MOUSE MAMMARY TUMOR VIRUS GENE EXPRESSION: REGULATION VIA CHROMATIN STRUCTURE AND GLUCOCORTICOID HORMONES

by

STUART CHARLES FEINSTEIN

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ABSTRACT

Initially, two mammary tumor virus (MTV) proviruses, integrated at different chromosomal loci in the genome of two clonal isolates of cultured rat hepatoma (HTC) cells, were investigated. One cell line, J2.17, expresses MTV RNA only in the presence of glucocorticoid hormones, whereas proviral genes in the other line, J2.15, are not transcribed in the presence or absence of glucocorticoids; nonetheless, proviral genes and cellular components that mediate hormone responses appear intact and normal. Mild DNase I digestion of chromosomes in isolated nuclei reveals that the J2.17 MTV DNA sequences are packaged in highly DNase I sensitive chromatin, whereas J2.15 MTV chromatin is relatively nuclease resistant. These results demonstrate that the same genetic element, located at two different chromosomal loci within a single cell, can differ in both chromatin structure and gene expression. Analysis of the chromatin structure of appropriate DNA sequences in uninfected HTC cells suggests that the difference in the chromatin structure of the two proviruses reflects a "spreading effect", in which integrating DNA is packaged into chromatin similar in configuration to surrounding chromatin. Thus, it is proposed that chromosomal position determines the folding pattern of newly introduced DNA sequences; this pattern in turn determines whether the genes can subsequently transcribe in response to the hormonal inducing signal.

In B13, another single copy MTV infected HTC cell derivative, glucocorticoid induction of MTV transcription is associated with a marked alteration in DNase I sensitivity of the proviral sequences. Taken together, these results with J2.17, J2.15 and B13 imply that at least two classes of both nuclease sensitive and resistant chromatin exist. In addition, kinetic analyses of the DNase I sensitivity of multiply infected mouse lymphoid cells suggest that MTV sequences integrated at different loci within a single cell are stably maintained in several apparently distinct folding configurations. Finally, analysis of a border between structural domains showed it to be sharply delineated; its position is unaffected by proximal MTV DNA integration (9000bp). Such studies begin to assess factors that govern establishment and maintenance of chromatin structural domains.

This dissertation is dedicated

to the memory of my father,

Joseph Feinstein

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MATERIALS AND METHODS

Cells and Tissue Culture

The rat hepatoma line HTC 4.1 (162) and its MTV infected clonal derivatives J2.15 and J2.17 have been described (128,129). B13 and B7 are both MTV infected HTC 4.1 clonal derivatives. Infections and clonal isolations were carried out as described above by Bonnie Maler. Line 71.12 is also an MTV containing HTC cell line, isolated by Vicki Chandler.

Cultures were propagated in monolayer in Dulbecco's modified Eagle's medium or in suspension in Swim's 77 (both obtained from GIBCO); in all cases, media were supplemented with 5-10% horse serum.

The W7 cell line and its infected subclone M4.12H (infected as above) are both derived from a Balb/c mouse lymphoma. T1M1 and VL3 are both derived from C57B1 mouse lymphomas. Lymphoma cells were grown in static suspension at 37°C. in Dulbecco's modified Eagle's medium supplimented with 10% horse serum. For procedures regarding cloning lymphoma cells and colony screening procedures, see Peterson and Yamamoto, (manuscript in preparation).

Nuclear Isolation

Unless otherwise stated, all procedures were performed at 0-4°C. HTC cells (2-3 X 10^8) were harvested by centrifugation, washed twice in 25 ml phosphate buffered saline (PBS) and

suspended in 20mM Tris HCl pH=7.4, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.3% Nonidet-P40 at 2 x 10^7 cells/ml. After swelling for 10 minutes, cells were disrupted using the tight fitting pestle of a Dounce homogenizer; greater than 99% breakage was confirmed by light microscopy. Nuclei were harvested by centrifugation at 300 x g for 7 minutes; the pellet was resuspended gently and washed once in DNase I buffer (10 mM Tris HCl pH=7.4, 10 mM NaCl, 3 mM $MgCl_2$), then resuspended in DNase I buffer to A_{260}=10 (measured following 1:20 dilution into 1% sodium dodecyl sulfate (SDS)).

Lymphoma and spleen nuclei were isolated by a slight modification of the above procedure. After washing in PBS, cells were suspended directly into DNase I buffer, allowed to swell 10 minutes on ice, and disrupted in the Dounce homogenizer. Nuclei were subsequently washed and treated as described above.

DNase I Digestion and DNA Isolation

Nuclear suspensions were brought to 37°C. and DNase I (Worthington DPFF) added to 1 µg/ml. At each time point, two aliquots were taken: (1) 250 µl of nuclei was removed and added to an equal volume of cold 7% perchloric acid, spun 2 minutes in an Eppendorf microcentrifuge, and the A_{260} of the supernatents recorded as a measure of extent of digestion; (2) for DNA isolation, a 0.5-1 ml aliquot was removed into one volume of 20 mM Tris HCl pH=7.4, 200 mM NaCl, 2 mM EDTA,

1% SDS, 0.2 mg/ml proteinase K. After overnight incubation at 37°C., DNA was purified by extraction with 1 volume neutralized phenol and 0.5 volume chloroform, followed by extraction with one volume chloroform; aqueous fractions were dialyzed against 10 mM Tris HCl pH=7.4, 1 mM EDTA, and DNA concentrations were determined colorimetrically.

Restriction Digests, Blotting and Autoradiography

Eco RI and Pst I were generous gifts from Pat Greene and Dave Peterson, and John Majors, respectively; Msp I was from New England Biolabs, Hpa II was from Boeringer-Manheim, and Hind III from Bethesda Research Laboratories. Digestion conditions were as described in the New England Biolabs catalogue; Bacteriophage lambda DNA was used to monitor completion of each reaction. Reaction volumes were 0.15-0.5 ml; after digestion, DNA was concentrated by ethanol precipitation. Specific DNA fragments were isolated from pBR322 by appropriate restriction enzyme digestion followed by either electrophoresis on Sea Plaque agarose (Marine Colloids) or sucrose gradient centrifugation on 10-40% gradients (1 M NaCl, 20 mM Tris HCl pH=7.4, 10 mM EDTA) in an SW 40 rotor, 48 hours, 32K at 15°C.

To label hybridization probes, DNA inserts were isolated. Nick translations were performed essentially according to Rigby et al. (163), employing 10 μM ^{32}P-dATP (New England Nuclear; 600 Ci/mmole) and 30 μM each of dGTP, dCTP and dTTP

in 25-100 µl reactions containing 50 mM Tris HCl pH=7.4, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 10 µg/ml DNA. A 1 mg/ml DNase I stock (Worthington DPFF) was freshly diluted 1:6000 into 50 mM Tris HCl pH=7.4, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 100 µg/ml bovine serum albumin, and added to 2% (v/v) to the reaction mixture. After incubation for 2 minutes at room temperature and 2 minutes at 16°C., E. Coli DNA polymerase I (Boehringer Mannheim, 4545 units/ml) was added to 2% (v/v) to the reaction, and incubation at 16°C. continued for 25 minutes. Reactions were stopped by addition of one volume of 200 mM Na_3EDTA followed by 10 minutes at 68°C. Unincorporated isotope was removed by chromatography on Sephadex G-50; specific activities were 2-4 X 10^8 cpm/µg.

Following fractionation by electrophoresis through 0.6% agarose (Seakem), DNA was transferred to nitrocellulose filters in 6 x SSC (1 x SSC = 150 mM NaCl, 15 mM Na citrate). Filters were air dried, baked in vacuo at 80°C. for 2 hours, and incubated in sealed plastic bags for 6-18 hours at 41°C. in 5 ml/200 cm^2 annealing mix (3 X SSC, 50% v/v formamide, 50 µg/ml denatured salmon sperm DNA, 5 X Denhardt's solution (0.1% each of bovine serum albumin, polyvinyl pyrollidone, and ficoll) prior to hybridization.

Hybridization with nick translated ^{32}P-DNA was started by adding 5 X 10^6 cpm/200 cm^2 filter to 10 ml annealing mix, heating for 5 minutes at 68°C., injecting the probe into the plastic bags containing the filters, and mixing thoroughly.

Incubation was continued at 41°C for three days; alternatively, hybridization buffer was supplemented with 10% (w/v) dextran sulfate, and annealed for 16-20 hours. Filters were washed for 1-2 hours with multiple buffer changes at 50°C. in 0.1 X SSC, 0.1% SDS, air dried and autoradiographed for 2-8 days at -70°C. with Kodak RP Royal X-omat film and a DuPont Cronex "Lightening Plus" intensifying screen.

Hybridization Reagents

Phage lambda 7.1a is a lambda gtWES recombinant carrying a fragment of MTV DNA that includes 85% of the sequences present in the intact virus; it was isolated and given to our laboratory by John Majors. Subsequently, the viral insert was subcloned into the Eco RI site of pBR322 and designated pMTV1. The Pst I "b" fragment of DNA was a subclone of lambda 71.a, which was subcloned into pBR322 by David Ucker. The recombinant plasmids p17.1, p17.2 and p15.1 were isolated by David Ucker and Susan Ross.

Data Evaluation

In principle, the autoradiographic data obtained in these experiments should be amenable to quantitative analysis, for example, by densitometric scanning; indeed, Bellard et al. (95) have described such an analysis. In our view, several practical limitations merit consideration in evaluating this approach. First, because different gene sequences residing in different

differentiated cell types are often compared in studies of this type, differences in DNase I sensitivity cannot be ascribed to differences in chromatin structure alone; however, our experiments focus on comparison of the chromatin structure of a single genetic element, MTV DNA, present at different loci within a single genetic and physiologic background, HTC cells. Second, the relative contributions of certain technical artifacts are difficult to assess. Thus, DNA fragments of different sizes diffuse from agarose gels with differential efficiencies, and bind to nitrocellulose with yet toher differential efficiencies. The effect of a third variable, in which fragments of different sizes present different substrate "targets" for DNase I, can be more readily estimated. The probability p of cleaving a given DNA fragment of length l is:

$$p = 1 - (1 - \frac{l}{L})^m$$

where L is the length of the entire genome, and m is the total number of cleavage events. Note that l/L <<1, so that

$$(1 - l/L)^m \simeq 1 - \frac{ml}{L} .$$

Thus, the probability function can be approximated by:

$$p = 1 - (1 - \frac{lm}{L}) .$$

Therefore, the relative probability of fragments l_1 and l_2 being cleaved in a digest of m cleavage events on genome L, is:

$$\frac{P_1}{P_2} = \frac{1 - (1 - \frac{l_1 m}{L})}{1 - (1 - \frac{l_2 m}{L})} = \frac{l_1}{l_2} .$$

That is, for two DNA fragments, the relative probability of cleavage is proportional to their relative lengths. We conclude that for chromatin fragments that differ in size by a factor of two or less, target size contributes in only a minor way to the persistence or disappearance of one fragment relative to the other.

Other Procedures

Solution hybridizations were performed as described (135), Densitometry was performed on an integrating Zeineh Soft Laser scanning densitometer.

INTRODUCTION

In this study, two interted questions have been approached. First, how do steroid hormones function to regulate eukaryotic gene expression? Second, how does chromatin structure (the supramolecular complex of protein and nucleic acids comprising the chromosomes) relate to the activity of eukaryotic genes?

TRANSCRIPTION AND REGULATION IN SIMPLE SYSTEMS

In prokaryotic organisms, gene expression is frequently regulated at the transcriptional level (1-3). Transcriptional promotors, those sequences where positive regulatory factors and RNA polymerase act, can be grouped into two distinct classes. Constitutive promoters function without intervention of positive regulatory factors whereas the second class of promoters contains insufficient sequence information to produce an efficient initiation complex without intervention of accessory factor(s). This mechanism allows for modulatory positive control of transcription by requiring specific DNA binding proteins as regulatory accessory factors. In addition, transcription of prokaryotic genes can be negatively regulated by the binding of specific repressor molecules to appropriate operator sequences.

The regulation of bacterial catabolic operons by the cyclic AMP receptor protein (CRP) appears particularly relevant to steroid hormone action (4-7). In response to diminished glucose levels, cellular cAMP levels rise, resulting in cAMP binding to CRP. cAMP binding likely results in a conformational change in the CRP, resulting in its increased affinity for specific DNA sequences within the promoters of the regulated operons (3).

An extraordinarily detailed mechanistic understanding of cAMP-CRP action has been derived from intensive biochemical and genetic analyses (4). In the lactose operon of E. coli, cAMP-CRP is a strong positive regulator of transcription, specifically binding at a sequence within the lac promotor that is precisely defined both genetically and biochemically (6-10). Importantly, basal and induced transcription initiates at the same nucleotide in vitro (10), suggesting that cAMP-CRP somehow increases the efficiency of initiation by RNA polymerase at a single promoter, rather than activating a second promoter; for example, both helix destabilizing activity and direct cAMP-CRP interaction with RNA polymerase have been proposed (4). Clearly, these models are not mutually exclusive.

Additionally, the lac operon is negatively controlled (11). The lac repressor normally binds to the lac operator, minimizing lac operon trancription. In the presence of β-galactosides which bind to the lac repressor, the repressor molecules release from the operator sequence, permitting

expression in combination with positive regulation by cAMP-CRP. Thus, both positive and negative regulatory mechanisms modulate the transcription of lac DNA.

Molecular understanding of regulated eukaryotic gene expression is considerably less detailed. At least in some cases, the general regulatory circuitry may be consistent with models of prokaryotic control. For example, during lytic infection by SV40, expression of the viral gene A product (T antigen) is required to initiate late transcription, as well as each new round of viral replication (12); T antigen autoregulates transcription of its own mRNA synthesis via a negative feedback mechanism (13). Recently, specific and sequential binding of T antigen to three tandem sites at the origin of replication and transcription has been demonstrated (14), remarkably reminiscent of lambda repressor and cro binding in prokaryotes (15). Finally, direct repression of transcription by T antigen has recently been demonstrated (16).

Another attractive system in which to examine regulated eukaryotic gene expression is yeast, which can be readily manipulated genetically. As an example, the genetic organization of gene products responsible for galactose utilization has been analyzed in some detail. There are three genetically linked structural genes, coding for galactokinase, transferase and epimerase. In addition, the unlinked gene products gal 4 and gal 80 appear to function as inducer and repressor proteins,

respectively. Hawthorne proposed a model where, in the absence of galactose, transcription at the locus of the gal 4 inducer protein is repressed via the constitutively expressed gal 80 product (17). The model further states that in the presence of galactose, the sugar binds and inactivates the gal 80 repressor, allowing synthesis of the gal 4 inducer; it is proposed that this results in the transcriptional induction of the galactose utilization structural genes. A more recent model has been suggested by Perlman and Hopper (18), designed to reconcile certain inconsistencies between the Hawthorne model and recent data. The new model suggests that the gal 80 product inactivates the gal 4 inducer protein by direct protein-protein interaction. In the presence of galactose, the sugar binds the gal 80 repressor, releasing the gal 4 product to subsequently induce transcription of the galactose utilization structural genes. Thus, regulatory circuitry in eukaryotic organisms may involve unique patterns distinct from those observed in prokaryotic organisms. This emphasizes the need to remain unbiased regarding the molecular interactions regulating eukaryotic gene expression.

STEROID HORMONES AND REGULATION OF EUKARYOTIC GENE EXPRESSION

Steroid hormones are fundamental effector molecules, eliciting important differentiative and developmental responses in essentially all animal tissues; expression of a few genes appears to be altered in a tissue specific manner. Though

physiological effects of steroid hormones have been known for many years, insights into molecular mechanisms have been forthcoming only relatively recently (19). All steroid hormones appear to function via a common mechanism (see figure 1). Upon entering the target cell, the steroid molecules specifically bind to soluble receptor proteins (20). This binding presumably alters the conformation of the receptor molecule allosterically, resulting in increased affinity for nuclear components. Rapid nuclear translocation of the hormone receptor complex ensues. At 37°C., only one minute is required from the time hormone is administered to cultured cells until the majority of receptors have migrated to the nucleus (21). The result is that steady state levels of a few specific mRNA species are altered in a tissue specific manner (22-27). Initial events subsequent to nuclear entry by the steroid receptor complex are largely unknown. In vitro reconstruction, coupled with genetics, will undoubtedly be required to dissect these molecular events.

That nuclear migration and binding of steroid receptor complexes is significant for appropriate biological responsiveness is demonstrated by several lines of evidence. Nuclear translocation occurs rapidly relative to observed biological responses (21). Anti-inducers, which bind receptor without causing nuclear translocation, do not evoke a biological response (28). Furthermore, variant S49 cells with altered receptors aberrant in nuclear translocation fail to respond normally to

steroid hormones (29).

Though it is widely believed that DNA is an important component of the hormone receptor complex nuclear binding site, the precise nature of this site is an issue of some controversy. In vitro, hormone receptor binding has been claimed to numerous nuclear components, including DNA (30), chromatin (31), nuclear membrane (32) and acidic (33) and basic (34) nuclear proteins.

Biochemical evidence supporting DNA as being an essential component of the nuclear binding site can be summarized as follows. DNase, but not RNase, releases hormone receptor complexes from nuclei (35,36). Several different steroid hormone-receptor complexes bind DNA cellulose (29,30). Further, a time and temperature dependant activation is required in vivo for the hormone receptor complex to translocate to the cell nucleus (37-38); in vitro, identical treatment is required for binding to DNA cellulose (30). In addition, anti-inducer compounds which bind receptor but fail to translocate to the nucleus in vivo do not stimulate hormone receptor complex binding to DNA in vitro (39). Genetic analysis, an essential tool providing in vivo significance for in vitro biochemical data (such as DNA cellulose chromatography), also suggests that DNA is an important component of the hormone receptor complex nuclear binding site. In one particularly important study, glucocorticoid receptor variants were selected in cultured cells (29). These variant cells were characterized

for *in vivo* receptor activities and *in vitro* DNA binding properties of their respective receptors. Receptors that bind hormone but do not accumulate in the nucleus *in vivo* displayed markedly reduced affinity for DNA *in vitro*. Conversely, variant receptors that bind hormone and translocate to the nucleus *in vivo* more avidly than wild type bound with greater affinity to DNA cellulose than wild type receptor. Thus, biological activity of receptor variants correlated remarkably well with *in vitro* DNA binding activity of these receptors.

In summary, steroid hormones enter the cell, bind specific soluble receptor proteins, and rapidly translocate to the nucleus. Within the nucleus, the hormone receptor complex binds to sites likely composed, at least in part, by DNA. The net result is altered transcriptional activity at a few precise loci.

Thus, steroid receptors appear functionally analagous to prokaryotic DNA binding regulatory proteins. However, prokaryotic transcriptional proteins display high affinity, sequence specific DNA binding properties, as well as lower affinity non-specific DNA binding. A model proposing the existance of a limited number of high affinity specific DNA binding sites for steroid receptors to mediate transcriptional regulation has been proposed (19,40). It has been suggested that up to 1000 specific DNA binding sites could exist, yet be experimentally masked from traditional approaches by a vast excess of non-

specific binding, analagous to properties observed for CRP (41), lambda repressor (42) and other DNA binding proteins. It is not particularly surprising that proteins with high affinity for a particular DNA sequence also have a reduced but measurable affinity for DNA of any sequence.

Recent work by Gronemeyer and Pongs in Drosophila provides strong cytological evidence that receptors for the steroid ecdysone bind specifically at or near target genes whose activities are directly affected (43). It should be noted that cytological evidence does not address the chemical nature of the binding site. However, the advent of recombinant DNA technology provides a means to isolate precise fragments of DNA. This allows a direct test of the proposed specific DNA binding properties of steroid receptors in the absense of presumed overwhelming, non-specific DNA binding noise. Very recent data (44) has clearly demonstrated specific binding of the glucocorticoid receptor protein to mouse mammary tumor virus DNA, a sequence whose transcription is strongly regulated by glucocorticoid hormones (see below).

Changes in the steady state level of specific mRNA species in response to steroid hormones can be viewed as falling into two broad classes (19). Primary responses result directly from the interaction between the hormone-receptor complex and its genomic binding sites; they occur with rapid kinetics, are sensitive to the action of transcriptional inhibitors such as actinomycin D, and are refractory to

translational inhibitors such as cycloheximide. Examples of such responses include glucocorticoid induction of mouse mammary tumor virus (24) and metallothionin (45) RNA's, as well as estrogen induction of vitellogenin RNA (46). Furthermore, glucocorticoid induction of tyrosine aminotransferase (TAT) also appears to be primary (47), though direct proof awaits cloning of TAT coding sequences for direct nucleic acid hybridization studies.

In contrast, secondary responses appear to involve a requisite accumulation of intermediate factor(s) for induction of the particular product being examined. Estrogen mediated induction of ovalbumin, an intensively investigated phenonenon, appears to fall into this catagory. Ovalbumin mRNA induction occurs only after a significant time lag, and does not occur in the presence of cycloheximide (48-50). Thus, estrogen may induce one or more intermediate proteins, which in turn induce ovalbumin transcription in a cascade of events. An alternative explanation has been presented by Palmiter et al., who suggest a rate limiting translocation of receptors from an initial non productive chromatin binding site to productive binding sites (48); this proposal does not readily account for the cycloheximide sensitivity of ovalbumin induction. In any event, this situation clearly makes a molecular analysis of steroid action in this system considerably more cumbersome and complex.

Remarkable visual demonstration of both primary and

secondary responses to steroid hormones can be observed in Drosophila, where individual genetic loci can be cytologically visualized in salivary giant chromosomes (51). Administration of the steroid hormone ecdysone initially produces a primary induction at several loci, sensitive to actinomycin D but not to cycloheximide. Shortly thereafter, successive waves of secondary inductions occur, sensitive to both actinomycin D and cycloheximide administration concurrently with ecdysone.

In summary, steroid hormone action is biologically important and appears amenable to experimental analysis. Effector molecules are known and available in labelled form, biological effects can be initiated synchronously in cell populations, and the kinetics and mechanisms of activation of specific genes analyzed.

CHROMATIN STRUCTURE AND REGULATION OF GENE EXPRESSION

Within a eukaryotic nucleus, DNA exists as a folded, nucleoprotein complex termed chromatin, rather than as naked DNA. Therefore, DNA recognition sequences for specific DNA binding proteins may be sensitive to modulation by the precise molecular nature of chromatin at the specific DNA recognition site. Further, in order to be physically confined within a nucleus, eukaryotic DNA must be compacted 10^5 fold into chromosomes, while at the same time remaining accessible for transcription and replication. Molecular analysis of chromosome structure has revealed an elementary subunit, the nucleosome.

It contains a proteinaceous center consisting of two each of four histone molecules (H2a, H2b, H3 and H4) that represent an octameric core structure, surrounded on the outside by approximately 146 base pairs of DNA (52,53). Nucleosomes are cylindrical (57 X 110 angstroms) with the DNA making roughly 1.75 turns around each subunit (54); these fundamental particles appear to be ubiquitous in all eukaryotic organisms. Between successive nucleosomes is a linker or spacer region of DNA (55), containing a single molecule of histone H1 (56); H1 is thought to be located on the linker DNA at the site where DNA enters and exits the nucleosome, perhaps to partially "lock" the DNA in position (57).

