Figure 12. Ku is required for repression of glucocorticoid induced transcription of MMTV.

Panel A: Schematic representation of the transcriptional regulatory elements in the MMTV LTR and the derived reporter constructs used for transient transfection assays. pHC421 carries the MMTV LTR sequence from –421 to +125, including NRE1, driving expression of the CAT gene. pHC364 carries the sequence from –364 to +125 and lacks NRE1. NRE1, negative regulatory element 1; GRE, glucocorticoid response element; Oct-1, octamer motif; NF1, nuclear factor 1 binding site; TATA; TATA box; CAT, chloramphenicol acetyltransferase reporter gene. Panel B: Wild type hamster V79 fibroblast cells (lanes 1-2) and the clonaly derived mutant V15B cells lacking functional Ku80 (lanes 3-6) were transfected with 2 μg of the rat GR expression vector p6RGR and 4 μg of MMTV reporter construct using the DEAE-Dextran technique. The Ku80 expression vector pBJ6-Ku80 was co-transfected in lanes 4 and 6. Cells were harvested 48 hr after dexamethasone (Dex) treatment, and assayed for CAT activity. Activity is presented as a percentage of maximum activity within each cell type and represents the means of three independent experiments, with two replications within each experiment. Error bars represent the standard error. In all transfections the transfection efficiency was assessed and corrected for by co-transfection of pRSVβ-gal and determination of β-gal activity.

the MMTV reporter construct led to an approximately four-fold repression of glucocorticoid induced transcription (Figure 13, compare lanes 1 and 2). By contrast, no repression was observed in the DNA-PK deficient SF7 cell line (compare lanes 3 and 4). This demonstrates that in addition to Ku, repression of glucocorticoid induced transcription of MMTV also requires the DNA-PK_{cs}.

3. Identification of DNA-PK phosphorylation sites in the glucocorticoid receptor

In wild type hamster fibroblast cells, as well as in Jurkat T-cells, the presence of NRE1 did not affect basal levels of MMTV transcription, but did repress the glucocorticoid-induced transcription (Figure 12, 13 and (Giffin et al., 1994)). The previous results demonstrated that both Ku and DNA-PK_{cs} are required for this repression. This lead us to hypothesize that NRE1-dependent phosphorylation by DNA-PK of another factor acting on the MMTV promoter modulates the response to glucocorticoids. The specificity of the repression for glucocorticoid-induced transcription suggests that one or more factors involved in the glucocorticoid responsiveness of MMTV are the likely downstream targets of DNA-PK. Indeed we had observed that both recombinant GR and OCT-1 are *in vitro* substrates for DNA-PK (Giffin et al., 1996). Furthermore, on closed-circular MMTV LTR templates, phosphorylation of these proteins required the presence of NRE1 (Giffin et al., 1996). GR appeared to be the most likely candidate since it directly mediates the effects of glucocorticoids and the repression was specific for glucocorticoid induced transcription.

We observed that a bacterially expressed fragment of GR containing the DBD (amino acids 407 to 568) fused at its N-terminus to GST (GR DBD), termed GST-

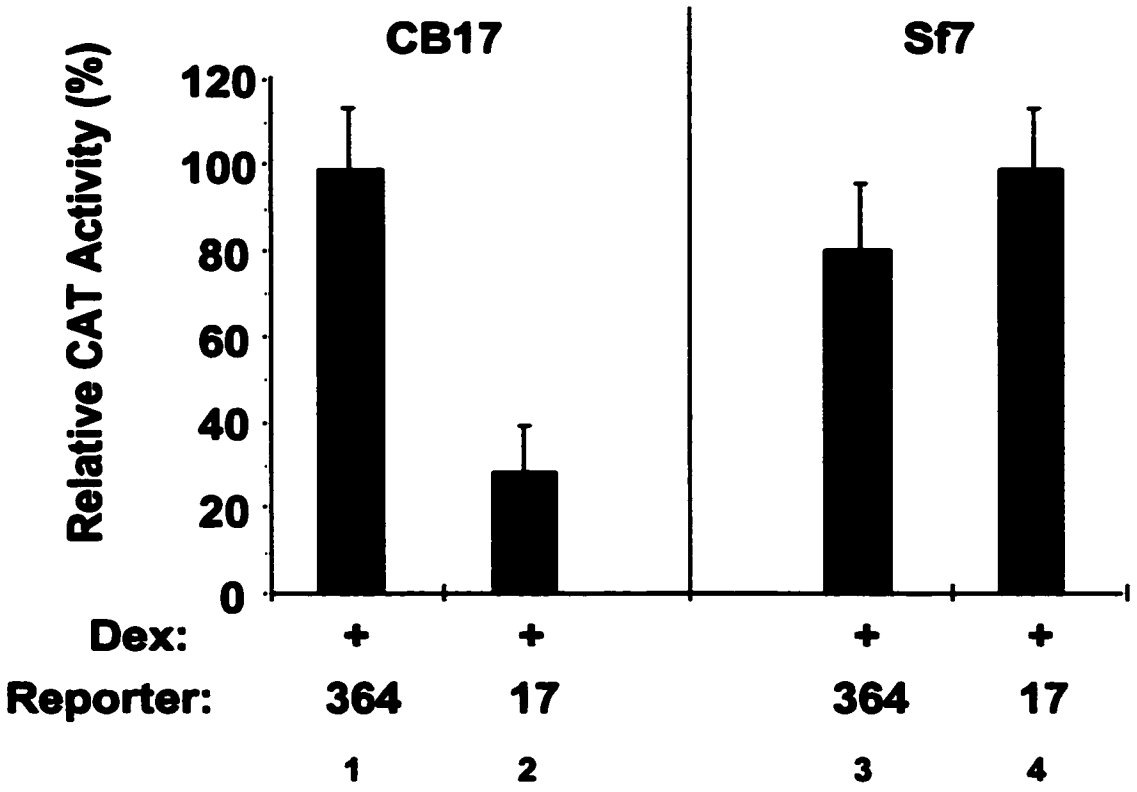

CB17
Sf7
Relative CAT Activity (%)
120
100
80
60
40
20
0
Dex:
+
+
+
+
Reporter:
364
17
364
17
1
2
3
4

Figure 13. DNA-PK is required for repression of glucocorticoid induced transcription of MMTV.

Wild type mouse CB17 fibroblast cells (lanes 1-2) and mutant Sf7 *scid* fibroblast cells lacking functional DNA-PK (lanes 3-4) were transfected with 2 μg of the rat GR expression vector p6RGR and 100 ng of MMTV reporter construct using Lipofectamine. The pHC17 and pHC364 reporters are described in Figure 12. Cells were harvested 48 hr after dexamethasone (Dex) treatment, and assayed for CAT activity. Activity is presented as a percentage of maximum activity within each cell type and represents the means of three independent experiments, with two replications within each experiment. Error bars represent the standard error. In all transfections the transfection efficiency was assessed and corrected for by co-transfection of pRSVβ-gal and determination of β-gal activity.

GR_{X568}, was also phosphorylated on closed-circular MMTV LTR DNA in a NRE1 dependent manner *in vitro* (Giffin et al., 1997). Trypsin digestion of this protein following phosphorylation by DNA-PK revealed only a single phosphorylated peptide. Phosphate release studies using Edman degradation on this peptide indicated that it was phosphorylated on the tenth amino acid (Giffin et al., 1997). Analysis of the GST-GR_{X568} sequence revealed only three potential tryptic peptides that contained amino acids that could be phosphorylated by DNA-PK (serine or threonine) at position 10. Treatment of the phosphorylated tryptic peptide with the AspN protease revealed that it contained an aspartic acid residue, while phosphate release studies on the AspN generated peptide indicated that the position of the phosphate remained unchanged at the tenth amino acid (Giffin et al., 1997). Of the three potential peptides with a serine or threonine at position 10, only one containing the serine corresponding to serine 527 could be digested with AspN and still be phosphorylated at position 10. We therefore concluded that serine 527 was the DNA-PK phosphorylation site on GST-GR_{X568} (Giffin et al., 1997). This presented the possibility that this residue was also phosphorylated in the full-length GR. However since this site was observed in an artificially produced subdomain of GR, we could not predict whether it would be conserved in the full-length GR nor could we rule out the presence of other DNA-PK phosphorylation sites. I therefore developed a strategy to identify all of the DNA-PK dependent phosphorylation sites within full-length rat GR. This would allow me to test the hypothesis that DNA-PK mediated phosphorylation of GR was responsible for the NRE1 mediated repression of MMTV by mutating the phosphorylation sites and observing the effects on transcription.

(i) Strategy for identification of DNA-PK dependant phosphorylation sites within the glucocorticoid receptor

To date it has not been possible to identify a consensus DNA-PK phosphorylation site. Several sites containing either SQ or TQ motifs have been identified, however not all such motifs are phosphorylated by DNA-PK (Anderson and Lees-Miller, 1992). Additionally sites lacking even the glutamine have been identified (Anderson and Lees-Miller, 1992; Zernik-Kobak et al., 1997). DNA-PK therefore appears to have little phosphorylation specificity. DNA-PK is also a relatively weak kinase, with a K_m of 200 μM (Anderson, 1993). We have therefore hypothesized that *in vivo* phosphorylation by DNA-PK requires the substrate to be co-localized to the DNA. Consistent with this it has been shown that *in vitro* phosphorylation of recombinant GR is 2-3 orders of magnitude stronger when localized in cis with DNA-PK than when in trans (Giffin et al., 1997). Therefore it was expected that only a small sub-population of GR which co-localizes with DNA-PK would be phosphorylated *in vivo*. This would likely make it exceedingly difficult to detect *in vivo* phosphorylation sites. I therefore developed a strategy in which I could identify DNA-PK specific phosphorylation sites *in vitro* and subsequently test for their effect on transcription *in vivo*.

The strategy used is represented in Figure 14. A stable Sf7 cell line, Sf7-mycGR, was created which over-expressed rat GR fused to an N-terminal 6X c-myc epitope tag (mycGR). Generation of this line is described in detail in the following section. The Sf7 *scid* cell line, lacking functional DNA-PK, was used to prevent the possibility that endogenous DNA-PK would phosphorylate the receptor. From these cells, Dex bound receptor was recovered then phosphorylated *in vitro* with purified DNA-PK in the

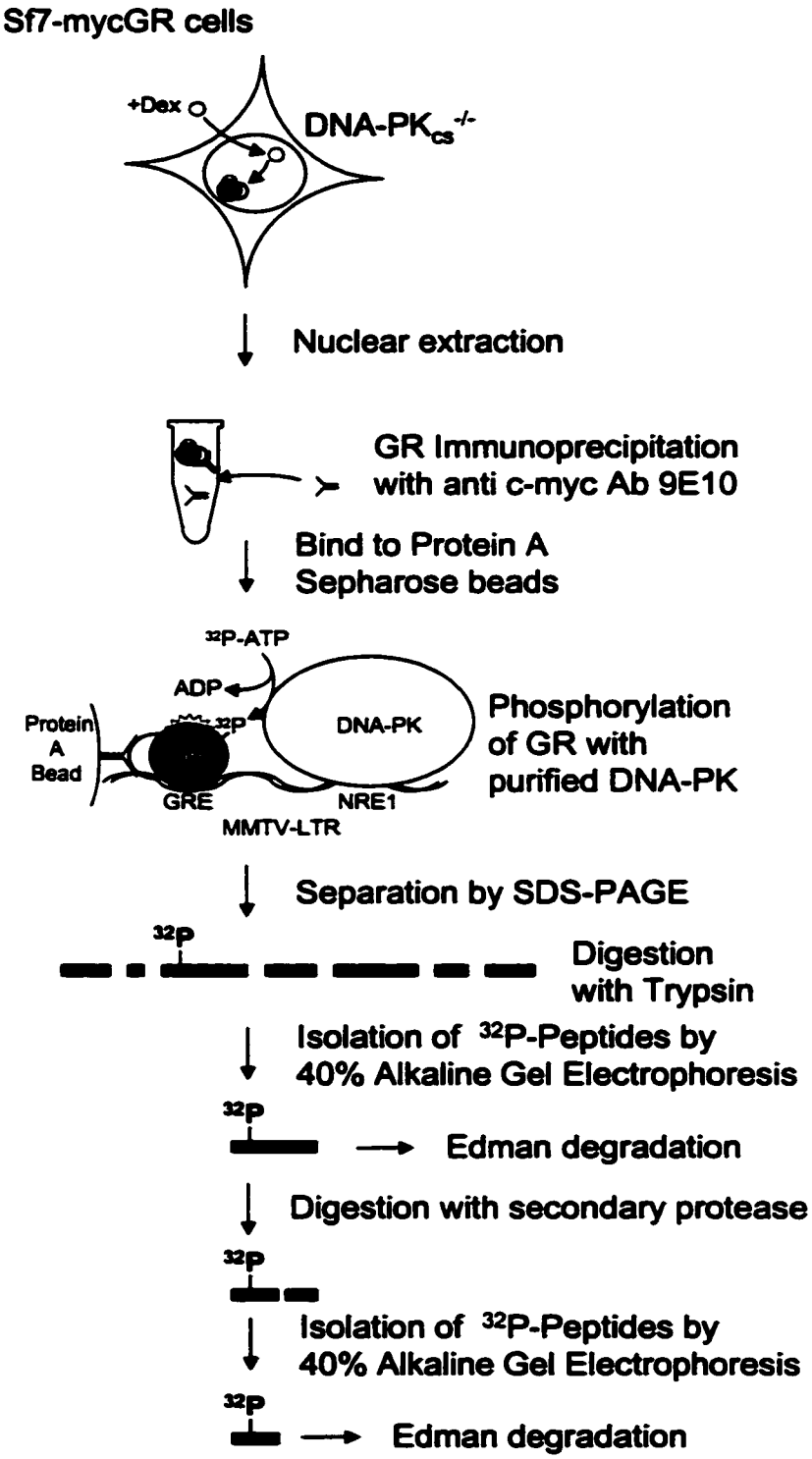

Sf7-mycGR cells
+Dex
DNA-PKcs-/-
Nuclear extraction
GR Immunoprecipitation
with anti c-myc Ab 9E10
Bind to Protein A
Sepharose beads
32P-ATP
ADP
32P
DNA-PK
Protein
A
Bead
GRE
NRE1
MMTV-LTR
Phosphorylation
of GR with
purified DNA-PK
Separation by SDS-PAGE
32P
Digestion
with Trypsin
Isolation of 32P-Peptides by
40% Alkaline Gel Electrophoresis
32P
Edman degradation
Digestion with secondary protease
32P
Isolation of 32P-Peptides by
40% Alkaline Gel Electrophoresis
32P
Edman degradation

Figure 14. Strategy for the identification of DNA-PK dependent phosphorylation sites on the rat glucocorticoid receptor.

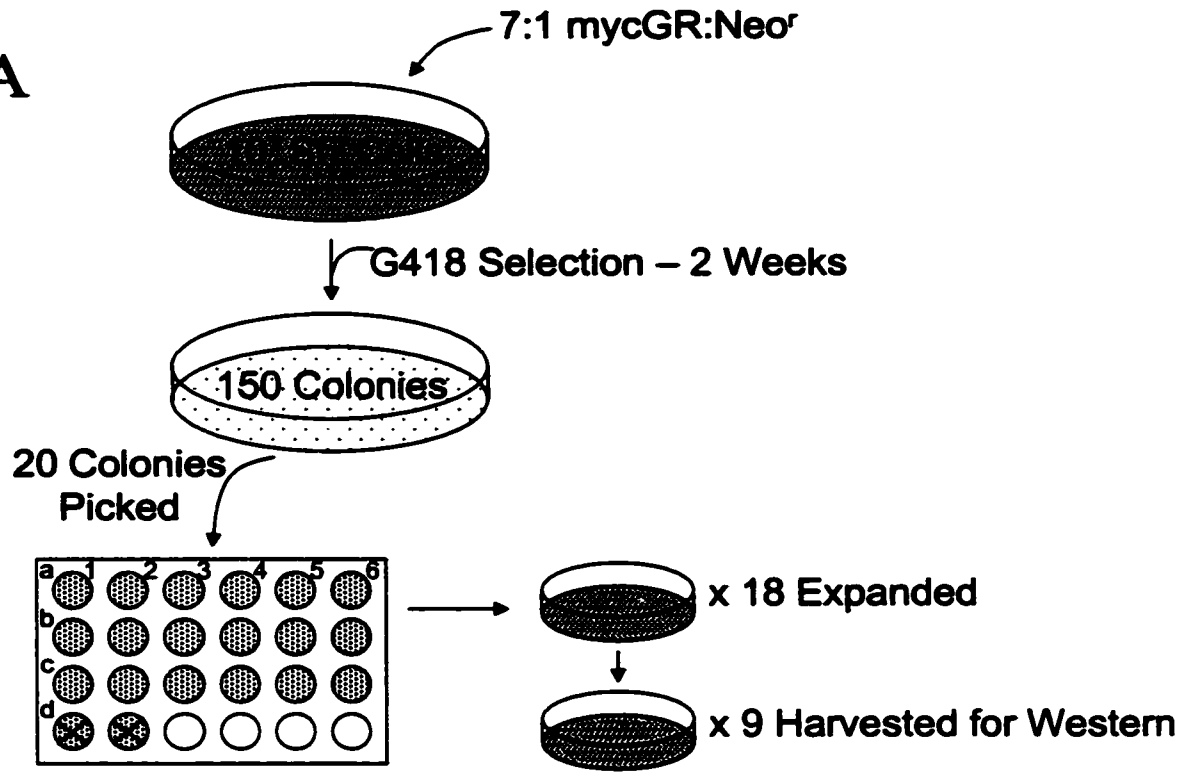
Sf7-mycGR cells were developed by stable transfection of the pTL2-mycGR plasmid into the *scid* Sf7 cell line. Sf7-mycGR cells were grown to confluency then treated for 1 h with 10^{-6} M Dexamethasone (Dex). High-salt nuclear extracts were prepared, diluted to 150 mM NaCl, then incubated with the anti c-myc antibody 9E10 overnight at 4^0C. Antibody-GR complexes were immobilized on protein A-sepharose beads, washed twice each with immunoprecipitation buffer and kinase buffer, then phosphorylated by DNA-PK by incubation with γ^{32}P-ATP and purified DNA-PK enzyme in kinase buffer. Beads were washed twice with immunoprecipitation buffer and subjected to SDS-PAGE. Gel slices containing γ^{32}P-GR were treated with TPCK-trypsin. γ^{32}P-tryptic peptides were separated by 40% alkaline gel electrophoresis and recovered by elution. They were then subjected to Edman degradation to identify the position of the γ^{32}P-amino acid within the peptide or digested with the secondary proteases AspN or GluC. Digested γ^{32}P-peptides were isolated by alkaline gel electrophoresis and subjected to Edman degradation to identify the new position of the γ^{32}P-amino acid. The mobility on alkaline gels, susceptibility to secondary protease digestion and the position of the γ^{32}P-amino acid were used to identify the phosphorylation site using a peptide map of GR (Appendix A).

presence of γ^{32}P-ATP. The radiolabeled GR was isolated and digested with trypsin. Trypsin specifically digests peptide bonds C-terminal to lysines and arginines (Walsh, 1970). ^{32}P-tryptic peptides were separated and recovered from 40% alkaline gels. To identify the amino acid position of the ^{32}P label the peptides were subjected to Edman degradation. This cyclical reaction cleaves a single amino acid from the N-terminus of the peptides. The radioactivity released with each amino acid was determined by scintillation counting. The peptides were also treated with secondary proteases to determine if they contained recognition sequences for these enzymes. The peptides digested by secondary proteases were also subjected to Edman degradation. By determining the susceptibility to secondary proteases and the amino acid position of the ^{32}P label, both within the trypsin-digested peptide as well as within the secondarily-digested peptides it was expected that the phosphorylation site could be identified using a peptide map of mycGR (Appendix A).

