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An enhancer LEF-1/TCF-1 site is essential for insertion site-independent transgene expression in thymus

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ABSTRACT

Transcriptional activation of eukaryotic genes involves assembly of specific multiprotein complexes on the promoters and enhancers of the genes. Recently, it has been proposed that the role of some of the proteins in the complex may be architectural, involving DNA bending, orchestration of protein–protein interaction and modulation of nucleosome structure. This role has been proposed for the HMG proteins LEF-1 and TCF-1. We examined the role of a LEF-1/TCF-1 binding site in the human adenosine deaminase (ADA) thymic enhancer. Mutational analysis demonstrated that a functional LEF-1/TCF-1 binding site is not required for enhancer-mediated transcriptional activation in transient transfection studies, but is essential for enhancer function in the *in vivo* chromatin context of transgenic mice. Mutation of the LEF-1/TCF-1 site destroyed the ability of the ADA enhancer/locus control region to specify high level, insertion site-independent transgene expression in thymus. DNase I and *Dpn*II accessibility experiments indicated dramatic changes in the chromatin organization of the ADA enhancer in transgenic mice with a mutated LEF-1/TCF-1 site. This supports the hypothesis that factors binding the LEF-1/TCF-1 site play an architectural role during the *in vivo* activation of the ADA enhancer, possibly involving chromatin modification.

INTRODUCTION

The adenosine deaminase (ADA) gene is expressed at high levels in human thymus, where it is required for proper maturation of developing thymocytes. A T cell-specific enhancer for the ADA gene is present within the first intron of both the human and mouse ADA genes (1–5). Six DNase I hypersensitive sites (HS) have been identified in the first intron of the human ADA gene and the thymic enhancer segment is associated with HS III. A core 225 bp segment within HS III is necessary and sufficient to activate the ADA promoter to drive high level expression of the chloramphenicol acetyltransferase (CAT) reporter gene in transient transfection experiments in the MOLT 4 T cell line (3), a line that expresses very high levels of endogenous ADA. In contrast, a larger 2.3 kb intronic fragment containing both HS II and HS III is required in transgenic mice to consistently drive high thymic CAT expression that is proportional to transgene copy number and independent of transgene insertion site. The additional functional sequences flanking the core enhancer are referred to as facilitators and they appear to play a role in establishing the proper chromatin structure at HS III (1,3).

Most studies on transcriptional regulation of eukaryotic gene expression to date have focused on sequence-specific transcription factors that bind their recognition sequence in the promoter or enhancer segment of a gene (6). After binding, many of these factors act as activators or repressors of transcription by interacting, either directly or indirectly, with the factors which assemble at the transcription start site to form the general transcription complex prior to initiation of actual gene transcription. When and where a particular gene is transcribed is determined by the specific array of factor binding sites present in the promoter and regulatory regions of the gene. At least part of this specificity is probably determined by the relative positions and orientations of the sites and interactions of the various bound factors. For example, stereospecific nucleoprotein complexes are thought to assemble on some, if not all, functional enhancer segments (7). Related to this is the proposal that the function of some sequence-specific transcription factors is to serve in an architectural role in formation of these complexes (7). An example of such a role would be bending of DNA to facilitate protein–protein interactions or to modulate nucleosomal structure.

Among the factors that have been shown to functionally bend DNA are members of the high mobility group (HMG) protein family. HMG proteins contain at least one moderately conserved region of ~80 amino acids that is required for DNA binding (8). The DNA specificity is variable among the members of the HMG protein family. For example, HMG-1, a non-histone chromosomal protein, binds DNA non-specifically, while others, such as LEF-1 and TCF-1, recognize a specific DNA sequence (8–10). LEF-1 and TCF-1 have evolved from a common ancestral gene, as shown by comparison with the single chicken homolog, chTCF (11). The HMG domains of LEF-1 and TCF-1 are virtually identical, differing by only three amino acids (11,12) and LEF-1 and TCF-1 bind the same sequence, (A/T)(A/T)CAAAG (9,13,14). LEF-1 and TCF-1 bind DNA in the minor groove and

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bend the DNA similarly (15–17). LEF-1 bends the DNA ~130°, causing the DNA to bend almost back on itself (15,18).

LEF-1 and TCF-1 are both expressed in T cells and binding sites for these proteins have been found in a number of T cell-specific genes, including the TCRα, TCRβ and TCRδ enhancers, the CD3 and CD4 enhancers and the HIV-1 enhancer (9,10,19–25). However, LEF-1 and TCF-1 are not classical activators of gene transcription. LEF-1 has been shown to function only in a context-dependent manner in the TCRα enhancer and multimerized LEF-1/TCF-1 binding sites are unable to augment basal activity from a linked promoter in transient transfections (9,10,12). Also, the LEF-1/TCF-1 binding site within the HIV-1 enhancer and the CD4 enhancer are not necessary for full enhancer activation *in vitro*, but the LEF-1/TCF-1 site is necessary for activation of the HIV-1 enhancer *in vivo* (20,26). These results suggest that one architectural role LEF-1/TCF-1 play in transcriptional regulation may be evident only *in vivo* and might involve modulation of nucleosomal structure. Gene activation has been shown in many systems to require the removal of nucleosomes in order for *trans*-acting factors to bind (27,28). This process of modifying, disrupting or displacing nucleosomes in the process of gene regulation is an area of active interest.

In this report, we investigate a LEF-1/TCF-1 binding site within the T cell-specific enhancer of the human ADA gene. The *in vitro* and *in vivo* systems that exist for studying the ADA gene were used to explore the role played by the LEF-1/TCF-1 site in the regulation of ADA expression. It was found, through mutational analysis, that the LEF-1/TCF-1 site was only necessary when transcription occurred in the context of nuclear chromatin. DNase I and *Dpn*II accessibility experiments performed on thymic nuclei from transgenic mice containing a transgene with a mutated LEF-1/TCF-1 site showed a dramatic difference in chromatin accessibility compared with a control. These results provide evidence that LEF-1 and/or TCF-1 are involved in modulating chromatin in hypersensitive site formation and activation of the ADA gene.

MATERIALS AND METHODS

Electrophoretic mobility shift assay

Mobility shift assays were performed with either bacterial extracts containing recombinant LEF-1 proteins or crude nuclear extract from the MOLT 4 human T cell line. The sequence of the normal oligonucleotide was 5′-GATGAAACTCAGTCTCCTTTGTTCC-CCTCCACCACC-3′ and the sequence of the oligonucleotide containing the LEF-1 binding site double point mutation was 5′-GA-TGAAACTCAGTCTCCATAGTTCCCCTCCACCACC-3′. The oligonucleotides were annealed to their complimentary strand and end-labeled using T4 polynucleotide kinase. Bacterial extracts containing either the induced recombinant full-length LEF-1 protein (bLEF-1) or the truncated LEF-1 protein (mLEF-1) were both generous gifts from Dr Marian Waterman (University of California, Irvine, CA) (10). The LEF-1-containing extracts were incubated with 0.1 ng of the labeled oligonucleotides under conditions described previously (10). Mobility shifts with crude nuclear extract from MOLT 4 cells were performed as described by Travis *et al.* (9), except that the probe was labeled as described above and the reaction contained 100 mM NaCl, instead of 50 mM. Anti-LEF-1 antiserum was provided by Dr Rudolph Groschedl and has been described previously (9). Crude nuclear extracts of MOLT 4 cells were prepared by an adaptation of the method described by Dignam *et al.* (29). The cells were collected, washed (PBS) and suspended in hypotonic buffer (10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF, 10 mM HEPES, pH 7.9). The swollen cells were homogenized (Dounce homogenizer) and nuclei were pelleted (3300 *g*, 15 min). The nuclei were suspended in low salt buffer (25% glycerol, 20 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM EDTA, 0.2 mM PMSF, 20 mM HEPES, pH 7.9) and extracted by slow, dropwise addition (with gentle mixing) of high salt buffer (low salt buffer with KCl increased to 1.2 M) to a final KCl concentration of 300 mM. Pelleting of the extracted nuclei yielded the crude nuclear extract.

