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Dissecting a Locus Control Region: Facilitation of Enhancer Function by Extended Enhancer-Flanking Sequences

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Using transgenic mice, we have defined novel gene regulatory elements, termed "facilitators." These elements bilaterally flank, by up to 1 kb, a 200-bp T-cell-specific enhancer domain in the human adenosine deaminase (ADA) gene. Facilitators were essential for gene copy-proportional and integration site-independent reporter expression in transgenic thymocytes, but they had no effect on the enhancer in transfected T cells. Both segments were required. Individual segments had no activity. A lack of facilitator function caused positional susceptibility and prevented DNase I-hypersensitive site formation at the enhancer. The segments were required to be at opposed ends of the enhancer, and they could not be grouped together. Reversing the orientation of a facilitator segment caused a partial loss of function, suggesting involvement of a stereospecific chromatin structure. *trans*-acting factor access to enhancer elements was modeled by exposing nuclei to a restriction endonuclease. The enhancer domain was accessible to the 4-cutter *Dpn*II in a tissue- and cell-type-specific fashion. However, unlike DNase I hypersensitivity and gene expression, accessibility to the endonuclease could occur without the facilitator segments, suggesting that an accessible chromatin domain is an intermediate state in the activational pathway. These results suggest that facilitators (i) are distinct from yet positionally constrained to the enhancer, (ii) participate in a chromatin structure transition that is necessary for the DNase I hypersensitivity and the transcriptional activating function of the enhancer, and (iii) act after cell-type-specific accessibility to the enhancer sequences is established by factors that do not require the facilitators to be present.

In contrast to the consistent behavior of most endogenous genes, *cis*-regulatory information carried in mammalian transgenes is subject to notorious variability generally referred to as the genomic position effect (26, 28, 49). The basis for the genomic position effect is poorly understood. One cause of it may be the inability of transgenes to control chromatin structure at genomic insertion sites (17, 63). A variety of studies with diverse eukaryotic systems indicate that the ability of *trans*-acting factors to function at *cis*-regulatory regions is strongly influenced by alterations of chromatin structure and organization (1, 4, 24, 30, 40, 42, 50). For example, nucleosomes and nucleosome positioning (29, 35, 36, 53, 55, 56, 61, 62), and specific histones (11) or histone motifs (16, 59), can dramatically impact the ability of transcriptional machinery to address underlying *cis* elements. The regulation of chromatin domain structure and accessibility to *trans*-acting factors may be a fundamental mechanism by which the genome can specifically and selectively express genes during developmental cell differentiation.

Some transgene constructions exhibit a high degree of consistency among independent mouse lines with regard to cell type specificity, level of expression, and in some cases, proportionality to gene copy number. Reporter expression that is proportional to gene copy number is a remarkable property that indicates the ability of each transgene to form and regulate an autonomously active locus. Sequences associated with the generation of these effects have been termed locus control regions (LCRs) (20, 27, 58). In light of their ability to dominantly control gene expression from any chromosomal region into which they are inserted, LCRs are appealing entities for mechanistic analyses of the elements and factors responsible for activating a chromatin domain. We have previously reported elements within the human adenosine deaminase (ADA) gene that bear analogy to LCRs. Human ADA deficiency causes severe combined immunodeficiency disease with a failure to develop T cells. ADA deficiency has been used as a model for corrective gene therapy based on gene introduction into autologous hematopoietic stem cells (5). The gene is normally expressed at a high level in thymocytes, and we have sought to identify and understand the regulatory elements responsible for controlling ADA cell type specificity and levels of expression in developing T cells.

Expression of the ADA gene in the thymus is controlled by *cis*-regulatory elements in its first intron (2). The intron contains six DNase I-hypersensitive sites. In transiently transfected T cells, positive-acting *cis* elements are localized in a 200-bp domain that corresponds to the limits of hypersensitive site III (HS III) hypersensitivity (3). In transgenic mice, a much larger 2.25-kb regulatory region was necessary for consistent position-independent transgene expression in the thymus, thus indicating the functional characteristics of an LCR. Transgenic mice made from constructions that lacked terminal sequences of the 2.25-kb regulatory region exhibited inconsistent gene expression. Whereas DNase I hypersensitivity was formed at the HS III enhancer in thymocytes of transgenic mice that contained up to 100 copies of a large ADA-CAT reporter gene (2), hypersensitivity was formed poorly or not all at the HS III enhancer by most or all copies of mouse lines that contained poorly expressed transgenes lacking sequences 5′ or 3′ of the enhancer domain (3). This led us to term these enhancer flank-

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ing sequences "facilitators" and to postulate (i) that their presence was required for the formation of hypersensitivity in most chromosomal integration sites and (ii) that there was an absolute requirement for the formation of the HS for enhancer function. In the present study we have analyzed the inconsistent behavior of the transgenes that lacked the flanking sequences, further dissected the flanking segments, and identified constraints that govern facilitator function. The results indicate that the facilitators can cooperate with the enhancer only if both segments are present and specifically positioned and oriented. It also appears likely that the segments function subsequent to the recognition of core elehancer elements by cell-type-specific factors.

MATERIALS AND METHODS

Transgenic analyses. Transgenic mice were made with the fertilized eggs of (C3H × C57BL/6)F_1 hybrid parents by procedures described by Hogan et al. (31). F_1 transgenic mice between 4 and 9 weeks of age were analyzed (2). Transgene copy numbers were determined by comparison of blot hybridizations of restriction endonuclease-digested DNA isolated from tails with known standards of human DNA and cloned plasmid DNA. For quantitative Southern analyses, autoradiograms were generally analyzed with a PhosphorImager device (Molecular Dynamics Inc., Mountain View, Calif.) or as previously described. Transgenic tissue chloramphenicol acetyltransferase (CAT) assays, protein concentration measurements, transgene copy number determinations, mouse dissections, in situ hybridization, and chromatin DNase I hypersensitivity analyses were performed as previously described (2, 3, 60).

Transgenes. Restriction fragments from various plasmids were isolated from agarose gels and prepared for microinjection (3). Transgenes were designated as follows: 5′ flanking DNA/hypersensitive site(s) or intron segment placed downstream of CAT. Restriction sites used for regulatory region subcloning are indicated in Fig. 1 and 3. The restriction sites used to generate short and long promoters were *Eco*RI for 0.2-kb, *Bss*HII for 0.3-kb, and *Nde*I for 3.7-kb promoter segments.