That the DNA is on the outside of the protein core is demonstrated by neutron scattering data (58) and by nucleolytic attack of chromatin. Certain nucleases cut the DNA at ten base pair intervals (59), likely related to the repeat of the helix (60). However, the conformation of DNA on the core is unknown. Models for kinks in the DNA at ten base pair intervals have been proposed (61,62), but no conclusive evidence supports this contention. Alternatively, based on energetic considerations, a gentle continuous bend of the duplex DNA around the core is also plausible (63). Nucleosomes are assembled in a linear array seen in the electron microscope as a 100 angstrom wide fibril (64,65); this structure is believed to condense into a 300 angstrom wide solenoidal helical structure, with 6-7 nucleosomes per turn (65-67). To

form a mitotic chromosome, this structure must be further condensed 100-fold. Huge loops have been proposed to provide this extra level of condensation (68-72).

Both transcriptionally active and inactive sequences are packaged in nucleosomes (73,74,79,80). Still, it is clear that all chromatin is not equivalent. Early light microscopy revealed diffuse euchromatin and highly compacted heterochromatin, subsequently shown to be transcriptionally active and inactive, respectively (75,76). Further, it was demonstrated that a given sequence could be euchromatic under one set of circumstances, and heterochromatic under other circumstances (77,78).

A significant advance toward understanding the inter-relationships between chromosome structure and transcriptional activity has been obtained by the utilization of nucleases as probes of chromatin structure. In 1975, Weintraub and Groudine demonstrated that, in chick erythrocyte nuclei, transcriptionally active globin genes are preferentially susceptible to nucleolytic attack by DNase I, whereas transcriptionally quiescent ovalbumin sequences are relatively resistent to such attack (79). Conversely, Garel and Axel showed that in oviduct, where ovalbumin is transcriptionally active and globin is inactive, the ovalbumin gene is DNase I sensitive while globin sequences are relatively resistant (80). That transcriptionally active sequences are DNase I sensitive now appears to be a general phenomenon. Other enzymes have been utilized as probes of chromatin structure, yielding results

generally analogous to those with DNase I. These include micrococcal nuclease (81,82), DNA polymerase (83), E. Coli RNA polymerase (84) and various restriction enzymes (85).

Altered ribosomal chromatin structure has been observed within ribosomal genes not actually undergoing transcription (86). Moreover, biochemical studies indicate that the DNase I sensitive structure is assembled on chromatin within three minutes of replication, even on rarely transcribed sequences (87). Thus it appears that DNase I sensitivity may actually precede and perhaps thereby facilitate transcription, rather than occurring simply as it consequence.

Is it the structure of the nucleosome itself, or the interaction of multiple nucleosomes in higher order structures that confers nuclease sensitivity? Weintraub and Groudine (79) reported that DNase I sensitivity can be demonstrated on isolated nucleosomes, whereas Garel and Axel (80) argued that preferential digestion of ovalbumin sequences was lost in isolated nucleosomes. However, Senear and Palmiter (88) recently showed that at 4°C., the ovalbumin sequences in isolated nucleosomes are indeed preferentially attacked by DNase I, but that this sensitivity is lost at 37°C.; thus, one view is that factors mediating DNase I sensitivity in isolated nucleosomes may be more labile in oviduct than in erythrocytes.

It appears that the high mobility group (HMG) proteins 14 and 17 are somehow involved in mediating DNase I sensitivity.

Removal of HMG proteins results in the loss of preferential sensitivity of globin sequences (89). DNase I sensitivity is restored when HMG 14 and 17 are reconstituted back onto chromatin (89). Further, in vitro studies show binding of HMG 14 and 17 to total isolated core particles (90).

Ongoing transcription itself does not seem essential for DNase I sensitivity. Garel, Zolan and Axel demonstrated that both rarely and frequently transcribed sequences are equally sensitive to DNase I (91). Further, in terminally differentiated erthrocytes or oviduct tubular gland cells, the globin and ovalbumin genes, respectively, remain DNase I sensitive even long after transcription ceases (79,80). Thus, DNase I recognizes the potential for transcription, rather than the transcriptional process per se.

Little is known regarding the nature of the "borders" that separate different domains of chromatin structure Chromatin fractionation procedures suggest that on the average, the so called active regions are 6000-7000 base pairs long (92). The actual significance of this is open to speculation. In one particular study, a border between DNase I sensitive and resistant regions was present within 3-5 nucleosomes of the termination of transcription (93). Similarly, recent data shows an abrupt change in chromatin structure in the flanking region adjacent to the active α globin genes (94). In contrast, the β globin and ovalbumin genes appear to be surrounded by vast regions of DNase I sensitivity (95,96).

Clearly, the mechanisms that establish and maintain these structural domains may be of tremendous importance for understanding development and differentiation.

In additon to the HMG proteins discussed above, several other structural features have been noted that could be involved in regulating eukaryotic gene expression. Among these are regions of extraordinary hypersensitivity to DNase I, nucleosome phasing, histone modification, and cytosine methylation. It should be emphasized that multiple mechanisms may be required to establish an appropriate strctural configuration at a given genomic location; there is no reason to presume a single mechanism is wholly responsible.

Development of an assay designed to detect and map regions of extreme hypersensitivity to DNase I has revealed that such sites reside at several positions in and around active globin genes (97), and five heat shock genes in Drosophila (98,99), most notably immediately 5' to the structural genes. Similarly, such a sensitive structure has been shown to exist adjacent to a rat insulin gene, in a tissue specific manner (100). These sites appear to represent a different kinetic class of DNase I sensitivity, and therefore likely possess a distinct molecular nature relative to the DNAse I sensitive structures thus far discussed. One interpretation of these data is that specific DNA recognition sequences exist and are especially accessible to nucleoplasm, available for an appropriate effector molecule(s) to initiate the transcriptional

process. Alternatively, a particular protein may be bound at such hypersensitive sites and confer extreme accessibility of the DNA to the nucleoplasm.

Phasing of nucleosomes, that is, their precise alignment with respect to specific sequences, could also potentially effect transcriptional activity of a locus. The transcriptional apparatus and/or specific regulatory proteins could require specific sequences reside either in a linker region or a particular position on the nucleosome in order to be functional. Evidence has been presented claiming that bulk chromatin is not phased (101,102). However, crucial regulatory regions, or even whole coding sequences could be phased, and the bulk chromatin experiments would not detect it. In fact, recent evidence in several systems suggest that nucleosomes are phased with respect to specific sequences (103-106). It should be emphasized that the current state of this field is strictly phenomenological, and the functional role of phasing is completely speculative; that micrococcal nuclease cleaves naked DNA with a marked sequence specificity (164) considerably complicates the situation.

Histone acetylation has also been suggested to be a modification responsible for altered transcriptional behavior (107). Acetylation abolishes the positive charge of lysine residues, which are likely to be significant in ionic histone:DNA interactions. In vivo, marked histone acetylation often precedes bursts of transcriptional activity (108)

In Tetrahymena, the transcriptionally active macronucleus shows markedly greater acetylation than the transcriptionally inactive micronucleus (109). Chromatin fractionation procedures also show enrichment for acetylated histones in the active fraction (110). Addition of butyrate to cultured cells produces a hyperacetylated state by blocking deacetylase activity (111); DNase I treatment of nuclei from such butyrate treated cells preferentially releases acetate (112). Although other butyrate effects have been reported, including induction of globin mRNA synthesis in Friend cells (113) and deinduction of ovalbumin mRNA in oviduct (114), no direct role for acetylation in gene expression has been demonstrated.

Lastly, methylation of cytosine residues in eukaryotic DNA has also been suggested as a means to regulate gene expression (115-116). Precise heritable methylation patterns could provide a means to both establish and maintain developmental patterns of transcription. An inverse relationship between the expression of specific DNA sequences and their extent of cytosine methylation at the tetranucleotide CCGG has been reported, and it has been proposed that extensive methylation of a given region may be at least in part responsible for preventing expression (117-119). The validity of this proposal is currently difficult to assess, as isoschizomer restriction enzyme analysis is a rather insensitive means to determine overall extent of cytosine methylation, and efficient assays to determine total methylation levels within a single gene do

not exist.

In view of the recent progress relating chromatin structure and transcription, it is worthwhile to consider a model for eukaryotic transcriptional regulation first proposed by Yamamoto and Alberts in 1976 (19). The model suggests that specific genes are activated by specific proteins recognizing specific DNA sequences. Binding of these proteins produces an altered chromatin structure along immediately proximal chromatin creating an "active patch". Some unidentified mechanism, for example histone acetylation, propagates this active patch along the chromatin for a finite distance. In order that only specific binding events induce transcription, the model proposes that multiple binding events must occur contiguously to produce a productive large patch of altered chromatin structure suitable for RNA synthesis to initiate. Nonspecific binding events with single binding proteins could produce smaller activated patches, but would be deficient at some stage of the pathway toward productive transcriptional activation. Interestingly, in the cases of T antigen (14), cro protein (11) and lambda repressor (11), specific binding sites accomodating three molecules of the particular protein are clustered at precise sites on the DNA.

REGULATED GENE EXPRESSION OF MOUSE MAMMARY TUMOR VIRUS

Since different chromosomal structures exist at different loci in the genome, can the interrelationships between position effects, chromatin structure and regulated gene expression be experimentally examined?

Mouse mammary tumor virus (MTV) is a type B retrovirus (for review, see 120); upon infection, its single stranded RNA genome is reverse transcribed into double stranded DNA approximately 9000 base pairs long, including two 1200 bp "long terminal repeats" (LTR), one at each end of the viral genome. This MTV DNA stably integrates into the host cell genome. Transcription products include genomic length (7.8 kb) RNA and a smaller spliced RNA species of approximately 3.8 kb (121). Viral transcripts are synthesized from integrated MTV proviral DNA by the cellular RNA polymerase II (122), which also synthesizes cellular mRNA.

All inbred mouse strains contain endogenous MTV proviruses in their germ line and somatic DNA, but these proviruses are rarely expressed (123). Mammary tumors have been shown to contain extra copies of integrated MTV DNA (124) in addition to their endogenous proviruses. Similarly, MTV can infect homologous or heterologous cell lines in culture (125-127). Upon infection, MTV DNA can integrate at many genomic sites, apparently at random (128,129), allowing analysis of a single DNA sequence integrated at different loci. Integration occurs at a unique site on the viral genome (128,129).

Infected heterologous cells are especially experimentally advantageous in that there is no background of endogenous sequences; moreover, it is possible to recover infected lines bearing only a single MTV provirus, so that the cell is haploid for the genes of interest (128,129).

Expression of MTV genes is strongly regulated at the transcriptional level both in homologously and heterologously infected cells. Viral RNA concentrations vary widely as a function of tissue, host and hormonal status (130). Importantly, all mouse mammary carcinoma explants and cell lines that synthesize MTV in culture are stimulated by dexamethasone, a synthetic glucocorticoid, to yield increased levels of viral RNA (132,133).

Basal transcriptional activity can be detected in certain infected cell lines (128,129). In these lines, the site of transcriptional initiation is identical both in the absence and presence of dexamethasone (133). Therefore, the steroid hormone receptor complex seems to act by specifically increasing the efficiency of transcriptional initiation events from a single site within the MTV genome, in striking analogy to cAMP-CRP activity in the lac operon. Current evidence suggests that dexamethasone does not alter the half life of viral RNA (134,135).

That MTV RNA induction is steroid receptor mediated has been demonstrated biochemically and genetically. Dose response curves demonstrate a direct correlation between percent

receptor bound by hormone and MTV RNA induction (136). Half maximal induction of MTV RNA occurs at the same steroid concentration as half maximal induction of TAT, a host glucocorticoid responsive enzyme in HTC cells (129). Progesterone, an anti-inducer that binds receptor without nuclear translocation, elicits no induction of viral RNA (129). Further, genetic selection for cells unable to respond to dexamethasone yields primarily cells with apparent lesions in the receptor protein (29,137).

Steroid mediated induction of MTV RNA is a primary response as defined earlier. In MTV infected HTC cells, the fully induced rate of viral RNA synthesis is achieved within 15 minutes of dexamethasone addition (135,136), with a $t_{\frac{1}{2}}$ of approximately 5 minutes (133). Further, the rapid induction of viral RNA in response to dexamethasone is unaffected by translational inhibitors such as cycloheximide, but is abolished by transcriptional inhibitors, such as actinomycin D (135,136).

It has been suggested that transcriptional promoters reside in the long terminal repeats (LTR) of the integrated provirus. Importantly, recent evidence demonstrates that MTV DNA itself contains the information necessary for its own glucocorticoid regulated transcription (133). However, different clonal isolates of MTV infected cells exhibit widely varying extents of MTV RNA expression (129). Neither the degree of inducibility nor the absolute level of expression is directly related to the number of integrated proviruses.

In the extreme, some infected cell lines synthesize no MTV RNA at all, although they appear to differ from cell lines inducibly synthesizing MTV RNA only by their sites of proviral integration (128,129). Thus, despite possessing the appropriate regulatory sequences necessary for MTV transcription, MTV expression is not assured simply by integration of MTV DNA into the host genome. It appears that other regulatory mechanisms must be involved. Candidate mechanisms include chromatin structure and effects imposed as a result of position within the host genome; clearly, these possibilities are not exclusive of one another.

In summary, MTV provides an excellent experimental system to study control of eukaryotic gene expression. It is a small, mobile DNA sequence, containing a single promoter regulated by a known and partially characterized hormone receptor molecule. Further, the MTV element can apparently integrate throughout the host genome, allowing analysis of position effects on a defined, discrete genetic element. It should therefore be feasible to address the questions originally proposed in this thesis. How do steroid hormones regulate eukaryotic gene expression and what role, if any, does chromatin structure play in the mechanism of steroid hormone action?

Figure 1. Generalized Mechanism of Steroid Action. Steroid (S) hormones enter the cell, where they bind specifically to soluble receptor proteins (R). Binding causes a presumed allosteric alteration in the receptor, such that the hormone-receptor complex (SR*) has an increased affinity for nuclear sites. This altered affinity results in nuclear translocation of the hormone-receptor complex.

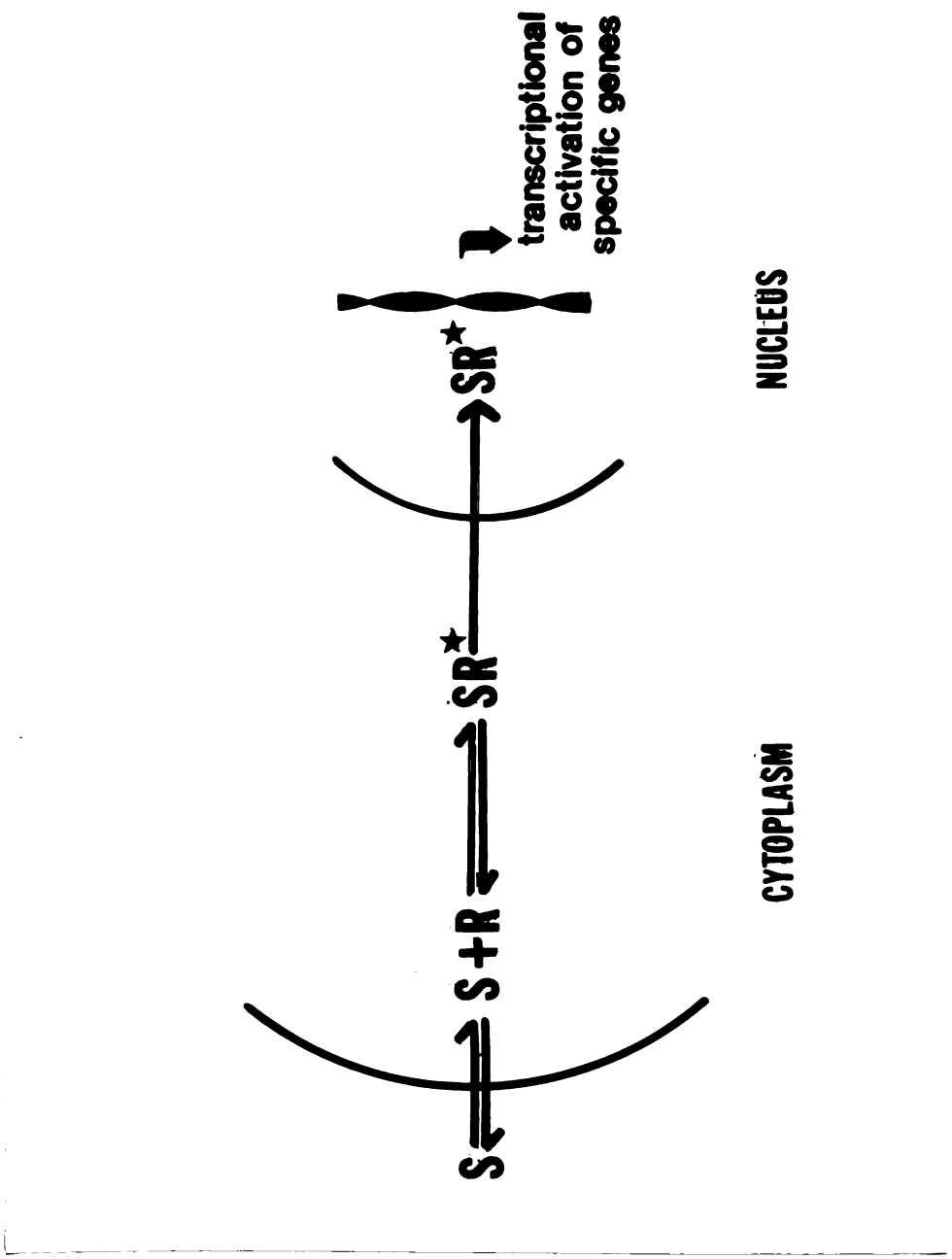
transcriptional activation of specific genes
NUCLEUS
CYTOPLASM
S+R
SR*
SR*
S

FIGURE 1

CHAPTER I

CHROMOSOMAL POSITION EFFECTS DETERMINE TRANSCRIPTIONAL POTENTIAL OF INTEGRATED MOUSE MAMMARY TUMOR VIRUS DNA

INTRODUCTION

It is likely that the regulation of specific gene transcription in eukaryotic cells occurs in some cases by mechanisms analogous to those that have been elucidated in prokaryotes. Steroid hormone receptors, for example, become associated with genomic sites upon binding a specific hormone ligand; as with regulatory proteins that act within prokaryotic operons, the interaction between the hormone receptor complex and the genomic sites somehow changes the activity of a few specific genes (for review, see 19). Indeed, recent evidence in Drosophila suggests that steroid receptors bind at or near genes whose activities are affected (43).

In addition, other levels of control appear to be involved in eukaryotic gene expression. As one example, cytogeneticists and developmental geneticists have described a phenomenon termed "position effect variegation", in which the expression of a given genetic locus depends upon aspects of the "chromosomal environment" in which it is located. Genetic rearrangements have been recovered that directly link a region of relatively uncondensed "euchromatin" with a portion of highly condensed "heterochromatin". In Drosophila, Lewis (77) established that an active euchromatic gene can be inactivated by a

stably inherited condensation that appears to spread from the nearby heterochromatic region. In mice, autosomal loci linked by translocation to the heterochromatic X chromosome are inactivated (78); as in Drosophila, loci proximal to the junction point are most strongly affected, and a gradient of inactivation, termed the spreading effect (77), can be observed on the autosome.

Expression of mouse mammary tumor virus (MTV) genes that are inserted via infection into the nuclear DNA of a cultured line of rat hepatoma (HTC) cells (128,129) have been investigated. The rate at which these genes are transcribed is rapidly and selectively stimulated by the steroid dexamethasone (133), a synthetic glucocorticoid hormone; this response is mediated by cell encoded steroid receptors (136,138). Although the precise site at which the receptors act has not been determined, recent results establish that MTV sequences are directly involved in their own regulated expression (133).

MTV proviruses integrate into the HTC genome as intact nonpermuted units of about 8.9 kilobase pairs (kbp); integration can occur at many host chromosomal loci (128,129). Interestingly, different clonal isolates of infected HTC cells, which contain MTV proviruses at different integration sites, display different extents of hormone stimulated viral RNA synthesis (128,129). In the extreme, certain lines contain MTV genes that fail to be expressed in the presence or absence of steroid, even though the proviruses themselves appear indistinguishable

from regulated and expressed counterparts integrated at other chromosomal loci (128,129).

For the purposes of this study, then, MTV can be regarded as a discrete regulatable DNA sequence located at different genomic sites in clonal derivatives of otherwise isogenic cells. Enzymatic structural probes and molecular hybridization have been employed to investigate the relationship between DNA modification, gene packaging in chromosomes, and the regulated expression of MTV genes; this approach may provide sensitive molecular probes of the nature of chromosomal position effects.

RESULTS

Experimental Strategy

Weintraub and Groudine (79) showed that when nuclei isolated from avian red blood cells are treated with DNase I, "active genes" (defined here as those with the potential to be expressed in that cell type) are preferentially digested; further studies have established that this phenomenon is a general one (80,139,140). It seemed conceivable that the clonal variation observed in the degree of expression of different MTV proviruses could reflect differences in chromosome structure that might be detectable with this enzymatic probe.

To approach this question, two clonal isolates of MTV-infected HTC cells, J2.15 and J2.17 (128,129) have been examined. Each line contains only a single MTV provirus per

cell; figure 2 shows the Eco RI and Pst I restriction endonuclease cleavage maps previously determined for the proviral and flanking cellular sequences (128). These data, as well as direct hybridization tests of cloned flanking sequences (133,141), establish that the MTV genes in these two lines have integrated into distinct loci in the HTC genome. Table 1 summarizes the results of earlier studies (128,129,133,141), showing that the MTV sequences in J2.17 cells are transcribed only in the presence of dexamethasone; in contrast, the viral genes in J2.15 cells fail to be expressed either in the absence or in the presence of the hormone. Dexamethasone appears to activate transcription of the entire MTV genome in J2.17, since the RNA produced anneals with all of the sequences in DNA probes complementary to intact virion RNA, and RNA corresponding to the full length viral genome is detected by blot (142) hybridization (143). Thus, these two HTC derivatives, isogenic except for site of insertion of the MTV DNA, allow examination of the chromatin structure of a single genetic element that is either inactive (J2.15), potentially active (J2.17 in the absence of dexamethasone), or active (J2.17 in the presence of dexamethasone).