Site-directed mutagenesis

The LEF-1/TCF-1 binding site within the ADA enhancer was mutated using the Altered Sites *in vitro* Mutagenesis system from Promega. A 2.3 kb *Sph*I fragment containing the ADA enhancer was cloned into the pAlter vector. The manufacturer's instructions were followed to produce the double point mutation using the 36 bp mutant oligonucleotide described above. Resulting ampicillin-resistant colonies were grown and plasmid DNA was isolated using magic miniprep columns purchased from Promega. The DNA was sequenced using Sequenase version 2.0 (US Biochemical) and the manufacturer's instructions for double-stranded sequencing. After confirmation of the desired mutation, the mutated plasmid construct was digested with *Sph*I and the 2.3 kb ADA enhancer fragment purified from a 1% SeaPlaque (FMC) agarose gel. This fragment was subsequently cloned into a plasmid vector containing the ADA promoter and the CAT reporter gene. The resulting plasmid was grown in *Escherichia coli* DH5α (BRL) and purified over a cesium chloride gradient. It is referred to below as 'Enhancer mutHMG'. The 2.3 kb *Sph*I fragment with a wild-type LEF-1/TCF-1 site was also cloned into the vector containing the ADA promoter and the CAT reporter gene and purified as above in order to serve as a control. This construct is referred to as 'Enhancer wtHMG'.

Transient transfection

ADA/CAT plasmids were transiently transfected into the T cell line MOLT 4 by a DEAE–dextran-facilitated method as previously described (2). Three independent transfections were done with duplicate samples to confirm the results. Quantitation of CAT activity in each extract was as previously described (2).

Transgene preparation

The Enhancer mutHMG plasmid was digested with *Bss*HII and *Hin*dIII to generate a 4.2 kb fragment referred to as the mutHMG transgene. The transgene was isolated from a 0.8% SeaPlaque agarose gel. The transgene was then purified over a Elu-tip D purification column (Schleicher & Schuell) using the basic protocol described by the manufacturer. A transgene containing the wild-type LEF-1/TCF-1 site was also prepared as described previously (3). This transgene was previously referred to as 0.3/II–IIIab, but will be referred to here as the wtHMG transgene for simplicity.

Transgenic analysis

Transgenic mice were prepared by pronuclear injection of the wtHMG or mutHMG transgenes as described (2). The thymic CAT

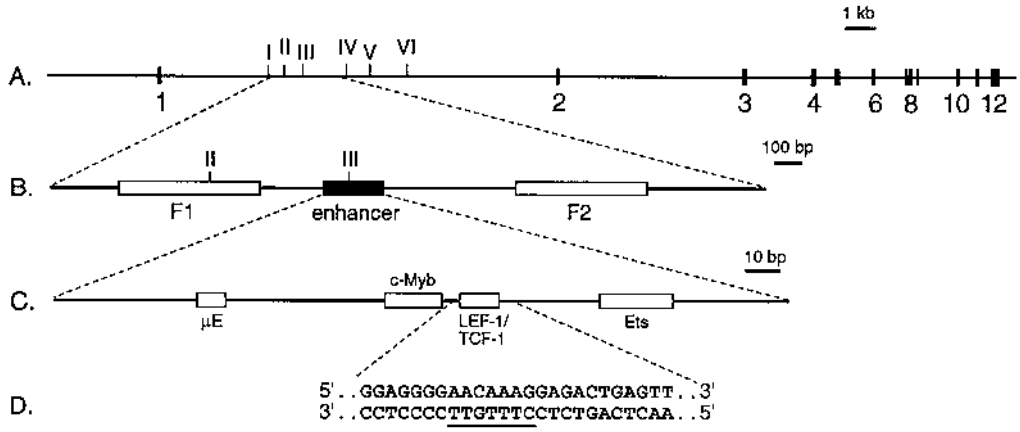

Figure 1. A schematic representation of the human ADA gene and enhancer region. (**A**) The 12 exons of the ADA gene are shown with the intervening sequences. Six HS identified in the first intron are represented by the Roman numerals I–VI. (**B**) An enlargement including HS II and III which shows the minimal region required for high level thymus-specific expression in transgenic mice. F1 and F2 represent facilitator segments that are required for position-independent expression in transgenic mice. The shaded box shows the 225 bp T cell-specific enhancer at HS III that was identified through transfection studies. (**C**) The 225 bp minimal enhancer is enlarged to show the regions protected in DNase I footprinting experiments. The consensus binding sequences for the known transcription factors such as theμE and LEF-1/TCF-1 sites were protected, along with a region containing consensus sequences for members of the Ets family. A central protected region was recently shown to functionally bind c-Myb (32). (**D**) The sequence spanning the LEF-1/TCF-1 site is shown. The 7 bp consensus recognition sequence of LEF-1 is underlined.

activity of F_1 offspring from established transgenic mouse lines was determined as previously described (2). The thymic CAT activity from at least two mice from each line was determined and expressed as pmol acetylated product/h/100 μg protein/transgene copy number. Transgene copy number was determined by standard Southern techniques. DNA was extracted from the liver of each mouse and digested with *Eco*RI. The hybridization signal of the *Eco*RI fragment was compared with that of an *Eco*RI fragment from a cloned plasmid the amount of which represented a known number of copies. The level of protein in each thymic extract was determined using the BioRad protein assay kit.

In situ hybridization

Probes for *in situ* hybridization were prepared from a pGEM-4Z plasmid containing a 550 bp *Hin*dIII–*Nco*I fragment from pSV0-CAT which encompasses the 5′-end of the reporter gene. ^{33}P-Labeled probes were synthesized from linear templates with T7 or SP6 polymerases using an *in vitro* transcription system (Promega). Thymic samples from transgenic mice were isolated, rinsed in 1× PBS and fixed in 4% paraformadehyde in 1× PBS at 4°C overnight. Tissues were frozen in M1 embedding matrix (Lipshaw) and cut into 10 μm sections. Tissue sections were then fixed, acetylated, prehybridized and hybridized (30,31) with a solution containing 5×10^5 c.p.m./μl ^{33}P-labeled CAT riboprobe. Following overnight hybridization at 48°C, sections were washed at high stringency and treated with RNase A (50 μg/ml; Worthington Biochemical Corp.) and RNase T1 (50 U/ml; Gibco BRL) at 37°C for 30 min. Sections were dehydrated and exposed to Kodak NTB-2 emulsion for 48 h at 4°C. Histological staining of sections was performed with hematoxylin and eosin. Sections were observed by light- and dark-field microscopy.

DNase I hypersensitivity

Nuclei were prepared from the thymus of 5- to 7-week-old transgenic mice as described (2), with a few modifications. The thymus was removed and submerged in 10 ml polyamine buffer and disrupted with a Pyrex tissue grinder. The polyamine buffer is that described (2) with the following modifications: 1 mM DTT instead of BME and 8 mM Pefabloc (Boehringer Mannheim) instead of PMSF. After disruption, the homogenates were filtered and centrifuged as described (2). The nuclear pellet was resuspended in wash buffer and the nuclei counted with a hemacytometer. Approximately 5×10^6 nuclei were aliquoted, pelleted and resuspended in 0.4 ml DNase I digestion buffer. Seven units of DNase I (Boehringer Mannheim) were added to each aliquot and incubated at 30°C for 5, 7, 10, 12, 15, 17, 20, 25, 30, 45 and 60 min. Zero time points were not subjected to DNase I. All reactions (including the zero time points) were stopped by lysing the nuclei by addition of 0.4 ml lysis buffer. Nuclear lysates were treated with RNase A and proteinase K and the DNA purified as described (2). The DNA was digested with *Bam*HI for 16 h, precipitated and quantitated. An equal amount of DNA was loaded per lane and electrophoresed through a 1% agarose gel, transferred to Nytran membrane (MSI) and hybridized to a 967 bp *Sca*I–*Bam*HI restriction fragment which was radiolabeled with random primers. Non-specific bound probe was removed by washing once for 15 min at 20°C in 2× SSC and 0.1% SDS, once for 15 min at 20°C in 0.1× SSC and 0.1% SDS and twice for 30 min at 65°C in 0.1× SSC and 0.1% SDS. Results were visualized after exposing to film with an intensifying screen for 2 days at –70°C.

Restriction enzyme accessibility

Thymic nuclei were isolated from transgenic mice as described above. Approximately 5×10^6 nuclei were aliquoted and digested with *Dpn*II for 1 h at 4°C as described (1). Nuclei were lysed using the lysis buffer described above for DNase I hypersensitivity. RNase A treatment, proteinase K treatment and DNA purification were also as described above. Purified DNA was digested with *Kpn*I overnight, precipitated and quantitated. An equal amount of DNA was loaded per lane and electrophoresed through a 4% FMC 3:1 gel. The DNA was transferred to Magna Charge membrane (MSI), washed and prehybridized according to the manufacturer's instructions. The 603 bp *Kpn*I fragment (Fig. 7)

was isolated from plasmid DNA, radiolabeled with random primers and used for hybridization to *Dpn*II fragments. Washes were as described above for the DNase I hypersensitivity procedure. *Dpn*II fragments were visualized after exposing to film at –70°C with an intensifying screen for 4 days.