***Dpn*II endonuclease accessibility assay.** Nuclei from cell lines or transgenic mouse tissues were prepared (2). Approximately 2.5×10^6 hemocytometer-quantitated nuclei per assay were suspended at 4°C in 0.2 ml of a buffer that contained 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 10 mM Tris HCl (pH 7.9 at 25°C). Various amounts of *Dpn*II per ml and 50% glycerol were added to a final concentration of 2% glycerol. Reactions were allowed to proceed on ice for 60 min. Reduced-temperature reaction conditions minimized nonspecific cleavage of the enhancer region, which was observed at room temperature. DNA was purified from the nuclei with sodium dodecyl sulfate and proteinase K, phenol, and RNase A. A 5-μg portion of DNA was cut with *Kpn*I and electrophoresed through a mixed agarose gel of 1.5% SeaKem LE and 1% NuSieve (FMC) in 1× TAE buffer. Southern blot transfer was to Magnagraph membranes (MSI, Westboro, Mass.). Hybridization was to a random-primer-labeled probe that encompassed the 603-bp *Kpn*I fragment from the regulatory region. Hybridizations and washes were done as recommended by the manufacturer.

Quantitative analysis of the relative intensities of the bands released by partial *Dpn*II digestion of the *Kpn*I fragment on Southern blots was accomplished by use of the PhosphorImager and ImageQuant software (Molecular Dynamics). Rectangles were drawn down the length of each lane and subjected to "linegraph" quantitation. Data were transferred to a spreadsheet file, graphed, and object pasted into a draw file with height-scale normalization of the parental band to assess relative cleavage with respect to gene copy number.

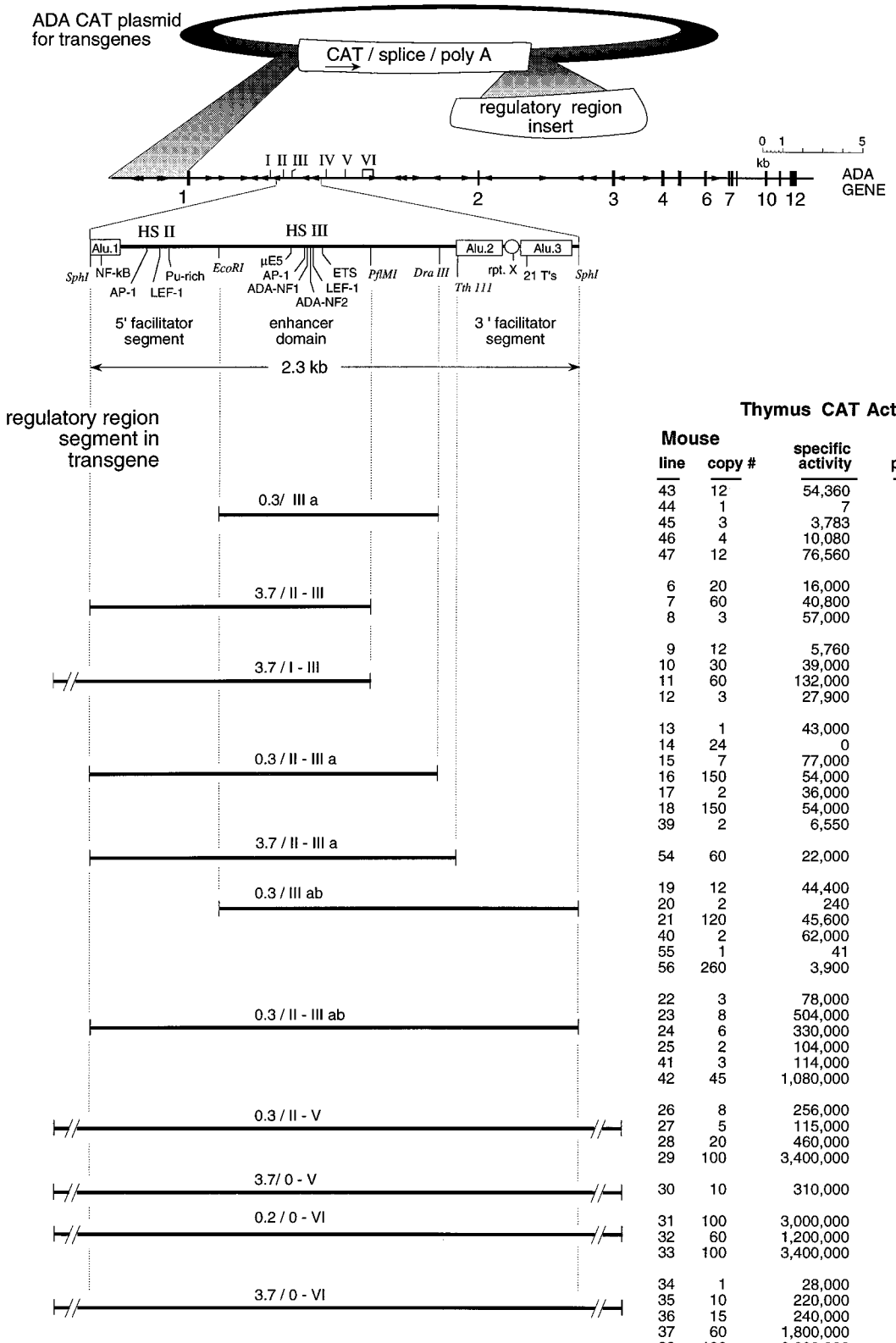
RESULTS

Transgenic analysis of the ADA thymic regulatory region. Segments of the human ADA gene responsible for the control of expression in the thymus were identified by analysis of a series of ADA-CAT reporter gene constructions in transgenic mice. The structures of the reporter genes and their expression in thymus tissue are shown in Fig. 1. Both total CAT specific activity and activity per gene copy are indicated. Mice that contained transgenes that included at least the 2.3-kb fragment (designated II-IIIab; lines 22 to 25 and 41 to 42) exhibited thymic CAT activity that was within the comparatively narrow range of 16,000 to 63,000 U per gene copy over the wide range of 1 to 100 gene copies. Deletion of sequences at either end of this 2.3-kb segment caused severe loss of consistency in levels of thymic expression per gene copy. In contrast, these terminal sequences had no effect in transfection transient-expression assays in lymphoid cell lines MOLT 4, CEM, and Raji (3). We have termed these terminal sequences facilitators. Since the effects of the facilitators are evident only in transgenic assays, this places practical limitations on their detailed characterization.