For the experiments to be described, nuclei were isolated and incubated with 1 μg/ml DNase I for various times. DNA was then purified, digested with either Eco RI or Pst I, and assayed for surviving MTV DNA containing fragments by electrophoresis in agarose gels and blot hybridization

(144); a similar procedure for analysis of DNase I sensitivity has been used independantly by others (97,139,140). The results are presented as kinetic analyses of specific DNA fragments remaining after increasing periods of digestion. To facilitate qualitative comparisons within and between experiments, the gel lanes bearing a 0.6-0.7% acid soluble digested sample (see Materials and Methods), as well as the undigested control have been marked in each figure. Quantitative assessment of data from these experiments is complicated by several factors (see "Data Evaluation" in Materials and Methods). It is worth noting, for example, that the relative probability of cleaving two fragments of pure DNA with DNase I is a direct function of their relative lengths (see Materials and Methods). Therefore, to minimize the target size problem, fragments that differ in size by less than a factor of two have been compared whenever possible.

In certain experiments (see figures 3 and 4), the chromatin structure within and near the insulin coding sequences was monitored with a cloned cDNA probe as a control; the insulin genes are inactive in these liver derived cells. These insulin containing fragments provide convenient internal markers against which the persistence or loss of a given test fragment can be measured at a given level of digestion. Although the location of the insulin coding sequences has not been mapped onto the two Eco RI and two Pst I HTC DNA fragments homologous to this probe, one fragment from each digest is

particularly resistant to DNase I attack, suggesting that those DNA segments are packaged entirely in nuclease resistant chromatin. The second fragment from each digest, which is neither highly sensitive nor fully resistant, may be packaged in a partially DNase I sensitive structure, or may contain subregions that are relatively sensitive.

Hormone Responsive MTV Genes are Highly Sensitive to DNase I

Is the activation of gene expression by the glucocorticoid-receptor complex in J2.17 cells accompanied by changes in chromosome structure that are recognized by DNase I? In the absence of dexamethasone, the MTV sequences in J2.17 are transcriptionally inactive at the level of detection of the assays employed; from estimates of the minimal detectable rate of transcription, calculations show that if the provirus is expressed at all, it is transcribed less than once every six generations. If this situation is analogous to that of the globin genes prior to their activation during development, it might be expected that a "naive" J2.17 provirus (as found in cells never exposed to dexamethasone) will be in a chromatin conformation that is relatively resistant to DNase I.

Nuclei from J2.17 cultures were treated with DNase I to give a range between zero and 1% digestion of total nuclear DNA. After cleavage with Eco RI, blots of the undigested samples revealed the expected MTV specific fragments of 9.3 and 5.5 kbp; however, brief exposure to DNase I rendered the

MTV containing DNA undetectable in samples from either dexamethasone treated (1 μM, 12 hours) or untreated cultures (figure 3B). On the same blot, fragments containing the insulin genes can be clearly detected even after greater than 1% digestion (figure 3B and 3C). Close inspection of these results suggests that the proviral sequences might be slightly more resistant to DNase I attack in the absence of hormonal stimulation than in its presence (see also figure 4); this interesting possibility is discussed further below. In any case, the important point here is that the MTV genes in J2.17 under either condition are highly DNase I sensitive relative to the insulin genes.

To assess the DNase I sensitivity of MTV sequences over the length of the whole provirus, the experiment above was repeated with J2.17 DNA that had been digested with Pst I instead of Eco RI; this enzyme cleaves MTV DNA into five fragments, of which four are strongly detected by the probe employed (figure 4, left lane). Figure 4 shows that each of these regions of the provirus is rapidly degraded by DNase I, being digested preferentially relative to the fragments containing the insulin sequences. It is therefore concluded that under the conditions of these experiments, all of the MTV DNA in J2.17 is packaged in a chromatin configuration that is highly susceptible to DNase I attack, independant of its hormone stimulated transcription.

Nonexpressible MTV Genes Are Not Highly Sensitive to DNase I

Restriction site mapping reveals that the J2.15 and J2.17 proviruses are integrated colinearly and contain identical internal cleavage sites, but are flanked by different cellular sequences (figure 2 and 127,133,141). Indeed, all proviruses examined after infection of HTC cells are indistinguishable with respect to viral gene arrangement (128). The failure of the MTV genes in J2.15 to be transcribed in either the absence or the presence of dexamethasone (table 1) does not reflect a general defect in hormone responsiveness, since several host enzyme activities retain normal inducibility by dexamethasone in these cells (129). Figure 3A shows that the 6.6 and 5.9 kbp MTV DNA containing Eco RI fragments in J2.15 are not highly sensitive to DNase I attack. Thus, consistent with their nonexpressed phenotype in these cells, the MTV sequences persist even after 1% digestion of total DNA and appear to be approximately (although not precisely) as sensitive to DNase I digestion as are the insulin sequences.

As a further control, the entire procedure of nuclear preparation, DNAse I digestion, DNA isolation, Eco RI restriction, fragment separation and analysis was carried out on a single mixed population of equal numbers of J2.15 and J2.17 cells. Figure 5 shows that, despite their apparently identical DNA sequences (see below), the J2.17 proviral fragments are again more rapidly degraded by DNase I than are those in J2.15. This experiment also establishes directly that the sensitivity

differences observed cannot simply reflect an artifact of target size, since the two resistant fragments are larger than one of the sensitive fragments.

Relative DNase I sensitivity of MTV sequences has been analyzed in fourteen independent cell lines. In the seven lines examined that produce MTV RNA in the presence of dexamethasone, all contain at least one proviral DNA insert that is DNase I sensitive; in contrast, in the seven lines tested that fail to transcribe MTV DNA, every MTV fragment is insensitive to DNase I digestion. Many of these lines are presented in subsequent chapters of this dissertation. Thus, it appears that the DNase I resistant configuration of chromatin may be sufficient to preclude expression of MTV DNA in HTC cells.

CCGG Sequences in MTV Proviral DNA Are Not Highly Methylated

These results define two correlated criteria by which the J2.15 and J2.17 MTV proviruses differ: transcriptional activation and chromatin structure. Why should a single genetic element, present at different loci in otherwise identical cells, differ in these ways? One hypothesis is that prior to MTV DNA integration, there exist two classes of unintegrated MTV DNA molecules that are identical in nucleotide sequence, but differ in some other heritable property, such as a covalent base modification or bound protein. Similar features within the normal cell genome could account for the

differential gene expression that distinguishes tissue and cell types; in particular, Holliday and Pugh (115) and Riggs (116) proposed that methylation of DNA bases might provide such a developmental determinant.

An inverse relationship between the expression of specific DNA sequences and their extent of cytosine methylation at the tetranucleotide CCGG has been reported in certain instances (117-119), and it has been suggested that extensive methylation of a given region may be at least responsible for preventing its expression. Therefore, the methylation of the MTV DNA sequences in cell lines J2.15 and J2.17 were compared. The isoschizomer restriction endonucleases Msp I and Hpa II (145) were used to digest DNA from both cell lines after growth in the presence and absence of hormone. Both enzymes cleave at 5' CCGG sequences, but only the former can act when 5-methylcytosine is substituted at the second position within the sequence; virtually all of the DNA methylation in higher cells occurs at the dinucleotide CpG (146). With J2.15 and J2.17 DNA, the two enzymes produce indistinguishable MTV sequence containing fragments (figure 6), indicating that none of the proviral CCGG sites detected is methylated. Hence, this experiment fails to support the idea that extensive DNA methylation at CCGG sequences is correlated with MTV gene expression.

The Two Preinsertion Fragments Differ in Chromatin Structure

An alternative explanation for the functional differences in the MTV sequences in J2.15 and J2.17 cells is that their different positions within the host genome affect directly their chromatin structure, and thereby their potential to respond to the hormonal stimulus. One simple view, for example, is that the chromatin structure of inserted MTV sequences is determined solely by a "spreading effect" in which the viral genes acquire the structural configuration of the region into which they integrate.

The specific HTC DNA sequences adjacent to the J2.15 and J2.17 proviruses have been cloned in recombinant vectors, and shown to detect the corresponding "preinsertion fragments" (figure 2) in uninfected HTC cells; interestingly, neither preinsertion fragment is transcribed at a detectable rate either in the absence of in the presence of dexamethasone (133,141). The minimal prediction of the "spreading effect" hypothesis is that in uninfected HTC cells, the 3.6 kbp Eco RI preinsertion fragment PF(15), which harbors a provirus in J2.15, should be relatively DNAse I resistant, whereas the 5.9 kbp preinsertion fragment PF(17), which is interrupted by a provirus in J2.17, should be DNase I sensitive.

Accordingly, nuclei from uninfected HTC cells were treated with DNase I; the DNA was then purified, digested with Eco RI and the two preinsertion fragments probed simultaneously on blots. Consistant with the hypothesis, the PF(17) fragment

is more sensitive to DNase I attack than is the PF(15) fragment (figure 7). Comparison of the relative DNase I sensitivity of the preinsertion and proviral chromatin from each region is complicated by the potential technical variables noted in the Materials and Methods section. Nevertheless, preliminary estimates from densitometric scans of autoradiographs (figure 8) appear consistent with the view that the J2.17 provirus and PF(17) may be degraded at similar rates, while the J2.15 provirus and PF(15) may display similar levels of resistance to DNase I attack. Thus, these results can be interpreted to suggest that the position effects on MTV gene expression in HTC cells reflect different chromatin structures imposed on the newly introduced DNA by flanking cellular chromatin.

Table 1. MTV gene expression in J2.15 and J2.17

Cell line	10^{-6}M dexamethasone	MTV RNA	
		Rate of Synthesis (ppm)	Steady State Level (molecules/cell)
J2.15	-	<4	<0.1
	+	<4	<0.1
J2.17	-	<4	<0.1
	+	43	70

Relative synthetic rates of MTV RNA (expressed as parts per million of total labeled RNA (ppm) were measured by hybridization of pulse labeled cell RNA to nitrocellulose filters bearing cloned MTV DNA (133,141). Steady state concentrations were determined from the kinetics of solution hybridization of unlabeled cell RNA to MTV ^{3}H-cDNA (135).

Figure 2. Eco RI(∇) and Pst I(Δ) sites within and adjacent to the J2.15 and J2.17 proviruses. The J2.15 and J2.17 maps are as derived by Ringold et al. (128). The preinsertion fragments predicted and detected in HTC cells are shown in the upper diagram of each panel. Recombinant clones carrying the inserts are denoted p15.1 and p17.1 (133,141).

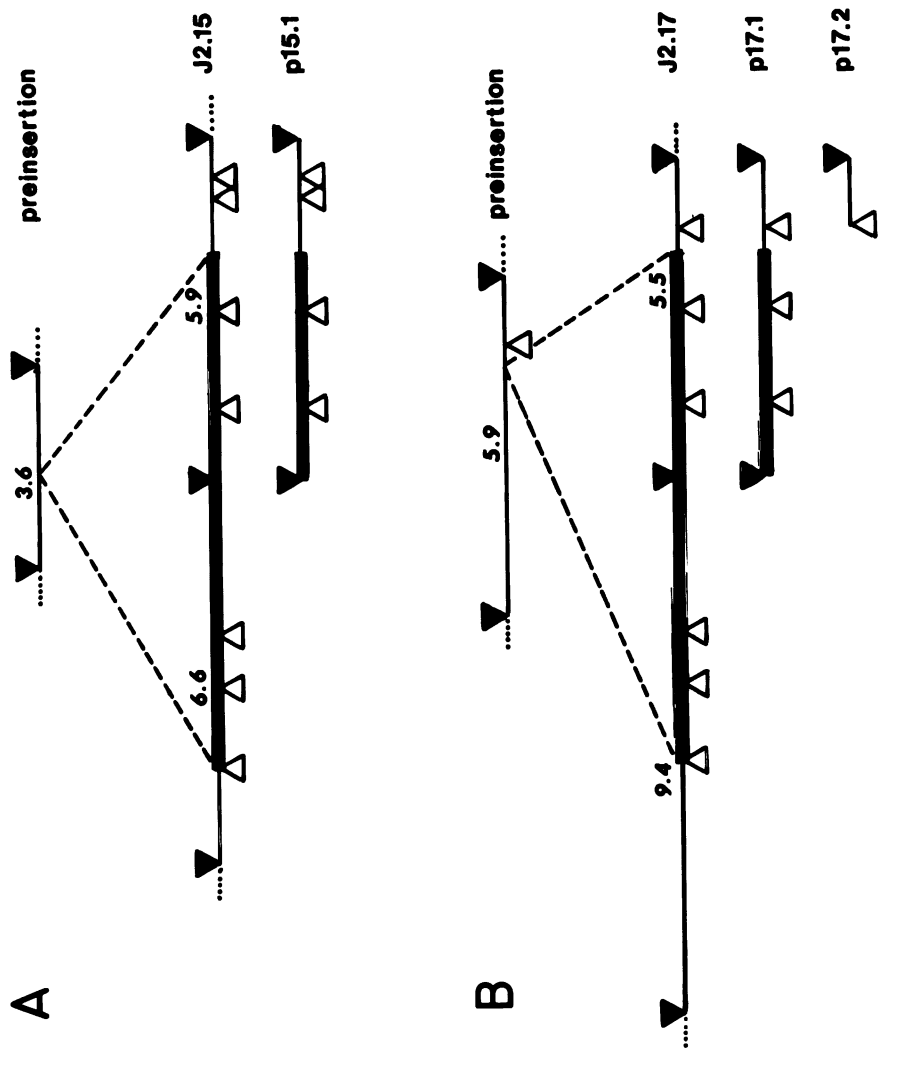
A
preinsertion
3.6
5.9
6.6
J2.15
p15.1
B
preinsertion
5.9
5.5
9.4
J2.17
p17.1
p17.2

FIGURE 2

Figure 3. DNase I sensitivity of proviral genes in J2.15 and J2.17. Cultures were propagated either in the absence or presence (1 μM, 12 hours) of dexamethasone. Nuclei were isolated and treated for various times with 1 μg/ml DNase I as described in Mateials and Methods. DNA was isolated, digested with Eco RI, fractionated on agarose gels, and transferred to nitrocellulose. The left hand lanes of panel A and B contain DNA undigested by DNAse I; subsequent lanes reflect increasingly extensive digestion (left to right): 0.3-0.4%, 0,6-0.7%, 0.9-1.0%, and 1.1-1.2% as monitored by perchloric acid solubility. Blots were probed with the recombinant insert from pMTV1, which includes 85% of the sequences present in the intact virus (see figure 4), and with cDNA prepared from cloned rat insulin sequences (gift of G. Bell). In this and subsequent figures, gel lanes bearing undigested and 0.6-0.7% digested samples are marked with stars.

(A) J2.15 DNA hybridized with pMTV1 ^{32}P-DNA

(B) J2.17 DNA hybridized with pMTV1 ^{32}P-DNA

(C) J2.17 DNA hybridized with rat insulin ^{32}P-DNA

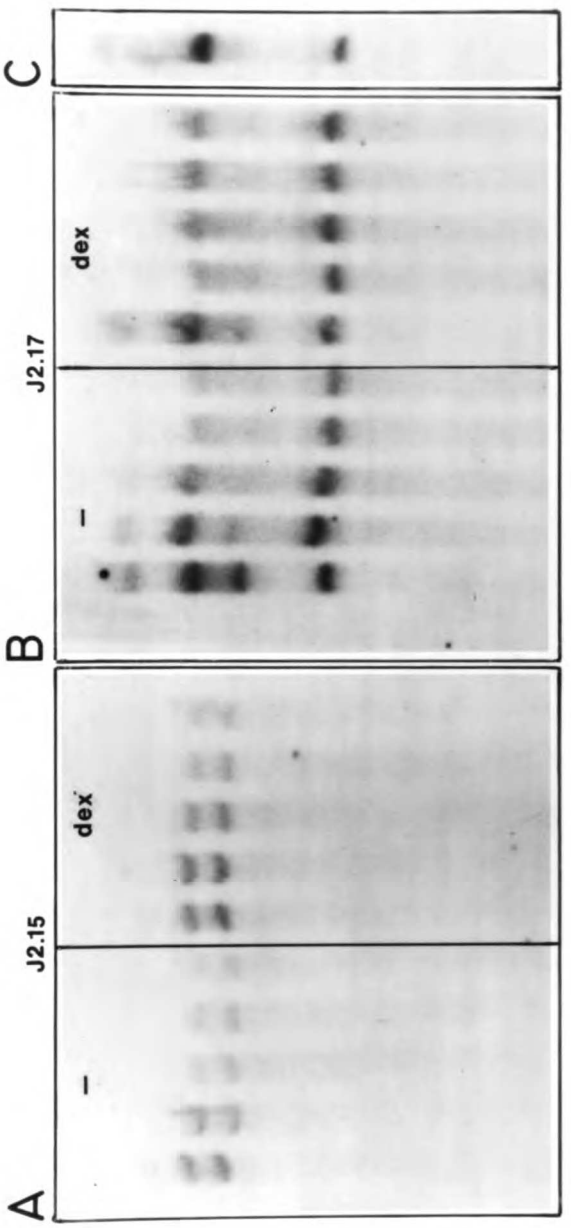

FIGURE 3

Figure 4. DNase I sensitivity of subregions of the J2.17 MTV provirus. Portions of the J2.17 DNAs prepared for figure 3 were digested with Pst I rather that Eco RI. The gel lanes, reflecting increasing DNase I digestion, are as in figure 3; after transfer of the DNA to nitrocellulose, the filter was hybridized with a mixture of pMTV1 ^{32}P-DNA and rat insulin ^{32}P-cDNA. The diagram shows the five MTV containing Pst I fragments in J2.17; the cloned pMTV1 probe used here detects all but the smallest (e) fragment. The bands denoted by the arrowheads at the righthand edge of the blot contain insulin sequences.

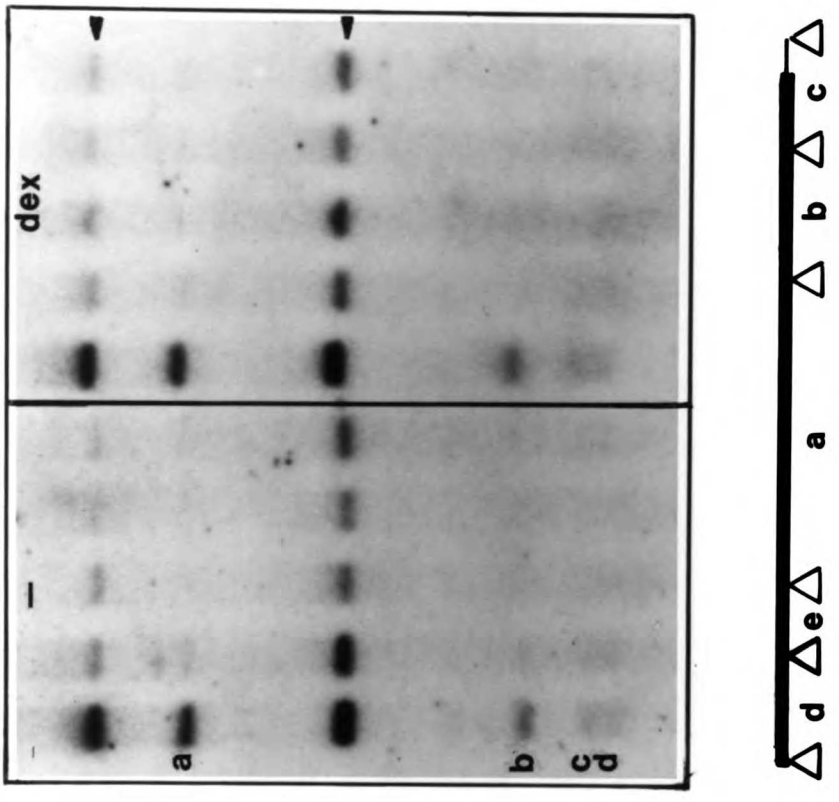

FIGURE 4

Figure 5. DNase I sensitivity of proviral genes in J2.15 and J2.17. The experiment was carried out exactly as described in figure 3 except that equal numbers of cells were mixed prior to nuclear isolation and all procedures were executed on the single preparation.

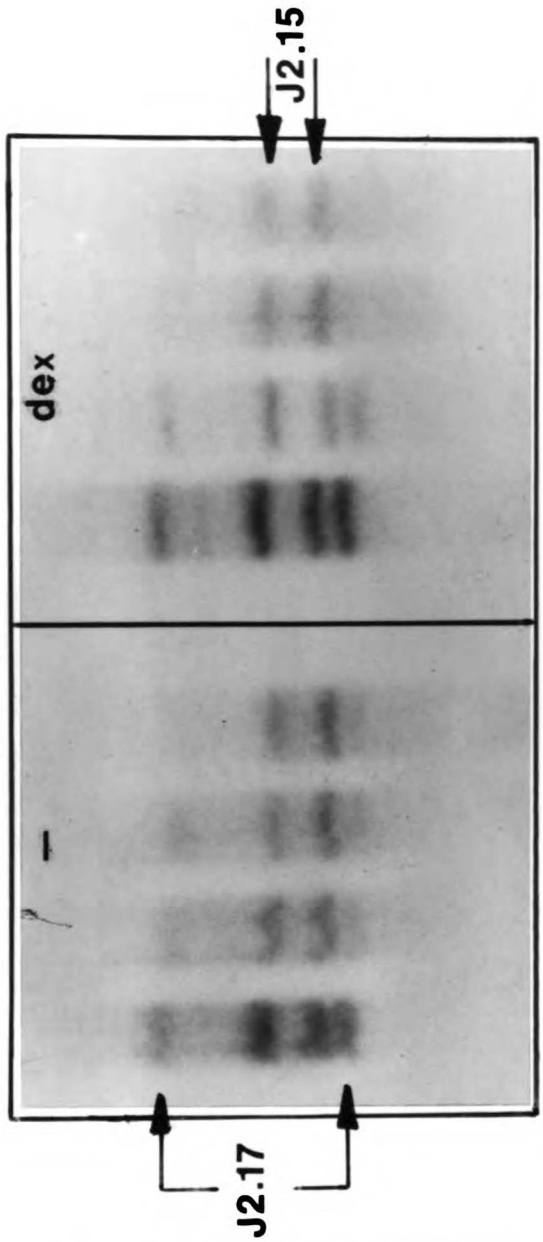

FIGURE 5

Figure 6. Comparison of the 5-methylcytosine content of CCGG sequences in J2.15 and J2.17 MTV proviruses. Cultures were propagated as in figure 3. DNA was isolated and digested to completion with either Msp I (right panel) or its 5-methycytosine sensitive isoschizomer Hpa II (left panel). After fractionation and transfer to nitrocellulose, the filter was hybridized with pMTV1 ^{32}P-DNA. a,b, J2.15; c,d, J2.17; a,c, cells prapageted in dexamethasone; b,d, cells propagated without hormone.