RESULTS

LEF-1/TCF-1 binding site within the ADA enhancer

During identification and characterization of a T cell-specific enhancer for the human ADA gene, six HS were identified within the first intron of the gene (Fig. 1A; 2). Deletional analysis defined a 225 bp minimal enhancer at HS III that is sufficient for high level T cell-specific expression in transient transfections (2,3). However, examination of ADA enhancer function in transgenic mice defined a much larger intronic fragment that is necessary for high level thymic CAT expression independent of insertion site (Fig. 1B; 3). This 2.3 kb fragment contains flanking facilitator segments in addition to the minimal enhancer. These facilitators are required for consistent transgene expression (1,3).

The 225 bp minimal ADA enhancer contains binding sites for a number of factors, as evidenced by DNase I protection footprinting (Fig. 1C; 2,3,32). One protected segment near the central core encompasses the sequence 5′-CTTTGTT-3′ (Fig. 1D), which matches the recognition sequence for the HMG proteins LEF-1 and TCF-1 (9,13). Both LEF-1 and TCF-1 have virtually identical DNA binding domains and bind the same DNA sequence (11). Electrophoretic mobility shift assays were done to evaluate binding to this recognition site within the ADA enhancer. Specific DNA binding was shown using bacterial extract containing human recombinant LEF-1 and a labeled 36 bp oligonucleotide containing the ADA LEF-1/TCF-1 binding site (Fig. 2A, lane 2). A double point mutation of the LEF-1/TCF-1 binding site (CTTTGTT to CATAGTT) within the 36 bp wild-type oligonucleotide completely abolished the binding of LEF-1 (Fig. 2A, lane 3). This mutation has been shown previously to abolish LEF-1 binding in other contexts (10). The specificity of the protein–DNA interaction was shown by the absence of the complex when the labeled 36 bp wild-type oligonucleotide was incubated with a bacterial extract that contains the LEF-1 protein without the HMG DNA binding domain (Fig. 2A, lane 4). The ability of factors in crude nuclear extract from the human MOLT 4 T cell line to bind was also examined (Fig. 2B). A single specific major band was observed when MOLT 4 nuclear extract was incubated with the normal 36 bp oligonucleotide probe (Fig. 2B, lane 2). Formation of this band was ablated by incubation with inhibiting antiserum (9) raised against purified human LEF-1 (Fig. 2B, lane 3). This polyclonal rabbit serum reacts with both LEF-1 and TCF-1 and perhaps with some other closely related members of this family.

Mutation of the LEF-1/TCF-1 binding site

To determine if LEF-1 and/or TCF-1 plays a functional role in thymic ADA expression, the LEF-1/TCF-1 binding site was mutated within the context of the 2.3 kb intronic fragment that gives a consistently high level of thymic CAT expression in transgenic mice (3). The mutation introduced was the double point mutation described above (CTTTGTT to CATAGTT). The mutated 2.3 kb fragment was placed downstream of the ADA promoter and the CAT reporter gene in a manner analogous to that used previously in examination of the normal enhancer segment (3). This mutated construct was tested both in transgenic mice and transient transfection studies to test its effect in different chromatin environments.

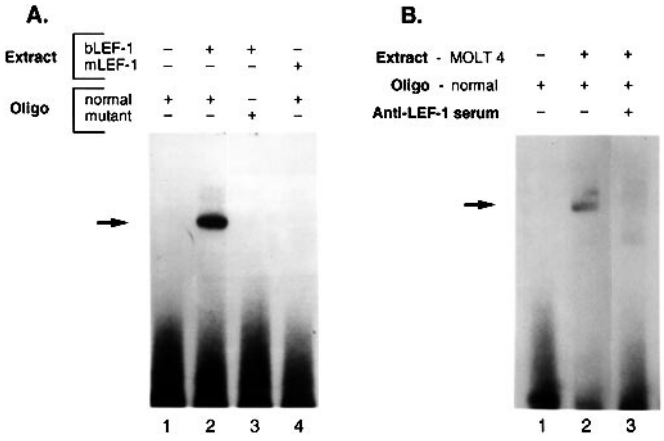

Figure 2. (**A**) Electrophoretic mobility shift assay showing binding of bacterially produced recombinant human LEF-1 to the consensus sequence present in the ADA enhancer. A protein–DNA complex formed when an aliquot of pLys bacterial extract containing recombinant LEF-1 protein (bLEF-1) was incubated with a labeled normal oligonucleotide (lane 2). Lane 1 contains the labeled normal oligonucleotide without bacterial extract added. No protein–DNA complex formed when bLEF-1 was incubated with a labeled mutant oligonucleotide (lane 3) or when the labeled normal oligonucleotide was incubated with bacterial extract containing a truncated form of bLEF-1 which does not contain the HMG DNA binding domain (mLEF-1, lane 4). (**B**) Electrophoretic mobility shift assay showing the binding of a LEF-1/TCF-1 type factor(s) in a nuclear extract from the human MOLT 4 T cell line. Lane 1 contains the labeled normal oligonucleotide probe described in (A) above, incubated in the absence of nuclear extract. Lane 2 contains the labeled probe incubated with MOLT 4 nuclear extract. Lane 3 shows the effect of incubating 0.5 µl undiluted anti-LEF-1 serum with the nuclear extract prior to incubation with the probe.

Mutation of the LEF-1/TCF-1 binding site has no effect in transient transfection experiments

To test the effect of the mutated LEF-1/TCF-1 site in the absence of a specific chromatin environment, non-replicating plasmids containing the mutated and normal LEF-1/TCF-1 site were transiently introduced into the MOLT 4 T cell line. It has been reported that replication of transfected plasmids is necessary to generate appropriately phased nucleosomes similar to that seen *in vivo* (33,34). No significant difference between the levels of CAT activity obtained for the construct containing the enhancer with the mutated LEF-1/TCF-1 site and the construct containing the enhancer with the normal LEF-1/TCF-1 site was observed (Fig. 3A). Each demonstrated enhanced expression of ~55- to 65-fold compared with the basal promoter. Three independent transfections were done, with samples carried out in duplicate, to confirm the results (Fig. 3B). Thus, LEF-1 and/or TCF-1 do not appear to be necessary components of the active enhancer complex nor are they essential for formation of the complex in the transient transfection system. As further support for this result, it was previously shown that multimerized copies of the 27 bp core of the ADA enhancer just upstream of the LEF-1/TCF-1 site, which included the c-Myb site, was sufficient for high level CAT expression in transient transfection experiments (3). The presence or absence of the LEF-1/TCF-1 site in the multimerized construct had little or no effect on the resulting CAT activity.

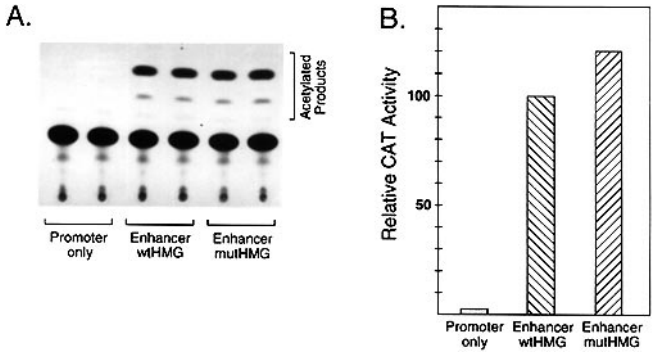

Figure 3. Results of transient transfections showing the effect of mutating the LEF-1/TCF-1 site. (**A**) Autoradiograph of a CAT assay done after transient transfection of plasmid constructs into MOLT 4 cells. In addition to plasmid and CAT sequences, the plasmids contained ADA promoter sequence only (Promoter only), the ADA promoter plus the 2.3 kb enhancer fragment (Enhancer wtHMG) or the ADA promoter plus the 2.3 kb enhancer fragment with the mutated LEF-1/TCF-1 site construct (Enhancer mutHMG). Transfections were carried out in duplicate, cell extracts prepared and the extracts assayed for CAT activity by incubation with [^{14}C]chloramphenicol and acetyl CoA. Levels of acetylated chloramphenicol products were used to calculate the CAT activity present. (**B**) Graphic representation of the relative CAT activity in MOLT 4 extracts after transient transfection of the plasmid constructs. CAT activity was first expressed as pmol product/h/pmol DNA transfected and then the CAT activity was normalized to that present in the 'Enhancer wtHMG' extract. Each bar represents the average of three independent transfections.