(ii) Generation of a stable DNA-PK$^{-/-}$ cell line expressing mycGR

The Sf7 cell line that stably expresses mycGR (Sf7-mycGR) was generated using the strategy outlined in Figure 15, panel A. Sf7 cells were transfected using the lipofectamine protocol. A 100 mm dish (approximately 10^7 cells) was transfected with 7 μg pTL2-mycGR and 1 μg pSV2NEO^r. 48 h post-transfection the media was aspirated from the cells and replaced with media containing 500 μg/ml G418. The cells were selected in this media for 2 weeks, with the media being replaced daily. Cells which had acquired stable integrations of the neomycin resistance gene developed colonies which by 2 weeks were approximately 1 mm in diameter. Twenty of these G418 resistant colonies were picked by transferring them on a sterile paper disc that had been soaked in trypsin

A


7:1 mycGR:Neo^r
G418 Selection – 2 Weeks
150 Colonies
20 Colonies Picked
a
b
c
d
1
2
3
4
5
6
x 18 Expanded
x 9 Harvested for Western

B

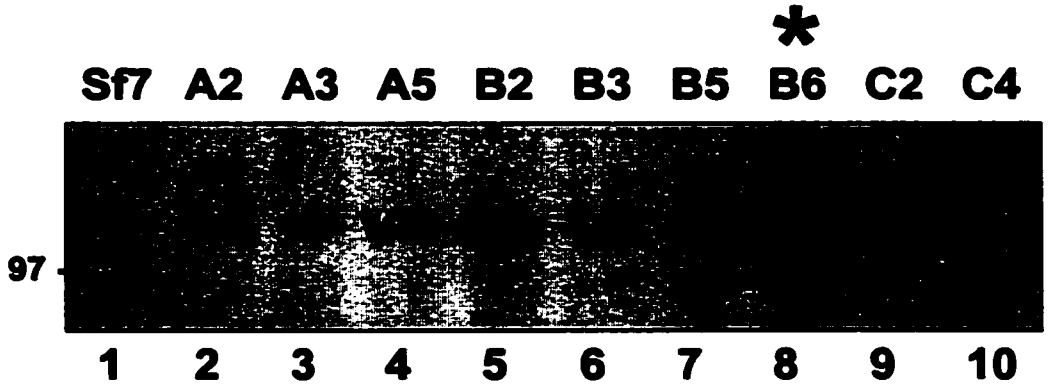

*
Sf7
A2
A3
A5
B2
B3
B5
B6
C2
C4
97
1
2
3
4
5
6
7
8
9
10

Figure 15. Development and characterization of a stable Sf7 cell line expressing mycGR.

Panel A: strategy for the development of a stable cell line Sf7-mycGR. 10^7 Sf7 cells were transfected with a 7:1 ratio of a vector expressing mycGR (pTL2-mycGR) to a vector expressing the gene for neomycin resistance (pSV2NEO^r). 48 h post-transfection cells were selected with 500 μg/ml G418. Selection was continued for 2 weeks after which time approximately 150 G418 resistant colonies were observed. Of these 20 were picked and transferred to individual wells of a 24 well plate. 18 clones survived the transfer and were expanded to 60 mm dishes. Of these, 9 were screened for expression of mycGR. The clones were named based on their position within the 24 well plate. Panel B: western analysis of G418 resistant clones for expression of mycGR. Whole cell extracts of the 9 clones were made and equal amounts of proteins were analyzed by western blotting. Expression of mycGR was detected using the anti-c-myc antibody 9E10. Clone designations are indicated above each lane. Lane 1 contained extract from untransfected Sf7 cells. Clone B6 (lane 8, *) was chosen as the highest expressing clone, designated Sf7-mycGR, and used for further analysis.

into individual wells of a 24 well tissue culture plate. Of these, eighteen survived and were expanded into 60 mm dishes. These clones were designated based on their position within the 24 well plate. Nine of the fastest growing clones were analyzed further by preparing whole cell extracts and determining the levels of mycGR expression using western analysis. Equal amounts of protein were loaded for each extract. To detect mycGR the anti c-myc antibody, 9E10 was used as a primary antibody. As can be seen in Figure 15, panel B the 9E10 antibody detected a protein of 120 kD in extracts from all nine of the resistant clones but not in extracts from untransfected Sf7 cells (compare lanes 2-10 with lane 1). Therefore all of the resistant clones expressed mycGR. Transient expression would not be expected to persist for 2 weeks therefore expression of mycGR in each of these clones was likely stable. The B6 clone expressed significantly more mycGR that any of the other clones (panel B, compare lane 8 with other lanes) therefore it was selected for further use and renamed Sf7-mycGR. Passage of this clone for >20 passages with no apparent decrease in expression of mycGR confirmed that the mycGR gene had stably integrated into these cells.

(iii) Full-length mycGR is specifically immunoprecipitated from Sf7-mycGR cells.

I next tested the specificity of immunoprecipitation of mycGR from Sf7-mycGR cells. I prepared nuclear extracts from Sf7-mycGR cells that had been treated with Dex for 2 h. 2.5 mg of extract was incubated with antibodies against GR, the c-myc tag or a control antibody against the GAL4 DBD. Antibody-protein complexes were recovered by incubating the samples with protein-A sepharose beads followed by centrifugation. The beads were washed then the immunoprecipitates were resolved on 6% SDS-PAGE

gels and proteins were visualized by silver staining (Figure 16, panel A). When either an anti c-myc (lane 3) or an anti GR (lane 4) antibody was used to immunoprecipitate, an approximately 120 kD band was observed which corresponded in size to mycGR. However this band was not observed when a non-specific antibody against the DBD of GAL4 was used (lane 5). Likewise when anti c-myc or GR antibodies were used to immunoprecipitate from nuclear extracts from Sf7 cells not expressing mycGR this band was also not observed (lane 1 and 2). This demonstrates that mycGR was specifically immunoprecipitated using our protocol. Other bands were observed on the silver stained gel, these included the IgG heavy chain which interacted with protein A and BSA which was used as a blocking agent on the protein A-sepharose beads to prevent non-specific binding. Other unidentified bands were observed inconsistently, and are likely due to non-specific interactions with the protein A-sepharose beads which were not eliminated by BSA blocking or washing. Clearly the 120 kD band corresponding to mycGR (lane 3 and 4) was separable from all other precipitated proteins indicating it could be easily isolated.

(iv) mycGR is specifically phosphorylated by DNA-PK *in vitro*.

I next tested whether immunoprecipitated mycGR could be phosphorylated *in vitro* by DNA-PK. To do this, I immunoprecipitated mycGR from Sf7-mycGR cells as described in the previous section using antibodies against either the c-myc tag, GR, or a control antibody against the GAL4 DBD. After washing the antibody-protein complexes imobilized on protein A-sepharose beads, they were rinsed in DNA-PK reaction buffer. They were then incubated with purified DNA-PK, γ^{32}P-ATP and linearized pHC17 plasmid which is derived from MMTV and contains the glucocorticoid response elements

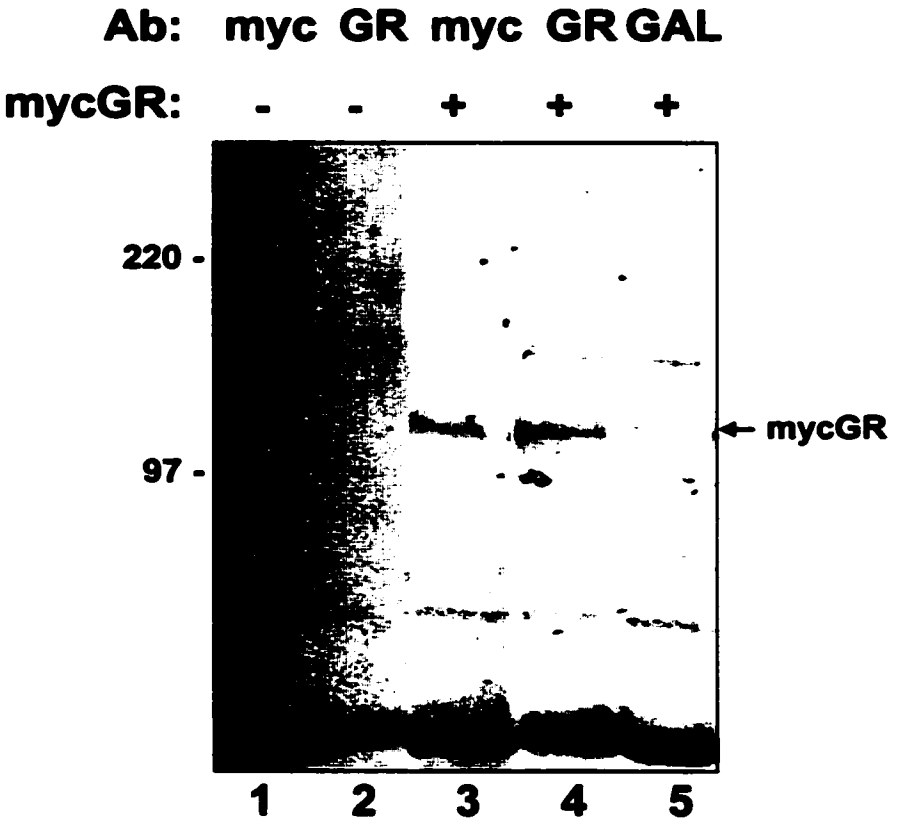

Ab: myc GR myc GR GAL
mycGR: - - + + +
220 -
97 -
mycGR
1 2 3 4 5

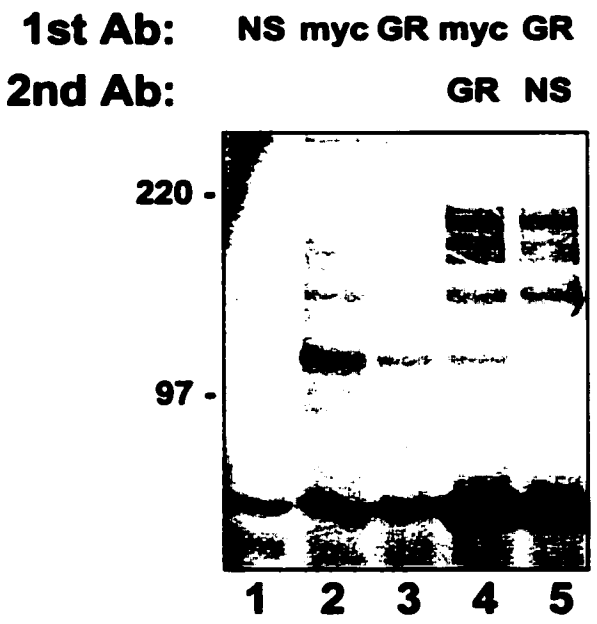
Figure 16. mycGR is specifically immunoprecipitated from stably expressing Sf7 cells.

Nuclear extracts from Dex treated Sf7 cells (lanes 1-2) or Sf7-mycGR cells (lanes 3-5) were incubated with the antibodies 9E10 against the 6 x c-myc tag (myc, lanes 1, 3) or BuGR against GR (GR, lanes 2, 4) to immunoprecipitate mycGR. As a non-specific control the antibody RK5C1 against the GAL4 DBD (NS, lane 5) was incubated. Antibody-protein complexes were separated through 6% SDS-PAGE gels. Gels were then silver-stained and photographed. This experiment was repeated 2 times with a representative experiment shown.

as well as NRE1, providing binding sites for both mycGR and Ku/DNA-PK. Following a 30 min incubation at 30°C the beads were washed again to remove any unincorporated ^{32}P-ATP. The immune complexes were then resolved on 6% SDS-PAGE gels and proteins were visualized by silver staining (Figure 17, panel A). Autoradiographs were then taken of the stained gels to assess the incorporation of ^{32}P (Figure 17, panel B). A 120 kD band corresponding in size to mycGR was observed following immunoprecipitation by the antibodies against c-myc and GR but not the GAL4 DBD (panel A, compare lanes 2 and 3 with lane 1) indicating that mycGR was specifically immunoprecipitated. Additionally mycGR was phosphorylated by DNA-PK since a ^{32}P-labeled protein with the identical mobility to mycGR was observed in the autoradiograph. This protein was also present when antibodies against c-myc and GR were used in the immunoprecipitation but not when the anti-GAL4 DBD antibody was used (panel B, compare lanes 2 and 3 with lane 1). In addition to mycGR a number of proteins which were present in the anti-myc and anti-GR immunoprecipitates following kinasing, were also observed to be substrates for DNA-PK (panel B, lanes 2 and 3). In particular a protein of approximately 160 kD was phosphorylated almost as strongly as mycGR (panel B, lanes 2 and 3) despite being much less abundant than mycGR in the immunoprecipitates as determined by silver staining (panel A, lanes 2 and 3). This suggested that this unidentified protein was a particularly good substrate for DNA-PK.

Due to the presence of extraneous phosphorylated proteins, I carried out a double-immunoprecipitation experiment to confirm the identity of the 120 kD band as mycGR as well as to attempt to increase the purification of mycGR. To accomplish this I immunoprecipitated mycGR using antibodies against c-myc or GR and kinased the

A


1st Ab:
NS myc GR myc GR
2nd Ab:
GR NS
220 -
97 -
1 2 3 4 5

B

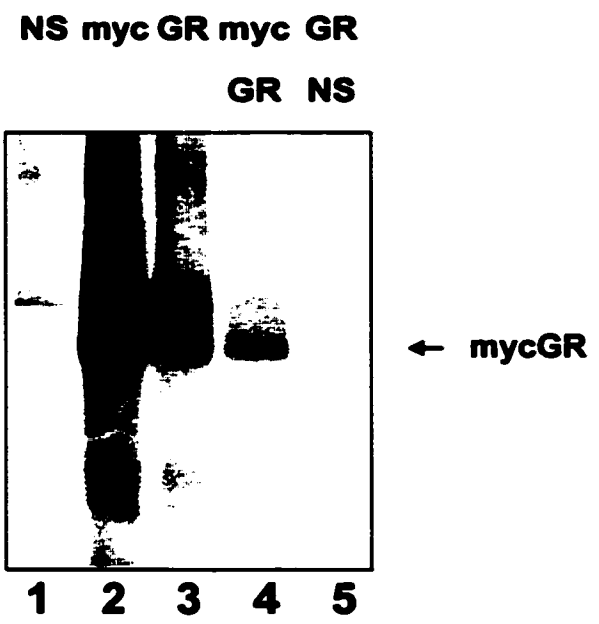

NS myc GR myc GR
GR NS
mycGR
1 2 3 4 5

Figure 17. mycGR is an *in vitro* substrate for DNA-PK.

Nuclear extracts from Dex treated Sf7-mycGR cells were incubated with the antibodies 9E10 against the 6 x c-myc tag (myc, lane 2, 4) or BuGR against GR (GR, lane 3, 5) to immunoprecipitate mycGR. As a non-specific control, the antibody RK5C1 against the GAL4 DBD (NS, lane 1) was incubated. The immunoprecipitates were rinsed in kinase buffer, then kinased with DNA-PK by incubating them with purified DNA-PK, γ^{32}P-ATP, and linearized pHC17 for 30 min at 30^{0}C. Following kinasing the immunoprecipitates were washed and antibody-protein complexes were either separated through 6% SDS-PAGE gels directly (lane 1-3) or subjected to a second immunoprecipitation (lane 4, 5). To perform a second immunoprecipitation, the immunoprecipitates were incubated in immunoprecipitation buffer with 0.2% SDS for 10 min at RT then dialyzed against immunoprecipitation buffer for 4 h at 4^{0}C to remove the SDS. Samples originally immunoprecipitated with 9E10 were then immunoprecipitated with BuGR (lane 4), while samples originally immunoprecipitated with BuGR were then immunoprecipitated with RK5C1 (lane 5). These immunoprecipitates were then resolved on 6% SDS-PAGE gels. The gels were silver stained and photographed to visualize the recovered proteins (Panel A) then autoradiographed to visualize the proteins labeled with ^{32}P (Panel B). This experiment was repeated 2 times with a representative experiment shown.

immunoprecipitated complex as described in the previous section. Following washing of the kinased complexes I incubated the samples in immunoprecipitation buffer containing 0.2% SDS for 10 min at room temperature then vortexed the samples for 10 sec to disrupt the immune complexes. The samples were centrifuged, the supernatants were recovered and then dialyzed against immunoprecipitation buffer at 4°C for 4 h. Following this, the sample immunoprecipitated with anti-myc antibody was re-immunoprecipitated using anti-GR antibody. The sample immunoprecipitated with anti-GR antibody was re-immunoprecipitated using antibody against the GAL4 DBD. These were then resolved on 6% SDS-PAGE gels. When mycGR was immunoprecipitated first with anti-myc antibody followed by anti-GR antibody a 120 kD protein with identical mobility to mycGR was observed in a silver stained gel (Figure 17, panel A, lane 4). This protein was not observed when immunoprecipitation with anti-GR was followed by immunoprecipitation with anti-GAL4 DBD (lane 5) indicating that mycGR was specifically immunoprecipitated by this procedure. Moreover, following double-immunoprecipitation with anti-myc followed by anti-GR antibodies, a band corresponding in size to mycGR was the only major ^{32}P-labeled protein (panel B, lane 4) indicating that the double-immunoprecipitation resulted in the loss of the extraneous phosphorylated proteins. In addition when samples were immunoprecipitated first with anti-GR antibodies followed by anti-GAL4 DBD antibodies no corresponding ^{32}P-labelled protein was observed (lane 5) confirming that the 120 kD ^{32}P-labeled protein was mycGR. Following double-immunoprecipitation the yield of mycGR was substantially reduced compared to single-immunoprecipitation (compare in panels A and B, lane 4 with lanes 2 and 3). In addition contaminating proteins were introduced (panel A, lanes 4

and 5) although they were not ^{32}P-labeled (panel B, lanes 4 and 5). Furthermore in single immunoprecipitations, despite the presence of extraneous phosphorylated proteins, the ^{32}P-labeled mycGR was clearly separable from these (panel B, lanes 2 and 3). For these reasons it was decided that single-immunoprecipitation was sufficient to recover ^{32}P-labeled mycGR and the double-immunoprecipitation was not pursued further.