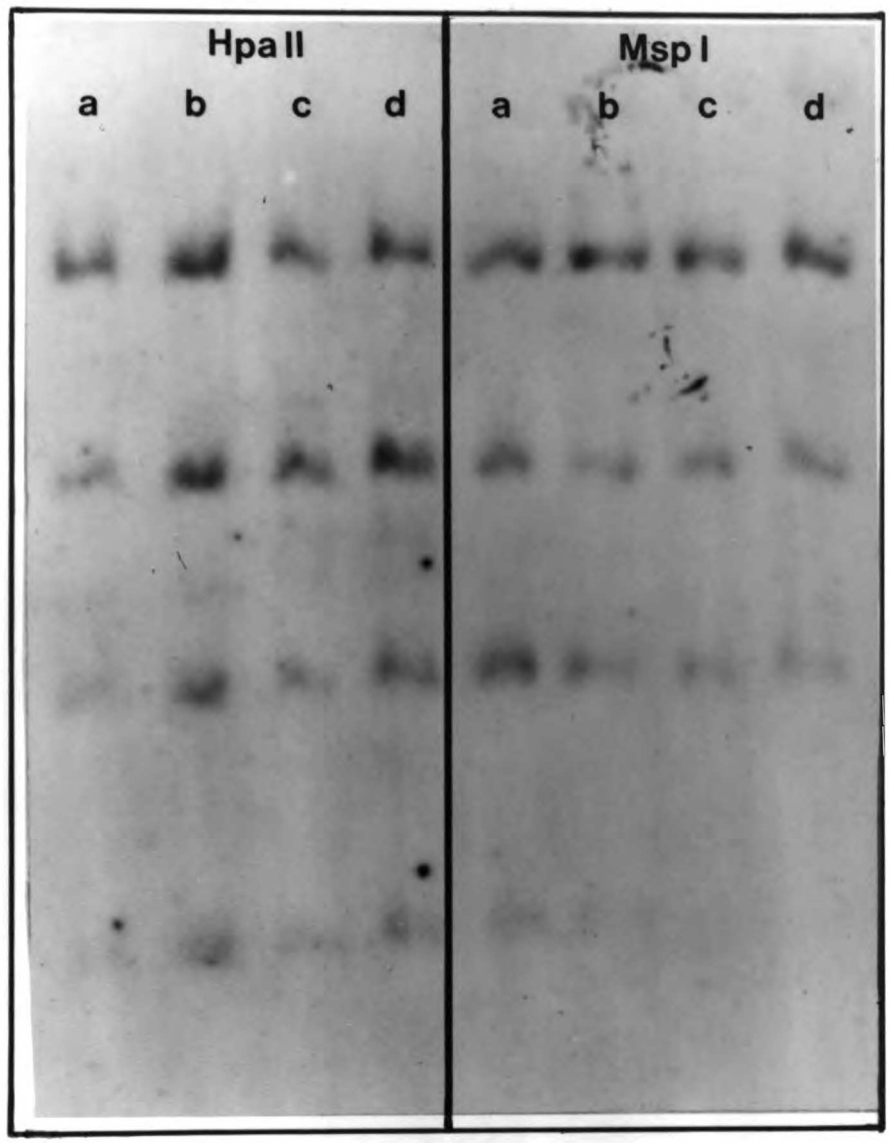

FIGURE 6

Figure 7. DNase I sensitivity of preinsertion fragments in HTC cells. All procedures were as described in figure 3 except that HTC cell DNA was hybridized with a mixture of p15.1 and p17.1 ^{32}P-DNA.

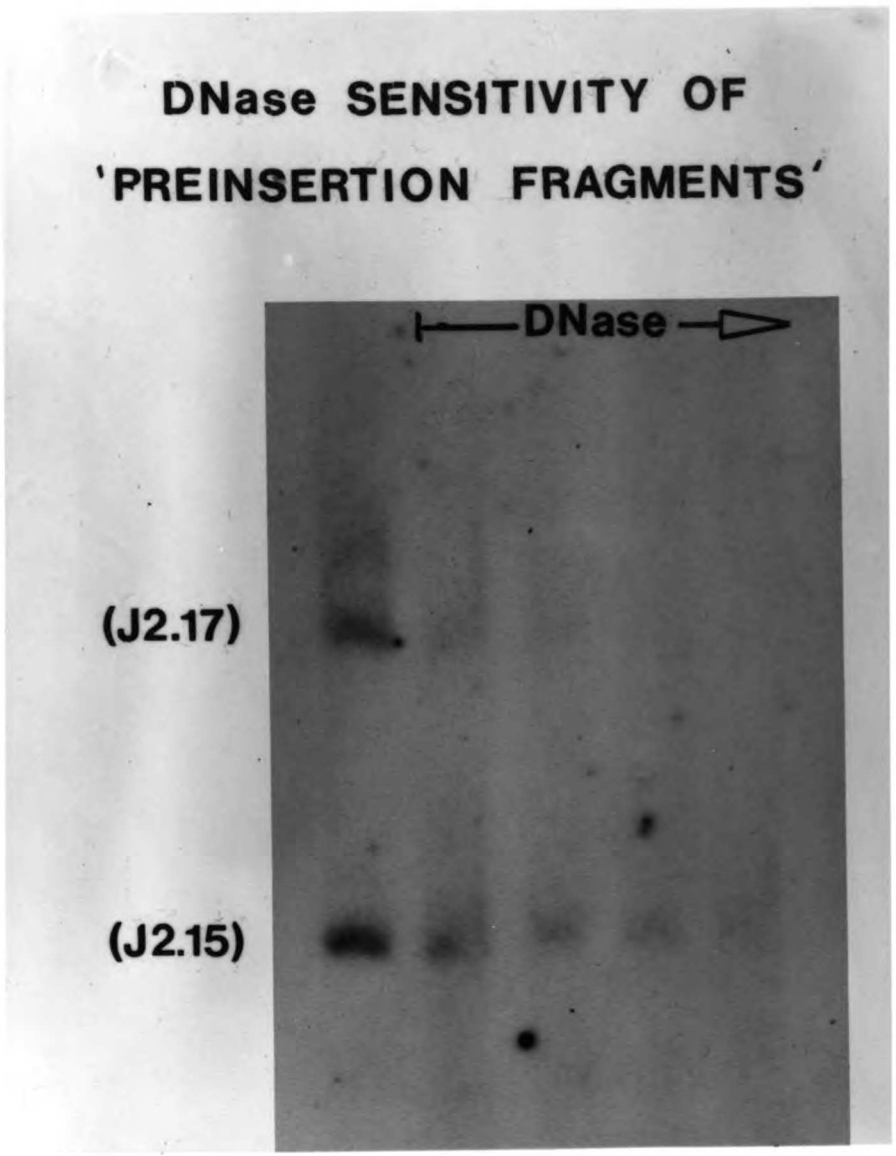
DNase SENSITIVITY OF
'PREINSERTION FRAGMENTS'
DNase
(J2.17)
(J2.15)

FIGURE 7

Figue 8. Relative DNase I sensitivity of proviral and preinsertion fragment chromatin. Autoradiographs of successive lanes of Southern blots, corresponding to 0, 0.25, 0.45, and 0.65% digestion with DNase I, were scanned on a Zeineh soft laser densitometer. Fragment sizes decrease from left to right.

(A) DNase I sensitivity of J2.15 and J2.17 proviruses. Autoradiograph shown in figure 5B was scanned. Fine vertical lines indicate fragments containing the J2.15 provirus; dashed lines indicated the J2.17 provirus.

(B) DNase I sensitivity of preinsertion fragments in HTC cells. Lanes 1-4 of autoradiographs shown in figure 7 were scanned. Vertical line indicates PF(15); dashed line indicates PF(17).

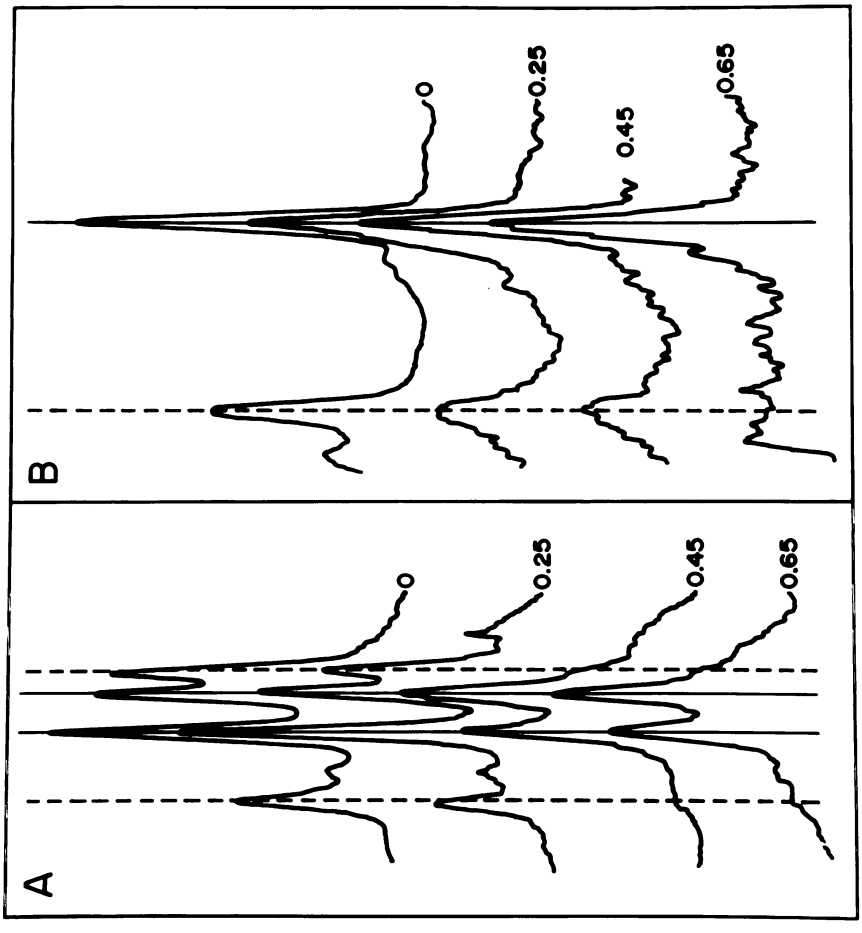

FIGURE 8

CHAPTER II

GENERALITY OF CHROMOSOME STRUCTURE AND TRANSCRIPTIONAL POTENTIAL CORRELATION - STEROID HORMONES CAN ALTER BOTH CHROMATIN STRUCTURE AND TRANSCRIPTIONAL ACTIVITY

INTRODUCTION

It is crucial to assess the generality of relationships between chromosome structure and function. In the previous chapter, transcriptional potential correlated with DNase I sensitivity. Additionally, administration of dexamethasone to the MTV RNA inducible cell line J2.17 may have resulted in a relatively subtle alteration of MTV proviral chromatin structure. DNase I analysis was therefore extended to several additional MTV containing HTC cell lines. This allows further correlation between chromosome structure and transcriptional activity. Also, it would be extremely useful to examine additional MTV RNA inducible cell lines for their chromosome structure response to steroid administration. Do other inducible cell lines all exhibit subtle chromatin structure alterations similar to that seen in J2.17? Do other chromatin structure responses to steroid hormones exist? In this chapter, several additional MTV containing HTC cell lines will be examined.

RESULTS

Steroid Inducible Chromatin Structure

B13 is an MTV infected HTC cell line which harbors a single MTV provirus integrated at a site distinct from either the J2.17 or the J2.15 proviruses; similar to J2.17, B13 synthesizes MTV RNA only in the presence of dexamethasone, accumulating a steady state level of approximately 10^2 molecules per cell (see table II and ref. 147). As seen in figure 9, the MTV provirus is resistant to DNase I in the absence of hormone, but sensitive in its presence. This result is markedly different from that obtained in the J2.17 cell line.

It is not particularly surprising that MTV sequences are DNase I sensitive in the presence of dexamethasone, as MTV RNA is actively synthesized. It is crucial to establish that MTV sequences are in fact resistant in the absence of hormone. To provide a positive control for this experiment, equal numbers of uninduced B13 and J2.17 cells were mixed prior to nuclear isolation and subsequent analysis. As shown in figure 10, the J2.17 MTV sequences are sensitive to DNase I in the absence of dexamethasone, whereas the B13 MTV sequences are DNase I resistant relative to J2.17. Thus, it appears that the MTV provirus in B13 is converted from a DNase I resistant packaging conformation to a DNase I sensitive structure in response to dexamethasone administration.

In the previous chapter, two distinct forms of chromosome structure were observed. In J2.15, MTV sequences were packaged in a relatively DNase I resistant structure and were transcriptionally quiescent. In contrast, MTV sequences in J2.17 were packaged in a relatively DNase I sensitive structure and were transcriptionally inducible. MTV sequences in B13 exhibit a third structure; in responce to dexamethasone, MTV chromatin structure shifts from a DNase I resistant to a sensitive form. Additionally, MTV transcription is induced. Thus, at least three different forms of chromatin structure have been observed. How many different structures might actually exist? This question will be addressed in the next chapter.

Chromatin Structure in Other Expressing Cell Lines

As another example, 71.12 is an MTV containing HTC cell line possessing three MTV containing fragments. 71.12 expresses MTV RNA in the presence of dexamethasone to a steady state level of approximatly 150-200 MTV RNA molecules per cell (see table II and 148). As can be seen in figure 11, only one of the three fragments is DNase I sensitive; MTV transcription therefore likely proceeds only from that DNase I sensitive sequence.

Altogether, seven MTV RNA inducible cell lines containing a total of 24 MTV proviruses have been examined. In every case, at least one MTV proviral sequence is DNase I sensitive.

Thus, the positive correlation between DNase I sensitivity and transcription is complete without exception. Of the total 24 proviruses in these responsive cells, eleven were sensitive. Of these eleven sensitive proviruses, only the B13 provirus exhibited a dramatic structural change in responce to dexamethasone administration. That all MTV proviruses in inducible cells need not be DNase I sensitive will be examined in greater detail in chapter III. It is interesting to consider whether all eleven sensitive proviruses have identical structures. Though DNase I digests each of them relatively rapidly, perhaps they have different molecular natures. It will ultimately be crucial to determine how many different kinds of chromatin structures actually exist, both for DNase I "sensitive" and "resistant" structures.

Chromatin Structure in Other Non-Expressing Cells

The MTV infected HTC cell line B7 expresses no MTV RNA in the presence or absence of dexamethasone (see table II and 147). As seen in figure 12, both MTV containing DNA fragments in these cells are DNase I resistant. Together with other analyses, seven nonexpressing MTV infected cell lines containing a total of sixteen proviruses have been examined. As a general rule, cells not expressing MTV RNA in either the presence or absence of dexamethasone contain MTV DNA packaged only in a DNase I resistant conformation.

Table 2. MTV gene expression in B13, 71.12, and M13B7

Cell line	10^{-6}M dexamethasone	MTV RNA (steady state) (molecules/cell)
B13	-	<0.1
	+	100
71.12	-	<0.1
	+	150-200
B7	-	<0.1
	+	<0.1

Steady state concentrations were determined (147,148) by solution hybridization of unlabeled cell RNA to MTV ^{3}H-cDNA (135).

Figure 9. DNase I sensitivity of MTV proviral genes in B13. Cultures were propagated either in the absence or presence (10^{-6}M, 12 hours) of dexamethasone. Nuclei were isolated, digested with 1 μg/ml DNase I, and DNA isolated as described in Materials and Methods. DNA was digested with Eco RI, fractionated on agarose gels, blotted and probed with pMTV1 ^{32}P-DNA. The left track of each panel contains DNA undigested by DNase I; subsequent tracks represent increasing DNase I digestion (left to right): 0.3%, 0.6%, 0.9%, 1.1% as monitored by perchloric acid solubility.

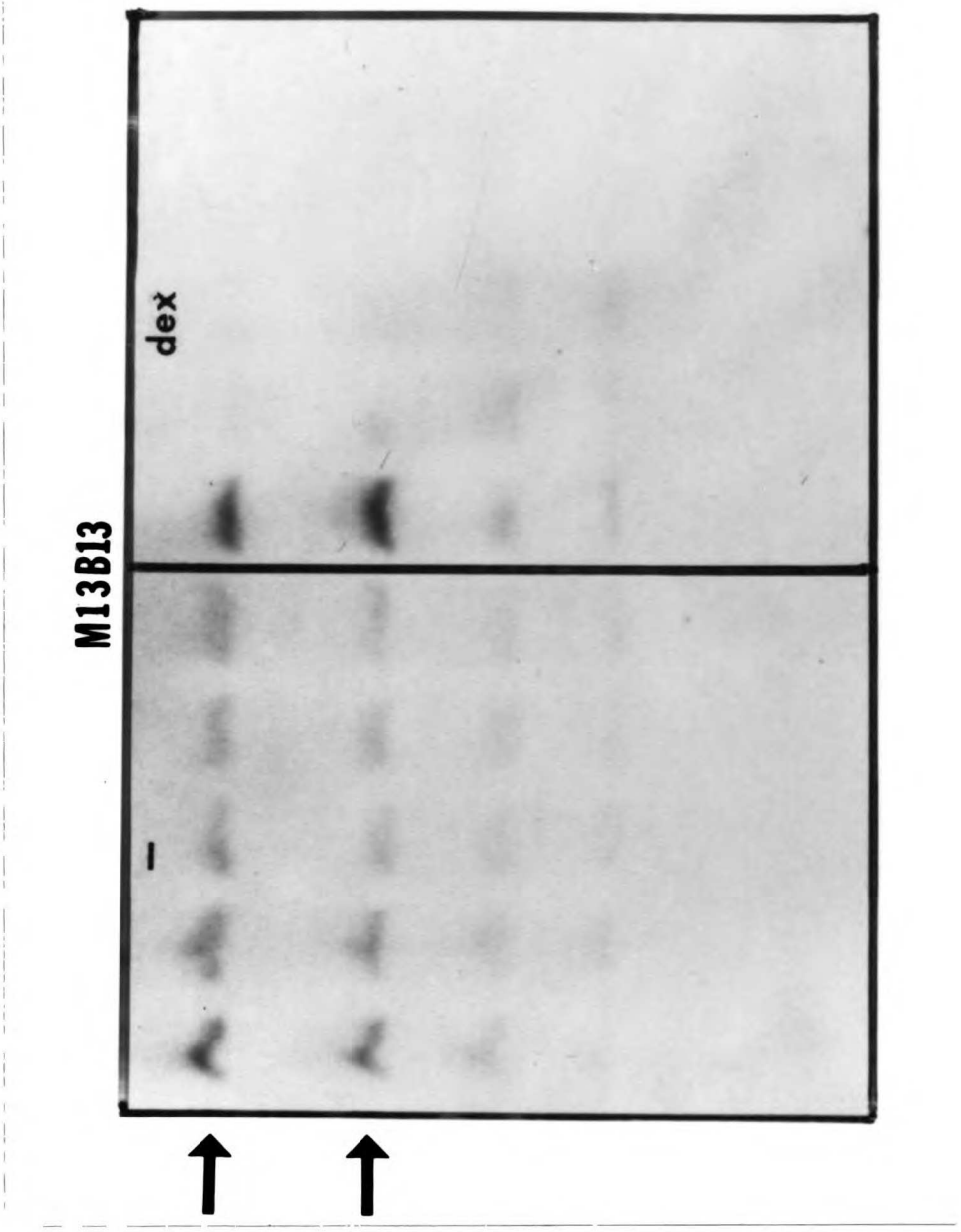

FIGURE 9

Figure 10. DNase I Sensitivity of MTV Proviral Genes in Mixed Cultures of B13 and J2.17. The experiment was carried out in the absence of dexamethasone exactly as that described in figure 9, except that equal numbers of B13 and J2.17 cells were mixed prior to nuclear isolation and all procedures were carried out on a single preparation. Panel A shows the time course of digestion. Extents of digestion were (left to right): 0.0%, 0.4%, 0.6%, 0.8%, 0.9%, 1.1%, 1.25%, 1.4%, 1.6%, 1.9% as monitored by perchloric acid solubility. Panel B is a lane containing only J2.17 DNA.

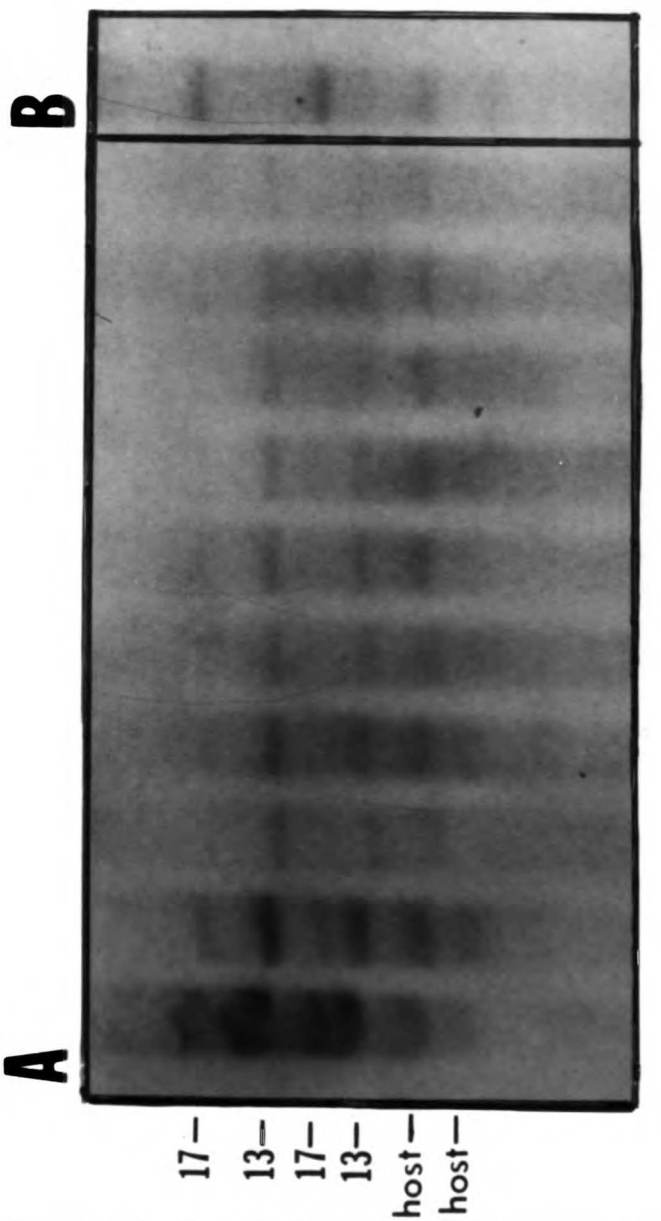

FIGURE 10

Figure 11. DNase I Sensitivity of MTV Sequences in 71.12 Cells. The experiment was carried out in the absence of dexamethasone exactly as described in figure 9. Extents of digestion are (left to right): 0.0%, 0.5%, 0.7%, 0.95%, 1.1%, 1.2% as monitored by perchloric acid solubility.