Mutation of the LEF-1/TCF-1 binding site disrupts position-independent expression in transgenic mice

Transgenic mice were utilized to examine the role of LEF-1 and/or TCF-1 in a chromatin environment. A fragment of ~4.2 kb was isolated from the mutated construct for microinjection to create transgenic mice. The fragment contained ~200 bp of the ADA promoter, the CAT reporter gene and the 2.3 kb of intronic fragment described above and is referred to as the mutHMG transgene (Fig. 4). This fragment is equivalent to the smallest transgene, referred to here as the wtHMG transgene, that was previously shown to express high level, copy number-dependent CAT activity independent of insertion site (3). F_1 offspring from established transgenic mouse lines were analyzed at 6–15 weeks of age. The results from mice containing the mutHMG transgene were compared with the results from mice containing the wtHMG transgene (Tables1 and 2). The thymus from eight lines of mice containing the wtHMG transgene demonstrated a consistently high level of CAT activity when normalized to transgene copy number. The CAT activity for these mice ranged from 24 000 to 63 000 U/gene copy number, with a mean of 41 000 ± 14 000 (Table 1). This high level of insertion site-independent CAT activity is observed not only in mice containing the wtHMG transgene, but also in mice with transgenes containing larger intronic fragments that encompass the 2.3 kb fragment. A total of 32 lines of mice of this type have been examined previously, with copy numbers ranging from 1 to 100 copies, and they gave a copy number-normalized mean CAT activity of 33 000 ± 13 000 (3). In strong contrast, mice containing the mutHMG transgene did not give consistently high levels of CAT activity. The actual level of CAT expression was highly variable and quite dependent on insertion site. The CAT activity for mice from five different lines ranged from 0.4 to 76% of the mean value of activity obtained for the wtHMG-containing mice, with four lines well outside the normal range (Table 2). Results with these four lines indicate that disruption of LEF-1 and/or TCF-1 binding severely impairs the ability to form a functional enhancer complex in many chromatin environments. However, mice from line 3 are capable of expressing thymic CAT activity from the array of 10 transgene copies at a level within the normal range, which suggests that a functional enhancer complex can form without LEF-1 and/or TCF-1 in some chromatin environments.

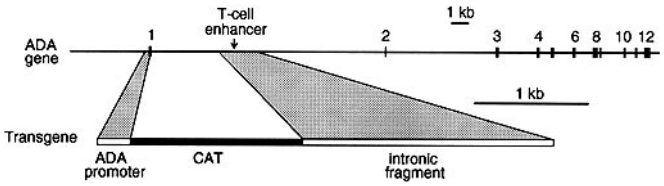

Figure 4. Scale diagrams depicting the ADA gene and the transgene used to make transgenic mice. The transgenes were made by fusing the CAT coding, splicing and polyadenylation sequences of pSVO-CAT to an ADA promoter segment and intronic segment as shown. The mutHMG transgene is identical to the wtHMG transgene except for a 2 bp mutation of the LEF-1/TCF-1 site in the T cell enhancer.

Table 1. Results from transgenic mice prepared by pronuclear injection of wtHMG transgene

Transgenic line[a]	Copy no.	Normalized thymic CAT activity[b,c]
1	3	26 000
2	8	63 000
3	6	55 000
4	2	52 000
5	3	38 000
6	45	24 000
7	2	30 000
8	5	37 000

[a]The wtHMG transgene described here is equivalent to the previously described transgene .3/II–IIIab (3). Lines 1–6 were previously characterized (1–3).
[b]Thymic CAT activity is ecpressed as pmol of acetylated product per hour per 100 µg of protein and normalized to transgene copy number. The CAT activity shown is the average activity from two mice.
[c]All mice analyzed were F_1.

Table 2. Results from transgenic mice prepared by pronuclear injection of mutHMG transgene

Transgenic line	Copy no.	Normalized thymic CAT activity[a,b]	Percent of normal mean[c]
1	45	170	0.4
2	55	940	2.3
3	10	31000	76
4	30	6400	16
5	1	8300	20

[a]Thymic CAT activity is expressed as pmol of acetylated product per hour per 100 µg of protein and normalized to transgene copy number. The CAT activity shown is the average activity from two mice except line 4 which is the mean of three mice with a S.D. of ±1400.
[b]All mice analyzed were F1 except line 5 which were F2 mice.
[c]The normal mean value is 41 000 ± 14 000.

Upon initial examination, there might be some concern about comparing results between wtHMG and mutHMG mice, due to copy number differences. Several of the mutHMG transgenic lines have a much higher copy number than most of the wtHMG lines (compare Table 1 and 2). It has been shown that some transgenes integrated in high copy numbers lose their copy number dependence or are silenced. In theory, this might explain all or part of the difference between wtHMG and mutHMG mice. This explanation seems highly unlikely, however, because of the previously mentioned results with transgenic mice containing the normal enhancer. Almost 100 mice from 32 independent lines, derived from eight different constructs, have been analyzed which contain the normal, functional, wild-type enhancer (3; unpublished results). These lines have copy numbers ranging from 1 to 100. All lines show full enhancer activity when normalized to transgene copy number (mean 33 000 ± 13 000, range 16 000–63 000). There is absolutely no evidence of loss of copy number dependence or silencing in the high copy number lines. The only difference within the entire enhancer domain between these mice and the mutHMG mice is the LEF-1/TCF-1 mutation. Therefore, it is clear that the loss of copy number proportionality in the mutHMG mice is due to the mutation, not the actual transgene copy number.

Regional CAT mRNA expression in the thymus of mutHMG mice

The normal human and mouse ADA genes are expressed at high levels in cortical thymocytes, with much lower expression in the medulla (2). Similarly, transgenic mice prepared from constructs with the normal enhancer also display high levels of thymic CAT expression in the cortex with much lower expression in the medulla (2,32). *In situ* hybridization experiments were used to compare thymic expression patterns of the CAT reporter genes in wtHMG and mutHMG transgenic mice. In evaluation of these results, the level of both total CAT expression and CAT expression/transgene copy number must be taken into account when examining the observed level and pattern of expression. In the thymus of wtHMG transgenic mice, the CAT mRNA is expressed at very high levels in cortical thymocytes with very little expression in the cells of the medulla (Fig. NO TAGA). This expression pattern is very analogous to that of the endogenous gene. The high expressing mutHMG line 3 mice also exhibit high level CAT expression specifically in the cortex (Fig. NO TAGB). Thus, wtHMG line 8 and mutHMG line 3 mice, which have similar levels of both total CAT expression and CAT expression/transgene copy, display a similar regional expression with a clear demarcation of the cortico-medullary junction. However, in the low expressing mutHMG mice, the enhanced level of CAT mRNA expression in the cortex compared with the medulla is lost. This is most evident in the mutHMG line 1 mice, which express a total CAT activity of only 7650 U (170 U/copy, 45 copies) compared with ~300 000 U (31 000 U/copy, 10 copies) of CAT activity in mutHMG line 3 mice. In mutHMG line 1 mice, the CAT mRNA is expressed at a very low, uniform level across both the cortex and medulla (Fig. NO TAGC). The mutHMG line 4 mice express a reasonably high level of total CAT activity at 192 000 U, but the expression level of 6400 U/copy, 30 copies represents only ~15–20% of normal activity. *In situ* studies with this line (Fig. NO TAGD) show cortical CAT expression that is clearly distinguishable as higher than medullary expression. However, the differential expression and demarcation of the cortico-medullary junction is significantly reduced from that of the normal and high expressing mutHMG transgenic lines. The relative level of signal observed in the medulla of each of the mouse lines correlates directly with copy number. It is probably a reflection of the low generalized expression observed previously in all tissues (2,3). However, the level of CAT mRNA seen by *in situ* hybridization in the cortex of each of the lines of mutHMG transgenic mice correlates with the level of normalized CAT expression observed for each line, indicating that the variable level of CAT expression is a reflection of the ability of the enhancer to function in cortical thymocytes.