Not evident in our prior analyses of fewer transgenic lines and constructions (3) was that all mice made from facilitator-deleted constructions that contained high-copy-number genes (e.g., lines 6, 7, and 16) exhibited low levels of expression per copy. The only facilitator-deleted mice that exhibited high levels of expression per copy (e.g., lines 8, 13, 17, and 40) contained low total copy numbers. This indicates that mice containing nonfacilitated constructions were unable to express multiple copies. A significant fraction of the mouse lines that lacked facilitator segments also had much lower levels of expression than would be expected of even a single copy of a facilitated transgene (e.g., lines 44, 14, and 20). Variability and inconsistency that occurred as a result of a several-hundred-base-pair facilitator deletion could not be prevented by the presence of an additional 3.4 kb of DNA isolated from 5′ flanking sequences of the ADA gene (lines 6 to 12 and 54). Within the context of the transgene concatemer, this represents the replacement of the 3′ sequences with the 5′ flanking DNA. Facilitator effects, therefore, are not caused by increasing spacing of transgenes. Rather, facilitators act via specific DNA sequences in the flanking segments.

Quantitative analysis of facilitator effects. To better illustrate the effects of the facilitator segments and gene copy number on transgene expression among independent mouse lines, the specific activity of CAT was plotted against the gene copy number for each mouse line (Fig. 2A). When these data are plotted in this fashion, transgenic mice very clearly separate into two groups. Mice that contained transgenes with both flanker segments exhibited a linear increase in expression with increasing copy numbers. In contrast, mice that lacked a flanking facilitator segment exhibited no increase in expression. However, these lines frequently did exhibit a level of expression consistent with the expression of one to two copies of a facilitated transgene.

Plotting expression per copy versus copy number on a log-log scale provides additional insight. The facilitator-deleted mouse lines appear to divide into two subpopulations (Fig. 2B). The first subpopulation includes about half of the independent mouse lines. Data from this group form a curve with a slope that approximates −1. This indicates that for each 10-fold increase in gene copy number there was a 10-fold decrease in per-copy gene expression. Additional transgene copies were completely inactive, and they only diluted the level of per-copy expression. In addition to the mouse lines that fell along the dilution curve, a separate group was distinguishable. Mouse lines in this group exhibited levels of expression much lower than would be expected if they conformed to the dilution curve. Their total thymic CAT activity was less than would be expected if even a single copy of the transgene was active. Thus, the lack of either one of the facilitators, in addition to causing an inability to express multiple copies, also caused a "position dependence" consisting of a variability in expression presumably associated with the transgene insertion site. In contrast, the consistency of expression among the various mouse lines when both facilitators are present suggests that each transgene copy is active and not nearly as susceptible to positional variation. Thus, the facilitators confer two different effects, (i) the ability to generate copy-proportional transgene activity and (ii) the ability to overcome negative position effects.



Thymus CAT Activity

Mouse line	copy #	specific activity	activity per copy
43	12	54,360	4,530
44	1	7	7
45	3	3,783	1,260
46	4	10,080	2,520
47	12	76,560	6,380
6	20	16,000	800
7	60	40,800	680
8	3	57,000	19,000
9	12	5,760	480
10	30	39,000	1,300
11	60	132,000	2,200
12	3	27,900	9,300
13	1	43,000	43,000
14	24	0	0
15	7	77,000	11,000
16	150	54,000	360
17	2	36,000	18,000
18	150	54,000	360
39	2	6,550	3,275
54	60	22,000	370
19	12	44,400	3,700
20	2	240	120
21	120	45,600	380
40	2	62,000	31,000
55	1	41	41
56	260	3,900	15
22	3	78,000	26,000
23	8	504,000	63,000
24	6	330,000	55,000
25	2	104,000	52,000
41	3	114,000	38,000
42	45	1,080,000	24,000
26	8	256,000	32,000
27	5	115,000	23,000
28	20	460,000	23,000
29	100	3,400,000	34,000
30	10	310,000	31,000
31	100	3,000,000	30,000
32	60	1,200,000	20,000
33	100	3,400,000	34,000
34	1	28,000	28,000
35	10	220,000	22,000
36	15	240,000	16,000
37	60	1,800,000	30,000
38	100	2,000,000	20,000

FIG. 1. CAT expression in thymus tissue from ADA-CAT transgenic mice. Total expression is indicated as specific activity and per-copy expression. Indicated segments of the human ADA gene first intron encompassing various portions of the 2.3-kb thymic regulatory region are placed downstream of the CAT reporter promoted by ADA gene 5′ flanking DNA. Constructs are designated by 5′ flanking DNA, HSs included, and additional flanking DNA. Roman numerals mark DNase-hypersensitive sites. Copy number equals the number of transgene copies per diploid genome as determined by Southern blot comparison of transgenic mouse liver DNA with similarly cut human DNA. Band intensities were analyzed by surface emission scanning (Molecular Dynamics PhosphorImager). Specific activity of CAT is given as picomoles of acetylated chloramphenicol per hour per 100 μg of protein. No mice with unrearranged transgenes were excluded from this chart.