I next tested the specificity of DNA-PK mediated phosphorylation of GR under these conditions. mycGR was immunoprecipitated from Sf7-mycGR cells and kinased by DNA-PK as described in the previous sections (Figure 18). To test that the phosphorylation was specifically by DNA-PK I included one reaction with no added DNA-PK. Alternatively, I added the DNA-PK inhibitors EtBr or wortmanin. The kinased immunoprecipitates were resolved on 6% SDS-PAGE gels and phosphorylated proteins were identified by autoradiography. When the anti c-myc antibody was used for immunoprecipitation a strongly phosphorylated band was observed at 120 kD corresponding in size to mycGR (Figure 18; panel A, lane 2). This band was not observed when a non-specific antibody was used confirming that this species corresponded to mycGR (lane 1). In the absence of added DNA-PK (lane 3) phosphorylation was substantially reduced indicating that very little contaminating kinase activity was present within the immunoprecipitates. In the presence of EtBr, which blocks the ability of DNA-PK to bind DNA and therefore prevents DNA-PK activity (lane 4); or in the presence of wortmanin, an inhibitor of DNA-PK kinase activity (lane 5) phosphorylation was also significantly reduced. These results indicate that under our kinase conditions mycGR is specifically phosphorylated by DNA-PK and not a contaminating kinase.

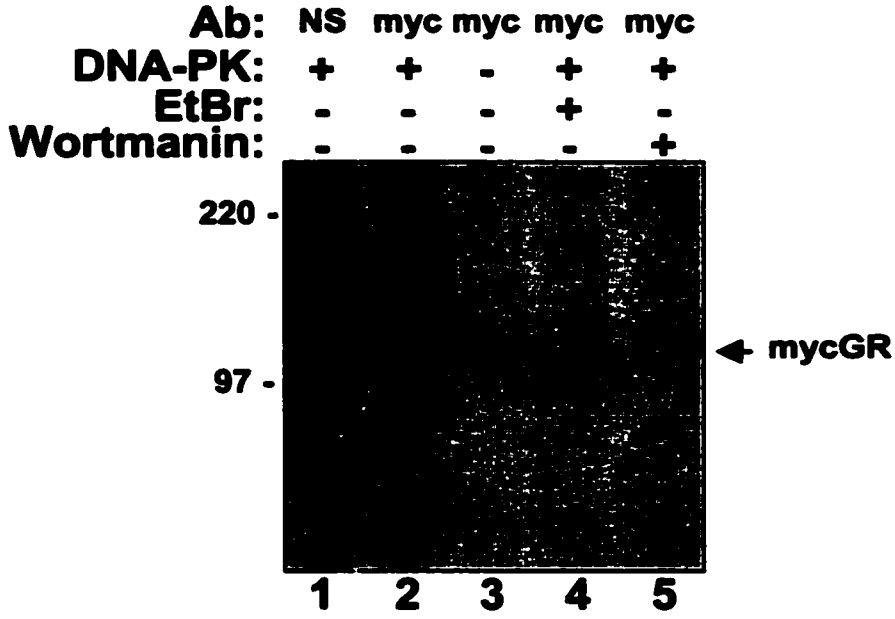

Ab: NS myc myc myc myc
DNA-PK: + + - + +
EtBr: - - - + -
Wortmanin: - - - - +
220 -
97 -
mycGR
1 2 3 4 5

Figure 18. mycGR is phosphorylated specifically by DNA-PK *in vitro*.

Nuclear extracts from Dex treated Sf7-mycGR cells were incubated with the antibody 9E10 against the 6 x c-myc tag (myc, lane 2-5) to immunoprecipitate mycGR. As a non-specific control, the antibody RK5C1 against the GAL4 DBD (NS, lane 1) was incubated. The immunoprecipitates were rinsed in kinase buffer, then kinased with DNA-PK by incubating them with purified DNA-PK, γ^{32}P-ATP, and linearized pHC17 for 30 min at 30^0C. To test the specificity of DNA-PK phosphorylation, no DNA-PK was added (lane 3) or kinase reactions were carried out in the presence of EtBr (lane 4), or wortmanin (lane 5). Following kinasing the immunoprecipitates were washed and antibody-protein complexes were separated through 6% SDS-PAGE gels. This experiment was repeated 3 times with a representative experiment shown.

(v) mycGR is specifically phosphorylated on two tryptic peptides.

To assess the number of DNA-PK phosphorylation sites within mycGR, I immunoprecipitated mycGR using the anti-myc antibody, kinased the immunoprecipitates with DNA-PK *in vitro*, and resolved the immunoprecipitated proteins on 6% SDS-PAGE gels as described in the previous sections. I stained the gels with amido black and excised the stained bands corresponding to mycGR. Autoradiographs were taken of the gel both prior to and after excising the amido black stained mycGR to confirm that mycGR was ^{32}P-labeled and that it was excised. As a control, bacterially expressed GST-GR$_{X568}$ which was known to be phosphorylated on serine 527 was also kinased with DNA-PK, resolved on SDS-PAGE gels, stained with amido black and excised from the gels. The gel slices were treated with trypsin, which allowed the tryptic peptides to elute from the gel. The efficiency of digestion and elution could be monitored by counting the gel slices and the elution liquid. Typically at least 70% of the counts were recovered in the elution liquid. After treatment with trypsin the elution liquid was dried, and the peptides were resuspended in alkaline gel sample buffer. The tryptic peptides were then resolved on 40% alkaline gels and visualized by autoradiography. Two major ^{32}P-labeled peptides were observed after digesting mycGR, indicating that it is phosphorylated on at least two sites by DNA-PK (Figure 19, lane 2). Interestingly the lower mobility peptide (designated peptide 1), corresponded in mobility to the peptide from GST-GR$_{X568}$ which is known to be phosphorylated on serine 527 (compare lanes 1 and 2), suggesting the possibility that full length mycGR was also phosphorylated on this residue. The higher mobility peptide (designated peptide 2), appeared to contain a novel phosphorylation site.

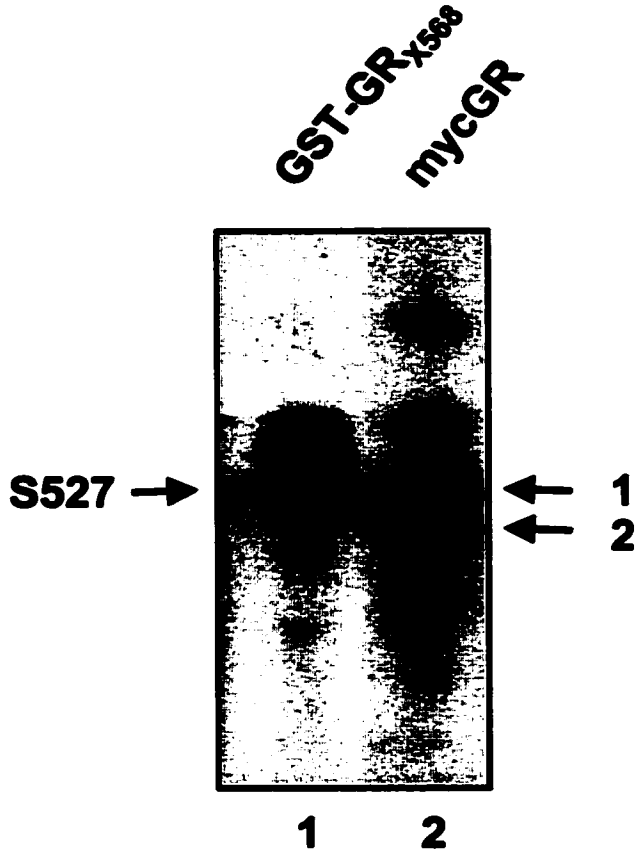

GST-GR$_{x568}$
mycGR
S527
1
2
1
2

Figure 19. Phosphorylation of mycGR by DNA-PK occurs on two major tryptic peptides.

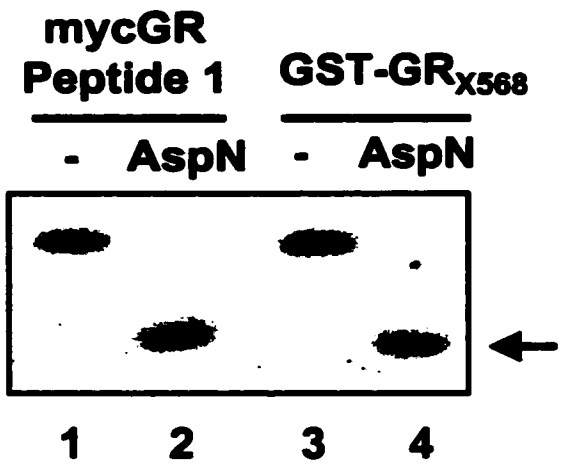
mycGR was immunoprecipitated from nuclear extracts from Dex treated Sf7-mycGR cells using the antibody 9E10 against the 6 x c-myc tag (lane 2). The immunoprecipitates were rinsed in kinase buffer, then kinased with DNA-PK by incubating them with purified DNA-PK, γ^{32}P-ATP, and linearized pHC17 for 30 min at 30^0C. As a control bacterially expressed GST-GR_{X568} immobilized on glutathione beads was also kinased (lane 1). Following kinasing the immunoprecipitates were washed and antibody-protein complexes were separated through 6% SDS-PAGE gels. Gel slices containing phosphorylated mycGR or GST-GR_{X568} were excised and treated with trypsin. Tryptic peptides were eluted, dried and resolved on 40% alkaline gels. ^{32}P-labeled peptides were visualized by autoradiography. This experiment was repeated 5 times with a representative experiment shown.

(vi) The phosphorylation site on peptide 1 corresponds to Ser 527

To determine whether peptide 1 contained aspartic acid or glutamic acid residues, it was eluted from the alkaline gel and digested with the specific secondary endoproteases AspN and GluC respectively. As a control the GST-GR_{X568} peptide was digested in parallel. Following treatment with AspN, which specifically digests peptide bonds N-terminal to aspartic acid residues, peptide 1 underwent an increase in mobility on a 40% alkaline gel relative to the untreated tryptic peptide (Figure 20; panel A, compare lanes 1 and 2). This indicates that this peptide contains at least one aspartic acid residue. The resulting AspN digested peptide was identical in mobility to the similarly digested peptide from the GST-GR_{X568}, providing further evidence that it corresponded to the serine 527 containing peptide (compare lanes 2 and 4). Digestion with the endoprotease GluC, which specifically cleaves peptide bonds C-terminal to glutamic acid residues, was weak due either to the relative inefficiency of this enzyme or the presence of contaminants. However cleavage did occur reproducibly in five independent trials, resulting in a higher mobility peptide (panel B, compare lanes 1 and 2). This indicates that this peptide contains at least one glutamic acid residue. Again the resulting cleaved peptide corresponded in mobility to the similarly treated peptide from the GR DBD further substantiating that these peptides were identical (compare lane 2 and 4).

To determine the position of the phosphorylated amino acid in peptide 1 the ^{32}P labeled tryptic and AspN digested peptides were eluted from the 40% alkaline gels and subjected to manual Edman degradation. Typically 1000-2000 cpm of the peptide were used for this analysis. First the peptide was immobilized on a solid-phase using a Sequelon-AA attachment kit (Millipore) by which peptides are covalently linked via the

A


mycGR
Peptide 1
GST-GR$_{X568}$
- AspN - AspN
1 2 3 4

B

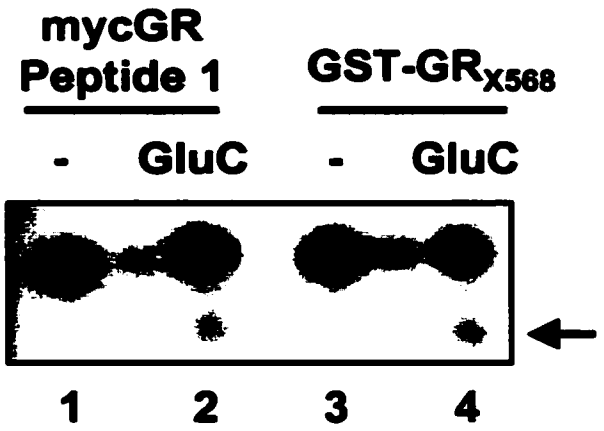
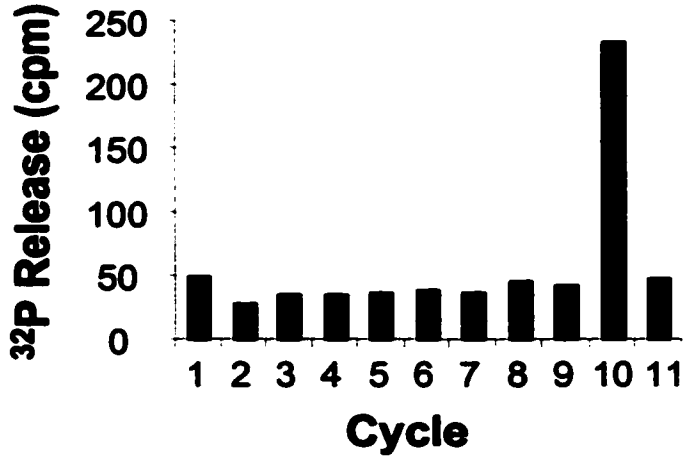

mycGR
Peptide 1
GST-GR$_{X568}$
- GluC - GluC
1 2 3 4

Figure 20. Peptide 1 contains aspartic acid and glutamic acid residues.

mycGR was immunoprecipitated from nuclear extracts from Dex treated Sf7-mycGR cells using the antibody 9E10 against the 6 x c-myc tag. The immunoprecipitates were rinsed in kinase buffer, then kinased with DNA-PK by incubating them with purified DNA-PK, γ^{32}P-ATP, and linearized pHC17 for 30 min at 30^{0}C. As a control bacterially expressed GST-GR$_{X568}$ immobilized on glutathione beads was also kinased. Following kinasing the immunoprecipitates were washed and antibody-protein complexes were separated through 6% SDS-PAGE gels. Gel slices containing phosphorylated mycGR or GST-GR$_{X568}$ were excised and treated with trypsin. Tryptic peptides were eluted, dried and resolved on 40% alkaline gels. For mycGR peptide 1 was recovered from the alkaline gel by elution. For GST-GR$_{X568}$ the single major ^{32}P-labeled peptide was recovered in the same manner. The isolated peptides were treated with the endoproteases AspN (Panel A; lanes 2 and 4) or GluC (Panel B; lanes 2 and 4) or were left untreated (Panels A and B; lanes 1 and 3). They were then resolved on 40% alkaline gels. mycGR (Panels A and B; lanes 1 and 2), GST-GR$_{X568}$ (Panels A and B; lanes 3 and 4). The arrows indicate the peptides resulting from AspN or GluC cleavage. AspN digestions were repeated three times with a representative experiment shown (Panel A). GluC digestions were repeated five times with a representative experiment shown (Panel B).

carboxyl group at their C-terminus or internal acidic groups to aryl amine groups on a PVDF disc by incubating the peptide on the disc in the presence of water-soluble carbodiimide. Linkage to the membrane was observed to occur with typically 30-60% efficiency. The peptide-linked discs were then subjected to manual Edman degradation. This is a cyclical reaction that cleaves a single amino acid from the N-terminus of a peptide in each cycle (Allen, 1989). The position of the ^{32}P label within the peptide was determined by scintillation counting the released amino acid after each cycle. Preliminary experiments using GST-GR_{X568} indicated that approximately 50% of the cpm linked to the membrane were irreversibly bound and would not release from the membrane during Edman degradation. In the experiment shown in Figure 21, for the trypsin digested peptide 1, of 1460 cpm of starting material, 630 cpm were linked to a membrane giving a linking efficiency of 43%. This was subjected to manual Edman degradation. The radioactivity released from the membrane in each cycle is indicated in Figure 21 (panel A). Through the first nine Edman degradation cycles the amount of radioactivity released from the membrane was only slightly above the background of 27 cpm. In cycle ten approximately 230 cpm were released from the membrane. This was significantly higher than observed in any of the previous cycles. Moreover, a significant decrease in the radioactivity remaining on the membrane also occurred in cycle ten. This dropped to 190 cpm. One further Edman degradation cycle was carried out and the amount of radioactivity released was again only slightly above background. These results indicate that peptide 1 is phosphorylated on the tenth amino acid. Furthermore, since less than 50% of the original counts remained on the membrane it appeared that these were irreversibly bound and no further phosphorylation sites existed on this peptide.

A


Peptide 1 - Trypsin
32P Release (cpm)
250
200
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11
Cycle

B

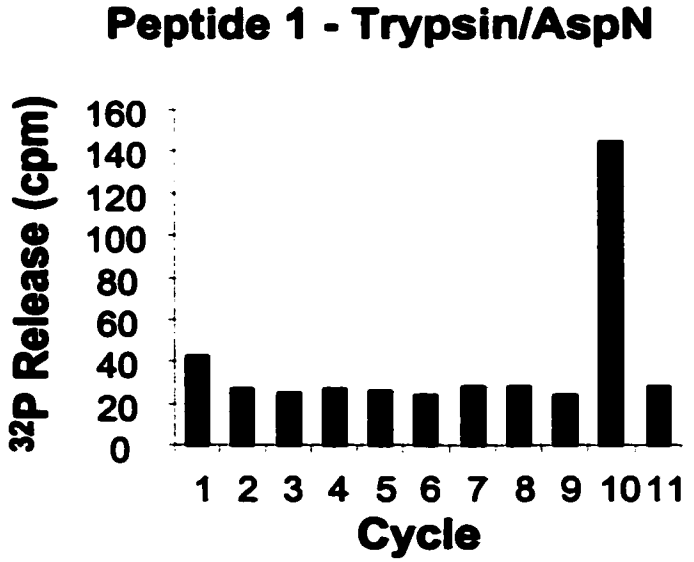

Peptide 1 - Trypsin/AspN
32P Release (cpm)
160
140
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11
Cycle

Figure 21. Phosphorylation of peptide 1 occurs on the tenth amino acid and the position is not altered by AspN cleavage.

mycGR was immunoprecipitated from nuclear extracts from Dex treated Sf7-mycGR cells using the antibody 9E10 against the 6 x c-myc tag. The immunoprecipitates were rinsed in kinase buffer, then kinased with DNA-PK by incubating them with purified DNA-PK, γ^{32}P-ATP, and linearized pHC17 for 30 min at 30^{0}C. Following kinasing the immunoprecipitates were washed and antibody-protein complexes were separated through 6% SDS-PAGE gels. Gel slices containing phosphorylated mycGR were excised and treated with trypsin. Tryptic peptides were eluted, dried and resolved on 40% alkaline gels. For mycGR, peptide 1 was recovered from the alkaline gel by elution. This was then used as is (Panel A) or secondarily digested with AspN and recovered again from 40% alkaline gels (Panel B). The isolated peptides were covalently coupled to aryl amine derivatized PVDF membranes at their C-terminus using a Sequleon AA reagent kit (Millipore). The membrane was subjected to 11 cycles of manual Edman degradation and the released amino acids were scintillation counted. Graphs show counts per minute released from the membrane following each cycle of Edman degradation. This experiment was repeated three times for peptide 1 and twice for peptide 1 digested with AspN.

Similarly peptide 1 digested with the AspN protease was also recovered from an alkaline gel, linked to a membrane and subjected to manual Edman degradation. Of 1020 cpm starting material, approximately 410 cpm of this peptide were linked to the membrane, indicating linking occurred with 40% efficiency. The amount of radioactivity released in each cycle of Edman degradation is shown in Figure 21 (panel B). As with the trypsin digested peptide, through the first nine cycles the amount of radioactivity released only slightly exceeded background. In cycle ten, I observed 150 cpm released from the peptide. This was accompanied by a corresponding decrease in the amount of radioactivity remaining on the membrane to approximately 130 cpm. One additional cycle of Edman degradation saw the amount of radioactivity released return to just slightly above background. This result indicates that cleavage of peptide 1 with AspN yields a peptide that is phosphorylated on the tenth amino acid and that this cleavage does not lead to a change of the position. Therefore the most N-terminal aspartic acid residue in peptide 1 must be C-terminal to the tenth amino acid since the number of amino acids N-terminal to the ^{32}P label was not altered by AspN cleavage.