Mice containing the mutHMG transgene have reduced hypersensitivity to DNase I

Removal of nucleosomes within the regulatory regions of a gene is essential in order for some *trans*-acting factors to bind and activate transcription. The removal of nucleosomes makes the DNA more susceptible to DNase I, forming what are known as DNase I hypersensitive sites (HS). Formation of HS III has been shown to be essential for proper T cell function of the ADA enhancer in transgenes (2,3). DNase I hypersensitivity experiments were done to examine the ability of mice with the wtHMG and mutHMG transgenes to form HS III. Mice from mutHMG lines 1–4 and wtHMG line 8 were tested. Thymic nuclei were isolated from transgenic mice and subjected to DNase I treatment for varying lengths of time. The purified DNA was digested with *Bam*HI and hybridized to a 967 bp *Sca*I–*Bam*HI fragment (Fig. 6A). As expected, a 3.2 kb band diagnostic of HS III was formed by cleavage of the 4.2 kb parental band by DNase I in wtHMG transgenic mice (Fig. 6B). However, formation of HS III was dramatically different in mice expressing the mutHMG transgene. Mice from mutHMG lines 1 and 2 showed very little or no formation of HS III (Fig. 6C). These are the lines of mice that express the lowest level of CAT activity. However, appearance of the 3.2 kb band in mutHMG line 3 mice indicates that HS III is fully formed in this line (Fig. 6C). This correlates with the high level of CAT expression observed for this line of mice. HS III is able to form in mutHMG line 4 mice, but with much lower efficiency than wtHMG mice (Fig. 6C). Thus the level of hypersensitivity at HS III correlates well with the level of CAT expression observed for each line of mutHMG mice, suggesting that LEF-1 and/or TCF-1 play a direct role in formation of HS III.

Reduced level of *Dpn*II accessibility in mutHMG transgenic mice

To further study the level of chromatin accessibility, thymic nuclei were digested with the specific restriction endonuclease *Dpn*II. *Dpn*II was chosen because the ADA enhancer contains three *Dpn*II sites within a 603 bp *Kpn*I fragment encompassing HS III (Fig. 7A). One of these *Dpn*II sites is in the center of HS III and was found to be more accessible than the flanking *Dpn*II sites in wtHMG mice (Fig. 7B; 1). This is evident from the relatively greater abundance of the 432 and 171 bp *Kpn*I–*Dpn*II fragments generated by cutting at the central *Dpn*II site. The other *Kpn*I–*Dpn*II fragments shown in Figure 7A are also present in DNA from wtHMG, but these fragments require more strenuous *Dpn*II digestion conditions to completely form and have a less intense signal than their size would warrant. For example, the 432 bp fragment formed by cutting at the central *Dpn*II site gives a more intense signal than the co-migrating 542/547 bp fragments liberated by cutting at either flanking *Dpn*II site.

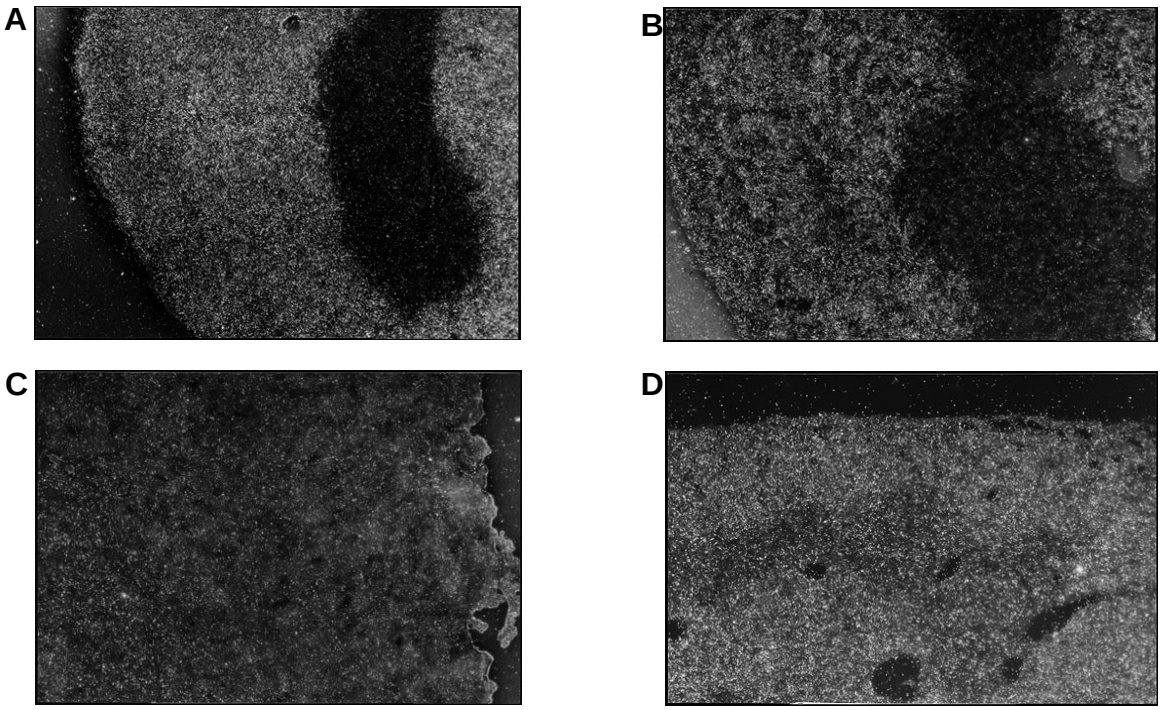

Figure 5. Results of an *in situ* hybridization analysis of CAT mRNA distribution in the thymus of transgenic mice. All *in situ* hybridizations were done using a ^{33}P-labeled RNA complimentary to the CAT mRNA. All slides were exposed for 48 h. The panels show CAT mRNA distribution in the thymus of a mouse from (**A**) wtHMG line 8, (**B**) mutHMG line 3, (**C**) mutHMG line 1 and (**D**) mutHMG line 4. All panels show similar thymic sections across both cortical and medullary areas. These discrete regions are most clearly defined in (A) and (B), which have heavy silver grain deposition over the cortex and much lower deposition over the medulla. All tissues were also probed with a ^{33}P-labeled sense CAT probe as a control and no significant silver grain deposition was seen in any of the panels (data not shown).

As observed with DNase I treatment, there is a significant difference in *Dpn*II cutting between the wtHMG and the mutHMG transgenic mice. There is significantly reduced *Dpn*II cutting within thymic nuclei from the low expressing mutHMG transgenic mice (Fig. 7C). This is especially evident in mutHMG lines 1 and 2 transgenic mice, in which production of the 432 and 171 bp *Kpn*I–*Dpn*II fragments is greatly reduced as compared with wtHMG mice. The 171 bp fragment, because of its size, was only evident in these lines of mice after a longer exposure to film (data not shown). In mutHMG line 2 mice, the co-migrating 542/547 bp fragments are liberated by cutting at the flanking *Dpn*II sites, but only with high *Dpn*II concentrations and then with significantly lower efficiency. In mutHMG line 4 mice, the 432 and 171 bp fragments are formed with greater efficiency than mutHMG lines 1 and 2, but much less well than in wtHMG mice. This again correlates with its level of expression. Also, in mutHMG line 4 mice, the 547/542 and 371 bp fragments, formed by cutting at the outer *Dpn*II sites, are much less evident than in the wtHMG mice. Significantly more *Dpn*II is required to generate these fragments in mutHMG line 4 mice as compared with wtHMG mice. This suggests that while the ADA enhancer region may be accessible to *Dpn*II, it is not as readily accessible as in wtHMG mice. The high expressing mutHMG line 3 mice generated all the *Kpn*I–*Dpn*II fragments, but with a slightly lower level of efficiency than the wtHMG mice and with a different profile. There is less preferential cutting at the central *Dpn*II site. However, all the *Kpn*I–*Dpn*II fragments were still generated with a higher efficiency than in any other line of mutHMG mice. Thus, among the mutHMG mice, *Dpn*II accessibility correlates with the level of CAT expression, in a manner similar to that observed for HS III formation.

DISCUSSION

Our results show that LEF-1/TCF-1 factors play an important role in thymic ADA expression, but not as a classical activator of transcription. Analysis of mutHMG transgenic mice shows that a role for LEF-1 and/or TCF-1 binding is evident only when the ADA enhancer is in the context of chromatin. Mutation of the LEF-1/TCF-1 site in the ADA enhancer significantly disrupts the ability to specify consistent, position-independent transgene expression, but has no effect on enhancer function in transient transfections. This occurs even in the presence of the facilitators, which were described earlier as playing a critical role in establishment of the proper chromatin environment for enhancer function (1,3).