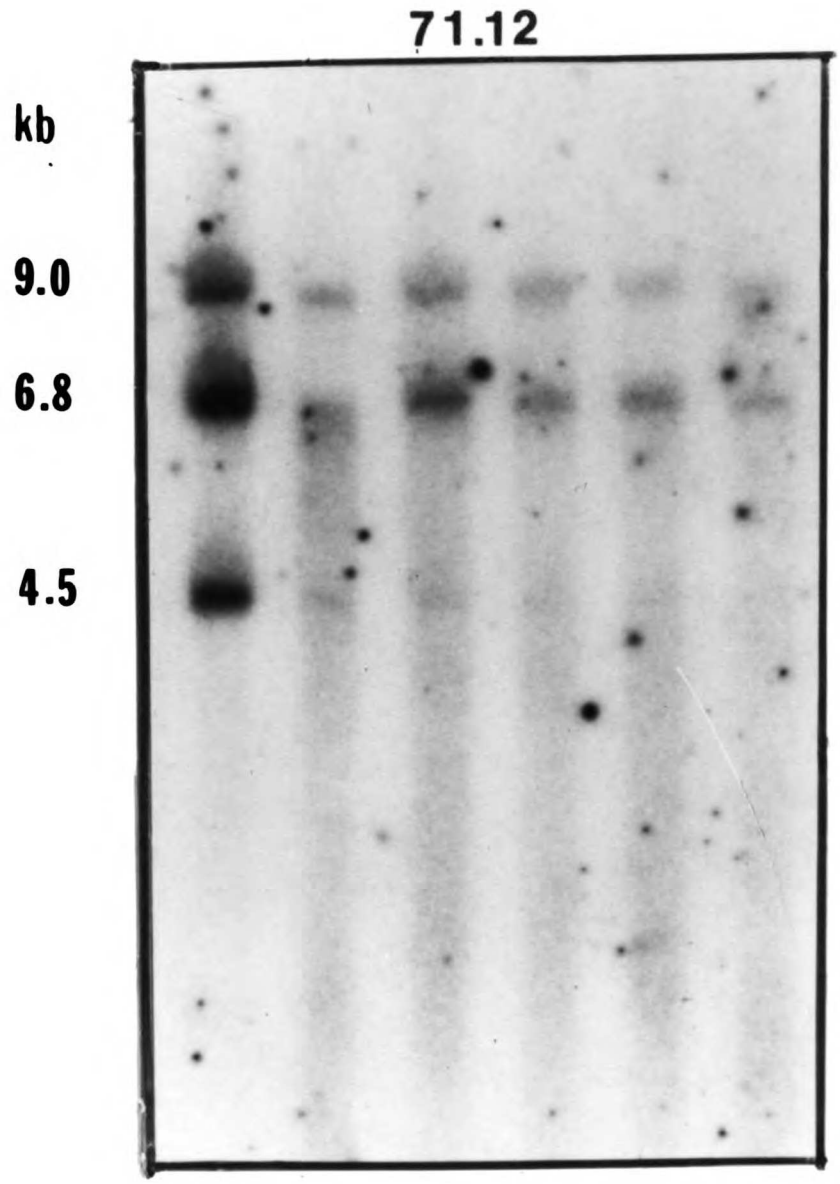

FIGURE 11

Figure 12. DNase I Sensitivity of Proviral Genes in M13B7. The experiment was carried out as described in figure 9. Uninduced nuclei were assayed at (left to right) 0.0%, 0.2%, 0.35%, 0.5%, 0.8%, 1.0% and dexamethasone induced nuclei assayed at 0.0%, 0.2%, 0.35%, 0.5%, 0.8% of digestion, as determined by perchloric acid solubility.

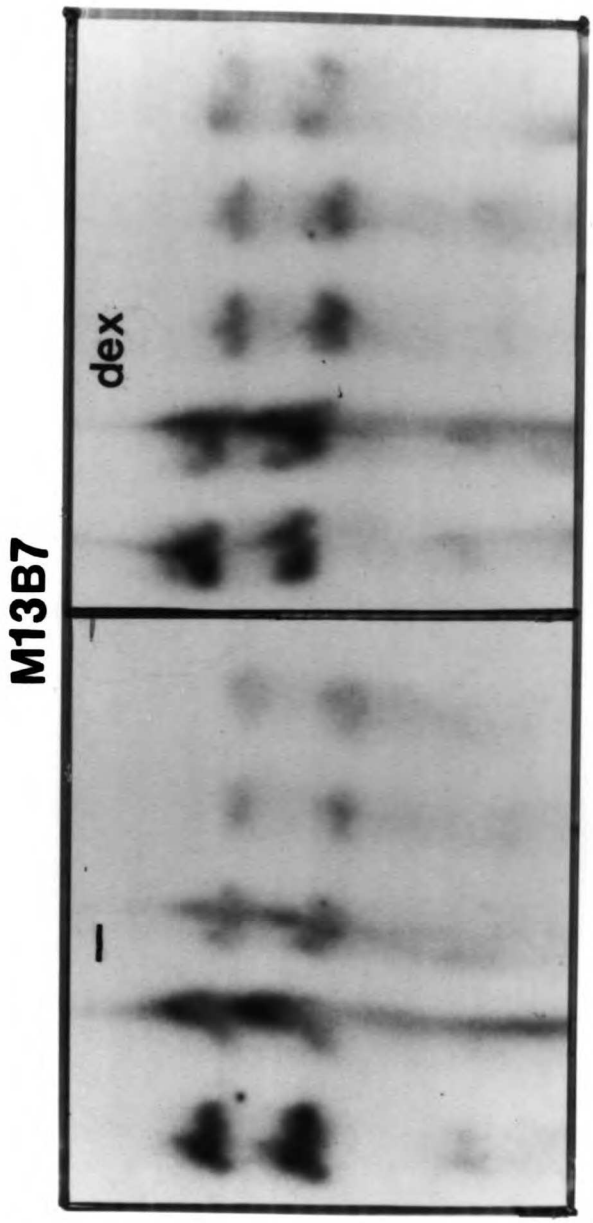

FIGURE 12

CHAPTER III

REGULATION OF CHROMOSOME STRUCTURE DETERMINATION ACTS IN CIS

INTRODUCTION

In chapter I, cell lines containing only a single MTV provirus were analyzed to assess the effects of integration site on the chromatin structure and expression of the MTV sequences. It was demonstrated that a single sequence can be packaged in different chromatin structures in different cells, and that DNase I resistant sequences are not expressed. In this chapter, MTV proviral sequences present in multiple copies per mouse cell are analyzed. Mouse cells employed in these studies contain multiple endogenous MTV proviruses. These proviral genes are normal components of the mouse genome, and are present at strain specific loci in all inbred mouse strains (122). Essentially all endogenous proviruses are transcriptionally inert. By analyzing both cells containing only endogenous MTV proviruses and cells subsequently exogenously infected by MTV virions, several important questions can be approached. Can a single sequence present in multiple copies within a single cell exist in different chromatin configurations? In this internally controlled situation, can multiple classes of structure be detected, or only binary sensitive and resistant? Finally, are endogenous proviruses DNase I resistant? Can their failure to be expressed be correlated with chromatin structure? Are they otherwise competant to respond?

Groudine et al. (149) have examined the DNase I sensitivity of RAV-0 endogenous and exogenously acquired Rous Sarcoma viral DNA, and concluded that endogenous genes are DNase I resistant and transcriptionallly inert due, most likely, to a cis acting control mechanism. However, these experiments utilized solution hybridization, making it impossible to distinguish the multiple proviruses present from one another, and therefore the results were an average of all proviruses. Furthermore, experiments were not performed on clonal lines, but rather on infected populations, which again necessitates averaging data from cells varying in innumerable ways. Thus, these experiments have an inherent lack of sensitivity and discrimination. In the present chapter, multiple proviruses are distinguished from each other by employing the DNase I assay described in chapter I, which utilizes Southern blotting. Further, all cells utilized in this study are clonal lines. Therefore, these experiments rigorously approach the questions originally asked by Groudine et al. (149), and in addition address the other questions presented above.

RESULTS

Analysis of endogenous MTV proviral expression was initiated on W7 cells, a line derived from a Balb/c mouse lymphoma. As seen schematically in figure 13, Hind III cuts MTV DNA at one site per provirus, generating two MTV specific fragments per provirus. The blot in figure 14 is annealed

with pMTV1 probe, which contains sequences homologous to both Hind III generated MTV containing fragments. Figure 14, lane 1, demonstrates that W7 cells contain two endogenous MTV proviruses. As seen in Table III, despite the presence of these MTV sequences, no MTV specific RNA is synthesized either in the presence or absence of glucocorticoid hormones (137). Why are MTV sequences not transcribed in these cells?

The lack of MTV transcription in W7 cells is not caused by a general incompetance of the cells to respond to glucocorticoids. Steroid binding experiments show that W7 cells possess specific dexamethasone receptor binding activity (137). Moreover, they are killed within 72 hours by physiological doses of glucocorticoids, a hormone receptor mediated response of mouse lymphoma cells (137). Thus, whatever mechanism precludes MTV expression appears to be selective for the MTV RNA induction pathway, rather than reflecting an overall loss of glucocorticoid responsiveness.

In attempting to determine if chromatin structure could be the mechanism responsible for the lack of transcriptional activity at these MTV proviral sequences, DNase I analysis was performed on W7 cell nuclei. Modification of the nuclear isolation procedure was necessary, as described in Materials and Methods. The result is shown in figure 16. This blot is annealed with probe made from the Pst "b" fragment, which hybridizes only with Hind III "b" MTV fragments (see figure 13). Despite digestion to greater than 1%, both endogenous

MTV proviral sequences are resistant to DNase I digestion. The Hind III "a" MTV fragments (see figure 13) are also resistant (data not shown). In view of the strong correlation between DNase I resistant chromatin structure and transcriptional inactivity, a reasonable interpretation of these data is that the failure of the endogenous MTV sequences in W7 cells to be transcribed reflects a chromosomal packaging structure incompatible with their expression. Alternatively, mutations in proviral sequences may have occurred at essential regulatory loci that preclude expression. However, recent results from other laboratories demonstrates that endogenous MTV proviruses can be cloned by recombinant DNA technology and used for DNA transformation experiments (150). Upon introduction into recipient cells, formerly transcriptionally quiescent endogenous sequences are found to be transcriptionally inducible by dexamethasone. This strongly argues for mechanisms other than mutations precluding expression of endogenous MTV proviruses.

How stable is the DNase I resistant structure of the W7 MTV endogenous proviruses? Is the restriction against expression of endogenous MTV sequences cis or trans acting? To examine these issues, W7 cells were infected exogenously with MTV virions. Dexamethasone induces a marked increase in the MTV RNA content within the resulting population of MTV infected cells (137).

Individual cells from the MTV infected population were cloned on agar plates and screened for inducibility by a

colony screening procedure (137). One such clone is M4.12H. As seen in figure 17, lane 1, M4.12H has acquired seven MTV proviruses exogenously via infection. To resolve the many Eco RI bands, this blot is annealed with the Pst "b" probe, which hybridizes to only Eco RI "b" generated MTV containing fragments (see figure 13). Therefore, each band corresponds to an individual provirus.

As seen in table III, M4.12H expresses MTV RNA only in the presence of dexamethasone to a steady state level of aproximately 10^2 MTV RNA molecules per cell. DNase I analysis will ascertain which proviruses are potentially capable of synthesizing MTV RNA, as well as which proviruses are DNase I resistant and therefore likely transcriptionally quiescent. Have the endogenous sequences been activated, or is transcription derived solely from exogenously acquired sequences, or some combination? Do all exogenously acquired proviruses have the same chromatin structure?

DNase I analysis of M4.12H is presented in figure 17. Several points are immediately apparent. First, several of the exogenously acquired proviruses are sensitive to DNase I and are therefore potentially transcribed. As endogenous proviral sequences remain packaged in a DNase I resistant structure and therefore are probably not transcriptionally active, transcription likely occurs only from that subset of the exogenously acquired proviral sequences that are packaged in DNase I sensitive structures. These results suggest that

transcriptional control at the level of chromatin structure functions in cis; trans acting positive or negative control elements would be expected to act on all proviral sequences uniformly. Further, a single DNA sequence, MTV DNA, exists in at least three different levels of sensitivity to DNase I, and hence different structures, within the same nucleus; this demonstrates that several different chromosomal packaging arrangements can exist for the same DNA sequence within a single cell. Alternatively, restriction fragments of intermediate sensitivity could contain varying amounts of resistant and sensitive structures to produce intermediate levels of sensitivity. Finally, the pattern of DNase I sensitivity is indistinguishable in the presence and absence of hormone. Thus, it appears that in M4.12H cells, the hormone receptor complex does not activate transcription via an alteration of chromatin structure that can be detected by DNase I digestion under these conditions.

Expression of Endogenous MTV Proviral Sequences

In contrast to uninfected W7 cells, and most other mouse lines containing only endogenous MTV proviruses, the TlMl 4D.17 lymphoma cell line (derived from the C57Bl strain) accumulates MTV RNA in the presence of glucocorticoid hormones; no MTV RNA is produced in the absence of hormone (see table III). The MTV RNA species synthesized are 35S and 24S, similar in size to the molecules produced by mouse mammary carcinoma cells and by infected HTC cells (121).

Figure 14, lanes 2-4, display Hind III digests of C57Bl DNA from three sources, TlMl 4D.17, VL3 and mouse spleen. Comparison with lane 1 demonstrates that C57Bl contains two MTV proviruses indistinguishable from those present in Balb/c (W7), plus an additional endogenous provirus. That C57Bl and Balb/c are closely related is also apparent from their very similar patterns of endogenous murine leukemia proviruses (152). As seen in figure 15, lanes 5-8, hybridization of the same filter with the Pst "b" MTV probe, specific for the Hind III "b" generated MTV containing fragments, establishes that bands at 12.0kb, 7.7kb and 4.7kb are "b" specific, implying that bands at 9.4kb, 8.4kb and 6.5kb are "a" specific. It should be noted that the extra provirus in C57Bl contains one extra "a" and one "b" fragment, consistent with it being an intact provirus.

The relative DNase I sensitivity of the individual MTV sequence containing Hind III fragments in TlMl 4D.17 cells is shown in figure 18. Fragments at 6.5kb and 7.7kb, "a" and "b" halves respectively, are both sensitive. Fragments at 9.4kb and 4.7kb, "a" and "b" halves respectively, are both resistant. It seems reasonable to assume therefore that the 7.7kb and 6.5kb fragments together comprise a single provirus while the 9.4kb and 4.7kb fragments constitute another. Making these assumptions, TlMl 4D.17 provirus 7.7kbp and 6.5kbp which is presumably identical to a DNase I resistant and nonexpressed provirus in W7 cells, is DNase I sensitive both in the presence

and absence of dexamethasone. Provirus 9.4kb and 4.7kb remains resistant in T1M1 4D.17, as in W7. The C57B1 specific endogenous provirus in T1M1 4D.17 cells (12.0kb and 8.4kb) is DNase I sensitive both in the presence and absence of dexamethasone (data not shown).

To test whether the DNase I sensitivity of specific endogenous MTV sequences in T1M1 4D.17 cells correlates with hormone responsive viral gene expression in that line, another C57B1 mouse lymphoma cell line, VL3 was examined. As seen in figure 14, lanes 2 and 3, and figure 15, lanes 1,2,5 and 6, the Hind III fragments containing MTV endogenous sequences in VL3 cells are indistinguishable from those in T1M1. Although the VL3 line appears not to express MTV RNA in either the presence or absence of dexamethasone (see Table III), two types of experiments showed that VL3 is nonetheless competant to respond to glucocorticoid hormones. First, as is the case for W7, M4.12H and T1M1, VL3 cells are killed within 72 hours of exposure to dexamethasone in a hormone receptor mediated response (137). Moreover, MTV infection of VL3 cells produces a cell population synthesizing MTV RNA in response to dexamethasone (137).

Figure 19 shows the results of a DNase I analysis of VL3. It is clear that two of the three VL3 endogenous MTV proviruses are DNase I resistant. Surprisingly, the C57B1 specific provirus (12.0kb and 8.4kb) is sensitive at a relatively advanced extent of DNase I digestion. Several possible

explanations exist for this result, elaborated upon in the discussion section. Among these, it is possible that this late sensitivity represents a different form of relatively DNase I resistant chromatin, as its sensitivity appears at a greater extent of nuclease digestion than the analagous T1M1 4D.17 provirus. However, it is also possible that VL3 actually synthesizes some low quantity of MTV RNA, but that it is not detectable by the steady state assays utilized because of rapid degradation coupled with a low synthetic rate. Rate determinations of transcription are therefore in progress. Yet another alternative is that this unusual provirus could be defective. Further analysis is necessary to properly interpret the VL3 data.

As a control, nuclei from C57B1 mouse spleen were subjected to DNase I analysis. Previous studies have shown that no MTV RNA is synthesized in this organ (130). As seen in figure 20, the six Hind III proviral fragments in the DNA of this tissue, identical to those seen in the cultured C57B1 cell lines (see figures 14 and 15), are all DNase I resistant, consistent with their nonexpressed phenotype.

In summary, T1M1 inducibly expresses MTV RNA, whereas W7 and C57B1 spleen cells appear not to do so. All endogenous MTV proviral sequences are DNase I resistant in W7 and C57B1 spleen cells, whereas some of the presumably identical sequences in T1M1 4D.17 are packaged in DNase I sensitive configurations. Thus, these results suggest that the failure of MTV endogenous

genes to transcribe can be attributed to chromatin structural constraints. T1M1 4D.17 can therefore be viewed as a variant in MTV chromosome structure determination, as normally DNase I resistant sequences have become DNase I sensitive.

Table 3. MTV gene expression in W7, M4.12H, T1M1 4D.17 and VL3

Cell line	10^{-6}M dexamethasone	MTV RNA (steady state) (molecules/cell)
W7	-	<0.1
	+	<0.1
M4.12H	-	<0.1
	+	150
T1M1 4D.17	-	<0.1
	+	100
VL3	-	<0.1
	+	<0.1

Steady state concentrations were determined (137) by solution hybridization of unlabeled cell RNA to MTV ^{3}H-cDNA (135).

Figure 13. Schematic map of Eco RI, Hind III and Pst I sites in MTV DNA. Size of Eco RI and Hind III "a" and "b" fragments is a function of location of host restriction sites. Probe to Pst I "b" fragment anneals only to the respective "b" fragments. "a" fragments include the 5' half of the viral RNA, while "b" fragments include sequences in the 3' portion of the viral RNA.

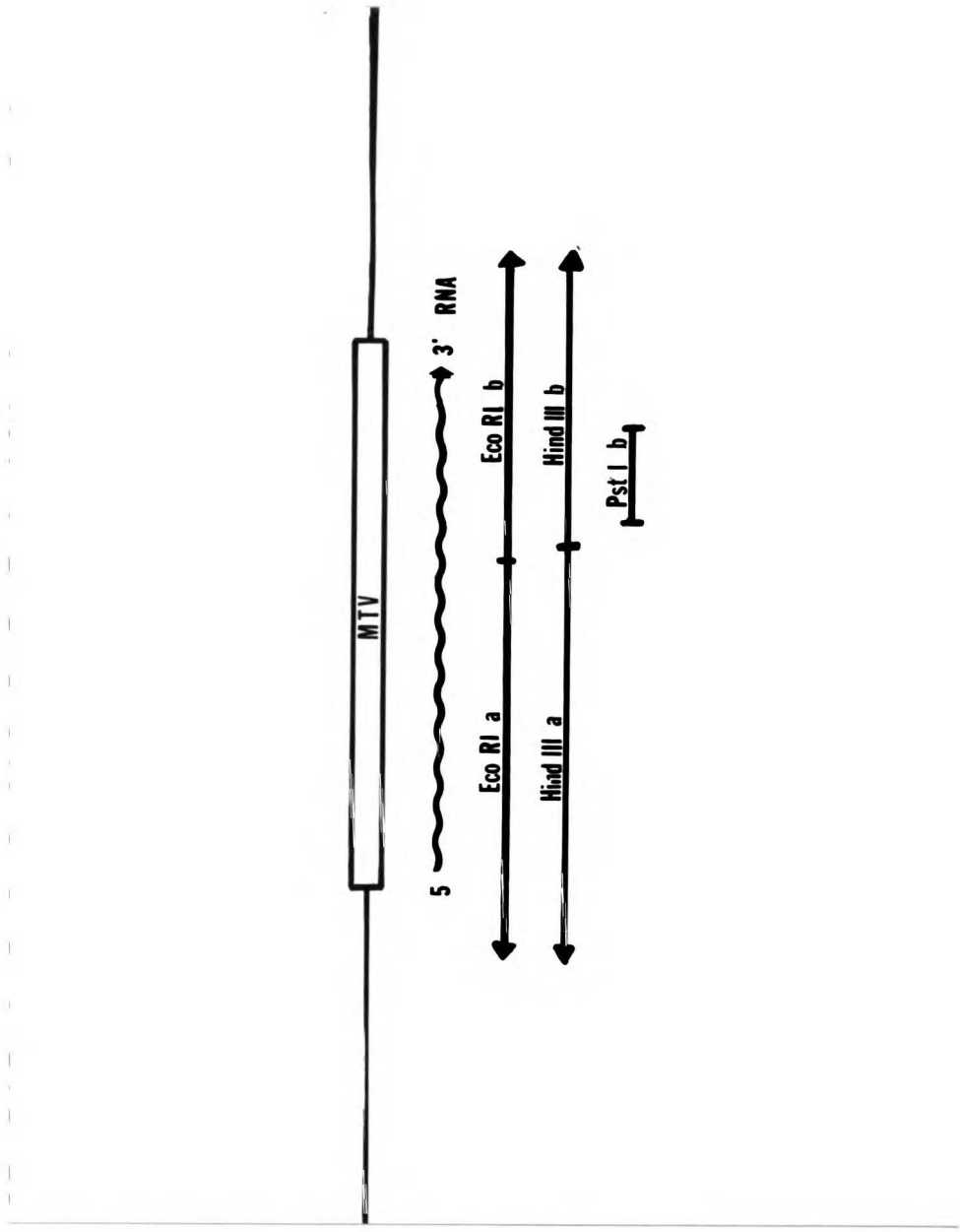

FIGURE 13

Figure 14. Blot hybridization of Hind III digested DNA from several mouse cells. High molecular weight DNA was prepared from appropriate cells, digested with Hind III, and subjected to Southern blot analysis. Lanes: (1) W7 DNA, (2) T1M1 4D.17 DNA, (3) VL3 DNA, (4) C57B1 mouse spleen DNA. The blot is annealed with pMTV1 (^{32}P) DNA, specific for both Hind III generated MTV containing fragments per provirus.