Trypsin digests peptide bonds on the C-terminal side of lysine or arginine residues (Walsh, 1970). A list of tryptic peptides representing all serine and threonine residues in mycGR is given in appendix A. After trypsin digestion there are seven possible peptides that contain either a serine or threonine as the tenth amino acid and these are summarized in Table 2. Of these seven, five contain aspartic acid residues that would be recognized by AspN. Since AspN treatment did not alter the position of the ^{32}P-labeled amino acid, the most N-terminal aspartic acid must be located C-terminally to the tenth position. This is the case for only two of the possible peptides: that containing sequences from 336-368,

Position in rat GR	Peptide Sequence
162 - 175	SSTSATGCATPTEK
217 - 227	DLEFSAGSPSK
336 - 368	MSAISVHGVSTSGGQMYHYDMNTASLSQQQDQK
387 - 406	CQGSGEDSLTSLGALNFPGR
420 - 438	PDVSSPPSSSSAATGPPPK
439 - 461	LCLVCSDEASGCHYGVLTCGSCK
518 - 536	GIQQATAGVSQDTSENPNK

Table 2. Tryptic peptides from mycGR containing serine or threonine at position 10.

All possible peptides generated by digestion of mycGR with trypsin and having serine or threonine ten amino acids from the N-terminus are shown. The corresponding positions within the wild type rat GR (without the myc tag) are indicated. The serines or threonine at position 10 are outlined while all aspartic or glutamic acid residues are indicated in bold.

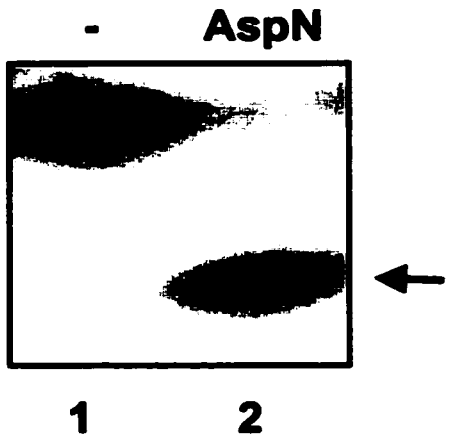
and that containing 518-536. The 336-368 peptide does not contain glutamic acid and therefore could not be cleaved by GluC as was observed with peptide 1 (Figure 20, panel B). By elimination therefore, the 518-536 peptide is the only possible peptide that could correspond to the digestion and Edman degradation data for peptide 1. The amino acid located at the tenth position within this peptide is serine 527, therefore it is concluded that this is a DNA-PK dependent phosphorylation site within mycGR. This is corroborated by the identical mobilities of peptide 1 and its digested products to the serine 527-containing peptide from the bacterially expressed GST-GR_{X568}. The peptide containing serine 527 also contains a serine located C-terminally at position 531 and a threonine at position 530. As was suggested by the less than 50% radioactivity remaining on the membrane following Edman degradation, these residues are not phosphorylated since AspN cleaves between these sites and position 527 and only a single ^{32}P-labeled peptide was observed following AspN digestion.

(vii) The second phosphorylation site is due to addition of the c-myc tag

To identify phosphorylation sites in peptide 2 an identical strategy was used. This peptide was eluted from alkaline gels and treated with the secondary endoproteases AspN and GluC (Figure 22). Treatment of peptide 2 with AspN resulted in an increase in mobility on a 40% alkaline gel relative to the untreated trypsin digested form (panel A, compare lanes 1 and 2) indicating the presence of an aspartic acid residue within this peptide. Digestion with GluC, again being weak, also resulted in an observed higher mobility species (panel B, compare lanes 1 and 2) indicating that peptide 2 also contained a glutamic acid residue.

A

mycGR
Peptide 2



B

mycGR
Peptide 2

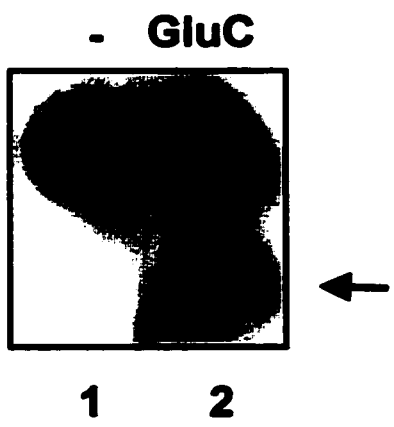


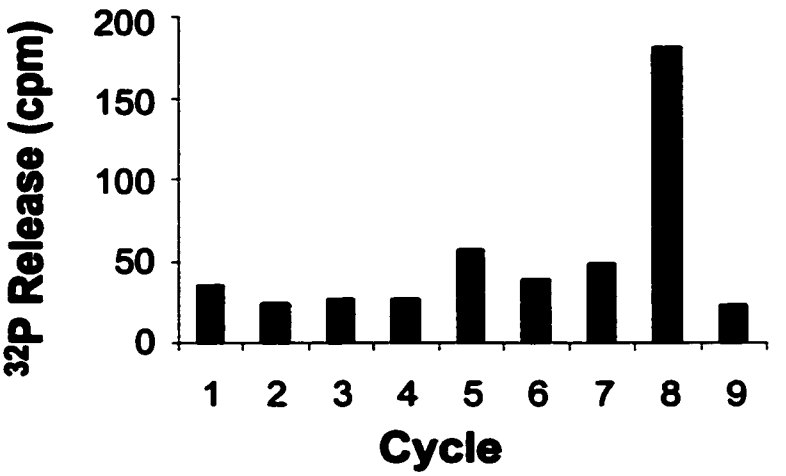
Figure 22. Peptide 2 contains aspartic acid and glutamic acid residues.

mycGR was immunoprecipitated from nuclear extracts from Dex treated Sf7-mycGR cells using the antibody 9E10 against the 6 x c-myc tag. The immunoprecipitates were rinsed in kinase buffer, then kinased with DNA-PK by incubating them with purified DNA-PK, γ^{32}P-ATP, and linearized pHC17 for 30 min at 30^{0}C. Following kinasing the immunoprecipitates were washed and antibody-protein complexes were separated through 6% SDS-PAGE gels. Gel slices containing phosphorylated mycGR were excised and treated with trypsin. Tryptic peptides were eluted, dried and resolved on 40% alkaline gels. For mycGR peptide 2 was recovered from the alkaline gel by elution. The isolated peptides were treated with the endoproteases AspN (Panel A; lanes 2) or GluC (Panel B; lanes 2) or were left untreated (Panels A and B; lane 1). They were then resolved on 40% alkaline gels. The arrows indicate the peptides resulting from AspN or GluC cleavage. AspN digestions were repeated two times with a representative experiment shown (Panel A). GluC digestions were repeated two times with a representative experiment shown (Panel B).

To determine the position of the ^{32}P label in peptide 2 both the trypsin-digested form and the AspN-digested forms were recovered from alkaline gels and subjected to manual Edman degradation. For the tryptic peptide, of 960 cpm starting material, 370 linked to the membrane indicating that linkage occurred with 39% efficiency. This was subjected to Edman degradation and scintillation counting revealed no significant release of radioactivity through the first seven cycles. In cycle eight a release of 180 cpm of ^{32}P from the membrane was observed (Figure 23; panel A), indicating this peptide was phosphorylated by DNA-PK on the eighth amino acid from the N-terminus. One additional cycle revealed that the amount of radiation released returned to just above background. Less than 50% of the linked counts remained on the membrane suggesting no additional phosphorylation sites were present. For peptide 2 digested with AspN, of 490 cpm starting material, 180 cpm linded to the membrane, indicating that linkage occurred with 37% efficiency. Again in the first seven cycles the amount of radioactivity released from the membrane was only slightly above background. In the eighth cycle 75 cpm of ^{32}P were observed to release from the membrane (panel B). This indicates that the phosphorylated residue remained eight amino acids from the N-terminus after AspN cleavage and that the most N-terminal aspartic acid residue in this peptide was located C-terminally to the eighth amino acid.

Examination of the tryptic peptide map in Appendix A shows that seven peptides in mycGR contain a serine or threonine eight residues from the N-terminus. These peptides are summarized in Table 3. Of these, five carry at least one aspartic acid and two of these, the peptides carrying sequences 301-313 and 387-406, contain at least one glutamic acid (Table 3). However in neither of these peptides is the AspN cleavage site

A


Peptide 2 - Trypsin
32P Release (cpm)
200
150
100
50
0
1 2 3 4 5 6 7 8 9
Cycle

B

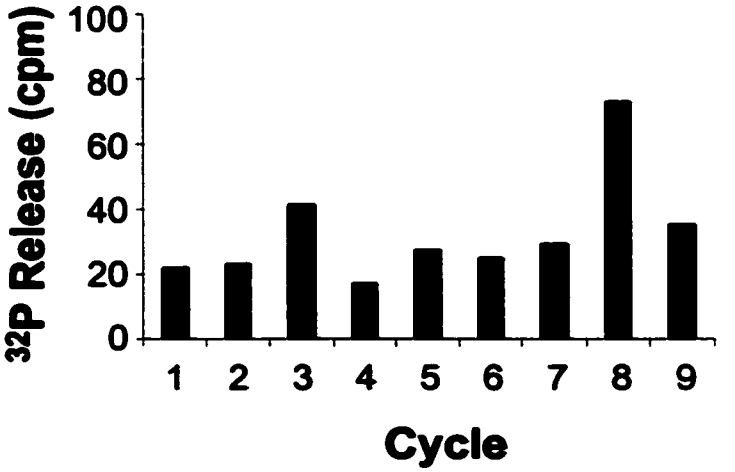

Peptide 2 - Trypsin/AspN
32P Release (cpm)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9
Cycle

Figure 23. Phosphorylation of peptide 2 occurs on the eighth amino acid and the position is not altered by AspN cleavage.

mycGR was immunoprecipitated from nuclear extracts from Dex treated Sf7-mycGR cells using the antibody 9E10 against the 6 x c-myc tag. The immunoprecipitates were rinsed in kinase buffer, then kinased with DNA-PK by incubating them with purified DNA-PK, γ^{32}P-ATP, and linearized pHC17 for 30 min at 30^{0}C. Following kinasing the immunoprecipitates were washed and antibody-protein complexes were separated through 6% SDS-PAGE gels. Gel slices containing phosphorylated mycGR were excised and treated with trypsin. Tryptic peptides were eluted, dried and resolved on 40% alkaline gels. Peptide 2 was recovered from the alkaline gel by elution. The isolated peptides were untreated (panel A) or treated with the endoproteases AspN and recovered from 40% alkaline gels. (panel B). The isolated peptides were covalently coupled to aryl amine derivatized PVDF membranes at their C-terminus using a Sequleon AA reagent kit (Millipore). The membrane was subjected to 9 cycles of manual Edman degradation and the released amino acids were scintillation counted. Graphs show counts per minute released from the membrane following each cycle of Edman degradation. Two repetitions of this experiment were performed for both the tryptic peptide and the AspN treated peptide. A representative experiment is shown.

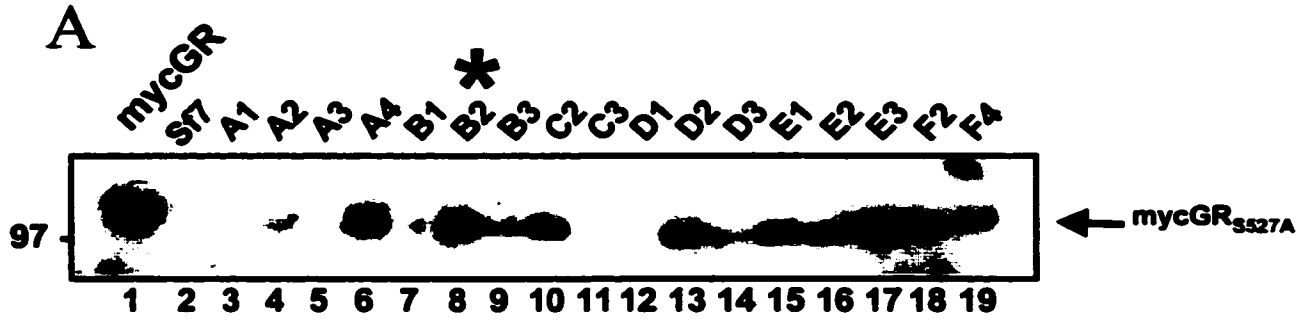
Position in rat GR	Peptide Sequence
204 - 216	LYPTDQSTFDLLK
217 - 227	DLEFSAGSPSK
279 - 297	DTGDTILSSPSSVALPQVK
301 - 313	DDFIELCTPGVIK
387 - 406	CQGSGEDSLTSLGALNFPGR
407 - 419	SVFSNGYSSPGMR
420 - 438	PDVSSPPSSSSAATGPPPK

Table 3. Tryptic peptides from mycGR containing serine or threonine at position 8.

All possible peptides generated by digestion of mycGR with trypsin and having serine or threonine eight amino acids from the N-terminus are shown. The corresponding positions within the wild type rat GR (without the myc tag) are indicated. The serines or threonines at position 8 are outlined while all aspartic or glutamic acid residues are indicated in bold.

located C-terminally to residue eight. The Edman degradation data are therefore inconsistent with the known amino acid sequence of mycGR.

A possible explanation for this is that a site created by addition of the c-myc tag to GR. To examine this possibility I took advantage of a HeLa cell line that stably expressed full-length wild type rat GR lacking the c-myc tag which had previously been isolated in our laboratory by Louise Pope. These cells were treated with Dex, nuclear extracts were prepared and these were used to immunoprecipitate Dex bound receptor. This immunoprecipitated GR was phosphorylated by DNA-PK and trypsinized as was done with the mycGR. The resulting peptides were then resolved on 40% alkaline gels. Visualization of the ^{32}P labeled peptides by autoradiography revealed that in native GR only a single major peptide was phosphorylated (Figure 24, panel B, lane 4). This peptide corresponded in mobility to peptide 1 of mycGR and the peptide from GST-GR_{X568} which contained serine 527 (Figure 21; compare lane 4 with lanes 1 and 2). The higher mobility peptide 2 was not phosphorylated when the c-myc tag was absent (lane 4). It is unlikely that the phosphorylation site on peptide 2 was kinased by endogenous DNA-PK from the HeLa cells prior to immunoprecipitation since phosphorylation of serine 527 was not significantly affected (compare lanes 1 and 2 with lane 4). In mycGR these two sites appear to be equally well recognized by DNA-PK as indicated by the equivalent level of labeling (lane 2 and Figure 16; panel C, lane 2). It is also unlikely that this phosphorylation site was masked by the anti GR antibody used for the immunoprecipitation since there was no effect of using this antibody on peptide 2 in mycGR. It is therefore concluded that the DNA-PK dependent phosphorylation site in


A
mycGR
S17
A1
A2
A3
A4
B1
B2
B3
C2
C3
D1
D2
D3
E1
E2
E3
F2
F4
97
mycGR$_{S527A}$
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

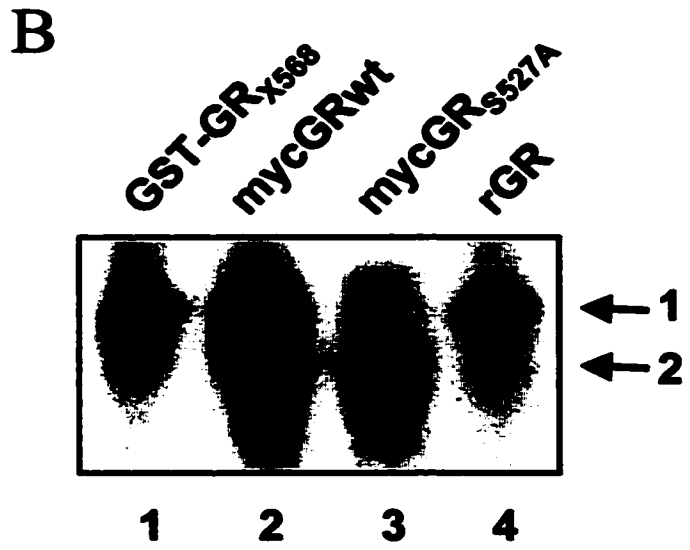

B
GST-GR$_{X568}$
mycGRwt
mycGR$_{S527A}$
rGR
1
2
1 2 3 4

Figure 24. Screening of mycGR$_{S527A}$ stable cell lines and analysis of DNA-PK phosphorylation of mycGR$_{S527A}$ and wild type GR.

Panel A: mycGR$_{S527A}$ was generated as described in Figure 25. It was stably introduced into Sf7 cells using the same strategy as for mycGR, as outlined in Figure 15, panel A. 10^7 Sf7 cells were transfected with a 7:1 ratio of a vector expressing mycGR$_{S527A}$ (pTL2-mycGR$_{S527A}$) to a vector expressing the gene for neomycin resistance (pSV2NEO^r). 48 h post-transfection cells were selected with 500 μg/ml G418. Selection was continued for 2 weeks after which time approximately 150 G418 resistant colonies were observed. Of these 20 were picked and transferred to individual wells of a 24 well plate. All clones survived the transfer and were expanded to 60 mm dishes. The clones were named based on their position within the 24 well plate. Of these, 17 were screened for expression of mycGR$_{S527A}$ by western analysis (shown). Whole cell extracts of the 17 clones were prepared and equal amounts of proteins were analyzed by western blotting. Expression of mycGR$_{S527A}$ was detected using the anti-c-myc antibody 9E10. Clone designations are indicated above each lane. Lane 1 contained extract from Sf7-mycGR cells while lane 2 contained extract from untransfected Sf7 cells. Clone B2 (lane 8, *) was chosen as the highest expressing clone and the cell line was designated Sf7-mycGR$_{S527A}$, and used for further analysis. This experiment was performed one time.
Panel B: mycGR (lane 2) and mycGR$_{S527A}$ (lane 3) were immunoprecipitated from Sf7-mycGR and Sf7-mycGR$_{S527A}$ cells respectively. Native rat GR without a myc tag was immunoprecipitated from stably expressing HeLa cells (lane 4). Immunoprecipitation was performed using the anti-GR antibody BuGR2. Bacterially expressed GST-GR$_{X568}$ was expressed in bacteria and recovered on glutathione-sepharose beads (lane 1). These proteins were rinsed in kinase buffer, then kinased with DNA-PK by incubating them with purified DNA-PK, γ^{32}P-ATP, and linearized pHC17 for 30 min at 30^{0}C. Following kinasing the immunoprecipitates were washed and antibody-protein complexes were separated through 6% SDS-PAGE gels. Gel slices containing the phosphorylated proteins were excised and treated with trypsin. Tryptic peptides were eluted, dried and resolved on 40% alkaline gels. ^{32}P-labeled peptides were visualized by autoradiography.

peptide 2 resulted from addition of the N-terminal c-myc tag, and is not present in native wild type GR.