It was previously shown that in ADA transgenes lacking the facilitator segments the enhancer domain is still accessible to *Dpn*II but HS III is unable to form (1). Based on this result, it was proposed that enhancer activation proceeds through a multi-step mechanism in which factors necessary for initial accessibility at HS III act to modulate the chromatin prior to the step at which the facilitators function (1). However this initial modulation is not sufficient for proper HS III formation without the facilitators. In the present study, the low expressing mutHMG mice show a marked decrease in *Dpn*II accessibility, suggesting that LEF-1 and/or TCF-1 may be one of the factors involved in this initial step

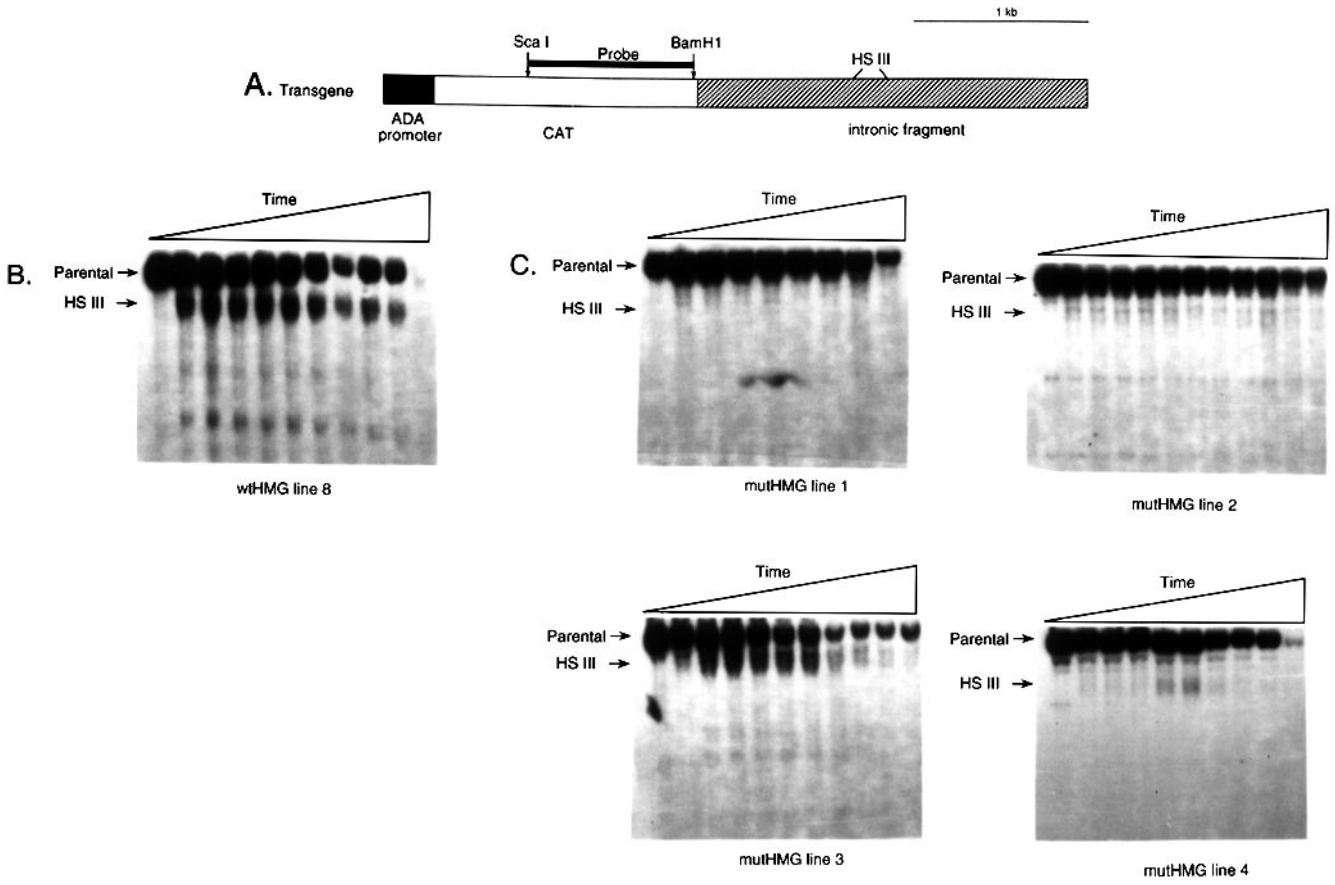

Figure 6. DNase I hypersensitivity in wtHMG- and mutHMG-expressing mice. (**A**) A schematic representation of the transgene microinjected in the preparation of mutHMG and wtHMG mice is shown. DNA from DNase I-treated thymic nuclei from transgenic mice was digested with *Bam*HI, electrophoresed and hybridized to the 967 bp *Sca*I–*Bam*HI fragment shown. The parental *Bam*HI fragment is 4.2 kb. Fragments of ~3.2 kb are generated by cleavage at HS III. (**B**) The near complete cleavage by DNase I at HS III in wtHMG line 8 mice is shown. The 4.2 kb parental and 3.2 kb HS band are indicated. Other HS bands in wtHMG line 8 mice are within the ADA promoter and CAT reporter gene. (**C**) The variable formation of HS III in mutHMG mice is shown. Low expressing lines 1 and 2 show virtually no cutting at HS III by DNase I. Line 4 shows a significantly reduced HS III band, while line 3 has near complete cutting at HS III, which is very similar to the results with wtHMG mice above.

of HS III formation leading eventually to active enhancer complex formation and thymic ADA expression.

This proposed role for LEF-1 and/or TCF-1 in ADA expression correlates well with the results of the DNase I and *Dpn*II accessibility experiments. The level of DNase I hypersensitivity at HS III parallels the level of CAT expression observed within the lines. The *Dpn*II accessibility generally correlated well with the level of expression in mutHMG mice. Low expressing lines were virtually inaccessible. The high expressing line 3 was less accessible to *Dpn*II compared with the wtHMG mice, but was much more accessible than any other line of mutHMG mice. It is postulated that the ability of the mutant ADA enhancer to establish initial accessibility and eventually form HS III is highly dependent on the chromatin environment in which the transgene array inserted within the mouse genome. The direct result is the variation in thymic CAT expression among the mutHMG mouse lines. Mice from mutHMG line 3 are able to express at near normal levels, presumably because of where the transgene inserted. This insertion site is amenable to formation of an active enhancer complex in the absence of a functional LEF-1/TCF-1 site, since LEF-1 and/or TCF-1 are not direct activators and are not required by the active complex. Line 3 mice establish an HS similar to HS III and express CAT normally. Subtle differences from the normal enhancer chromatin domain may be reflected in the decreased level and different pattern of accessibility to *Dpn*II.

The nature of the underlying differences in chromatin environment at the transgene insertion site that result in the highly varied expression among the mutHMG transgenes in the different lines is unclear. The functionality of these differences is probably related to the mechanism of HS III formation. There are two principal types of mechanisms proposed for tissue-specific HS formation. One mechanism occurs at or near the time of replication and involves a competition for binding between factors involved in HS formation and those involved in nucleosome deposition. The other proposes an active disruption or remodeling of nucleosomal structure. At different transgene insertion sites there may be differences in the local chromatin environment that effect the inherent stability of nucleosomes or the kinetics of their formation. Such conditions might determine the relative requirement for LEF-1/TCF-1 and the effect of a non-functional LEF-1/TCF-1 site in the transgenes at that insertion site.