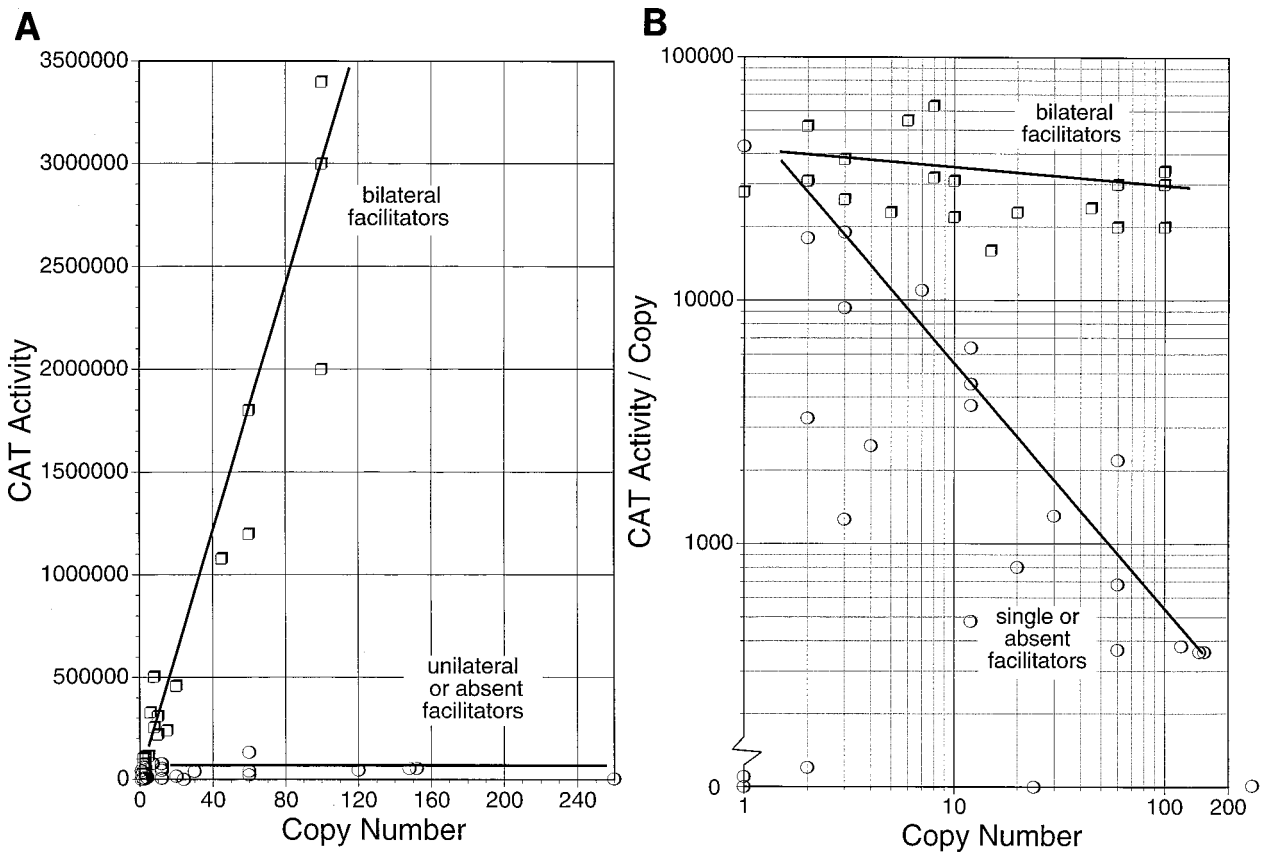
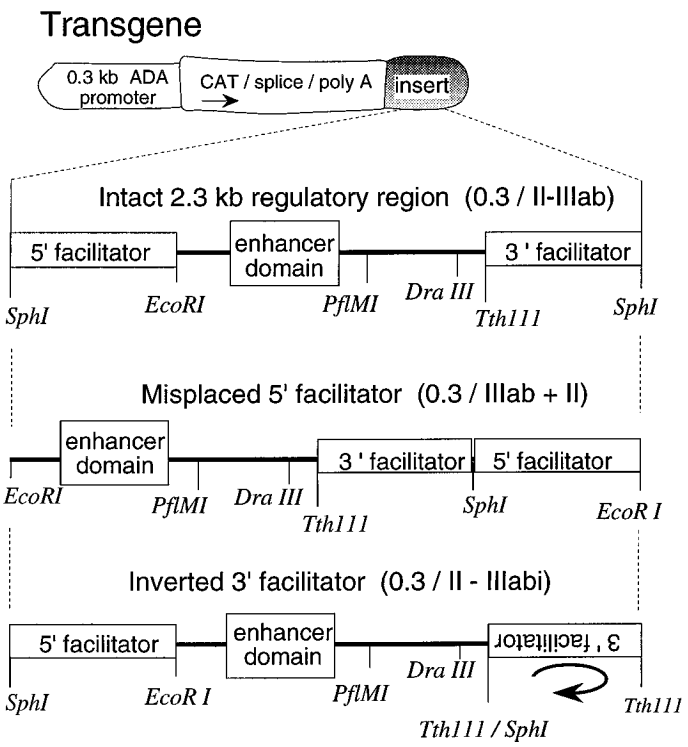


FIG. 2. Bilateral facilitators generate copy number-proportional gene expression and prevent insertion site-dependent suppression. Transgene constructions with two flanker segments (squares) were contained in lines 22 to 38, 41, and 42. Transgene constructions with only one flanker segment (circles) were contained in lines 6 to 21, 39, 40, 43 to 47, and 54 to 56. (A) Expression versus copy number. Unweighted linear regression analyses indicate that for group 1 CAT activity equaled $28{,}700X - 10{,}000$ ($r^2 = 0.93$) and that for group 2 CAT activity equaled $184X + 34{,}100$ ($r^2 = 0.085$), where X equals the copy number. (B) Expression per copy versus copy number, log-log scale. The symbols are the same as those for panel A. The line drawn through group 2 datum points has a slope of -1.0, and it depicts the unweighted best fit of datum points, excluding those for mice with less than 25% of the expression expected for a single facilitated copy. This argument is intended to indicate that there are two populations of group 2 mice, those that conform roughly to the dilution curve and those that produce values significantly under the curve (see the text).

On the basis of our previous analyses, it was possible that unilateral facilitator segments were essential for the albeit partial function of facilitator-deleted transgenes such as 0.3/II-IIIa and 0.3/IIIab. To test for this possibility, a construct missing both the 5′ and 3′ required facilitator segments was made (0.3/IIIa; Fig. 1). As shown in Fig. 1, two of the lines containing this construct (43 and 47) exhibited per-copy expression equivalent to that of 1 or 2 facilitated copies, despite having approximately 12 copies each. In addition, three other mouse lines exhibited levels of expression lower than would be expected if even a single copy were active. Thus, the bilateral facilitator-deleted construction exhibits the same expression capability and susceptibility to insertion site-dependent suppression as other nonfacilitated lines. This implies that neither the 5′ nor the 3′ facilitator alone has a detectable activity in transgenic assays. The activity of each facilitator segment appears to be dependent on the presence of the other segment. We therefore hypothesize that the facilitated regulatory region functions as a structure or unit that forms only when all components are present.