(viii) Confirmation of serine 527 as the phosphorylated residue in peptide 1

To confirm the identification of this phosphorylation site a point mutation was generated in mycGR in which serine 527 was mutated to alanine (referred to as S527A). A description of the generation of this mutant is given in section 4. A Sf7 cell line stably expressing this mutated GR was generated using the strategy outlined in Figure 15, panel A. Sf7 cells were transfected using the lipofectamine protocol. A 100 mm dish (approximately 10^7 cells) was transfected with 7 μg pTL2-mycGR$_{S527A}$ and 1 μg pSV2NEO^r. 48 h post-transfection the media was aspirated from the cells and replaced with media containing 500 μg/ml G418. The cells were selected in this media for 2 weeks, with the media being replaced daily. Cells, which had acquired stable integrations of the neomycin resistance gene, developed colonies that by 2 weeks were approximately 1 mm in diameter. Twenty of these G418 resistant colonies were picked by transferring them on a sterile paper disc that had been soaked in trypsin into individual wells of a 24 well tissue culture plate. All of these survived and were expanded into 60 mm dishes. These clones were designated based on their position within the 24 well plate. Seventeen of the fastest growing clones were analyzed further by preparing whole cell extracts and determining the levels of mycGR expression using western analysis. As a positive control, extracts from the Sf7-mycGR cells were used. As a negative control extracts from untransfected Sf7 cells were used. Equal amounts of protein were loaded for each extract. To detect mycGR the anti c-myc antibody, 9E10 was used as a primary antibody. As can be seen in Figure 24, panel A, the 9E10 antibody detected a protein of 120 kD in

extracts from twelve of the seventeen resistant clones (see lanes 4, 6, 8-10 and 13-19) but not in extracts from untransfected Sf7 cells (compare with lane2). This protein was identical in mobility to the band attributed to mycGR from Sf7-mycGR cells (see lane 1). Clone B2 expressed the highest levels of this protein and was therefore selected for further analysis and this cell line was designated Sf7-mycGR$_{S527A}$. Expression of mycGR$_{S527A}$ in this cell line was significant but considerably less than the expression of mycGR in Sf7-mycGR cells (compare lane 8 with lane 1).

mycGR$_{S527A}$ was recovered from these cells by immunoprecipitation, phosphorylated by DNA-PK and digested with trypsin using the established protocols. After separation of the resulting peptides by electrophoresis on 40% alkaline gels only a single band was observed (Figure 24; panel B, lane 3). This peptide was identical in mobility to peptide 2 from mycGR (compare lane 3 with lane 2). No band corresponding in size to peptide 1 of mycGR or the serine 527 containing peptide of GST-GR$_{X568}$ was observed (compare lane 3 with lanes 1 and 2). This indicated that ^{32}P-labelling of peptide 1 containing alanine 527 in the S527A mutant did not occur. That peptide 1 was not phosphorylated in the S527A mutant shows that this mutation eliminated the DNA-PK phosphorylation site in this peptide. It was therefore confirmed that serine 527 is a DNA-PK phosphorylation site in mycGR and this is the only site in wild type GR.

4. Functional analysis of the role of DNA-PK mediated phosphorylation of the glucocorticoid receptor

The previous results demonstrate that both Ku and DNA-PK are required for the NRE1 mediated repression of the glucocorticoid-induced transcription of MMTV. In

addition, DNA-PK was shown to phosphorylate GR, the primary factor involved in the activation of MMTV by glucocorticoids, on only a single serine residue at position 527 *in vitro*. It then became important to determine if the phosphorylation of serine 527 by DNA-PK was relevant to the NRE1 mediated repression.

(i) Generation of serine 527 mutants in mycGR

As a first step, point mutations of serine 527 were generated by PCR-mediated site-directed mutagenesis. By this method serine 527 was mutated to alanine, aspartic acid or glutamic acid (referred to as S527A, S527D and S527E respectively) in the mycGR construct. Substitution of serine to alanine results in the loss of the side chain hydroxyl group of serine preventing phosphorylation by protein kinases. By contrast mutations to aspartic acid or glutamic acid result in the addition a constitutive negative charge, which in some cases has been shown to mimic phosphorylation (Xiao et al., 1997; Shao et al., 1998).

The strategy to generate these mutants is outlined in Figure 25. The mutations were generated using oligonucleotides to PCR amplify an 833 bp PstI/BamHI fragment from pTL2-rGRwt which contained the rat GR coding sequence from amino acid positions 525 to the C-terminal 795 (panel A). For each of the mutations the 5' primers used for the PCR contained an alteration in the 527 codon which specified the desired amino acid. The PCR reaction therefore amplified a fragment which contained the desired mutation at codon 527. The amplified fragment was then digested with PstI/BamHI to generate complimentary ends and was ligated back into the corresponding site of pTL2-rGRwt. Sequencing was performed to confirm that the resulting clones had acquired the desired mutations (panel B). To introduce these mutations into the mycGR

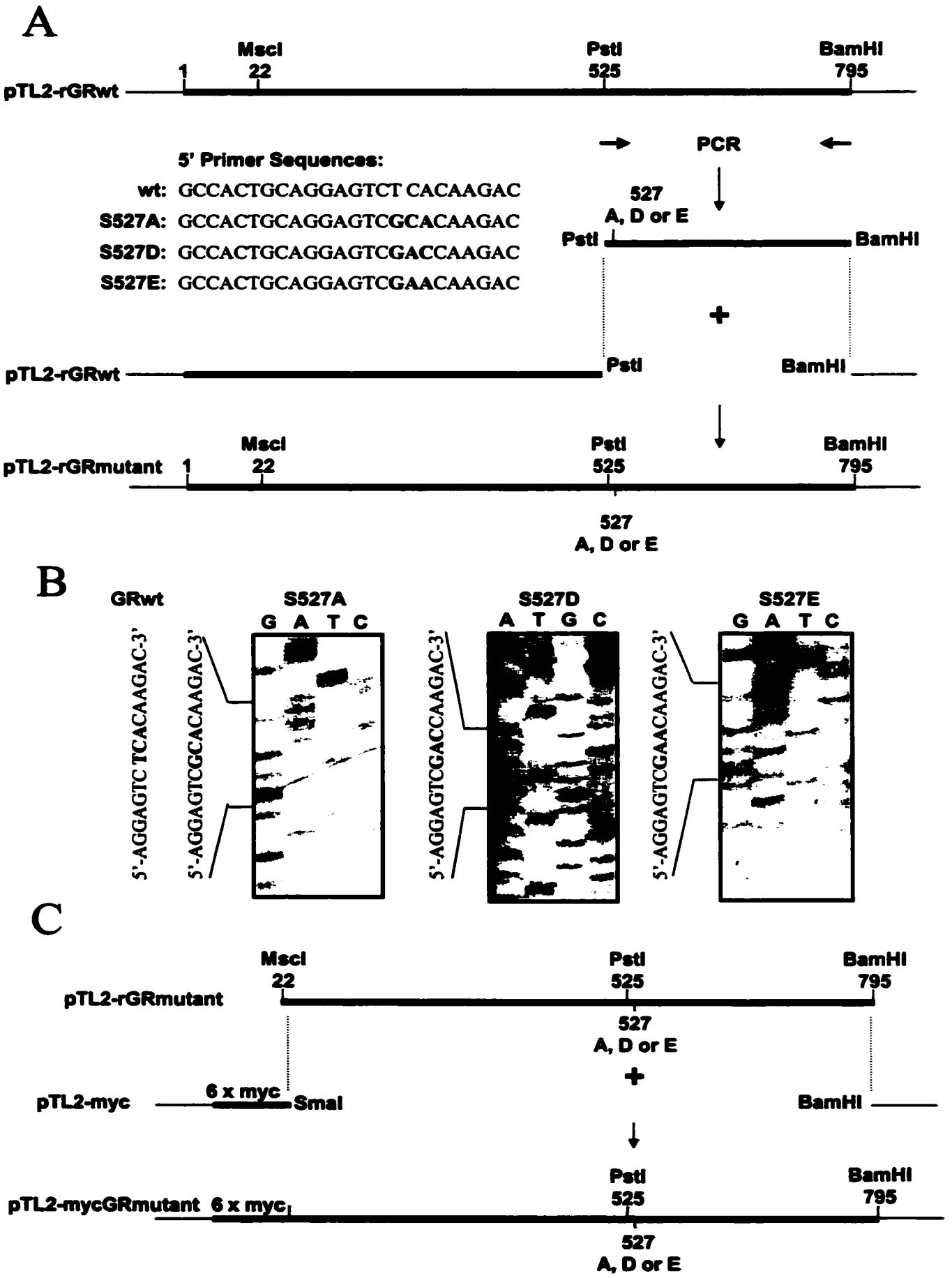

A
MscI
PstI
BamHI
1
22
525
795
pTL2-rGRwt
PCR
5' Primer Sequences:
wt: GCCACTGCAGGAGTCT CACAAGAC
S527A: GCCACTGCAGGAGTCGCACAAGAC
S527D: GCCACTGCAGGAGTCGACCAAGAC
S527E: GCCACTGCAGGAGTCGAACAAGAC
527
A, D or E
PstI
BamHI
pTL2-rGRwt
PstI
BamHI
MscI
PstI
BamHI
pTL2-rGRmutant 1
22
525
795
527
A, D or E
B
GRwt
S527A
S527D
S527E
G A T C
A T G C
G A T C
5'-AGGAGTC TCACAAGAC-3'
5'-AGGAGTCGCACAAGAC-3'
5'-AGGAGTCGACCAAGAC-3'
5'-AGGAGTCGAACAAGAC-3'
C
MscI
PstI
BamHI
22
525
795
pTL2-rGRmutant
527
A, D or E
pTL2-myc
6 x myc
SmaI
BamHI
PstI
BamHI
pTL2-mycGRmutant 6 x myc
525
795
527
A, D or E

Figure 25. Mutation of serine 527 to alanine, aspartic acid and glutamic acid.

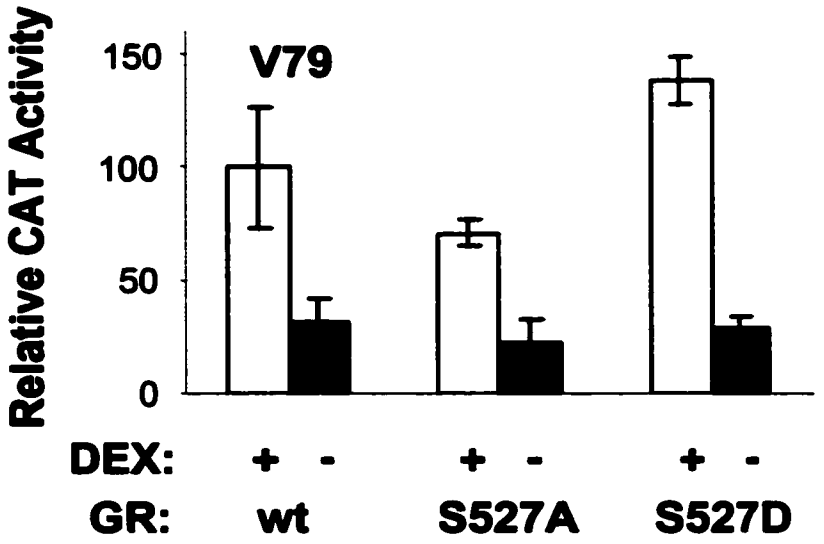
Panel A: Strategy for site-directed mutagenesis of serine 527 in rat GR. The PstI/BamHI fragment of rat GR (525 to 795) was amplified by PCR using a 5' primer containing a mutation within the codon for serine 527. The annealing positions of the PCR primers within pTL2-rGRwt is indicated by the arrows. The sequences of the 5' primers used for each mutation are shown with the mutated codons indicated in bold. The amplified fragments containing each mutation were digested with PstI/BamHI and subcloned into the corresponding site of pTL2-rGRwt. Panel B. The resulting clones were sequenced to confirm the incorporation of the desired mutation. Panel C. To introduce the mutations into mycGR, MscI/BamHI fragments of the pTL2-rGR mutants were subcloned into the SmaI/BamHI site of pTL2-myc. The resulting vectors were pTL2-mycGR$_{S527A}$, pTL2-mycGR$_{S527D}$ and pTL2-mycGR$_{S527E}$.

context the mutant pTL2-rGR clones were digested with MscI/BamHI (panel C). MscI cleaves within the GR sequence at position 21, while BamHI cuts immediately adjacent to the C-terminal amino acid 795. This fragment was sub-cloned into the SmaI/BamHI site of pTL2-myc which generated a fusion protein with the 6 x c-myc tag fused to amino acid 22 of the GR mutants.

(ii) Mutation of serine 527 in GR has no significant effect on transcription of a simple glucocorticoid responsive synthetic promoter.

To determine if mutation of serine 527 had any affect on the intrinsic ability of GR to activate transcription a transient transfection assay was used using a simple synthetic glucocorticoid responsive promoter. The reporter construct used contained a GRE located upstream of the thymidine kinase TATA box. This was cotransfected with either the wild type or mutant GRs into both wild type V79 cells and Ku deficient V15B cells. On this promoter, glucocorticoid treatment resulted in an approximately four-fold activation of transcription with wild type mycGR in both cell types (Figure 26, Panel A and B, compare lane 1). The S527A mutation had no significant effect on the level of transcription relative to the wild type receptor, either for glucocorticoid induced or basal transcription. The S527E mutation slightly activated transcription, however this effect was minor. This mutation had the same effect in wild type cells and Ku deficient cells (Panel A and B, compare lanes 2 and 3 with lane 1). This result indicates that substitution of serine 527 for a neutral alanine or a negatively charged aspartic acid results in receptors which are competent to activate transcription from a simple synthetic promoter. Therefore these mutations likely do not have any effects on the intrinsic activities of GR.

A


Relative CAT Activity
150
100
50
0
V79
DEX: + - + - + -
GR: wt S527A S527D

B

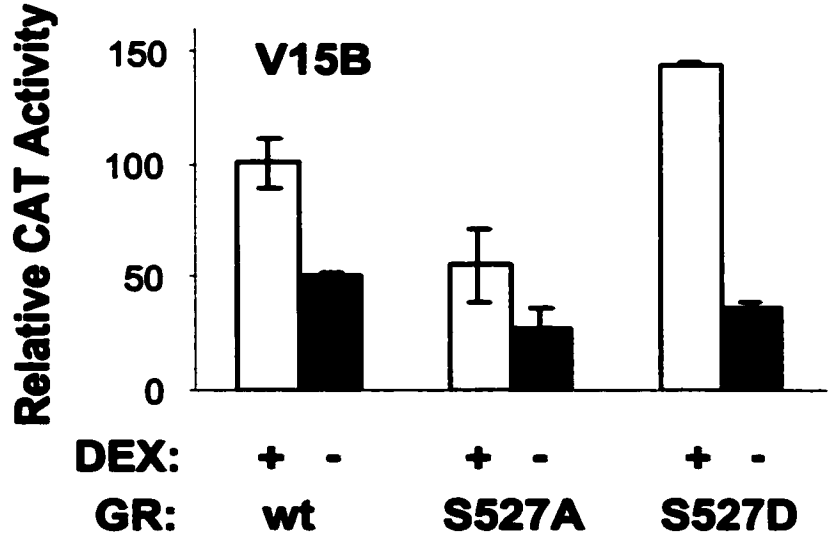

Relative CAT Activity
150
100
50
0
V15B
DEX: + - + - + -
GR: wt S527A S527D

Figure 26. Mutation of serine 527 does not affect transcription from a simple synthetic GRE containing promoter.

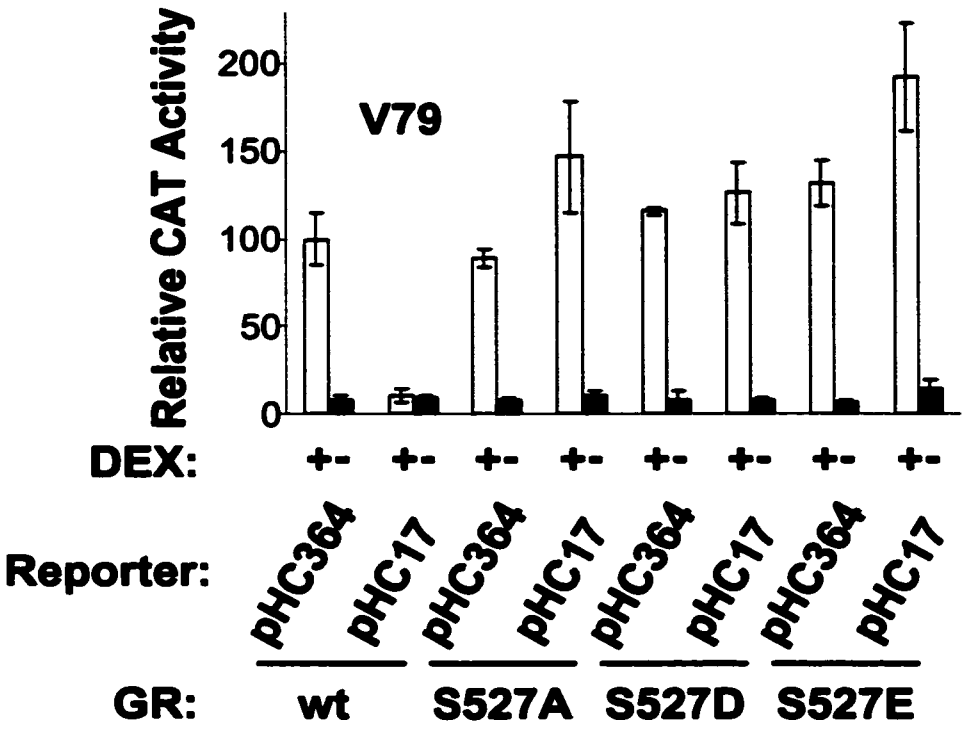
Expression plasmids for mycGR (wt) and the mutants with serine 527 altered to alanine (S527A) or aspartic acid (S527D) were transiently transfected into wild type hamster V79 fibroblast cells (Panel A) and the clonally derived mutant V15B cells lacking functional Ku80 (Panel B). 50 ng of the GRE-TK-CAT reporter construct was cotransfected. Cells were harvested 24 hr after dexamethasone (Dex) treatment, and assayed for CAT activity. Activity is represented as a percentage of activity of wild type GR following Dex treatment. In all cases the transfection efficiency was assessed and corrected for by co-transfection of pRSVβ-gal and determination of β-gal activity. Activities represent the means of three independent experiments, with two replications within each experiment. Error bars indicate standard error.