There is a formal possibility that the ADA enhancer is not fully functional in mutHMG line 3 mice, but that the array of transgenes in line 3 simply inserted into a genomic site near a potent transcriptional activator sequence. This would seem unlikely, since

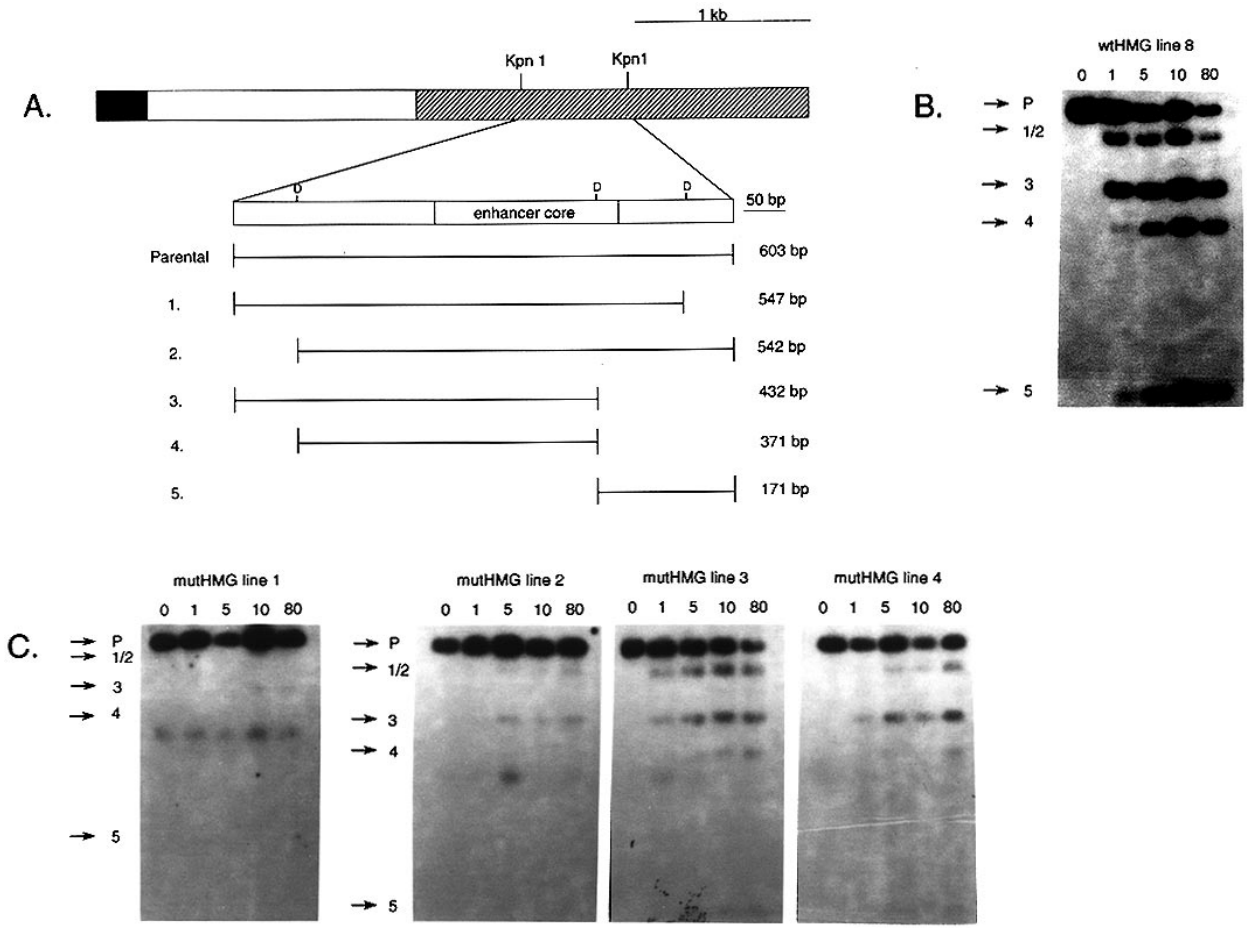

Figure 7. *Dpn*II accessibility in wtHMG- and mutHMG-expressing mice. Nuclei were isolated from thymus of wtHMG and mutHMG mice and treated with increasing amounts of *Dpn*II as described in Materials and Methods. The *Dpn*II-digested DNA was purified and digested with *Kpn*I, electrophoresed and hybridized to a labeled aliquot of the 603 bp *Kpn*I fragment. (**A**) A schematic representation of the transgene is shown depicting the location of the two *Kpn*I sites within the enhancer. The region between the two *Kpn*I sites is enlarged to show the location of the *Dpn*II sites. The expected *Kpn*I–*Dpn*II fragments and their sizes are indicated. (**B**) *Dpn*II accessibility within the enhancer is shown for wtHMG line 8 mice. All expected *Kpn*I–*Dpn*II fragments described above are present. These fragments are indicated by numbers corresponding to the number given to the fragments in (A). (**C**) The *Dpn*II accessibility within the enhancer is shown for each line of mutHMG mice. The expected migration of each *Kpn*I–*Dpn*II fragment described in (A) is indicated by the appropriate number. mutHMG lines 1, 2 and 4 have significantly reduced accessibility to *Dpn*II, while mutHMG line 3 shows much better accessibility.

the sequence would have to activate transgene expression in a tissue-specific pattern very similar to that observed with the ADA enhancer (i.e. high level expression only in the thymus) and cause formation of a discrete HS III at the enhancer site. The *in situ* hybridization experiment also argues against an outside transcriptional activator driving the observed CAT expression in mutHMG line 3 mice, since the CAT mRNA expression pattern in the thymic cortex and medulla mimics the CAT expression seen with the wtHMG mice.

Other systems provide supportive evidence that LEF-1 and TCF-1 function at an early stage in gene expression and that this function occurs at the level of chromatin structure. Deletion of LEF-1/TCF-1 binding sites within the HIV enhancer had little or no effect in a transient transfection experiment, but the virus was unable to replicate in peripheral blood lymphocytes and certain native T cell lines when analyzed for viral replication using a mutant viral stock (20,35). These results led to a more extensive study of the function of the LEF-1/TCF-1 site within the HIV-1 enhancer using an *in vitro* chromatin reconstitution system in which the level of *in vitro* transcription was measured using a naked DNA template and a chromatin packaged template (26). It was found that *in vitro* packaging of the HIV-1 enhancer into chromatin drastically repressed *in vitro* transcription. Alleviation of this repression was dependent on the addition of LEF-1. However, when HIV-1 transcription was measured from naked DNA, high level transcription occurred even in the presence of a mutated LEF-1 site, strongly supporting the hypothesis that LEF-1 is only necessary in the context of chromatin (26). In another study, mutation of the LEF-1/TCF-1 binding site within the CD4 enhancer had very minor effects on enhancer activity in a transient transfection experiment, but the mutation has not been studied for its effect in an *in vivo* system (21). It is possible that LEF-1 and TCF-1 play a chromatin-related role in expression of other T cell-specific genes. Since LEF-1/TCF-1 sites are present in several T cell-specific genes required for proper T cell development it has even been postulated that LEF-1 and TCF-1 act as a developmental switch to initiate T cell development (36,37).

The importance of TCF-1 in T cell development is shown by the phenotype observed in mice deficient for the TCF-1 protein. Mice deficient for the TCF-1 protein are blocked at an early stage in thymocyte development at the transition from the CD8+

immature single positive to the CD4+/CD8+ double positive stage (37). However, the blockage is not complete. A small percentage of functioning mature T cells are present and are enough to keep the mice healthy. The 'leakiness' of this mutation could be due to redundant function by LEF-1. LEF-1 and TCF-1 are both expressed early in development, but LEF-1 is expressed at a much lower level. Therefore, it may only be able to partially compensate for the loss of TCF-1.

The phenotype of LEF-1-deficient mice may indicate a similar role for HMG proteins in development of other systems as well (38). Unlike the TCF-1-deficient mice, the LEF-1-deficient mice have no effect on their lymphoid system, perhaps again because of redundant function by TCF-1 or other factors. However, mice homozygous for a mutation of the LEF-1 gene that eliminates its protein expression were found to lack teeth, mammary glands, whiskers, hair and the mesencephalic nucleus of the trigeminal nerve (38). This phenotype may be explained by the embryonic expression pattern of LEF-1. LEF-1 and TCF-1 are both widely expressed during embryogenesis, with expression of both in many tissues (16). However, LEF-1 is uniquely expressed in brain, inner ear and developing hair follicles, perhaps partially explaining the phenotype of the LEF-1-deficient mice (16,38). Recently, it was found that LEF-1/TCF-1 binding sites are present in a number of human keratin gene promoters (39). An initial study examining the role of LEF-1 in expression of keratin-specific genes revealed that LEF-1 expression precedes expression of keratin-specific genes and overexpression of LEF-1 in oral epithelium resulted in large hairs erupting from the gum (39). These results, together with the results of the LEF-1-deficient mice, suggest that LEF-1 could be an initiator of keratin-specific gene expression within hair follicles. Disruption of chromatin to make these promoters accessible was postulated as a possible mechanism and is currently being studied (39).