Perturbations of the facilitator segments. To determine the constraints that affect the ability of the facilitators to function, flanking segments were rearranged. The 5′ facilitator segment was repositioned (Fig. 3) to determine whether facilitator segments must bilaterally flank the enhancer domain. To do this, sequences critical for 5′ facilitator activity were moved to a position downstream of the 3′ facilitator segment. None of three transgenic lines generated exhibited an appropriate level of expression. The line with the best expression (no. 48) contained a single copy that was 25 to 50% as active as expected for a single-copy facilitated transgene. The other two lines exhibited very low levels of expression, indicating that rearrangement destroyed the ability of the facilitator sequences to insulate the transgene from insertion site-dependent suppressive effects. This shows that the complete sequences of the 2.3-kb regulatory region were insufficient for facilitated transgene function unless arranged in a specific fashion. Further, the ability of the facilitated regulatory region to be insulated from positional effects could not be conferred by unilaterally grouped facilitator sequences.

A potentially less perturbed construction, in which the orientation of a segment from the 3′ facilitator was inverted, is also shown in Fig. 3. Eight transgenic lines that contain the inverted construct exhibited considerable variability in expression, with both high and low levels of per-copy activity. Unlike the deletion series, a number of these mice attained a total level of expression that would be expected of multiple copies of a facilitated transgene. However, it is clear that facilitator activity was neither complete nor consistent, especially in the lines that contained high-copy-number genes. Like the reposi-



CAT Activity

Mouse line	copy #	Thymus specific activity	Thymus activity per copy	Spleen specific activity	Spleen activity per copy
22	3	78,000	26,000	110	37
23	8	504,000	63,000	1,600	200
24	6	330,000	55,000	1,560	260
25	2	104,000	52,000	2,800	1,400
41	3	114,000	38,000	360	120
42	45	1,080,000	24,000	112,500	2,500
48	1	10,360	10,360	540	540
49	4	44	11	4	1
50	6	750	125	12	2
51	50	36,000	720	2,000	40
52	200	6,000	30	600	3
53	50	180,000	3,600	3,300	66
57	24	194,000	8,880	16,530	690
58	3	256,000	85,400	1,700	560
59	8	498,000	62,250	9,700	1,210
60	36	319,000	8,860	48,300	1,340
61	50	399,000	8,000	15,400	310
62	12	312,000	2,600	3,440	290
63	2	12	6	0	0

FIG. 3. Facilitator sequences interact with the enhancer in a position- and orientation-specific manner. Perturbed facilitator segments are incapable of conferring position independence or gene copy-proportional expression in the thymus. Spleen activities are considerably more variable than thymus activity, and the lack of facilitation does not appear to account for variability in spleen expression. CAT activity units are the same as those in Fig. 1.

tioned construct, the inverted construct contained all of the sequences of the entire regulatory region. Thus, despite the presence of the 3′ facilitator segment sequences, misorientation prevented the formation of a consistently active facilitated regulatory region.

Effects of facilitators on cell-type-specific expression. We have previously reported that a large facilitated transgene (ADA CAT 4/0-VI) is expressed at high levels in transgenic mouse cortical thymocytes, as is the normal human ADA gene (2). To better understand defective and variable expression by nonfacilitated transgenes, we performed in situ hybridization analysis of CAT mRNA distribution within the thymus. A lack of facilitation could destroy the normal program of transgene expression during differentiation of thymocytes or cause a variegated expression among thymocyte types, perhaps reflecting a stochastic activation process under facilitator control. As shown in Fig. 4a through d, CAT mRNA was detectable at high levels in the cortical thymocytes of four mouse lines: no. 35 (3.7/0-VI; facilitated), 47 (0.3/IIIa; unfacilitated; 5′ and 3′ segments deleted), 40 (0.3/IIIab; unfacilitated; 5′ segment deleted), and 48 (0.3/I1.6 + II; misplaced 5′ facilitator). In all of these lines expression was strong in cortical thymocytes and strongly diminished or absent in most medullary thymocytes. This pattern of expression is identical to that of ADA mRNA in the human thymus as detected by in situ hybridization (data not shown) and that of ADA enzyme activity as previously shown (2).

Not all unfacilitated lines, however, exhibited appropriate cortical-medullary expression (Fig. 4e through g). Line 54 (3.7/II-IIIa) exhibited an abnormally high level of medullary thymocyte expression that was in addition to a strong signal in cortical thymocytes. Expression in medullary thymocytes was even more exaggerated in line 15 (0.3/II-IIIa). This line also exhibited the expected signal in cortical thymocytes. One mouse line, 21 (0.3/IIIab), exhibited variegated expression in both cortical and medullary thymocytes. This line contains 120 copies of the 5′ facilitator-deleted construct pADA CAT 0.3/IIIab and expresses CAT mRNA at a level comparable to that of one copy of a line made from pADA CAT 0.3/II-IIIab (Fig. 1). Silver grains were evident over only 10 to 30% of its cortical thymocytes, with no detectable expression in the remainder of the cortical thymocytes. The cells expressing high levels of CAT mRNA were not homogeneously interspersed with nonexpressing cells. Rather, expressing and nonexpressing cells occurred in clusters that contained 5 to 25 cells per cluster per cross section. Expressing clusters were distributed randomly throughout all of the thymus lobules. Variegated expression was not likely due to the absence of specific elements in the 5′ facilitator because line 40, made from the same construct, expressed CAT mRNA as expected. The variegated pattern of line 21 was most likely caused by the local chromatin environment unique to its site of integration (position effect).