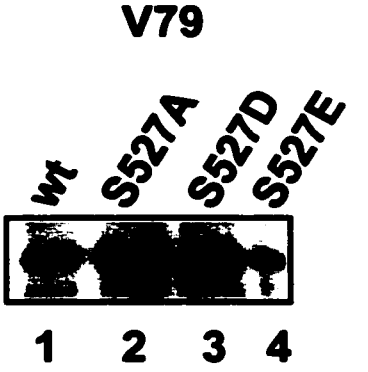
(iii) Mutation of serine 527 in GR abolishes NRE1 mediated repression of glucocorticoid induced transcription of MMTV

To determine the effects of these mutations on MMTV transcription the mutants were used in transient transfection assays in wild type V79 and Ku deficient V15B hamster fibroblasts. The mycGR mutants were co-transfected with the pHC17 and pHC364 reporter constructs used previously, which contain or lack the NRE1 element respectively. That the wild type and mutant GRs were expressed at equivalent levels was confirmed by western analysis of whole cell extracts of the transfected cells (Figure 27). As was observed with native rat GR, the induction of transcription by Dex with wild type mycGR was repressed approximately 10 fold on an MMTV LTR containing NRE1 relative to one lacking NRE1 (Figure 27, panel A, compare lane 3 with lane 1). In cells deficient in Ku, and by extension DNA-PK kinase activity, the NRE1 mediated repression was lost (panel B, compare lanes 3 with lane 1). By contrast, with the S527A mutation, NRE1 had little effect on glucocorticoid induced transcription either in cells with or without functional Ku (Panel A and B, compare lanes 5 and 7). This indicates that the hydroxyl group on serine 527 was essential for NRE1 mediated repression and suggests that phosphorylation of this residue results in a decreased transcriptional activity of the receptor. Importantly, the complete abolition of repression by this mutation in wild type cells suggests that GR is the only downstream target of DNA-PK relevant for the effect of NRE1.

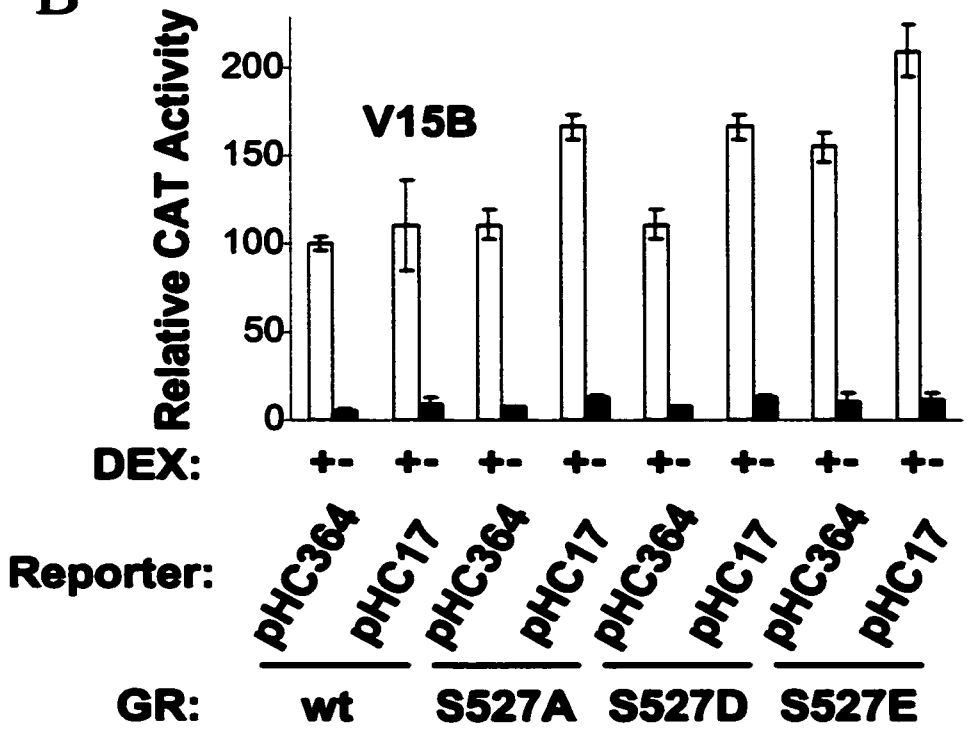
The S527D and S527E mutations were each fully able to activate transcription on the MMTV LTR in the absence of NRE1 in both wild type and Ku deficient cells (Panel A and B, compare lanes 9 and 13 with lane 1). This indicates that a negative charge at

A


Relative CAT Activity
200
150
100
50
0
V79
DEX:
+- +- +- +- +- +- +- +-
Reporter:
pHC364 pHC17 pHC364 pHC17 pHC364 pHC17 pHC364 pHC17
GR:
wt
S527A
S527D
S527E


V79
wt
S527A
S527D
S527E
1 2 3 4

B


Relative CAT Activity
200
150
100
50
0
V15B
DEX:
+- +- +- +- +- +- +- +-
Reporter:
pHC364 pHC17 pHC364 pHC17 pHC364 pHC17 pHC364 pHC17
GR:
wt
S527A
S527D
S527E

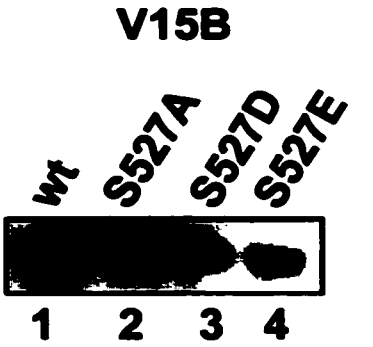

V15B
wt
S527A
S527D
S527E
1 2 3 4

Figure 27. Mutation of serine 527 to alanine abolishes NRE1 mediated repression of glucocorticoid induced transcription of MMTV.

mycGR mutants with serine 527 mutated to alanine (S527A), aspartic acid (S527D) or glutamic acid (S527E) were generated by PCR mediated site-directed mutagenesis. Wild type hamster V79 fibroblast cells (Panel A) and the clonally derived mutant V15B cells lacking functional Ku80 (Panel B) were transiently transfected with 250 ng of expression plasmid for the mutants and the wild type mycGR (wt) using Exgen. 50 ng of the MMTV reporters plasmid pHC17 (containing NRE1) or pHC364 (lacking NRE1), described in Figure 13, were cotransfected. Cells were harvested 24 hr after dexamethasone (Dex) treatment, and assayed for CAT activity. Activity is represented as a percentage of the activity of pHC364 with wild type mycGR and Dex treatment. In all transfections the transfection efficiency was assessed and corrected for by co-transfection of pRSVβ-gal and determination of β-gal activity. An equivalent level of expression of each of the receptors was confirmed by western analysis using an anti c-myc antibody. Activities represent the means of three independent experiments, with two replications within each experiment. Error bars indicate standard error.

position 527 per se was not sufficient to repress the receptors ability to activate transcription, suggesting that following phosphorylation, the precise structure of the region containing phosphoserine 527 is critical for the decrease in transciptional activity of the receptor. As with the S527A mutation, the presence of NRE1 did not result in a decrease in transcriptional activity with either the S527D or S527E mutations in wild type cells (Panel A, compare lane 11 with 9 and lane 15 with 13). The fact that none of the mutations used support phosphorylation at position 527 provides further evidence that a structural feature conferred by phosphorylation is required for the decrease in transcriptional activity.

IV. DISCUSSION

1. Ku Binding to NRE1

(i) Kinetics of binding

Binding to NRE1 by factors in Jurkat crude nuclear extracts occurred relatively quickly with on-rates comparable to those of other transcription factors such as GR, PR (Schauer et al., 1989) and Oct-1 (Malmborg et al., 1995; van Leeuwen et al., 1997). There was a clear difference in the rate of binding to double-stranded DNA as compared to single-stranded DNA. The on-rate for double-stranded NRE1 was 7-fold longer than binding to the upper-strand, while it was almost 4-fold longer than binding to lower-strand (see Figure 4). This kinetic preference for the single-stranded forms of NRE1 could reflect the increased flexibility of the single-strands, potentially allowing stronger contacts with the binding proteins.

Alternatively, given that the double-stranded NRE1 binding protein has been identified as the Ku autoantigen, the delay in association with the double-strand could reflect a requirement for single-stranded contact by Ku. Several lines of evidence suggest that Ku can induce an alteration in the double-stranded helix. Ku has been reported to have an ATP-dependent helicase activity (Cao et al., 1994; Ochem et al., 1997; Tuteja et al., 1997; Tuteja et al., 1994). Upon binding to NRE1, Ku is able to induce structural transitions in the DNA, reflected by hypersensitivity to both double and single-stranded DNA cleavage agents (Giffin et al., 1999; Giffin and Hache, 1995; Giffin et al., 1996). This hypersensitivity is dependent upon the presence of ATP and Mg^{2+}. Furthermore, in

the absence of ATP and Mg^{2+} Ku70 contacts the upper-strand of double-stranded NRE1 while Ku80 is absent (Giffin et al., 1999). ATP and Mg^{2+} induce Ku80 to make intimate contacts with the upper-strand of double-stranded DNA. On the single upper-strand of NRE1 however both Ku80 and Ku70 make contacts (Giffin et al., 1999; Torrance et al., 1998). Together these observations suggest that in the presence of ATP and Mg^{2+} Ku perturbs the structure of the DNA double helix, likely reflecting some degree of strand separation. The EMSA reactions used to measure the kinetics of binding to NRE1 were done in the presence of EDTA, indicating that very little free Mg^{2+} was present. Under these conditions Ku would be inefficient at inducing structural changes to the DNA, and Ku80 would not contact the upper strand, possibly explaining the relatively slow on-rate on double-stranded NRE1 as compared to the single-strand. Interestingly, DNA-PK activity has been suggested to require both single- and double-stranded DNA (Leuther et al., 1999; Suwa et al., 1994).

In contrast to the on-rates, the rates of dissociation from NRE1 were very slow (Figure 5). Dissociation from double-stranded NRE1 had a half-time of 60 min, while that for upper-strand was 30 min. The unidentified lower-strand binding factor did not release from the DNA under the standard competition conditions, and addition of 5 times the amount of competitor resulted in only a weak competition after 12 h. Interestingly, dissociation of Ku from DNA appears to require DNA ends. When Ku is allowed to bind linear DNA and the DNA is then converted to a closed-circular form by ligation, the Ku-DNA complex becomes highly resistant to dissociation (Paillard and Strauss, 1991). This suggests that ends act as exit points as well as entry points for Ku on linear DNA. Furthermore, this means that Ku must be able to translocate to the end in order to

dissociate. When bound to NRE1, Ku appears to also be able to translocate along the DNA, as indicated by its ability to induce DNaseI and $KmNO_4$ hypersensitive sites in regions flanking NRE1 in footprinting assays (Giffin et al., 1999; Giffin et al., 1996). Interestingly this hypersensitivity is dependent on Mg^{2+} and ATP. Therefore, it seems likely that in EMSA in the absence of Mg^{2+}, Ku bound at NRE1 would be unable to efficiently translocate to the end and dissociate from the DNA, possibly explaining the extended off-rates. It will be interesting to determine if addition of Mg^{2+} or ATP has any effect on the off-rates.

Protease sensitivity experiments suggested that Ku has a protease resistant DNA binding core. However, the stability of binding by this core was significantly less than binding of the intact protein (Giffin et al., 1999). This suggests a model for the structure of Ku, in which a protease-sensitive domain or domains extend from the DNA binding core, and stabilize the interaction with the DNA.

Importantly, the single-stranded binding has been suggested to be important for the transcriptional control mediated by NRE1 since the MT element, which is not bound in the single-stranded form, could not repress transcription when cloned in front of a heterologous promoter (Giffin et al., 1994) or when it replaced NRE1 in the MMTV promoter (Giffin et al., 1999). Binding to the double-stranded MT element was observed to occur with a half time of 4 min (Figure 4). This is more comparable to the rates of binding to the single-stranded forms of NRE1 than to the double-strand. This is interesting as MT does not support Mg^{2+} dependent translocation, hypersensitivity to DNA digestion agents or cross-linking of Ku80 (Giffin et al., 1999). Therefore the rapid rate of binding to MT likely does not involve structural changes in the DNA or single-

stranded DNA contacts. Since MT has only a single copy of the polypurine/polypyrimidine direct repeat of NRE1 it is possible that there is a competition in the full-length element between binding to these repeats. In MT, with only one copy of the repeat such a competition would not occur, potentially explaining the faster on-rate. The off-rate for MT was found to be 4 hours. This is considerably slower than the off-rates observed for the double- or upper-stranded NRE1. This would be consistent with the model in which translocation of Ku from the binding site is necessary for dissociation from the DNA, since MT does not support translocation of Ku even in the presence of Mg^{2+} or ATP (Giffin et al., 1999).

The kinetics of binding of recombinant purified Ku to upper NRE1 were also measured. Purified Ku bound to the upper NRE1 with a half-time of 1.6 minutes (Figure 10), which agreed closely with the result obtained with crude extracts. The dissociation of purified Ku from the upper strand occurred with a half-time of 68 min (Figure 11), which was approximately two-fold longer than that observed using crude extracts. Whether this discrepancy was due to differences in the conditions used to prepare the purified Ku and the crude extracts or reflected an effect of a factor in the crude extracts is unclear. Interestingly, in addition to Ku, a second unidentified factor was observed during purification of the upper-NRE1 binding activity (Torrance et al., 1998). This factor would be present in the Jurkat crude nuclear extracts and therefore may influence the ability of Ku to dissociate from the DNA. It appears unlikely that this factor is binding the DNA directly, however, as it was observed to migrate at a lower mobility than Ku in EMSA experiments, and such a slower migrating complex was not observed while determining the kinetics of binding to NRE1 by factors in Jurkat nuclear extracts.

(ii) The Affinity of Ku for NRE1

Following the identification of NRE1 as a sequence-specific DNA binding site for Ku it became important to determine the relative affinities of Ku for NRE1 and DNA ends. To this end a quantitative Scatchard analysis was performed by measuring binding of purified Ku to a double-stranded DNA microcircle containing the NRE1 element and lacking ends in EMSA. Using this technique the equilibrium binding constant was determined as K_d = 0.84+/-0.24 nM (Figure 8) which is comparable to the binding constants of many transcription factors such as GR, AR (Chalepakis et al., 1990; Rundlett and Miesfeld, 1995) and Oct-1 (Verrijzer et al., 1990). This value is three-fold higher than what has been reported for Ku binding to DNA ends (K_d = 2.4 nM) in an EMSA system (Blier et al., 1993). This is in contrast to competition results which suggested that the affinity of Ku for NRE1 was at least an order of magnitude higher than that to DNA ends (Giffin et al., 1997; Giffin et al., 1996). The discrepancy may reflect differences in the conditions used to perform the Scatchard analysis. Regardless this demonstrates that NRE1 is the highest affinity binding site reported for Ku to date.

Following the identification of Ku as the upper-strand NRE1 binding activity it became important to determine the affinity of Ku for this form of DNA. Therefore another Scatchard analysis was performed by measuring Ku binding to a linear NRE1-containing oligonucleotide. Ku does not appreciably bind to single-stranded DNA ends, thus only Ku binding to the single-stranded NRE1 sequence would be detected in our assay (Griffith et al., 1992; Mimori and Hardin, 1986; Tuteja et al., 1994). With this technique we determined the equilibrium binding constant for Ku binding to single-

stranded NRE1 was K_d = 3.5 +/- 1.3 nM (Figure 9). This is marginally higher than what had been reported for Ku binding to double-stranded DNA ends (Blier et al., 1993). Despite this it was demonstrated using competition experiments that upper-stranded NRE1 was bound by Ku with higher affinity than double-stranded ends when compared under identical conditions (Torrance et al., 1998). This discrepancy is again likely due to differences in the conditions used to perform the Scatchard analysis. Taking together the results of the competition assays and the equilibrium binding constants we have concluded that double-strand NRE1 is the highest affinitiy Ku binding site, while upper-strand is bound with intermediate affinity. Double-stranded DNA ends are bound with lower affinity.

(iii) Binding of Jurkat nuclear factors to NRE1 in a southwestern assay

The southwestern blotting assay was originally carried out in an attempt to establish conditions to screen an expression library for the NRE1 binding factors. Using double stranded NRE1 as a probe in these blots revealed 4 NRE1 binding factors of 185, 145, 88 and 67 kD (Figure 6). Given the eventual identification of the Ku heterodimer as the double-stranded NRE1 binding factor it is tempting to speculate that the two smaller NRE1 binding factors correspond to Ku80 and Ku70 respectively. If this holds true it would suggest that both Ku subunits can interact with NRE1 in isolation. This would further distinguish NRE1 binding from end binding as Ku80 in isolation is unable to bind DNA ends while Ku70 has been observed to interact with DNA ends in southwestern assays and immunoprecipitation assays (Wang et al., 1994a; Wang et al., 1994b) but not in EMSA where both Ku subunits are required (Griffith et al., 1992; Ochem et al., 1997; Ono et al., 1994; Wu and Lieber, 1996). In UV cross-linking assays only Ku70 cross-

linked to the double-stranded DNA in the absence of Mg^{2+} however addition of Mg^{2+} and ATP induced Ku80 to also make double-stranded contact (Giffin et al., 1999). In the southwestern assay the hybridizations were performed in the presence of 2 mM Mg^{2+}. Therefore the binding of the 67 and 88 kD factors to NRE1 in the southwestern assay correlate with the contact with NRE1 by Ku70 and Ku80 under similar conditions. Both Ku subunits were demonstrated to interact with a minimal replication origin (Ruiz et al., 1999), another proposed sequence-specific Ku binding site, in southwestern assays. Ku however does not recognize this site when incorporated into microcircles[1].

Interestingly ligated copies of the MT element were recognized by only the 76 kD species. This correlates with the fact that only Ku70 can cross-link to the MT element, even in the presence of Mg^{2+} and ATP. This result suggests that using the ligated MT element to screen an expression library would have been successful at identifying Ku70 as a MT binding factor.

The identity of the two larger species binding to NRE1 is unknown but it would appear that they are binding with at least some specificity as they are not competed by the 500-fold excess of salmon sperm DNA in the hybridization reactions, nor do they recognize a non-specific probe (Figure 7). By using EMSA to compare the mobility shift of double-stranded NRE1 by factors in Jurkat nuclear extracts with purified Ku, we determined that Ku was the only factor in crude extracts binding to double-stranded NRE1 (Giffin et al., 1996). Therefore these higher molecular weight factors are not likely relevant for NRE1 binding.

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[1] W. Giffin and R. Haché, unpublished observation

(iv) Cross recognition of NRE1 binding factors with an octamer motif

The observation that the NRE1 binding factors in crude nuclear extracts could cross recognize an octamer motif, in both southwestern assays (Figure 6) and EMSA (Figure 7) was at first surprising but following the identification of Ku as the NRE1 binding factor an explanation became evident. Ku, or a Ku-like protein, had previously been reported to bind to the octamer motifs of the immunoglobulin genes (May et al., 1991). We later determined that an octamer motif was not bound directly by Ku when incorporated into a DNA microcircle (Giffin et al., 1997). Nevertheless it appears to stably associate with this motif as its interaction is not competed by an excess of competitor DNA in either of the two assays. Likely what this reflects is that the octamer motif is a 'pause' site for Ku. In this scenario, following binding of Ku to the DNA ends, it translocates to the octamer motif where it pauses due to a more stable interaction with the DNA.

2. Ku and DNA-PK are required for the NRE1 mediated repression of glucocorticoid-induced MMTV transcription

Since NRE1 proved to be a specific binding site for the Ku autoantigen I set out to determine what role Ku and DNA-PK had in the repression mediated by NRE1. Using mutant cell lines I demonstrated the role of Ku and DNA-PK_{CS} in the repressive effect of NRE1 on glucocorticoid-induced MMTV transcription. The V15B cell line is a mutant cell line belonging to X-ray cross complementation group IR5. This cell line had an inactivating mutation in the Ku80 gene (Errami et al., 1996). In the parental cell line V79, NRE1 repressed the glucocorticoid-induced transcription by approximately 10 fold.