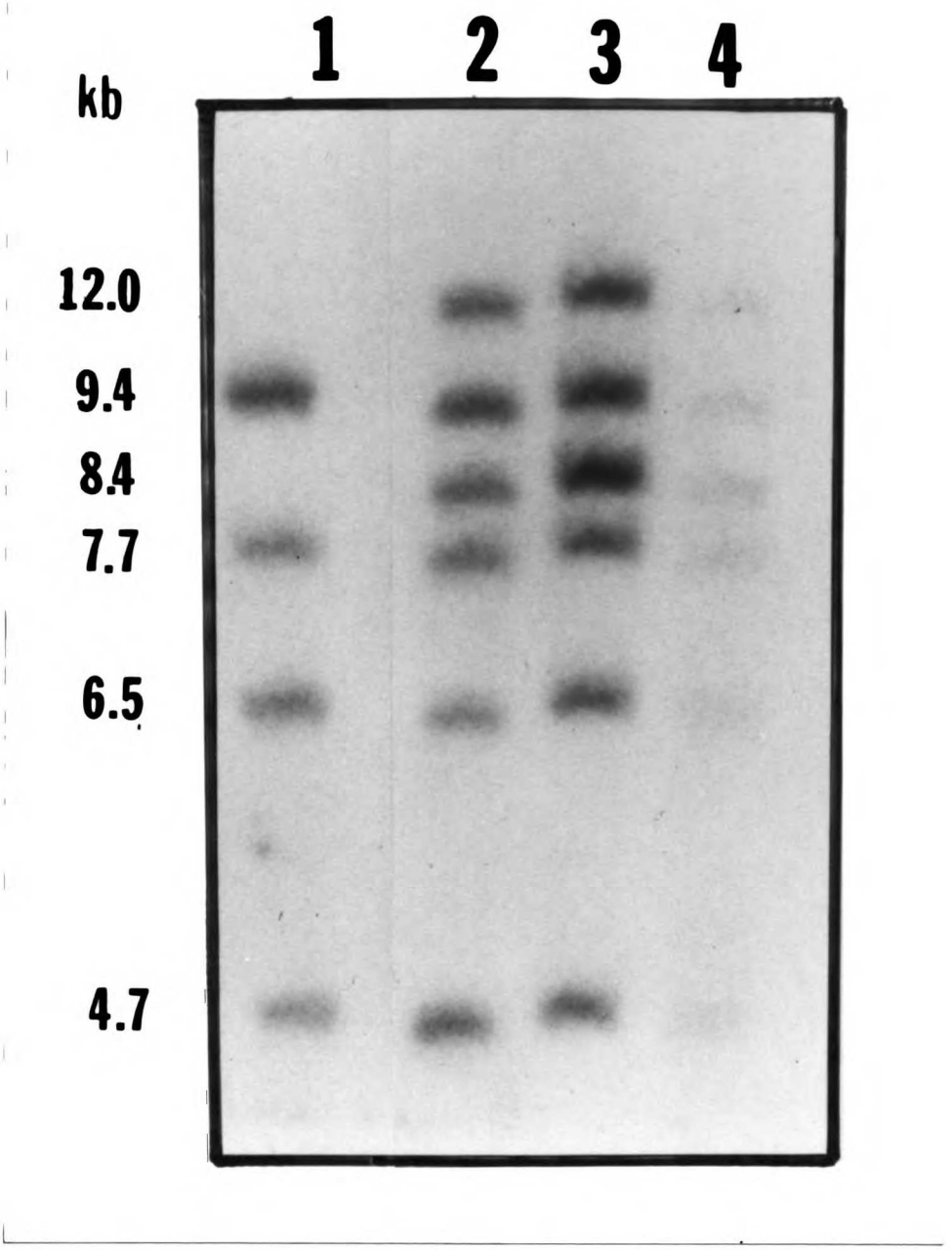

FIGURE 14

Figure 15. Blot hybridization of several C57Bl mouse cell lines. High molecular weight DNA was isolated, Hind III digested and analyzed by the procedure of Southern. Lanes 1-4 are annealed with pMTV1 (^{32}P) DNA. Lanes 5-8 are annealed with Pst I "b" (^{32}P) DNA, specific for only Hind III "b" MTV containing fragments. Lanes (1 and 5), T1M1 4D.17; Lanes (2 and 6), VL3; Lanes (3 and 7), C57Bl mouse spleen; Lanes (4 and 8), PG19 (another C57Bl mouse cell line).

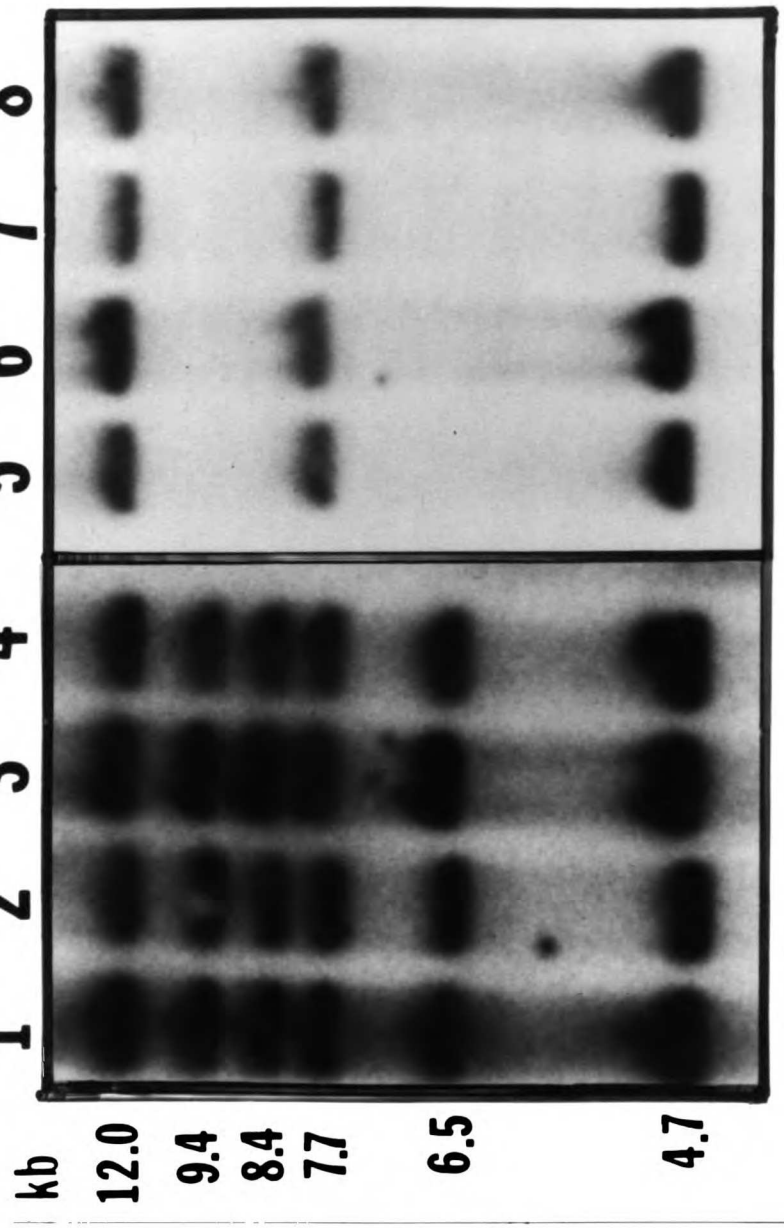

FIGURE 15

Figure 16. DNase I sensitivity of MTV sequence containing Eco RI fragments in W7 cells. DNase I analysis was carried out as described in figure 9. Time points were taken at (left to right): 0 seconds, 50 seconds, 2 minutes, and 4 minutes. Assuming 0.5-0.6% digestion per minute, as assayed by perchloric acid solubility, this corresponds to 0.0%, 0.4%, 1.0%, 2.0% digestion.

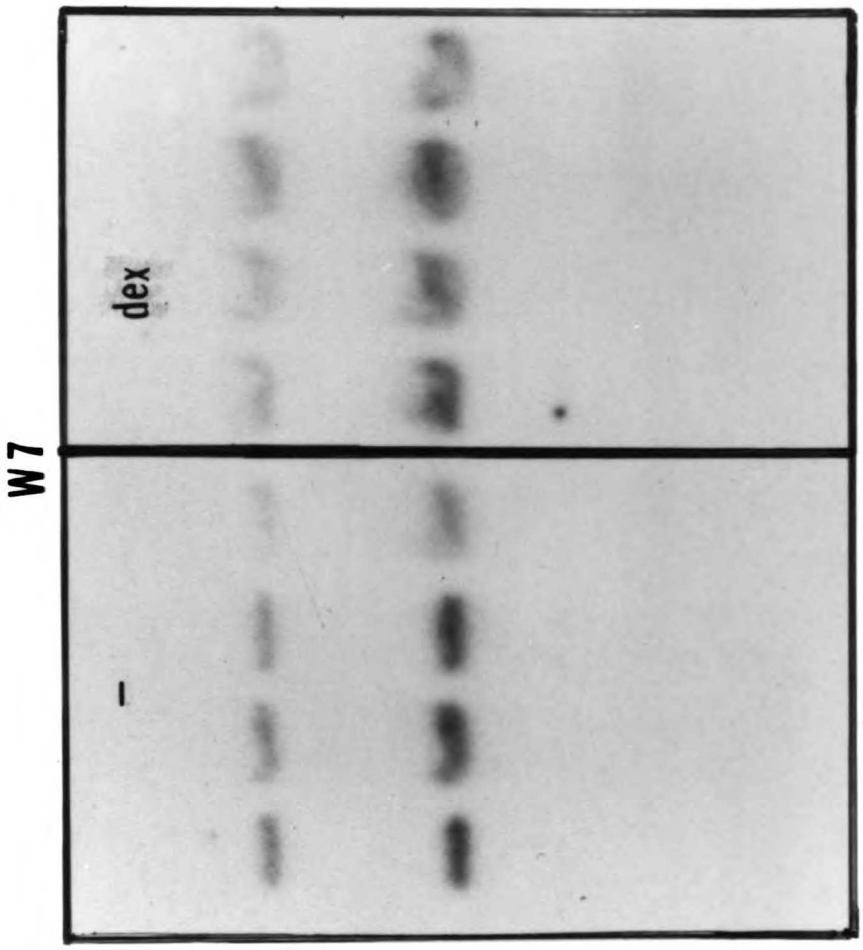

FIGURE 16

Figure 17. DNase I sensitivity of MTV sequence containing Eco RI fragments in M4.12H. The experiment was carried out as described in figure 9. Time points were taken at (left to right) 0 seconds, 15 seconds, 35 seconds, 50 seconds, 75 seconds, 95 seconds, 2 min, 2.5 min, 3 min, 4 min. Assuming 0.5-0.6% digestion per minute, as assayed by perchloric acid solubility, this corresponds to (left to right): 0.0%, 0.1%, 0.3%, 0.5%, 0.6%, 0.75%, 1.0%, 1.25%, 1.5%, 2.0%.

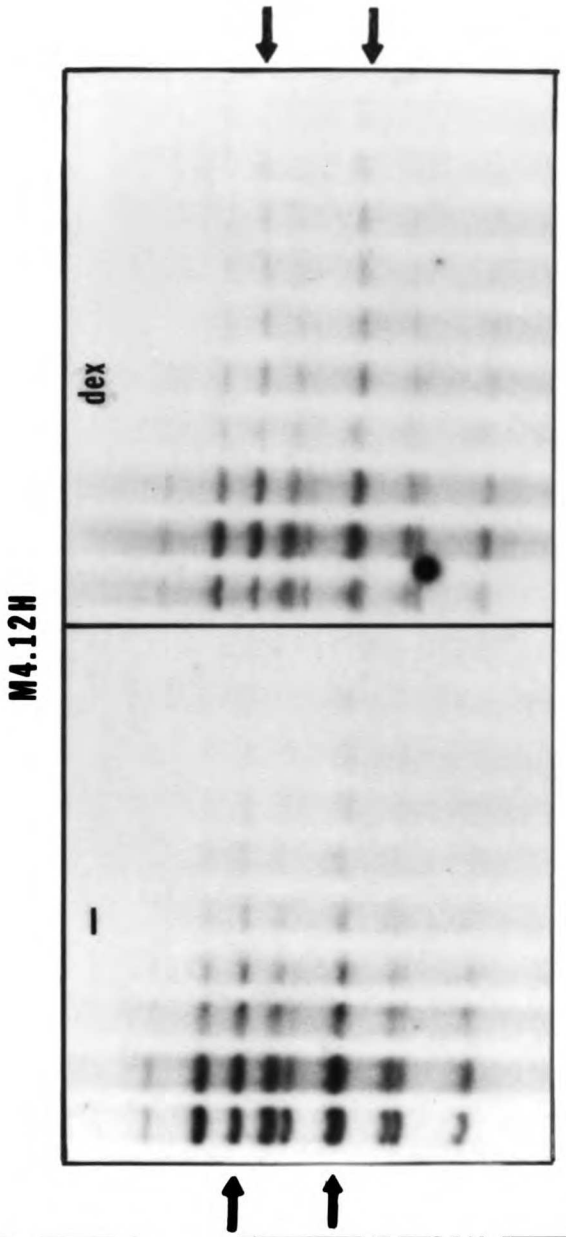

FIGURE 17

Figure 18. DNase I sensitivity of MTV sequence containing Hind III fragments in T1M1 4D.17. The experiment was carried out as described in figure 9, except that Hind III was substituted for Eco RI. Only data from dexamethasone induced nuclei is shown; uninduced nuclei gave identical results. Extents of digestion were (left to right): 0%, 0.75%, 1.75%, 3.0%, 4.1%, 5.7%, 8.4%, 11.0% and 13.0%, as assayed by perchoric acid solubility.

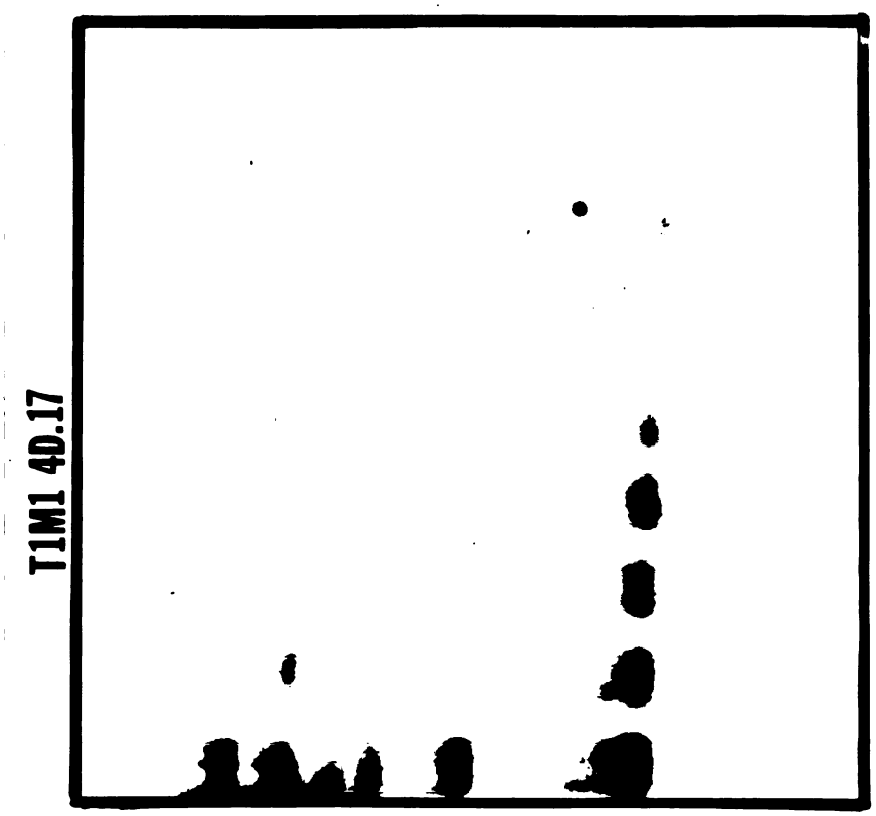

FIGURE 18

Figure 19. DNase I sensitivity of MTV sequence containing Hind III fragments in VL3 cells. The experiment was performed as described in figure 9, utilizing Hind III instead of Eco RI. Extents of digestion (left to right): (no dexamethasone) 0%, 0.5%, 1.8% and 5.0%; (dexamethasone) 0%, 0.4%, 0.75% and 3.0%, as assayed by perchloric acid solubility.

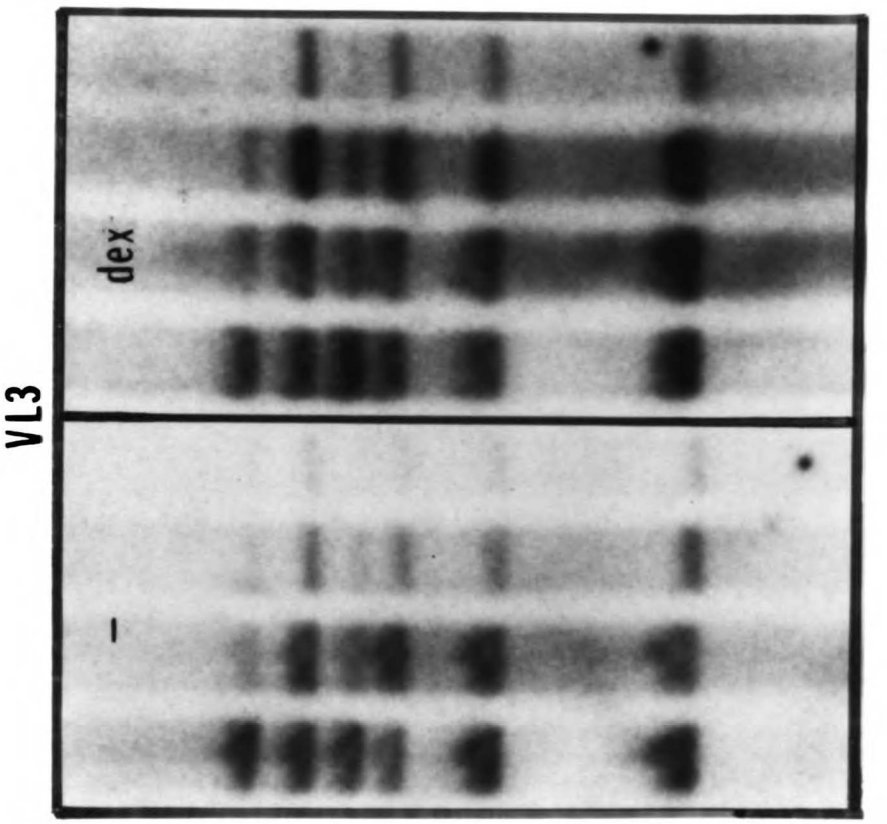

FIGURE 19

Figure 20. DNase I sensitivity of MTV sequence contain Hind III fragments in C57Bl mouse spleen cells. Four C57Bl mouse spleens were removed and cells were dispersed by puncturing and mincing the organ with razor blades. Nuclei were prepared identically to lymphoma nuclei, and DNase I treated. Restriction digests were performed with Hind III. Time points were taken at (left to right): 0 sec, 20 sec, 45 sec, 75 sec, 2 min, 4 min, and 6 min. Assuming 0.5-0.6% digestion per minute, as assayed by perchloric acid solubility, this corresponds to (left to right): 0.0%, 0.2%, 0.4%, 0.7%, 1.0%, 2.0%, 3.0%.

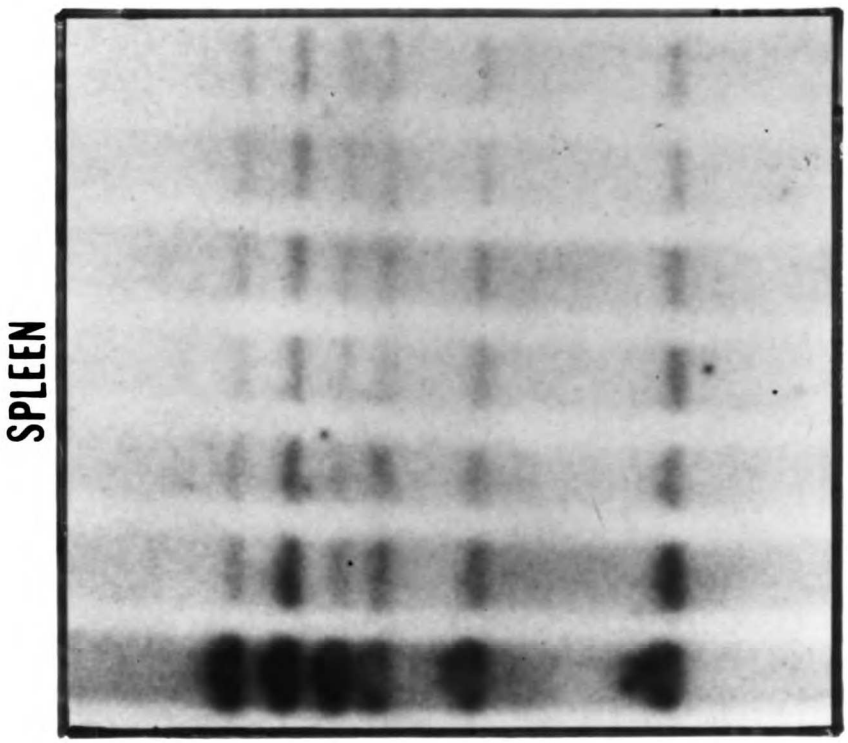

FIGURE 20

CHAPTER IV

BORDERS DEFINING DOMAINS OF CHROMATIN STRUCTURE

INTRODUCTION

It now seems apparent that the genome is organized into different packaging configurations, and can therefore be considered a structural mosaic. What might be the nature of the borders that define these structural domains? Are they sharp and distinct, or gradual structural transitions? That integrating MTV DNA is subject to chromosome structure spreading effects of adjacent host sequences, as proposed in chapter I, implies that this incoming DNA does not markedly effect the chromosome structure within the borders of the structural domain into which it integrates. However, might the newly acquired DNA effect the borders of the domain themselves? Moreover, in cases where dexamethasone alters chromatin structure of MTV proviruses, does the structural change respect the borders of the existing structural domain or rather establish new borders? As described below, the DNase I assay provides a sensitive procedure with which to define borders and begin to characterize their establishment and maintenance.

RESULTS

The DNase I procedure monitors the survival of a DNA fragment of choice. Any sensitive domain will confer sensitivity

upon an entire fragment. Therefore, a DNA fragment designated as resistant must be resistant throughout its entire length, whereas a DNase I sensitive fragment can have both DNase I sensitive and resistant domains. Fragments must be molecularly dissected utilizing various restriction enzymes to assess the sensitivity of each portion of the original fragment. This underlies the strategy employed to detect borders.

J2.17 cells contain a single hormone inducible, DNase I sensitive MTV provirus (see chapter I). The cloned Eco RI "b" fragment (3' half; see figure 13) from J2.17, which extends 1.9 kbp into the host sequences and is designated p17.1, can be utilized as a probe of adjacent chromatin structure on the downstream (3') side of the integrated provirus. The Eco RI "b" J2.17 fragment is DNase I sensitive (see chapter I). Additionally, a Pst I site resides in host DNA about 0.2 kbp downstream from the end of the viral sequences (see figure 13). Thus, by utilizing probes specific for the host Eco RI-Pst I fragment, chromatin structure of host sequences just downstream from the MTV provirus can be examined.