DNase I hypersensitivity. We have previously shown that DNA in thymic nuclei from lines 37 and 38 was virtually completely cleaved by DNase I at HS III (2). This implies that each copy of HS III present in the 60 and 100 copies of the facilitated transgenes in these lines, respectively, was present in a hypersensitive configuration. A similarly complete cleavage by

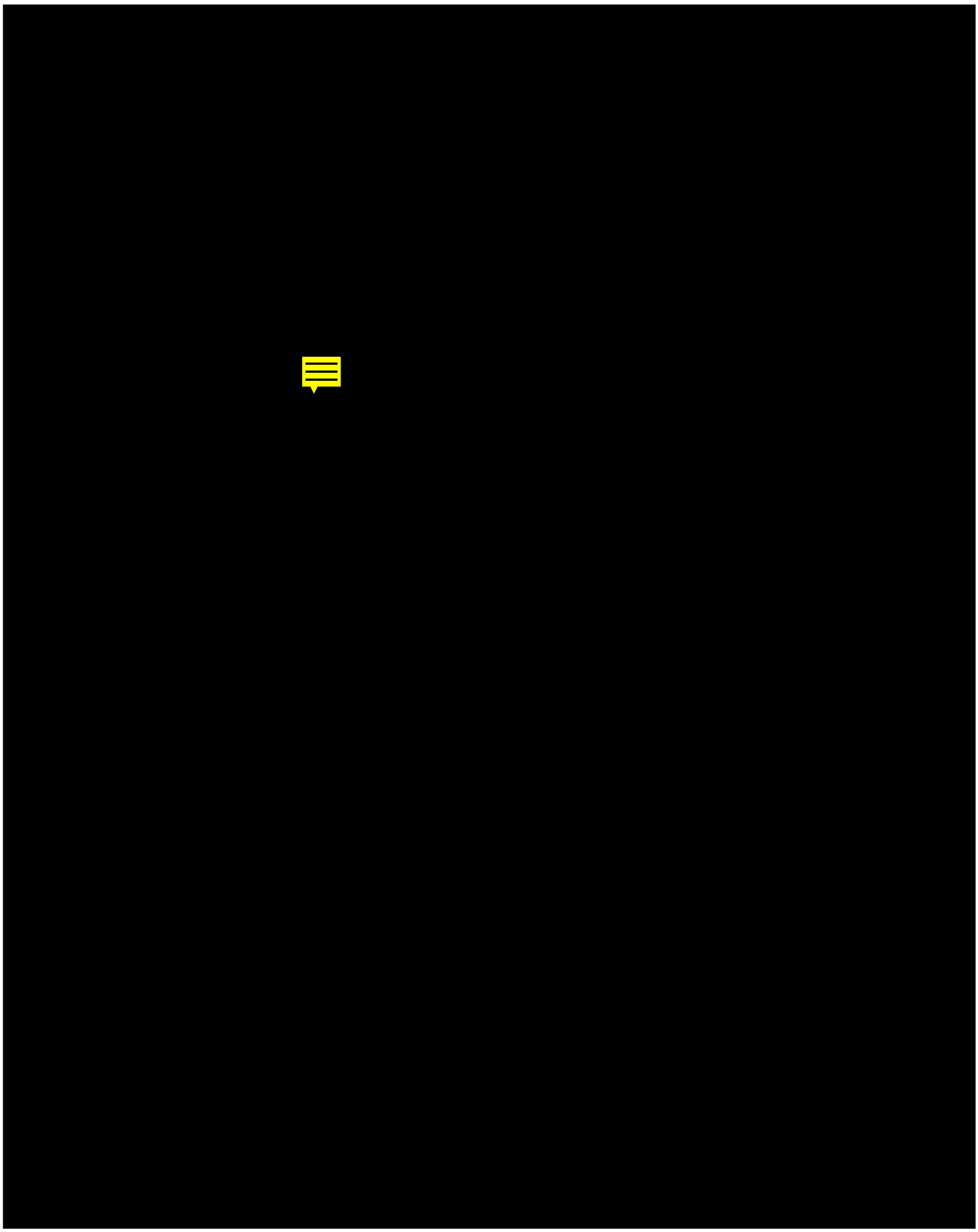


FIG. 4. Facilitation does not alter cell type specificity of expression, but there can be inappropriate regulation in a fraction of the unfacilitated mouse lines. Results of in situ hybridization analysis of CAT mRNA distribution in thymus tissue from several transgenic mouse lines are shown. Dark-field illumination demonstrates a signal as pinkish white grains over sites of mRNA accumulation. (a through d) Mouse lines 35 (3.7/0-VI), 43 (0.3/IIIa), 40 (0.3/IIIab), and 48 (0.3/IIIab + II). Expression is uniform among the cortical thymocytes (indicated by c) and much reduced in medullary thymocytes (indicated by m). (e through g) Mouse lines 15 (0.3/II-IIIa), 21 (0.3/IIIab), and 54 (3.7/II-IIIa). These panels demonstrate atypical expression with a significant signal in medullary thymocytes. In addition to medullary expression (indicated by m), there is a uniform signal in cortical thymocytes shown in panels e and g (indicated by c) and a much reduced signal in medullary thymocytes. (h) Higher-power magnification of panel f. Expression is clearly nonuniform among the cortical thymocytes (indicated by c), with a signal present in 10 to 30% of the cortical thymocytes. A somewhat higher percentage of the medullary thymoyctes shown in panel f are positive, though the percentage is not as uniformly high as that for panel e. Slides were exposed for various lengths of time (5 to 28 days) to optimize signal discrimination, counterstained with hematoxylin and eosin, and photographed at a final magnification of ×32 (a through g) or ×64 (h).