In the V15B cells however this repressive effect was lost. Introduction of a gene for Ku80 into these cells rescued the NRE1 mediated repression (Figure 12). This demonstrates that Ku is required for the repression through NRE1. Neither the presence nor absence of NRE1 or Ku had a significant effect on the basal levels of transcription in these cells. A similar experiment was performed using the *scid* cell line Sf7. The *scid* phenotype is a result of an inactivating mutation of the DNA-PK_{CS} gene (Araki et al., 1997; Blunt et al., 1996; Danska et al., 1994). In wild type CB17 cells NRE1 repressed glucocorticoid induced transcription by 4 fold on MMTV reporters (Figure 13). In *scid* cells there was no significant effect of NRE1. I was unable to perform a complementation experiment, in this case since at the time the experiment was performed there was no cDNA for DNA-PK available. The basal activities of the MMTV reporters in these cells were too low to measure, however we can conclude the repression of glucocorticoid-induced transcription is lost in Sf7 cells and thus this appears to require DNA-PK.

The observation that the repressive effect of NRE1 was specific for glucocorticoid-induced transcription, at least in V79/V15B cells, suggested that Ku and DNA-PK_{CS} had a repressive effect on glucocorticoid-activated transcription. The DNA-activated kinase activity of DNA-PK provided an obvious potential regulatory mechanism. The downstream target of this regulation would most likely be a factor involved in glucocorticoid-activated transcription, the most likely being the GR itself, as it directly mediates the effects of glucocorticoids. For this reason I concentrated my efforts on determining what role DNA-PK kinase activity had on glucocorticoid activated transcription on the MMTV promoter. Although GR was the most likely target of

regulation by DNA-PK there were several others, such as Oct-1 which synergizes with GR to activate transcripition or GRIP-1, SRC-1 or other coactivators involved in transmitting the transcriptional response to glucocorticoids. It was also possible that DNA-PK acted in a manner independent of its kinase activity.

Since DNA-PK only efficiently phosphorylates its substrates when they are co-located to the same DNA molecule (Gottlieb and Jackson, 1993) it suggested that only the sub-population of GR that bound to the MMTV GREs at the same time as DNA-PK bound to NRE1 would be phosphorylated. There are two implications of this: first it would suggest that any repressive effects mediated by the DNA-PK kinase activity would likely be highly promoter-specific. These would occur only on promoters which contained binding sites for DNA-PK and its phosphorylation targets. The second implication was that if GR were an *in vivo* target of DNA-PK only the sub-population of GR that co-occupied the MMTV promoter with DNA-PK would be phosphorylated. Thus it would likely be difficult to identify DNA-PK specific phosphorylation sites using an *in vivo* approach. I therefore utilized the strategy outlined in Figure 14.

3. Phosphorylation of serine 527 of rat GR by DNA-PK

(i) Identification of serine 527 as a DNA-PK specific phosphorylation site

The *in vitro* strategy used to identify the DNA-PK mediated phosphorylation sites on GR proved successful and a single *in vivo* phosphorylation site on the native wild type rat GR was identified. This site was comprised of a DNA-PK consensus SQ motif and is proximal to acidic residues that appear to comprise preferred DNA-PK phosphorylation sites (Anderson and Lees-Miller, 1992). This unique site was identical to the site

identified in a recombinant GR DBD peptide (GST-GR_{X568}) (Giffin et al., 1997). This was somewhat surprising as the full-length GR had several SQ motifs in addition to serine 527 however, none were recognized by DNA-PK.

Serine 527 of rat GR is highly conserved among mammalian glucocorticoid receptors, being conserved in species from tree shrew to human. The region surrounding serine 527 including the following glutamine 528, which forms the DNA-PK consensus motif, and the glutamic acid 529 which provides an acidic charge. This conservation suggests that regulation by phosphorylation at serine 527 may occur in other species as well. If this is true, it would indicate that other mechanisms are involved as the MMTV promoter is specific for mice. Such regulation may or may not involve DNA-PK. It appears however that this would be specific for mammals as neither Xenopus nor trout GRs display this conservation.

The identification of an extraneous phosphorylation site in the mycGR demonstrates the problems that are inherent to using fusion proteins. The source of this phosphorylated peptide is unknown as it did not correspond to any site within GR or the myc tag. A detailed examination of the peptide map of mycGR determined it was not due to incomplete trypsinization or phosphorylation of tyrosine residues. A possibility may have been a myc interacting protein that co-immunoprecipitated with mycGR and was recognized by DNA-PK. Phosphorylation of wild type GR not carrying the myc tag demonstrated that whatever the source of this phosphorylated peptide, it was not present on the native receptor.

(ii) The effect of serine 527 phosphorylation on GR transcription

Mutation of serine 527 of GR to alanine resulted in a complete loss of NRE1 mediated repression of glucocorticoid-induced transcription (Figure 27). Therefore blocking phosphorylation at the site which was mapped as the *in vitro* DNA-PK phosphorylation site correlates to the repressive effect which was shown to require DNA-PK (Figure 13) and which is mediated by a specific DNA binding site for Ku/DNA-PK_{CS} (Giffin et al., 1999). This provides strong evidence that DNA-PK mediated phosphorylation of GR on the MMTV promoter results in decreased GR transcriptional activity. No direct evidence yet exists that demonstrates *in vivo* phosphorylation of serine 527 by DNA-PK. According to our hypothesis, only GR molecules which co-occupy the MMTV promoter with DNA-PK will be phosphorylated on serine 527. Therefore, only a sub-population of GR would be expected to be phosphorylated on this site *in vivo*. Thus it is not surprising that phosphoserine 527 has not been previously observed. Two lines of evidence suggest that additional *in vivo* phosphorylation sites exist. First, in the HPLC profiles of the phosphotryptic peptides of mouse GR a number of minor peaks were observed that were not identified (Figure 3, panel 3) (Bodwell et al., 1991; Bodwell et al., 1998). Moreover, the seven phosphorylation sites identified in mouse GR account for only 80% of the total receptor phosphate (Bodwell et al., 1991; Mason and Housley, 1993). This suggests that additional minor phosphorylation sites occur *in vivo*.

Two approaches can be used to obtain direct evidence that serine 527 is phosphorylated *in vivo*. First, if the tryptic peptide containing serine 527 does represent one of the minor peaks observed in the HPLC profiles, then blocking phosphorylation at that site by mutation to alanine should result in the disappearance of that peak in the

HPLC profile. Moreover, if this phosphorylation is indeed specific for DNA-PK then the same peak should not be observed in DNA-PK deficient cells. A second approach would be to generate an antibody specific for phosphoserine 527. Such an antibody could be used in immunofluorescence assays *in vivo* to visualize GR phosphorylated at serine 527 within the cell.

Interestingly, substitution of serine 527 for aspartic acid or glutamic acid resulted in a receptor that could activate transcription of MMTV in response to glucocorticoids regardless of the presence of NRE1 (Figure 27). Two conclusions can be drawn from this observation. First, the presence of a negative charge at position 527 of rat GR is insufficient for the NRE1 mediated repression. If a negative charge introduced by phosphorylation were responsible for the repression mediated by NRE1 the S527D and S527E mutations would have been expected to result in a diminished activity in either the presence or absence of NRE1. Instead, these mutant receptors appeared fully active in either case. Repression therefore appears to require the precise conformation of GR at position 527 provided by phosphoserine. This result is not surprising as a number of cases have been described where substitution of a phosphorylation site with aspartic acid or glutamic acid have not mimicked the effect of phosphorylation (Parker et al., 1998). Additionally, this observation provides indirect evidence that a negative charge at this position per se does not affect the intrinsic properties of GR such as DNA binding, dimerization or ligand binding since the S527A and S527E mutants were fully able to activate transcription in response to glucocorticoids.

In the functional assays to determine the effect of mutating serine 527 of GR (Figure 27), the wild type and mutant receptors carried the c-myc epitope tag used in

mapping the DNA-PK phosphorylation sites on GR. It was demonstrated in Figure 24, panel B that these tagged receptors have a phosphorylation site that isn't present on native GR. This raises the question of whether this extraneous phosphorylation had any effect on the transcriptional activity of the receptors and influenced the results of the functional assays. Although this can not be ruled out, in these assays mutation of serine 527 to alanine resulted in a transcriptional activity approximately equal to that of the wild type receptor on the MMTV promoter lacking NRE1. Therefore, mutation of the single phosphorylation site on native GR completely restored the repressive effect of NRE1. This indicates that the extraneous phosphorylation site was not involved in the NRE1 mediated repression. It was observed that the fold-induction of transcription by glucocorticoids was lower with the tagged receptors as compared to the native receptors. For example wild type mycGR essentially did not induced transcription in response to Dex on the reporter containing NRE1. The native receptor however was observed to induce transcription on this same reporter in the same cell type (compare Figure 27, panel A with Figure 12). This suggests that, in comparison to the native receptors, either the ability of the myc-tagged receptors to induce transcription in response to Dex was diminished or that they increased basal transcription. This effect may have been due to the presence of the c-myc tag, the presence of the extraneous phosphorylation site or the absence of the N-terminal 21 amino acids of GR in these receptors.

(iii) How does phosphorylation of serine 527 affect transactivation by GR?

In keeping with the previous model in which co-occupancy of the MMTV LTR by DNA-PK and GR is required for phosphorylation, then a requirement for any model explaining the effect of serine 527 phosphorylation on GR would be the initial binding to

DNA by GR. In this light the ability of GR to activate transcription in response to glucocorticoids could be affected by serine 527 phosphorylation via a number of mechanisms. These are described schematically in Figure 28. One possibility is that an activity intrinsic to GR could be affected (panel A). For example, since serine 527 occurs in the hinge region of GR in close proximity to the DBD it is possible that the DNA binding ability of GR could be affected. This could happen by two mechanisms. First, following the initial binding to the GRE the subsequent phosphorylation by DNA-PK could destabilize the base-specific contacts made by the N-terminal Zn^{2+} finger in the DBD (Luisi et al., 1991), causing a dissociation of GR from the GRE. DNA binding by GR has been proposed to follow a 'hit-and-run' mechanism where it is continuously binding and dissociating from its binding sites (Fletcher et al., 2000; McNally et al., 2000). If this is true, then phosphorylation would result in an increased off-rate. Alternatively, since the C-terminal Zn^{2+} finger in the DBD contains the amino acids responsible for dimerization (Dahlman-Wright et al., 1992; Danielian et al., 1992; Luisi et al., 1991; Umesono and Evans, 1989) DNA binding could be influenced indirectly by a decrease in the stability of the GR homodimers, again resulting in GR dissociating from the GRE. Another possibility is that phosphorylation of serine 527 could decrease the ligand binding affinity. The resulting dissociation of the ligand from the receptor would lead to the loss of the AF-2 activity in the LBD as well as dissociation from the GRE and eventual recycling into the hsp/immunophilin complex. A final intrinsic property of GR that could potentially be affected by this phosphorylation is its intracellular localization. Although NL1 is located in close proximity to serine 527, and DNA-PK has been shown to influence nuclear localization of the SV40 T-Ag which has a NLS similar to NL1

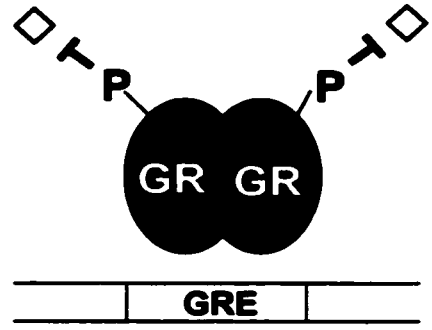
A

Base Specific

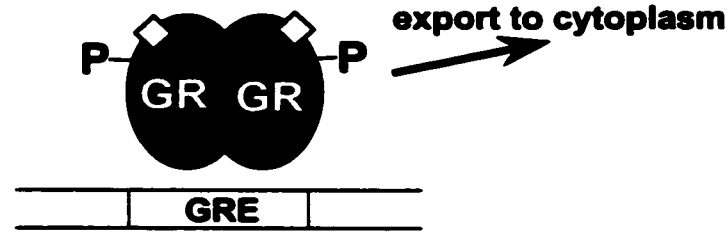
Dimerization

DNA Binding:

Ligand Binding:



Nuclear Export:



B

Recruitment

Interference

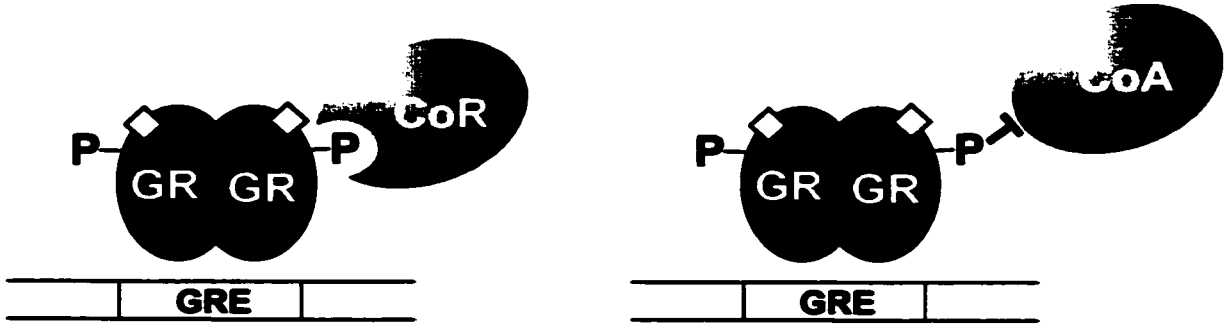


Figure 28. Possible mechanisms to explain how phopshorylation of serine 527 of GR by DNA-PK decreases trancriptional activity of GR.

Phosphorylation of serine 527 of GR by DNA-PK leads to a repression of the glucocorticoid activated transcription of MMTV. A number of mechanisms explaining the possible effects on GR are shown. Phosphorylation could have an effect on the intrinsic activities of GR (Panel A). Phoshorylation could affect the DNA binding either directly by interfering with base specifc contacts, or indirectly by interfering with homodimerization. The ligand binding activity could also be impared, or the rate of nuclear export increased. Alternatively phosphorylation of serine 527 of GR could affect a target downstream of GR (Panel B). Two possibilities are shown, phosphorylation could mediate a recruitment of a corepressor (Co-R) or it could interfere with recruitment of a coactivator (Co-A).

(Xiao et al., 1996; Xiao et al., 1997), nuclear import is not the likely effect of serine 527 phopshorylation. The reason for this is that for GR to be phosphorylated by DNA-PK it must already be nuclear and bound to DNA. It is formally possible, however, that phosphorylation of GR at serine 527 could increase the rate of nuclear export thereby preventing it from activating transcription.

Phosphorylation of serine 527 could also affect interactions between GR and downstream acting factors thereby decreasing its transactivation potential. Two possible models are immediately apparent. In the first, phosphorylation at serine 527 would block the interaction between GR and a coactivator such as GRIP-1 or SRC-1. In this 'interference' model (Figure 28, panel B) the coactivators would be unable to mediate transcriptional activation. Its important to note that in the transcription assays using transiently transfected DNA, the MMTV LTR reporter genes do not form organized chromatin (Archer, 1993; Archer et al., 1991; Ogryzko et al., 1998) and therefore the transactivation observed is independent of chromatin. Nevertheless, phosphorylation at serine 527 could inhibit the ability of coactivators to recruit TBP or RNA pol II. To date the region in proximity to serine 527 has not been observed to mediate interactions between GR and coactivators. However it is interesting to note that this region has been reported to contain a weak transactivation activity (Hollenberg et al., 1987; Miesfeld et al., 1987; Schena et al., 1989) suggesting coactivator interactions could occur here.

Alternatively, serine 527 phosphorylation could promote the interaction with a repressive activity. In this 'recruitment' model, a factor binding in a phosphoserine 527 dependent manner could inhibit the ability of GR to activate transcription. This factor could be a corepressor that actively inhibits transcription. Corepressors such as NCoR

and SMRT are components of complexes which contain histone deacetylases and mediate the opposite effect of HATs (Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997). Although the repressive effect of serine 527 phosphorylation appears to be independent of chromatin, a corepressor could mediate repression of GR via a novel mechanism. To date no corepressors have been identified that interact with GR, however it is interesting to note that corepressors bind to the hinge region of TR (Horlein et al., 1995) and that serine 527 occurs in the homologous region of GR. Another possibility is that following phosphorylation of serine 527 a factor is recruited to GR that inhibits transcriptional activity through a mechanism involving interference or stearic hindrance. In this scenario the factor would bind to GR through an interaction involving phosphoserine 527 and prevent coactivators or other downstream factors from interacting with GR.

When the effects of phosphorylation rely on the precise conformation of the phosphorylated domain, substitution of the phosphorylated residue with an acidic residue is typically not phosphomimetic. This is because the acidic residues, although providing a compensating negative charge, do not precisely mimic the conformation of the phosphorylated amino acid. This is observed in the interaction between the kinase inducible domain (KID) of CREB and the KIX domain of CBP (Parker et al., 1998). Phosphorylation of serine 133 in the KID is required for the recruitment of CBP through the KIX domain and subsequent transcriptional activation (Cardinaux et al., 2000). Conformational studies reveal that the KID domain is largely unstructured in the free state but adopts a precise helix-linker-helix conformation upon binding to KIX (Radhakrishnan et al., 1997). Substitution of serine 133 with glutamic acid blocks this

conformation from forming and prevents both binding by KIX and transcriptional activation (Parker et al., 1998).

The fact that a constitutive negative charge at position 527 does not interfere with the ability of GR to induce transcription in response to glucocorticoids indicates that the conformation of the region surrounding phosphoserine 527 is the determining factor in the repressive effect of this phosphorylation. The carboxylate group of aspartic or glutamic acid can not mimic the phosphate group on phosphoserine 527. The charge in and of itself is insufficient to mediate the repressive effect of phosphorylation. Conceptually this argues for the 'recruitment' model over the 'interference' model. One would predict that the formation of a protein-protein interaction, as in the 'recruitment' model, would be dependent on the conformations of the binding surfaces of the involved proteins. In this case the interaction could be sensitive to subtle conformational changes within these faces such as those introduced by the substitution of phosphoserine for aspartic or glutamic acids. In the 'interference' model on the other hand, the protein-protein interaction occurs in the absence of phosphorylation of serine 527 and introduction of a phosphate at that residue disrupts the interaction. In this case the binding surface of GR would undergo a more severe alteration due to the introduction of a negative charge and one would predict that negative charges introduced by aspartic or glutamic acids would have a similarly severe effect.

Although this hypothesis remains to be proven, preliminary evidence suggests that 'recruitment' may occur; *in vitro* following phosphorylation of GR by DNA-PK, DNA-PK has been observed to remain bound to GR[2]. If this were to occur *in vivo* DNA-

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[2] W. Giffin and R. Haché, unpublished observation

PK, due to its large size, would likely stearically interfere with the binding of coactivators to GR thereby inhibiting transcriptional activation. Supporting this hypothesis is a recent report describing an interaction between the DBD of PR and DNA-PK (Sartorius et al., 2000).