Aliquots of J2.17 DNA samples utilized in chapter I, figures 3 and 4, were digested with both Pst I and Eco RI and blots of the fractionated fragments annealed with probe specific for the Pst I-Eco RI host fragment (p17.2; see figure 2). The result is shown in figure 21B. The fragment at 1.6 kbp is clearly resistant to nuclease digestion. As this is the same J2.17 DNA used in chapter I, figures 3 and

4, MTV sequences serve as a positive control for this experiment. In chapter I, all Pst I MTV containing fragments were DNase I sensitive. This implies that a structural border exists between the most 3' Pst I site in the provirus and the region of the most proximal host Pst I site downstream from the provirus. It is possible that DNase I sensitivity could extend past the Pst I site a short distance into the Pst I-Eco RI fragment; since it is at the end of the fragment being examined, such sensitivity would not grossly alter the overall resistance of the fragment, but rather may be detected as a reduction in molecular weight. As no obvious change in size is observed during the time course, it is unlikely such sensitivity, if it exists at all, extends more than 100-200 base pairs past the Pst I site. The distance from the 3' most MTV Pst I site to 200 base pairs past the host Pst I site equals 1.6 kbp. However, most of these sequences are transcribed and therefore likely to be DNase I sensitive. Recent data suggest that the site of polyadenylation is about 240 base pairs from the end of the MTV genome (133). Therefore, the border is likely to be located within a region that is a maximum of about 600 base pairs, or the equivalent of 3 nucleosomes from the site of transcriptional termination. This implies that the transition between DNase I sensitive and resistant domains for the sequences being studied is sharply delineated.

Upon integration, did MTV DNA establish this border

immediately downstream from itself? Alternatively, did a border already exist in that region of HTC chromatin prior to the MTV DNA integration event? Did MTV integration cause the border location to be altered? Recall from chapter I that the J2.17 preinsertion fragment is DNase I sensitive (see figure 7). HTC DNA utilized in chapter I (figure 7) was digested with Eco RI and Pst I, and probed with p17.2 DNA. Figure 21A shows that the Pst I-Eco RI HTC fragment downstream from the future site of MTV integration is DNase I resistant. Thus, to a first approximation, integration of 9000 base pairs of MTV DNA has not altered the presence of the chromatin structure border. This result is consistant with results in chapter I which suggest that MTV DNA is passive with regard to establishing chromatin structure and merely adopts the structure of its preinsertion fragment. Figure 22 schematically summarizes the above data.

In order to more fully understand border formation, it would be extremely useful to define the position of the border upstream from the J2.17 provirus. Attempts to clone sequences upstream from MTV proviruses have been severely hindered by a segment of DNA within the MTV genome which is not compatible with growth in E. Coli, the so called "poison sequence". Further, it appears that repeated DNA sequences exist just upstream from the MTV element, making Southern blotting very difficult. Nonetheless, alternative approaches

are being considered to define the J2.17 border upstream from the MTV element.

Figure 21. DNase I sensitivity of proviral flanking sequences. HTC (A) and J2.17 (B) DNA analyzed in figures 7, and 3 and 4, respectively, was digested with both Eco RI and Pst I, fractionated on agarose gels, blotted and probed with p17.2 (^{32}P) DNA (see figure 2) specific for the HTC Pst I-Eco RI fragment downstream from the J2.17 proviral site of integration. Extents of digestion were (left to right): 0.0%, 0.6%, 0.9%, 1.1% as assayed by perchloric acid solubility.

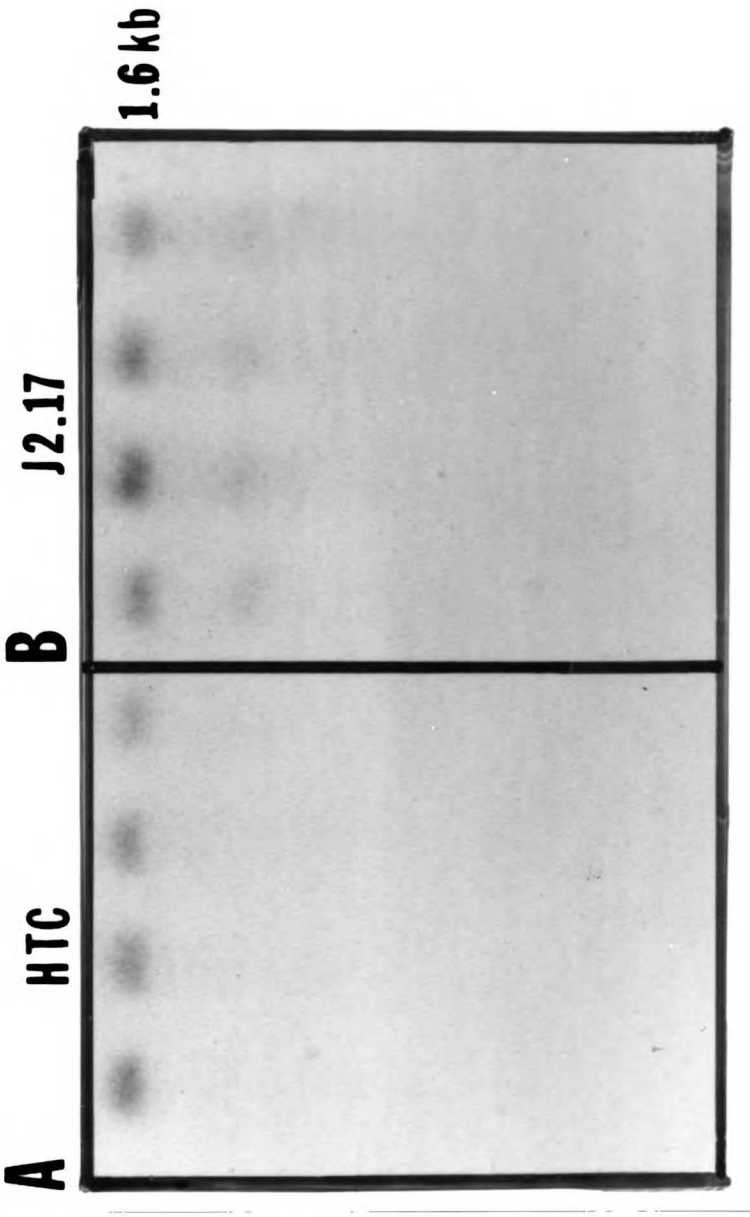

FIGURE 21

Figure 22. Schematic summary of border analysis. Eco RI(∇) and Pst I(Δ) sites are depicted. Dashed lines (- - - -) represent DNase I sensitive fragments. Heavy solid lines (———) represent DNase I resistant fragments. The region defined as "border region" assumes DNase I sensitivity extends at least to the site of polyadenylation.

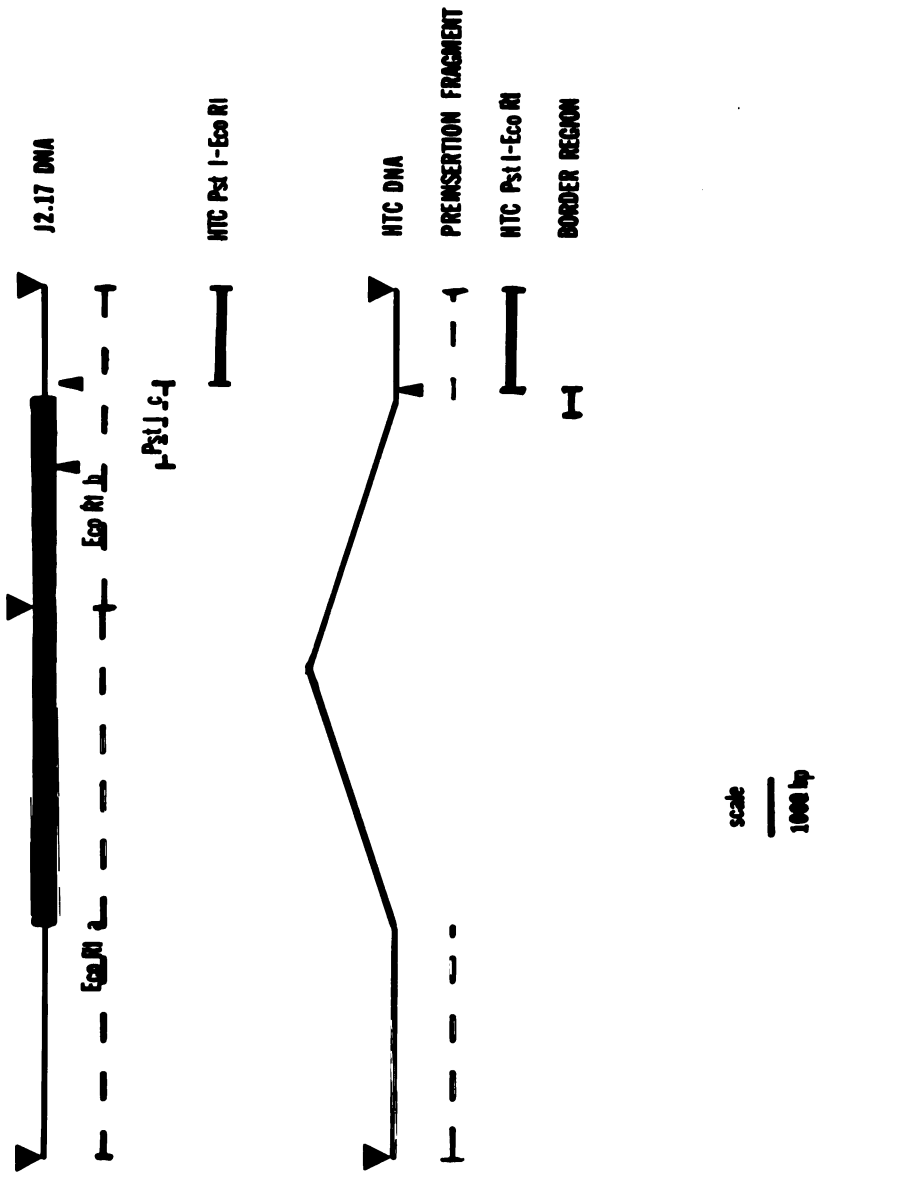
J2.17 DNA
NTC Pst I-Eco RI
NTC DNA
PREINSERTION FRAGMENT
NTC Pst I-Eco RI
BORDER REGION
scale
1000 bp

FIGURE 22

CHAPTER V

DISCUSSION

To assess the role of chromatin structure in steroid mediated regulation of MTV gene expression, an assay was devised in which the action of DNase I upon specific sequences in isolated nuclei was monitored by the blotting procedure of Southern (144); a similar procedure has been described by others (97,139,140). This dramatically improves resolution relative to solution hybridization, allowing visualization of individual fragments and subfragments of choice, rather than averaging multiple fragments. One limitation of the procedure is that identical genes on homologous chromosomes are scored together as an average, as they produce identical restriction enzyme patterns. That is, if one identical homolog were sensitive and the other resistant, the assay will define the gene as relatively resistant. This limitation is not relevant to MTV proviruses acquired by exogenous infection, since they presumably integrate into only one of the homologous chromosomes at a given site.

The possibility that hormone mediated MTV gene expression simply reflects integration into steroid responsive loci has been ruled out by data with the MTV provirus in J2.17, where it was demonstrated that the preinsertion fragment in uninfected HTC cells is transcriptionally quiescent both in the presence and absence of dexamethasone (133). Rather, sequences required

for steroid mediated transcription appear to be present within the MTV element itself. These regulatory sequences will be designated as the "local" regulatory element(s). However, gene dosage alone does not account for the magnitude of steroid mediated induction of MTV RNA in various infected tissue culture cell lines; integration of MTV DNA into the host genome is not itself sufficient to insure expression (128,129). Thus, it seems likely that additional regulatory mechanisms are involved. How might such mechanisms function?

Packaging As A Determinant Of Expression

Analysis of MTV proviruses exogenously introduced via infection, which differ only by their integration site into the host genome, demonstrates that the same DNA sequence can be packaged in different structures. In a general way, these structures correlate with transcriptional potential, as shown by non-expressing cells such as J2.15 and expressing lines such as J2.17. In addition, the same DNA sequence, located at the same loci in closely related cells, can be packaged differently. This is observed with proviral DNA in T1M1 4D.17, W7 and spleen cells. Again, the correlation between DNase I sensitivity and transcriptional competance holds true. These results are analagous to tissue specific expression and structure analysis of differentiated genes such as globin and ovalbumin (79,80), with the advantage that MTV sequences are differentially packaged in T1M1 4D.17, VL3 and W7, all derived

from closely related cell types.

The failure of MTV proviral sequences to transcribe in J2.15 and W7 appears to be the result of cis acting constraints. The cells themselves are competant to respond to glucocorticoid hormones. For example, in J2.15 cells, dexamethasone induces glutamine synthetase and TAT, two host glucocorticoid inducible enzymes, indistinguishably from uninfected parental cells (129). W7 cells are killed by physiological doses of glucocorticoids, a hormone receptor mediated event (137). Furthermore, superinfection of J2.15 (153) and W7 (137) with MTV virions produces cells capable of synthesizing MTV RNA in response to glucocorticoids. Thus, it appears that all diffusible factors requisite for MTV RNA induction are present, and that no trans acting repressors of MTV RNA induction are present in these cells.

It is conceivable that these inactive proviruses have sustained mutations that preclude expression; this possibility can be tested by DNA transformation experiments, or ultimately by direct sequence analysis. Technical difficulties dicussed earlier (a small "poison sequence" in the MTV genome incompatible with growth in E. Coli) prevent cloning of the integrated J2.15 MTV provirus, currently making this experiment relatively difficult. However, lymphoma endogenous proviruses are clonable and therefore amenable for DNA transformation experiments. It is significant that in the fourteen independant cell lines tested to date, the DNase I sensitivity of proviral sequences

is fully correlated with the potential for MTV gene expression, with the possible exception of VL3, which will be discussed in more detail below. Thus, while mutations within the viral sequences may account for some fraction of the proviruses that display altered expression, it seems likely that chromatin structure is a major determinant of the potential for MTV hormone responsiveness.

The present results suggest that DNase I sensitive chromatin packaging is essential, but not sufficient for expression, whereas DNase I resistance may be sufficient, but not necessary to preclude expression. Regulation at the level of chromatin structure is here designated "packaging" control, in contrast to "local" control defined earlier (for example, specific regulatory sequences). It is proposed that both permissive packaging and proper local regulatory elements must be present in order for transcription of a locus to occur; both packaging and local regulatory elements act in cis, and can be responsive to trans acting regulatory factors. Thus, the failure of J2.15, W7 and M13B7 to transcribe MTV sequences in the presence of dexamethasone is attributed to nonpermissive packaging, while these elements likely possess appropriate local regulatory elements. Recently published data support this interpretation. A presumably transcriptionally inert endogenous MTV provirus was cloned intact and introduced into other cells by DNA transformation (150). The recipient

cells were then capable of inducing MTV RNA in response to dexamethasone.

Clearly, DNase I provides only a crude measure of chromatin structure; packaging seems unlikely to act as a simple binary control mechanism. Considering the vast number of possible histone modification arrangements and nonhistone chromosomal protein interactions with core structures and linker regions, it seems very possible that a large number of structures exist. The complex pattern visualized in the polytene chromosomes of Drosophila larval salivary glands implies that this is so. One interpretation of the DNase I studies presented in this dissertation is that under the conditions employed, DNase I is capable of discriminating one group of chromatin structures from another, but that it cannot readily distinguish structures within a group. For example, it is not clear whether the active J2.17 provirus has precisely the same chromatin structure as other active proviruses. Additionally, an argument can be presented proposing the existance of multiple functional forms of transcriptionally active chromatin, i.e., forms which all produce transcripts, but at markedly different rates. For example, M1.54 is an MTV infected HTC cell line containing about 10 MTV proviruses (154); it has an induced rate of MTV RNA synthesis at least fifty fold greater that J2.17 (133). Thus, rate of MTV transcription per provirus in M1.54 must be greater than in J2.17. Additionally, if some M1.54 proviruses are inactive, then the rate of MTV RNA

synthesis per functional provirus is even greater. Further, that MTV expression is observed in the absence of hormone demonstrates a functional difference between J2.17 and at least one M1.54 provirus. Ultimately, assays capable of higher resolution, in conjunction with genetic manipulation of cell lines, will be required to determine precise relationships between structure and function.

In principle, increased rates of transcription could reflect readthrough transcription from an active cellular promotor upstream of the proviral integration site. However, in the case of M1.54, MTV RNA is strongly dexamethasone inducible (128,129), viral transcripts are identical in size to those seen in J2.17 (143), and recent mapping data shows that all MTV RNA synthesized in M1.54 initiates at a single site within the MTV genome. Thus, the simplest interpretation is that different transcriptionally active proviruses have differing levels of activity, which could be mediated by different packaging arrangements. Multiple packaging structures have been clearly seen in M4.12H. A hierarchy of distinct gene packaging arrangements corresponding to different levels of transcriptional activity may exist. This possibility can be examined by biochemical and genetic approaches. Beginning with cell lines infected by a single MTV provirus, variants synthesizing MTV RNA at higher or lower rates could be isolated, and correlations to gene packaging arrangements determined. Also, more cell lines harboring single inducible proviruses

should be isolated. Do they all synthesize MTV RNA at equivalent rates? Do their structures differ? How do the J2.17 and B13 proviruses differ?

Structural and transcriptional differences observed between endogenous MTV proviruses in spleen, W7 and T1M1 4D.17 demonstrate that altered states of chromatin structure are heritable. The data are consistent with the notion that an alteration has occurred in chromosome structure determination for T1M1 4D.17 MTV proviruses, in that normally non-expressing and DNase I resistant proviruses have converted to DNase I sensitive and are transcriptionally active. Successful DNA transformation experiments with cloned spleen endogenous proviral sequences will be essential to proving that local regulatory elements are intact, but inactive in these proviral sequences. The mechanism of such a structural alteration could be very important to the questions of tissue specificity and developmental activation of genes.

With respect to the molecular nature of different packaging arrangements, it is noteworthy that micrococcal nuclease produces identical results as does DNase I in J2.15 and J2.17 nuclei (155), utilizing the assay described in this dissertation. This suggests that the distinctions between these packaging arrangements is not limited to the nucleosome core, as micrococcal nuclease preferentially attacks linker DNA while DNase I preferentially attacks core DNA. It is possible that differences

manifested at the primary level of chromosome structure could have consequences for the ultimate higher order structure of that region of chromosome.

The Spreading Effect

Position effect variegation was originally observed following chromosomal translocations that brought active genetic loci into proximity with highly condensed "constitutive heterochromatin" (77); those experiments provided the first suggestion that a segment of DNA may be folded into different configurations independent of its specific sequence, and that those folding patterns can profoundly affect the potential for expression of that segment. However, detection of this gradient or "spreading effect" of gene inactivation by cytological observation is limited in resolution; only gross structural transitions affecting very large genetic distances can be monitored. As such, these events were not readily amenable to detailed molecular analysis.

Studies to date have not directly approached the nature of position effects. For example, Weintraub and Groudine (79) compared either different genes at their respective loci in a single cell type or a single gene at a single locus in different cell types. In the first case, both gene sequence and position differ, so the source of the difference in activity is ambiguous; in the second, the use of different cell types introduces multiple variables. By analyzing a

single sequence at different positions within the genome of a single cell type, the issue of specification of structure can be approached.

The present experiments concern the structure and activity of a single DNA sequence located at different chromosomal sites within a single differentiated cell type. Therefore, because chromosomal position is the only variable in these lines, its effects on expression of a discrete genetic element can be unequivocally assessed. Moreover, uninfected HTC cells provide the true genetic null; in effect, a "deletion mutation" lacking the MTV genes only. As such, these experiments appear to approach at a finer level of discrimination the phenomenon originally described by Lewis (77); the results are consistent with the view that different types of chromatin structure can "spread" across a newly introduced sequence, and that the structure is an important determinant for subsequent expression.

These experiments suggest that MTV DNA itself does not determine its own chromatin structure and that it acquires the packaging arrangement of the region into which it integrates by a spreading effect. In one view, chromatin can be regarded as a mosaic crystalline construction in which different domains of the chromosome have distinct "crystalline" structures. Upon integration of a new segment of DNA within such a domain, crystal packing of nucleosomes acquired by the inserted DNA would determine its chromatin structure, thereby resulting

in the spreading effect. It should be noted that only two cell lines have been tested. Experiments to test the generality of this spreading effect are in progress.

The spreading effect could have important regulatory implications. Consider, for example, mating type determination in yeast. Elegant genetic analysis has given rise to the cassette model (156). This model proposes that yeast cells contain silent copies of both a and α mating type genes, while also possessing an active copy of a or α at the MAT locus, which governs the mating type of the cell. Copies of the silent cassettes can integrate at MAT, replacing the previous resident DNA and become transcriptionally active. Most importantly, it has been genetically demonstrated that the inert a and α genes contain potentially functional local regulatory elements (157). Thus, perhaps the silent a and α cassettes are packaged nonpermissively whereas MAT is packaged in a permissive chromatin structure. Upon translocation from the silent locus to MAT, the integrating fragment of DNA adopts the permissive packaging arrangement present at MAT by a spreading effect, and transcription can thereby proceed.

Another example of mobile DNA relating to expression involves regulation of immunoglobulin synthesis. Transcription could be activated after rearrangement by the spreading of a permissive chromatin structure from the new structural environment into the immunoglobulin sequences. Recent data regarding the V_K chain are consistent with this notion (158). This gene is

packaged in DNase I resistant chromatin in the germline locus, whereas it is DNase I sensitive at the rearranged somatic locus. Interestingly, the C_K gene is DNase I sensitive both at germ line and rearranged loci. Speculatively, one could imagine that the germ line V_K gene contains a promotor, but expression is precluded by packaging regulation. The germ line C_K gene may be packaged permissively, but is not transcribed for lack of a promotor, until somatic rearrangement aligns it with the V_K gene.

Finally, it should be noted that MTV is an ideal genetic element with which to study the spreading effect. MTV DNA, containing its own regulatory sequences, can integrate randomly throughout the genome, and therefore presumably adopt any chromatin structure that exists. Effects on expression can then be determined. This allows an in vivo assessment of any chromatin structure configuration that the cell uses on a single, regulatable genetic element.

Functional Organization of MTV Genes and Mechanism of Receptor Action

DNase I resistant MTV sequences appear to be packaged in at least two phenotypically distinct structures. Those in J2.15, W7 and spleen are not hormone responsive and fail to be transcribed. In contrast, the resistant structure found in B13 in the absence of hormone converts to a DNase I sensitive form in the presence of dexamethasone and transcription is

observed. An analagous chromatin effect has been reported in Drosophila, in which a heat shock reversibly alters the DNAse I sensitivity of a set of inducible genes (140). If the structural transition were to cause the activation of expression, then the structural alteration must precede the initiation of transcription. Arguments can be made favoring altered structure leading to altered function, as presented in the introduction. Experiments to analyze this issue are underway. Two approaches are planned. First, the kinetics of structural alteration will be determined relative to the increased rate of transcriptional activation. Second, transcriptional inhibitors will be employed to determine if the alteration ofstructure is blocked concurrently with trancription, or alternatively, if altered structure can proceed in the absence of induced transcription.