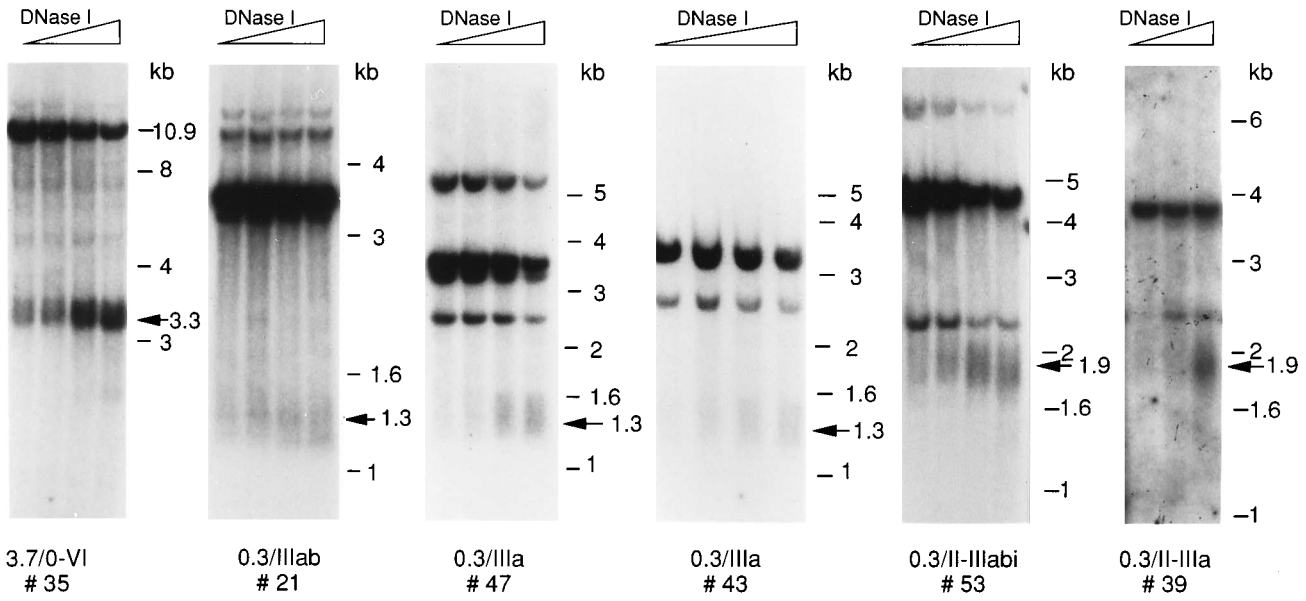


FIG. 5. DNase I-hypersensitive site formation at HS III is limited or absent in nonfacilitated transgenic mouse lines. Cutting at HS III is shown by an arrow for each of the indicated mouse lines. Control line 35 exhibits a nearly complete cleavage of its parental 10.9-kb band to generate a 3.3-kb band, whereas none of the unfacilitated lines exhibit comparable digestion. Each lane contains 7 μg of purified genomic DNA. After DNase I digestions (0 to 20 U/ml, 10 min, 30°C), purified DNAs were digested with *Bam*HI (line 35) or *Sca*I (lines 21, 47, 43, 53, and 39), electrophoresed through agarose gels, and probed with fragments corresponding to the 5′ ends of released fragments. *Sca*I cuts only once per transgene and therefore generates junctional fragments that are higher or lower than the main parental band. Molecular sizes were determined from the migration of adjacent markers.

DNase I at HS III was also observed in MOLT 4 and CCRF-CEM T-lymphoblastoid cell lines. In contrast, thymocytes from the nonfacilitated mouse lines 6 and 8 exhibited very limited hypersensitivity, with most transgene copies resistant to DNase I cleavage. Of the limited HS III hypersensitivity that was detectable, it was severalfold greater in line 6, which expressed CAT mRNA at higher levels, than in line 8 (3). We have now extended these analyses to other variably expressing nonfacilitated constructions in mice. As shown in Fig. 5, each of the unfacilitated constructions exhibited reduced hypersensitivity at HS III, with only a small fraction of the transgene copies cleaved at their HS III sites. Interestingly, some junctional bands appeared to be more DNase I sensitive than the main concatemer band. This was most apparent in lines 47 and 53. The band intensities of the DNase I-released fragment are similar for lines 21, 47, and 43, which all express the equivalent of one to two copies of the transgene. In contrast, consistent with its high level of total activity expression by a nonfacilitated construct, line 53 exhibited severalfold more HS III product than did the other nonfacilitated mouse lines. However, the extent of digestion was less than that expected of facilitated transgenes. These results indicate that the formation of DNase I hypersensitivity at the HS III enhancer region is associated with the active status of the regulatory region and that the defect in the function of the regulatory region occurs prior to or at the stage of the formation of the DNase I-hypersensitive site in thymic nuclear chromatin. The facilitators are necessary for more than a single copy of the transgene to form hypersensitivity and achieve copy-proportional expression.

Factor access to nuclear chromatin assessed with a restriction endonuclease. To test the potential accessibility of *cis*-active elements of the regulatory region to cell- and sequence-specific DNA binding proteins, we used a restriction endonuclease as a model of a nuclear factor. Some restriction endonuclease sites are highly accessible for cleavage in areas of active nuclear chromatin (44). Nuclei were partially fractionated, suspended in restriction buffer, and treated with the restriction enzyme. We first examined the accessibility of sequences in nuclei from human MOLT 4 T-lymphoblastoid cells and Raji B-lymphoblastoid cells. In MOLT 4 cells there is strong expression of the endogenous human ADA gene coupled with full HS III DNase I hypersensitivity, whereas in Raji cells there is low-level expression of ADA and essentially absent HS III DNase I hypersensitivity (2). Of three restriction enzymes tested, *Dpn*II, *Sty*I, and *Hin*fI, only *Dpn*II cut well under the conditions utilized. Of three *Dpn*II sites, one was cut strongly and two were cut weakly in MOLT 4 cell nuclei (Fig. 6). The strongest cutting site was the central *Dpn*II site, located within the HS III domain. Cutting at this site generates a 432-bp band. The two weaker cut sites are outside the limits of DNase I hypersensitivity (Fig. 6A), and they generate 542- and 547-bp bands which are indistinguishable by single cutting. However, significant production of the 371-bp band and retention of the 171-bp band in MOLT 4 cells indicate that the region was also susceptible to double digestion at the central and 5′ *Dpn*II sites and that the 5′ site is more accessible than the 3′ site. In Raji cells, there was weak cutting of multiple sites, indicating a minimal accessibility to endonuclease despite the absence of DNase I hypersensitivity. As a function of *Dpn*II concentration (Fig. 6, panel B), equivalent releases of the 432-bp band from Raji nuclei exposed to 80 U of *Dpn*II per ml and from MOLT 4 nuclei exposed to 1 U/ml occurred. This indicates a striking difference in the accessibility of these domains in nuclear chromatin. The preference for cutting at the central *Dpn*II site seen in MOLT 4 nuclei was also less pronounced in Raji cells. The ratio of band intensities at 432 bp to those at 540 bp was about 10:1 in MOLT 4 cells and less than 2:1 in Raji cells. When limited amounts of *Dpn*II were used to cut naked DNA purified from MOLT 4 and Raji cells, there was no preferential cutting of any of the sites (data not shown). This indicates that increased sensitivity to cleavage at the central *Dpn*II site in