The data above support the hypothesis that LEF-1 and TCF-1 play an architectural role in initiation of gene expression. Studies with the TCRα enhancer have identified a different type of architectural function for LEF-1/TCF-1 in formation of an active enhancer complex. The minimal TCRα enhancer was defined as a 116 bp fragment located downstream of the TCRα gene (40). This fragment contains DNA binding sites for two other proteins in addition to LEF-1. A member of the CREB family binds a site upstream of the LEF-1 site and an Ets-type binding site is located downstream of the LEF-1 site (9,10,40). LEF-1 facilitates the protein interactions between the various components in formation of the enhancer complex, which can then stimulate gene transcription (7,9,10,15,41–43). Mutations in the LEF-1 site or changes in the site's relative location within the enhancer context both had dramatic effects on enhancer function (9,10). The context-dependent function of a LEF-1/TCF-1 site may allow it to play significantly different roles in the relative context of different enhancers. This is supported by results of studies involving the immunoglobulin κ3′ enhancer, in which LEF-1 is postulated to play a negative role in gene expression by bending the DNA and preventing protein–protein interaction (44). It is also possible that different members of the HMG family may bind LEF-1/TCF-1 sites preferentially in specific sequence contexts with different functional consequences. Whatever the explanation for any differences there may be in the function of LEF-1/TCF-1 sites present in different genes, it becomes increasingly clear that some transcription factors have a vital function in regulated gene expression by playing an architectural role.

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REFERENCES

1 Aronow,B.J., Ebert,C.A., Valerius,M.T., Potter,S.S., Wiginton,D.A., Witte,D.P. and Hutton,J.J. (1995) *Mol. Cell. Biol.*, **15**, 1123–1135.
2 Aronow,B., Lattier,D., Silbiger,R., Dusing,M., Hutton,J., Jones,G., Stock,J., McNeish,J., Potter,S., Witte,D. and Wiginton,D. (1989) *Genes Dev.*, **3**, 1384–1400.
3 Aronow,B.J., Silbiger,R.N., Dusing,M.R., Stock,J.L., Yager,K.L., Potter,S.S., Hutton,J.J. and Wiginton,D.A. (1992) *Mol. Cell. Biol.*, **12**, 4170–4185.
4 Brickner,A.G., Gossage,D.L., Dusing,M.R. and Wiginton,D.A. (1995) *Gene*, **167**, 261–266.
5 Winston,J., Hong,L., Akroyd,S., Hanten,G., Waymire,K., Overbeek,P. and Kellems,R.E. (1995) In Sahota,A. and Taylor,A. (eds), *Purine and Pyrimidine Metabolism in Man.* Plenum Publishing, New York, NY, Vol. VIII, pp. 579–584.
6 Mitchell,P.J. and Tjian,R. (1989) *Science*, **245**, 371–378.
7 Tjian,R. and Maniatis,T. (1994) *Cell*, **77**, 5–8.
8 Giese,K., Amsterdam,A. and Grosschedl,R. (1991) *Genes Dev.*, **5**, 2567–2578.
9 Travis,A., Amsterdam,A., Belanger,C. and Grosschedl,R. (1991) *Genes Dev.*, **5**, 880–894.
10 Waterman,M.L., Fischer,W.H. and Jones,K.A. (1991) *Genes Dev.*, **5**, 656–669.
11 Castrop,J., Hoevenagel,R., Young,J.R. and Clevers,H.C. (1992) *Eur. J. Immunol.*, **22**, 1327–1330.
12 Clevers,H.C., Oosterwegel,M.A. and Georgopoulos,K. (1993) *Immunol. Today*, **14**, 591–596.
13 Waterman,M.L. and Jones,K.A. (1990) *New Biologist*, **2**, 621–636.
14 Oosterwegel,M., van de Wetering,M, Dooijes,D., Klomp,L., Winoto,A., Georgopoulos,K., Meijlink,F. and Clevers,H. (1991) *J. Exp. Med.*, **173**, 1133–1142.
15 Giese,K., Cox,J. and Grosschedl,R. (1992) *Cell*, **69**, 185–195.
16 Oosterwegel,M., van de Wetering,M, Timmerman,J., Kruisbeek,A., Destree,O., Meijlink,F. and Clevers,H. (1993) *Development*, **118**, 439–448.
17 van de Wetering,M. and Clevers,H. (1992) *EMBO J.*, **11**, 3039–3044.
18 Ferrari,S., Harley,V.R., Pontiggia,A., Goodfellow,P.N., Lovell,B.R. and Bianchi,M.E. (1992) *EMBO J.*, **11**, 4497–4506.
19 Clevers,H., Lonberg,N., Dunlap,S., Lacy,E. and Terhorst,C. (1989) *EMBO J.*, **8**, 2527–2535.
20 Kim,J.Y., Gonzalez,S.F., Zeichner,S.L. and Alwine,J.C. (1993) *J. Virol.*, **67**, 1658–1662.
21 Sawada,S. and Littman,D.R. (1991) *Mol. Cell. Biol.*, **11**, 5506–5515.
22 Jones,K. and Peterlin,B. (1994) *Annu. Rev. Biochem.*, **63**, 717–743.
23 Gottschalk,L.R. and Leiden,J.M. (1990) *Mol. Cell. Biol.*, **10**, 5486–5495.
24 Redondo,J.M., Hata,S., Brocklehurst,C. and Krangel,M.S. (1990) *Science*, **247**, 1225–1229.
25 Ho,I.C. and Leiden,J.M. (1990) *Mol. Cell. Biol.*, **10**, 4720–4727.
26 Sheridan,P.L., Sheline,C.T., Cannon,K., L.,V. M., Pazin,M. J., Kadonaga,J.T. and Jones,K.A. (1995) *Genes Dev.*, **9**, 2090–2014.
27 Simpson,R.T. (1990) *Nature*, **343**, 387–389.
28 Archer,T.K., Cordingley,M.G., Wolford,R.G. and Hager,G.L. (1991) *Mol. Cell. Biol.*, **11**, 688–698.
29 Dignam,J.T., Lebovitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475–1479.
30 Harper,M., Masselle,L., Gallo,R. and Wong-Staal,F. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 772–776.
31 Hayashi,S., Gilliam,I.C., Delaney,A.D. and Tener,G.M. (1977) *J. Histochem. Cytochem.*, **26**, 677–679.
32 Ess,K.C., Whitaker,T.L., Cost,G.J., Witte,D.P., Hutton,J.J. and Aronow,B.J. (1995) *Mol. Cell. Biol.*, **15**, 5707–5715.

33 Archer,T.K., Lefebvre,P., Wolford,R.G. and Hager,G.L. (1992) *Science*, **255**, 1573–1576.
34 Almouzni,G., Clark,D.J., Mechali,M. and Wolffe,A.P. (1990) *Nucleic Acids Res.*, **18**, 5767–5774.
35 Zeichner,S.L., Kim,J.Y. and Alwine,J.C. (1991) *J. Virol.*, **65**, 2436–2444.
36 Leiden,J.M. (1993) *Annu. Rev. Immunol.*, **11**, 539–570.
37 Verbeek,S.,Izon,D., Hafhuls,F., Robannus-Maandag,E., te Riele,H., van de Wetering,M., MacDonald,H.R. and Clevers,H. (1995) *Nature*, **374**, 70–74.
38 van Genderen,C., Okamura,R.M., Farinas,I., Quo,R.-G., Parslow,T.G., Bruhn,L. and Grosschedl,R. (1994) *Genes Dev.*, **8**, 2691–2703.
39 Zhou,P., Byrne,C., Jacobs,J. and Fuchs,E. (1995) *Genes Dev.*, **9**, 570–583.
40 Ho,I.C., Yang,L.H., Morle,G. and Leiden,J.M. (1989) *Proc. Nati. Acad. Sci. USA*, **86**, 6714–6718.
41 Carlsson,P., Waterman,M.L. and Jones,K.A. (1993) *Genes Dev.*, **7**, 2418–2430.
42 Giese,K. and Grosschedl,R. (1993) *EMBO J.*, **12**, 4667–4676.
43 Giese,K., Kingsley,C., Kirshner,J.R. and Grosschedl,R. (1995) *Genes Dev.*, **9**, 995–1008.
44 Meyer,K.B. and Ireland,J. (1994) *Nucleic Acids Res.*, **22**, 1576–1582.