4. Influence of NRE1 on viral expression and tumorigenesis

To date the role of NRE1 in the life cycle of MMTV remains unexplored. NRE1 could potentially influence both viral expression and tumorigenesis. It is especially tempting to speculate that NRE1 is important in MMTV induced T-cell lymphoma, given that in all such cases that have been examined, deletions of the LTR spanning NRE1 have been observed (Dudley and Risser, 1984; Michalides and Wagenaar, 1986; Michalides et al., 1982; Yanagawa et al., 1990). Previously these deletions have been shown to contain a negative regulatory element (Hsu et al., 1988; Yanagawa et al., 1990). Moreover, an infectious provirus clone containing this deletion induced T-cell lymphoma but not mammary tumors (Yanagawa et al., 1993). Thus these deletions appear to lead to a deregulation of MMTV expression in T-cells.

If NRE1 is a regulator of MMTV that is important to the life cycle of the virus, then mutagenesis of this sequence could be expected to influence viral expression and tumorigenesis. This could be assessed by infecting mice with a viral clone harboring a mutation in NRE1 that blocks Ku binding (Giffin et al., 1999), but does not alter the coding sequence of the SAg. Systems for generating infectious MMTV and infecting mice have been described (Qin et al., 1999; Shackleford and Varmus, 1988). The effect of blocking Ku binding to NRE1 could then be determined by monitoring infected mice

for tumor development. Any effect on viral expression could also be determined by comparing the viral levels in target tissues with those generated by wild-type virus.

The role of Ku and DNA-PK in any NRE1 mediated effects could be addressed directly by infecting Ku- or DNA-PK-deficient mice with either the NRE1 mutant or wild-type virus. The high level of occurrence of T-cell lymphoma in $Ku70^{-/-}$ (Gu et al., 1997; Li et al., 1998) or $DNA\text{-}PKCS^{-/-}$ (Jhappan et al., 1997) mice would preclude these strains from being used. More appropriate would be $Ku80^{-/-}$ mice in which no lymphoma has been observed (Nussenzweig et al., 1996) or *scid* mice in which low levels of T-cell lymphoma occur (Custer et al., 1985).

V. CONCLUSIONS

1. Characterization of a negative regulatory element in the MMTV LTR (NRE1) revealed nuclear factors binding to the double-stranded form, as well as each of the single strands. A truncated form of this element (MT) was also bound in the double-stranded form but not the single-stranded forms. Kinetic studies showed binding occurred to the ds, up, lo and MT forms with $t_{1/2}$s of 11, 1.5, 3 and 4 min respectively. The off-rates were determined to be 60 min, 30 min, 12 h and 4 h respectively. The binding occurs at rates typical for transcription factors but is unusually stable. Moreover the kinetics of binding to each form of NRE1 are clearly distinguishable.

2. Four factors were observed to bind NRE1 in southwestern analysis, while only one was observed binding to MT. The four factors appeared to cross-react with an octamer motif. The dsNRE1 binding factor was confirmed to recognize an octamer motif in EMSA. This is consistent with the identification of Ku as the NRE1 binding factor which has been reported to bind to octamer motifs.

3. The double-stranded NRE1 binding factor is the Ku autoantigen heterodimer. Ku was demonstrated to bind directly to NRE1 with an affinity of K_d = 0.84 +/- 0.24 nM. Ku also bound upper NRE1 with an affinity of K_d = 3.5 +/- 1.3 mM. Ku bound to upper NRE1 with an on-rate of $t_{1/2}$ = 1.6 min while the off-

rate was $t_{1/2}$ = 68 min. This demonstrated that double-stranded NRE1 was the highest affinity binding site, upper-stranded NRE1 was bound with intermediate affinity, while DNA ends and structures were bound at a lower affinity.

4. Transient transfection assays using MMTV reporters either containing or lacking NRE1 were performed. Using mutant cell lines not expressing functional Ku or its associated factor DNA-PK, it was demonstrated both factors were required for the NRE1 mediated repression of glucocorticoid-induced transcription of MMTV.

5. DNA-PK was shown to specifically phosphorylate a mycGR on serine 527 *in vitro*. A second phosphorylation site on mycGR was determined to result from the addition of the myc tag, while a native receptor was phosphorylated only on serine 527. Therefore, serine 527 is the only relevant DNA-PK phosphorylation site in native GR. Transient transfections using serine 527 mutants of GR demonstrated that subsitution of serine 527 for alanine abrogated NRE1 mediated repression. Substitutions for aspartic acid and glutamic acid also abrogated this effect.

6. From these results I propose a model in which Ku binds the NRE1 element in the MMTV LTR, and recruits DNA-PK_{CS}. Upon activation by glucocorticoids, GR binds to the HRE in the MMTV LTR and is phosphorylated by DNA-PK on serine 527. This phosphorylation results in a decrease in the transcriptional activation by GR.

VI. REFERENCES

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VII. APPENDIX A

Table 4. Map of phosphorylation sites within the tryptic peptides of mycGR.

All peptides containing serine or threonine generated by digestion of mycGR with trypsin are listed in order of the position of the serine or threonine residues within the peptide. Peptides may be listed multiple times depending on the number of serine or threonine residues within the peptide. The sequence of the peptide is followed by the corresponding position of the N-terminus of the peptide within native rat GR, lacking the myc tag (N-term). Peptides within the myc tag are indicated by myc followed by a number representing the position within the tag. The release cycle indicates the position of the serine or threonine within the peptide, which would be indicated by a release of ^{32}P in Edman degradation should that residue be phosphorylated. The S/T position indicates the position of the corresponding serine or threonine within native rat GR lacking the myc tag. Serines or threonines within the myc tag are indicated by myc followed by the number representing the position within the tag. The # of S/Ts indicates the total number of serines or threonines within the peptide and hence the number of times that peptide is listed in the map. AspN, GluC, Chymo and CnBr indicate the position of the corresponding serine or threonine within the tryptic peptide after digestion with the secondary proteases AspN, GluC, chymotrypsin or cyanogen bromide respectively. These are only listed for tryptic peptides with serine or threonine at positions 8 or 10. Two classes of chymotrypsin digestion sites exist, those which are strongly cleaved (F, Y, W) and those which are weakly cleaved (L, M, A, D, E). The position of the serine or threonine after strong cleavage is indicated in regular script while the position after weak cleavage is indicated in subscript.

mycGR Tryptic Peptides Containing S/T	N-Term	Release Cycle	S/T Position	# of S/T's	Asp-N	Glu-C	Chymo	CnBr
SLR	33	1	33	1				
STSVPENPK	153	1	153	3				
SSTSATGCATPTEK	162	1	162	7				
THSDASSEQQNR	180	1	180	4				
SQTGTNGGSVK	193	1	193	4				
SDLLIDENLLSPLAGEDDPFLLEGNTNEDCK	236	1	236	3				
TEK	298	1	298	1				
SVFSNGYSSPGMR	407	1	407	4				
TIVPAALPQLTPTLVSLLEVIEPEVLYAGYDSSVPDSAWR	537	1	537	7				
SYR	630	1	630	1				
TLLLLSSVPK	686	1	686	3				
SQELFDEIR	700	1	700	1				
TMSIEFPEMLAEIITNQIPK	762	1	762	3				
GSVMDFYK	25	2	26	1				
VSASSPSVAAASQADSK	42	2	43	6				
GSTSNVQQR	69	2	70	3				
STSVPENPK	153	2	154	3				
SSTSATGCATPTEK	162	2	163	7				
DTNESPWR	228	2	229	2				
DTGDTILSSPSSVALPQVK	279	2	280	6				
MSAISVHGVSTSGGQMYHYDMNTASLSQQQDQK	336	2	337	7				
QSSGNLLCFAPDLIINEQR	633	2	634	2				
MSLPCMYDQCK	652	2	654	1				
MTYIK	709	2	710	1				
YSNGNIK	782	2	783	1				
LISEEDLNEMEQK	myc 5	3	myc 7	1				
LISEEDLNEMEQK	myc 18	3	myc 20	1				
LISEEDLNEMEQK	myc 31	3	myc 33	1				
LISEEDLNEMEQK	myc 44	3	myc 46	1				
LISEEDLNEMESLGDLTMEQK	myc 57	3	myc 59	3				
LISEEDLNSCSPQGR	myc 78	3	myc 80	3				
GSTSNVQQR	69	3	71	3				
AVSLSMGLYMGETETK	102	3	104	4				
STSVPENPK	153	3	155	3				
SSTSATGCATPTEK	162	3	164	7				
THSDASSEQQNR	180	3	182	4				
SQTGTNGGSVK	193	3	195	4				
IMTTLNMLGGR	577	3	579	2				
QSSGNLLCFAPDLIINEQR	633	3	635	2				
TMSIEFPEMLAEIITNQIPK	762	3	764	3				
GGATVK	36	4	39	1				
VSASSPSVAAASQADSK	42	4	45	6				
GSTSNVQQR	69	4	72	3				
SSTSATGCATPTEK	162	4	165	7				
LYPTDQSTFDLLK	204	4	207	3				
CQGSGEDSLTSLGALNFPGR	387	4	390	4				

SVFSNGYSSPGMR	407	4	410	4				
PDVSSPPSSSSAATGPPPK	420	4	423	7				
IMTTLNMLGGR	577	4	580	2				
LQVSYEEYLCMK	674	4	677	1				
EGNSSQNWQR	727	4	730	2				
LLDSMHEVVENLLTYCFQTFLDK	739	4	742	3				
VSASSPSVAAASQADSK	42	5	46	6				
AVSLSMGLYMGETETK	102	5	106	4				
LLEESIANLNR	142	5	146	1				
SQTGTNGGSVK	193	5	197	4				
DLEFSAGSPSK	217	5	221	3				
DTNESPWR	228	5	232	2				
DTGDTILSSPSSVALPQVK	279	5	283	6				
MSAISVHGVSTSGGQMYHYDMNTASLSQQ QDQK	336	5	340	7				
PDVSSPPSSSSAATGPPPK	420	5	424	7				
EGNSSQNWQR	727	5	731	2				
FYQLTK	733	5	737	1				
ILLDFSK	62	6	67	1				
SSTSATGCATPTEK	162	6	167	7				
THSDASSEQQNR	180	6	185	4				
LCLVCSDEASGCHYGVLTCGSCK	439	6	444	4				
GIQQATAGVSQDTSENPNK	518	6	523	4				
HMLFVSSELQR	663	6	668	2				
TLLLLSSVPK	686	6	691	3				
VSASSPSVAAASQADSK	42	7	48	6				
THSDASSEQQNR	180	7	186	4				
LYPTDQSTFDLLK	204	7	210	3				
PLILPDTK	267	7	273	1				
HMLFVSSELQR	663	7	669	2				
TLLLLSSVPK	686	7	692	3				
LYPTDQSTFDLLK	204	8	211	3	3	-	$5_{,3}$	-
DLEFSAGSPSK	217	8	224	3	-	2	$4_{,2}$	-
DTGDTILSSPSSVALPQVK	279	8	286	6	5	-	$_{1}$	-
DDFIELCTPGVIK	301	8	308	1	7	3	$5_{,2}$	-
CQGSGEDSLTSLGALNFPGR	387	8	394	4	2	2	$8_{,1}$	-
SVFSNGYSSPGMR	407	8	414	4	-	-	1	8
PDVSSPPSSSSAATGPPPK	420	8	427	7	7	-	$_{6}$	-
LISEEDLNSCSPQGR	myc 78	9	myc 86	3				
SQTGTNGGSVK	193	9	201	4				
DTGDTILSSPSSVALPQVK	279	9	287	6				
LGPVYCQASFSGTNIIGNK	317	9	325	3				
SVFSNGYSSPGMR	407	9	415	4				
PDVSSPPSSSSAATGPPPK	420	9	428	7				
NLHLDDQMTLLQYSWMFLMAFALGWR	604	9	612	2				
SSTSATGCATPTEK	162	10	171	7	-	10	$_{5}$	-
DLEFSAGSPSK	217	10	226	3	-	7	$6_{,4}$	-
MSAISVHGVSTSGGQMYHYDMNTASLSQQ QDQK	336	10	345	7	10	-	$10_{,7}$	9
CQGSGEDSLTSLGALNFPGR	387	10	396	4	4	4	$10_{,3}$	-
PDVSSPPSSSSAATGPPPK	420	10	429	7	9	-	$_{8}$	-
LCLVCSDEASGCHYGVLTCGSCK	439	10	448	4	4	2	$10_{,2}$	-
GIQQATAGVSQDTSENPNK	518	10	527	4	10	10	3	-
LISEEDLNSCSPQGR	myc 78	11	myc 80	3				
SDLLIDENLLSPLAGEDDPFLLEGNTNED CK	236	11	246	3				

DTGDTILSSPSSVALPQVK	279	11	289	6				
LGPVYCQASFSGTNIIGNK	317	11	327	3				
CQGSGEDSLTSLGALNFPGR	387	11	397	4				
PDVSSPPSSSSAATGPPPK	420	11	430	7				
TIVPAALPQLTPTLVSLLEVIEPEVLYAG YDSSVPDSAWR	537	11	547	7				
LISEEDLNEMESLGDLTMEQK	myc 57	12	myc 68	3				
VSASSPSVAAASQADSK	42	12	53	6				
SSTSATGCATPTEK	162	12	173	7				
DTGDTILSSPSSVALPQVK	279	12	290	6				
MSAISVHGVSTSGGQMYHYDMNTASLSQQ QDQK	336	12	347	7				
AVSLSMGLYMGETETK	102	13	114	4				
LGPVYCQASFSGTNIIGNK	317	13	329	3				
PVFNVIPPIPVGSENWNR	369	13	381	1				
GIQQATAGVSQDTSENPNK	518	13	530	4				
TIVPAALPQLTPTLVSLLEVIEPEVLYAG YDSSVPDSAWR	537	13	549	7				
PDVSSPPSSSSAATGPPPK	420	14	433	7				
GIQQATAGVSQDTSENPNK	518	14	531	4				
NLHLDDQMTLLQYSWMFLMAFALGWR	604	14	618	2				
LLDSMHEVVENLLTYCFQTFLDK	739	14	752	3				
AVSLSMGLYMGETETK	102	15	116	4				
TMSIEFPEMLAEIITNQIPK	762	15	776	3				
VSASSPSVAAASQADSK	42	16	57	6				
TIVPAALPQLTPTLVSLLEVIEPEVLYAG YDSSVPDSAWR	537	16	552	7				
LISEEDLNEMESLGDLTMEQK	myc 57	17	myc 73	3				
VMGNDLGYPQQGQLGLSSGETDFR	118	17	134	3				
VMGNDLGYPQQGQLGLSSGETDFR	118	18	135	3				
LCLVCSDEASGCHYGVLTCGSCK	439	18	456	4				
LLDSMHEVVENLLTYCFQTFLDK	739	19	757	3				
VMGNDLGYPQQGQLGLSSGETDFR	118	21	138	3				
LCLVCSDEASGCHYGVLTCGSCK	439	21	459	4				
QQQQQQQQQQQQQQQQQQQPGLSK	78	23	100	1				
MSAISVHGVSTSGGQMYHYDMNTASLSQQ QDQK	336	23	358	7				
MSAISVHGVSTSGGQMYHYDMNTASLSQQ QDQK	336	25	360	7				
SDLLIDENLLSPLAGEDDPFLLEGNTNED CK	236	26	261	3				
MSAISVHGVSTSGGQMYHYDMNTASLSQQ QDQK	336	27	362	7				
TIVPAALPQLTPTLVSLLEVIEPEVLYAG YDSSVPDSAWR	537	32	568	7				
TIVPAALPQLTPTLVSLLEVIEPEVLYAG YDSSVPDSAWR	537	33	569	7				
TIVPAALPQLTPTLVSLLEVIEPEVLYAG YDSSVPDSAWR	537	37	573	7				

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Publications

Heather Torrance, Ward Giffin, **David J. Rodda**, Louise Pope and Robert J. G. Haché. **(1998).** Sequence-Specific Binding of Ku Autoantigen to Single-Stranded DNA. *Journal of Biological Chemistry*. **273**: 20810-20819.

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Ward Giffin, Heather Torrance, **David J. Rodda**, Gratien G. Préfontaine, Louise Pope and Robert J.G. Haché. **(1996).** Sequence-Specific DNA Binding by Ku Autoantigen and its Effects on Transcription. *Nature*. **380**: 265-268.

David J. Rodda, Ward Giffin and Robert J.G. Haché. **(1995).** Multi-Strand Binding of Nuclear Factors to a Repressor of Mouse Mammary Tumor Virus Transcription can be Distinguished Kinetically. *Biochemical and Biophysical Research Communications*. **209**: 379-384.

David J. Rodda and Hiroshi Yamazaki. **(1994).** Poly(vinyl alcohol) as a Blocking Agent in Enzyme Immunoassays. *Immunological Investigations*. **23**: 421-428

Conference Presentations

David J. Rodda, Ward Giffin and Robert J. G. Hache. Distortion of DNA Structure Upon Factor Binding to a DNA Sequence Element that Represses Glucocorticoid Induced MMTV Transcription. *The Endocrine Society Annual Meeting*, **June 1995**.

Heather Torrance, **David J. Rodda**, Gratien G. Prefontaine, Ward Giffin and Robert J. G. Hache. Phosphorylation of the Glucocorticoid Receptor on the MMTV Promoter by DNA-PK is Directed Through a Sequence-Specific Binding Site for Ku Autoantigen. *The Endocrine Society Annual Meeting*, **June 1995**.

Research Experience

DNA: subcloning, minipreps, maxipreps, PCR, site-directed mutagenesis, sequencing.

Protein: SDS-PAGE, Western blotting, Immunopreciptitation, Kinase Assays, Protease sensitivity assays, Bacterial expression, Phospho-amino acid analysis.

Peptides: Alkaline Gel Electrophoresis, HPLC, Manual Edman degredation.

DNA/Protein Interactions: Electrophoretic mobility shift assays, affinity and kinetic assays, South-western blotting, DNA

pulldown assasys.

Protein/Protein Interactions: GST-pulldown assays, Co-immunoprecipitation.

Tissue Culture: Culture of numerous cell types, Transient and stable transfections, CAT assays.

Current Research Project

My research has focused on characterizing a transcriptional negative regulatory element (NRE1) in the long terminal repeat of the mouse mammary tumour virus (MMTV). Early during my research we identified the factor binding specifically to this element as the Ku autoantigen. I proceeded to characterize the binding of Ku to its binding site using electrophoretic mobility shift assays. Ku is known to be the DNA binding subunit of the DNA-dependent protein kinase (DNA-PK). By performing transient transfection assays in wild type and mutant cell lines I showed that both Ku and DNA-PK were required for the transcriptional repression of MMTV mediated by the NRE1 element. MMTV transcription is strongly activated by glucocorticoids. Interestingly the NRE1 mediated repression is specific for glucocorticoid-activated transcription, while it has little effect on basal transcription. I showed that the glucocorticoid receptor (GR) is a substrate for DNA-PK. This suggested that DNA-PK specific phosphorylation of GR results in a decrease in the trans-activation potential of GR. To test this hypothesis I mapped the DNA-PK specific phosphorylation site on GR to a serine in the hinge domain of the receptor using an approach involving specific protease digestion and manual Edman degredation. I then used site-directed mutagenesis to alter the amino acid at this site. Interestingly, when the serine is replaced with an amino acid that can't be phosphorylated, the NRE1 mediated repression is lost. This is significant in that it is the first demonstration of an in vivo effect of the kinase activity of DNA-PK as well the first demonstration of an in vivo effect of a phosphorylation site in GR. Currently I am conducting a series of experiments designed to characterize the mechanism by which phosphorylation of GR at this site results in the loss of trans-activation potential.