At least two phenotypic classes of sensitive chromatin have been observed. In one class seen in J2.17, in two of the T1M1 4D.17 proviruses and in at least some of the M4.12H proviruses, the MTV sequences are sensitive, but inactive in the absence of steroid. In the presence of dexamethasone, these still sensitive genes are transcribed actively.

Results in VL3 could create a third phenotype of DNase I sensitive structure, one sensitive but not transcribed in the presence of dexamethasone. Such a structure may also exist in other cells with multiple sensitive proviruses, such as M4.12H, but currently it is impossible to determine which of the DNase I sensitive sequences are in fact transcriptionally

active. However, further analysis is required to precisely determine the situation in VL3 cells. As discussed above, the preferential sensitivity of the C57Bl specific provirus is apparent only late in the time course. Thus, this may be an example of a "less resistant" resistant chromatin structure, representing a subclass of transcriptionally inactive, resistant structures. The C57Bl specific provirus appears to be markedly more resistant than its TlMl 4D.17 counterpart. Alternative and testable possibilities exist. VL3 cells could have undetectably low steady state levels of MTV RNA, caused by "leaky" expression and/or a relatively rapid rate of MTV RNA degradation. This possibility can perhaps be assessed by determining the rate of MTV RNA synthesis in the cells. Alternatively, the DNase I sensitive provirus could be an example of permissive packaging in the absence of appropriate local regulation, i.e., a mutation in regulatory sequences. This possibility can be tested by cloning the appropriate proviral sequence and utilizing it for DNA transformation experiments. Both rate of synthesis and cloning experiments are in progress. Interpretation of VL3 results therefore awaits the outcome of these experiments and a more definitive kinetic analysis of VL3 and TlMl 4D.17.

The present data indicate that hormone receptor mediated alteration of chromatin structure, as defined by DNase I sensitivity, is not essential for transcriptional activation; however, detectable changes do occur in some cases. Combining

these data with the previously described spreading effect data, the following models are suggested. MTV DNA integrates into a given genomic locus, adopting the packaging structure of the adjacent chromatin and thereby transcriptional potential is determined. If the acquired structure is comparable to that of B13, a marked effect of hormone on the DNase I sensitivity of its chromatin will be observed. Alternatively, if the structure is comparable to J2.17, then the observed effect of dexamethasone on proviral DNase I sensitivity will be minimal or non existant; receptor may alter only certain forms of chromatin structure. Alternatively, structural changes not detected by DNase I could in fact accompany activation of such proviruses, but are not detectable under these experimental conditions. In this view, the receptor molecule acts as a site specific chromatin structure determining element (i.e. an element capable of mediating packaging regulation). Alternatively, if the altered chromatin structure in B13 were to be only a consequence of transcriptional activity, the receptor may simply act only at local regulatory sequences. A third possibility is that the receptor facilitates both packaging and local regulatory effects, directly or indirectly. According to this scheme, transcriptional activation of sequences in B13 requires both packaging and local regulatory functions of the receptor, whereas the structure in J2.17 might be activated by a local regulatory function alone. Higher resolution assays of structure, <u>in vitro</u> reconstruction

of the hormone response, and genetic approaches will likely be necessary to elucidate the actual mechanism.

Different genes related to one another by either function or time of expression may have characteristic packaging arrangements. For example, constitutively expressed functions could be organized with simple promotors in permissive packaging configurations. Transcription could proceed without need of additional trans acting regulatory factors functionally analagous to CRP. Terminally differentiated functions in nonexpressing cells could be packaged in an unresponsive nonpermissive chromatin structure comparable to that found for the MTV provirus in J2.15, with their local regulatory elements intact. In expressing cells, the same genes could be packaged in a permissive structure either with or without a trans acting regulatory factor requirement. Tissue specific transiently expressed genes, such as inducible functions, would be packaged in unresponsive, nonpermissive structures in nonexpressing tissues. Conversely in potentially expressing cells, transiently expressed functions would be packaged permissively for transcription (DNase I sensitive or resistant), with an obligatory requirement for a trans acting regulatory factor to modulate expression via packaging and/or local regulatory control. Trans acting regulatory molecules, such as the glucocorticoid receptor in B13, could thus act as chromatin structure determining elements.

Modulation of gene expression is crucial for the numerous

molecular events essential for proper differentiation and development. If the regulatory functions of chromosome packaging arrangements are superimposed upon inducer and repressor molecular mechanisms of control, a vast array of potential levels of expression can be imagined. Perhaps of consequence, it should be recalled that in prokaryotes, a given gene product will vary up to 10^3 fold between repressed and induced states. In marked contrast, a given eukaryotic gene product can show as great as 10^7 to 10^8 fold different levels of expression in cells from the same species (159). This great range may be required for two reasons. The vast size of the eukaryotic genome may demand highly efficient, tissue specific repression of differentially expressed genes. A small quantity of each of a great many gene products could present intolerable energetic expense and functional inefficiency for the cell. Second, highly specialized differentiated cells often make tremendous quantities of a few gene products. To attain these levels, perhaps an extremely amenable chromatin structure can markedly increase the rate of transcription by synergistically interacting with the local regulatory elements.

Tissue Specificity and Structural Borders

Steroid hormones display two distinct levels of specificity. Only a small subset of all genes are responsive to a given steroid hormone. Further, only a subset of these genes are responsive in a given cell type. Current data support the

idea that a given steroid receptor is the same gene product is all cells (160); thus, tissue specificity of the hormone response is apparently not achieved via tissue specific receptor proteins. An alternative possibility is that selectivity operates by allowing only specific subsets of steroid responsive genes in a given cell type to acquire a chromosome structure permissive for expression. Thus, hormone bound receptor molecules might interact functionally only with that subset of putatively responsive loci that is determined at the level of chromatin structure.

Given this view of tissue specificity, how are tissue specific domains of chromatin structure established and maintained? Possible mechanisms can be imagined. Domains of chromatin structure may be established by interactions between repeated DNA sequences and tissue specific members of a general class of chromatin structure determining proteins. Specific proteins establish borders and/or the configuration within the structural domains. As differentiation proceeds, different arrays of structure determining proteins either alter existing domains or establish new domains, in a tissue specific manner. These domains are stable and heritable, perhaps by a templating mechanism at replication forks which provides that each daughter DNA molecule be packaged identically as the parental molecule. Domains can be altered only by superceding structure determining proteins synthesized at

appropriate times by the developing cells. It may not be necessary that such determination factors be constitutively present to maintain domain structures if domains are stably maintained with high efficiency. This could avoid direct competition between different structure determining factors. By this scheme, co-ordinate developmental activation of banks of genes required at given developmental stages would require a relatively small number of structure determining proteins expressed sequentially.

Regulation of gene expression during differentiation and development can therefore be imagined to be a cascade of structure determining proteins, which present the appropriate limited subset of genes and local regulatory elements to the cellular transcriptional apparatus. In this view, transcription is modulated both by packaging control and local regulatory elements, both of which can be modulated by specific trans acting regulatory elements.

At a mechanistic level, how might these packaging conformations and domain borders be determined? Several models can be imagined. On the one hand, both border location and packaging conformation might be determined by a single mechanism. Two extreme models can be proposed for such a process, as seen in figure 23, A and B. In case A, a chromatin structure organizing center produces a translocatable effect along the chromosome a given distance in either direction. Such a model has been suggested by Cattanach (78), based on X chromosome translocation

data. Governed by such a mechanism, borders of chromatin structure are likely to be gradual and diffuse. Differentiation proceeds by cascades of chromatin determining proteins creating successive waves of organizing centers.

In case B, asymetric structure determining elements establish structure in one form to the left and another to the right. Theoretically, "hybrid" packaging conformations could exist if the two adjacent asymmetric structure determining elements demarcating a single structural domain specified different structures; such a situation would vastly increase the possible number of structural, and perhaps functional, states in which a domain could exist. In contrast to model A, this mechanism predicts sharp, distinct borders, which could, but need not, alter location during development. Differentiation proceeds by a cascade of asymetric chromatin structure determining proteins.

A different type of model suggests that mechanisms determining domain border location could be completely distinct from mechanisms specifying structural conformation within that domain (see figure 23C) Thus, location of borders could be determined by sequence specific DNA binding proteins, and/or particular higher order DNA structures. Additionally, independantly acting chromatin structure determining proteins specify the packaging conformation within borders, perhaps acting via a chromatin stucture organizing center such as that in model A. Depending on the specificity of factors

determining border locations, these positions may or may not vary during differentiation. Again, differentiation could proceed by a cascade of chromatin structure determining proteins, plus perhaps factors altering border location. Clearly, combinations of models A, B, and C are possible.

Perhaps relevant to model C is data regarding giant chromosomes in Diptera. With few exceptions, the band-interband pattern of the chromosomes is identical in cells from different tissues (161). Further, the number and sequence of bands and interbands is invariant in all examined somatic tissues, and is remarkably well conserved evolutionarily (161). Differences observed between tissues tend to be localized within the confines of a band or interband (161). This data could be interpreted as suggesting that, at least at the cytological level, border locations are invariant in chromosomes, but that structure within a domain is alterable while respecting the invariance of the borders.

Data presented in chapter IV of this dissertation, defining a structural border in J2.17 and uninfected parental HTC cells, favors models B and C, inasmuch as integration of 9000 base pairs of DNA does not alter the location of a packaging border. Further, the border appears to be very sharp and distinct. Clearly, it is crucial that more examples of borders be isolated for further analysis.

Future Directions

The mechanism of chromosome structure changes can be directly analyzed in the B13 cell line, where MTV sequences convert from DNase I resistant to DNase I sensitive in response to dexamethasone. Toward a better understanding of the establishment and function of chromatin structural domains, the kinetics of chromatin alteration relative to transcriptional activation will be determined. Further, drug inhibitor studies will attempt to separate the steroid induced alteration of chromatin structure from transcriptional initiation. These experiments will help determine whether chromosome structure is a determinant or a consequence of transcriptional activity.

In order to understand more fully the construction of borders, it will be crucial to fine structure map the J2.17 and HTC borders. Is the border identical in the cell lines? How sharp is the demarcation? Is a border present at the same position in other rat cell lines?

If borders can be defined for the DNase I sensitive T1M1 4D.17 MTV proviruses, direct comparison to appropriate spleen positions can be made. Does spleen chromatin have a detectable border of any sort at the appropriate position? Is there any sequence homology between borders? Are the borders sharp and distinct, or gradual?

Possible relationships between borders and nucleosome phasing will also be investigated. Might nucleosomes be phased with respect to specific sequences at border locations?

This could account for the sharpness of the observed HTC border, though other models can also do so. Similar experiments can be performed on T1M1 4D.17 and spleen, when borders are defined in those cells.

Might nucleosomes be phased within MTV chromatin and be important for expression? Are phasing patterns altered co-ordinately for inducible proviruses in response to hormonal stimulation? Are there different phasing patterns for potentially active proviruses as opposed to nonresponding proviruses? Analysis of phasing patterns in J2.15 and J2.17 are in progress.

Genetic approaches can also be designed. As examples, screening of hormone treated nonresponding cell lines such as J2.15 or W7 can be performed to isolate genetic variants expressing MTV RNA. It would be predicted that the MTV chromatin structure in such variants must convert to DNase I sensitivity. When these variants are available, rates of MTV transcription can be compared to nuclease sensitivity. Can structural differences be detected among the variants? Perhaps higher resolution probes will be able to distinguish different structures correlating to different transcriptional rates. Have phasing relationships been altered or established? Once borders are defined in the variants, structural and sequence comparisons can be made both to parental and uninfected HTC cells. Alternatively, non-responding J2.17 of B13 variants can be recovered and similarly examined.

Analysis of chromatin structure and its role in eukaryotic gene expression is yet at an early stage of development. It certainly appears that packaging regulation is an important factor in the control of eukaryotic gene activity. As described in the introduction, there exists a large variety of potential mechanisms by which to mediate packaging regulation. Development of higher resolution assays of chromatin structure, efficient *in vitro* reconstruction systems and genetic manipulation of cells will be essential to obtain precise molecular details, yielding the data required for an understanding of the role of chromatin structure in the regulation of eukaryotic gene expression.

Figure 23. Models for border formation and structural determination. In (A), an organizing center exists which propagates a given packaging arrangement a given distance from itself symetrically. In (B), asymmetric elements determine a given structure in one direction, and another structure to the other side. In (C), the limits of the domain are demarked by border determinants, while the chromatin conformation within the domain is independantly determined by a structure determining factor(s).

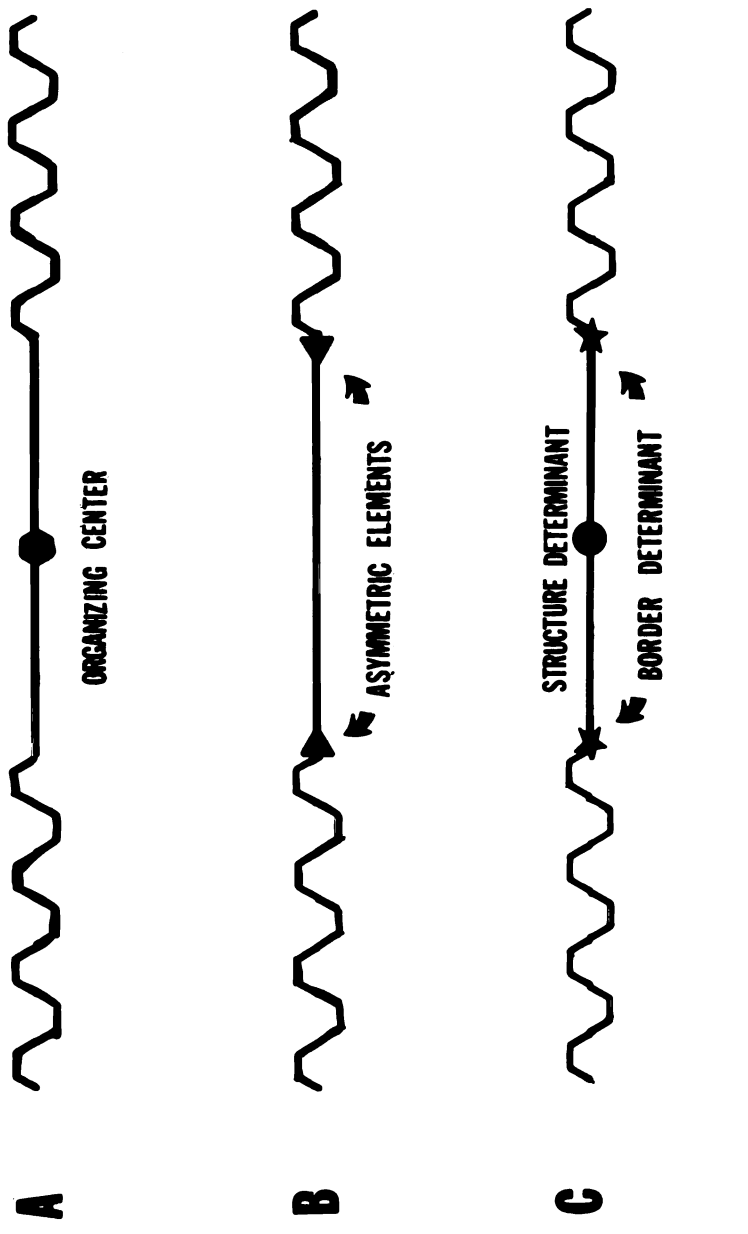
A
ORGANIZING CENTER
B
ASYMMETRIC ELEMENTS
C
STRUCTURE DETERMINANT
BORDER DETERMINANT

FIGURE 23

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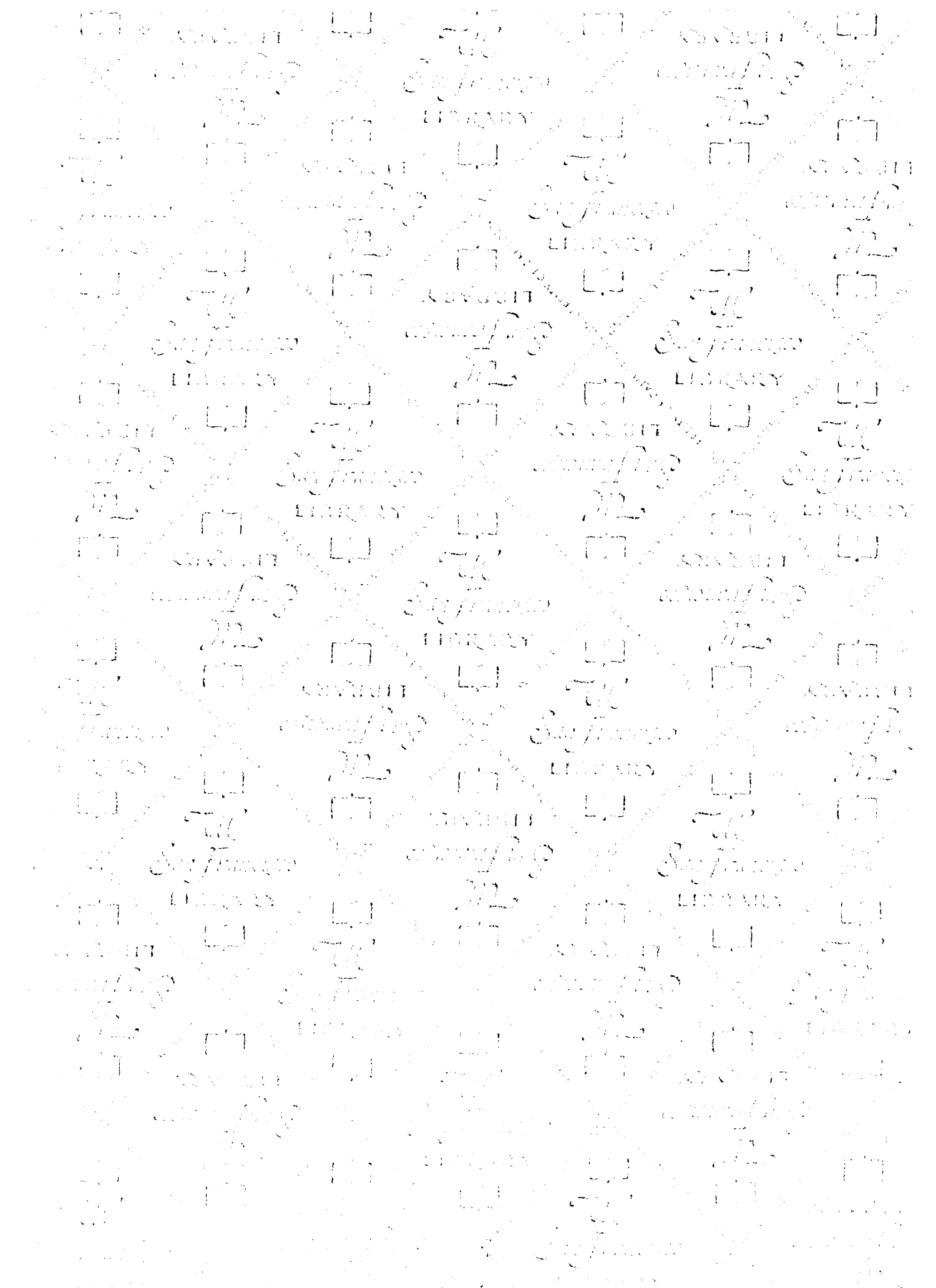

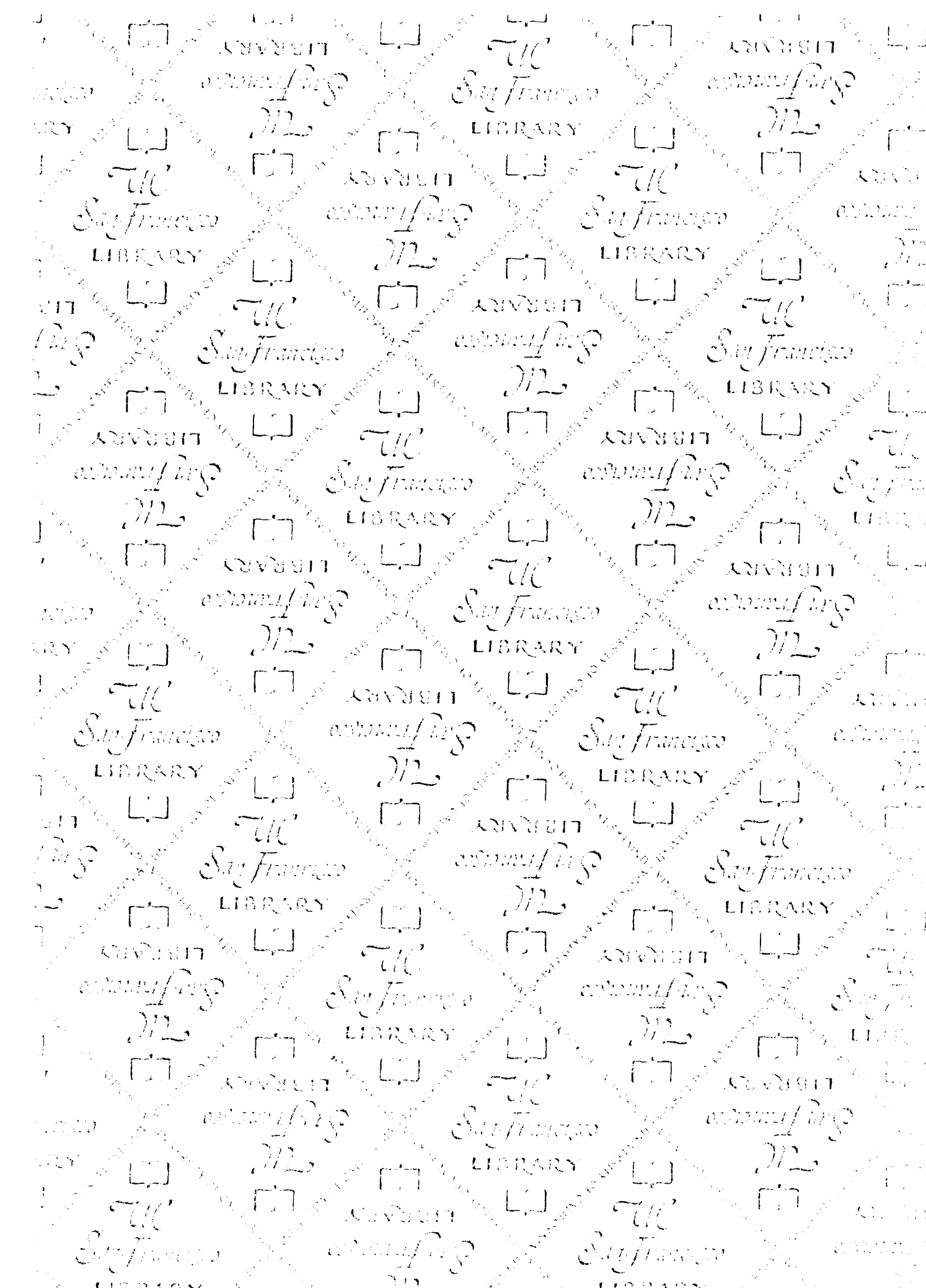
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