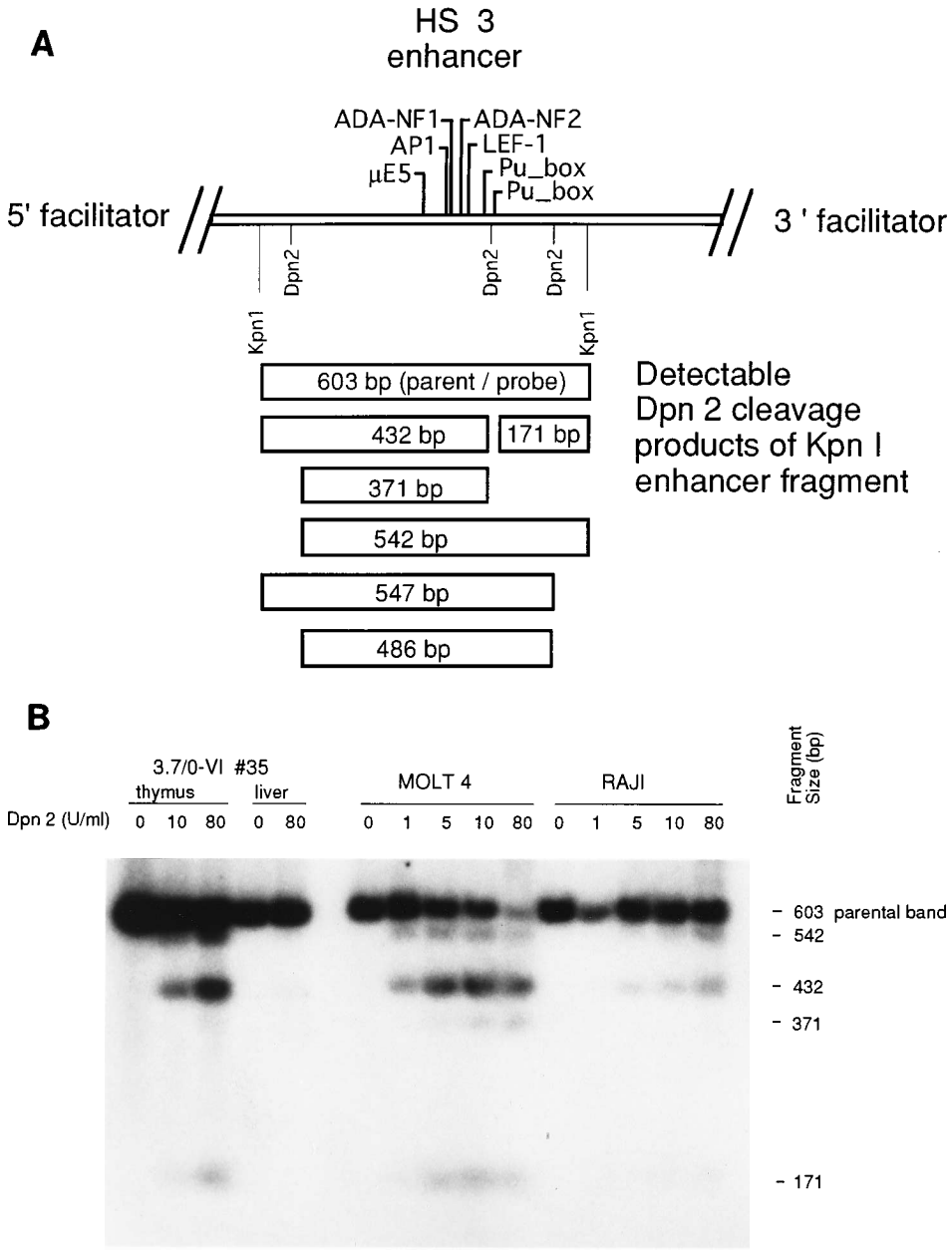


FIG. 6. Cell-type- and tissue-specific access to enhancer domain sequences in nuclear chromatin. Nuclei from MOLT 4, CEM, and transgenic 3.7/0-VI 35 thymus and liver cells were subjected to digestion with various concentrations of *Dpn*II. DNA was purified, restricted with *Kpn*I, electrophoresed (7 μg) through a high-percentage gel, blot transferred, and probed with the entire *Kpn*I fragment. (A) Map of the relative positions of the enhancer domain, previously delineated elements of the enhancer, *Dpn*II and *Kpn*I cut sites, and the sizes of the expected bands. (B) Autoradiogram showing the fragmentation of the regulatory region as a function of increasing concentration of *Dpn*II. Similar fragmentation occurs in transgenic thymus and MOLT 4 cells, but there is very little accessibility of the regulatory region to *Dpn*II in Raji cells and transgenic liver nuclei.

MOLT 4 cells is the result of its conformation within nuclear chromatin.

To determine the accessibility of enhancer sequences in transgenic mice, nuclei from several facilitated and nonfacilitated lines were exposed to *Dpn*II. We first compared nuclei from thymus and liver tissue from the facilitated line 35 (Fig. 6B). *Dpn*II accessibility was highly tissue specific. There was no detectable cleavage in nuclei from liver tissue, a tissue that expresses very little ADA or CAT and lacks HS III DNase I hypersensitivity (2). However, nuclei from thymus tissue of line 35 exhibited a high degree of cleavage by *Dpn*II. As shown in Fig. 7, the relative intensities of the bands were almost identical to those for MOLT 4 nuclei. This suggests that each of the 10 copies integrated in line 35 is in a conformation similar to that in which it occurs in MOLT 4. The nonfacilitated lines also exhibited a substantial degree of *Dpn*II accessibility (Fig. 7A). However, significant differences were evident. As shown best in the densitometric-type PhosphorImage linegraph analysis (Fig. 7B), the ratio of the 432-bp bands to the 542- and 547-bp bands was reduced in the nonfacilitated lines compared with MOLT 4 or thymocytes that contained facilitated transgenes. There was also more double-cut 371-bp band in the nonfacilitated

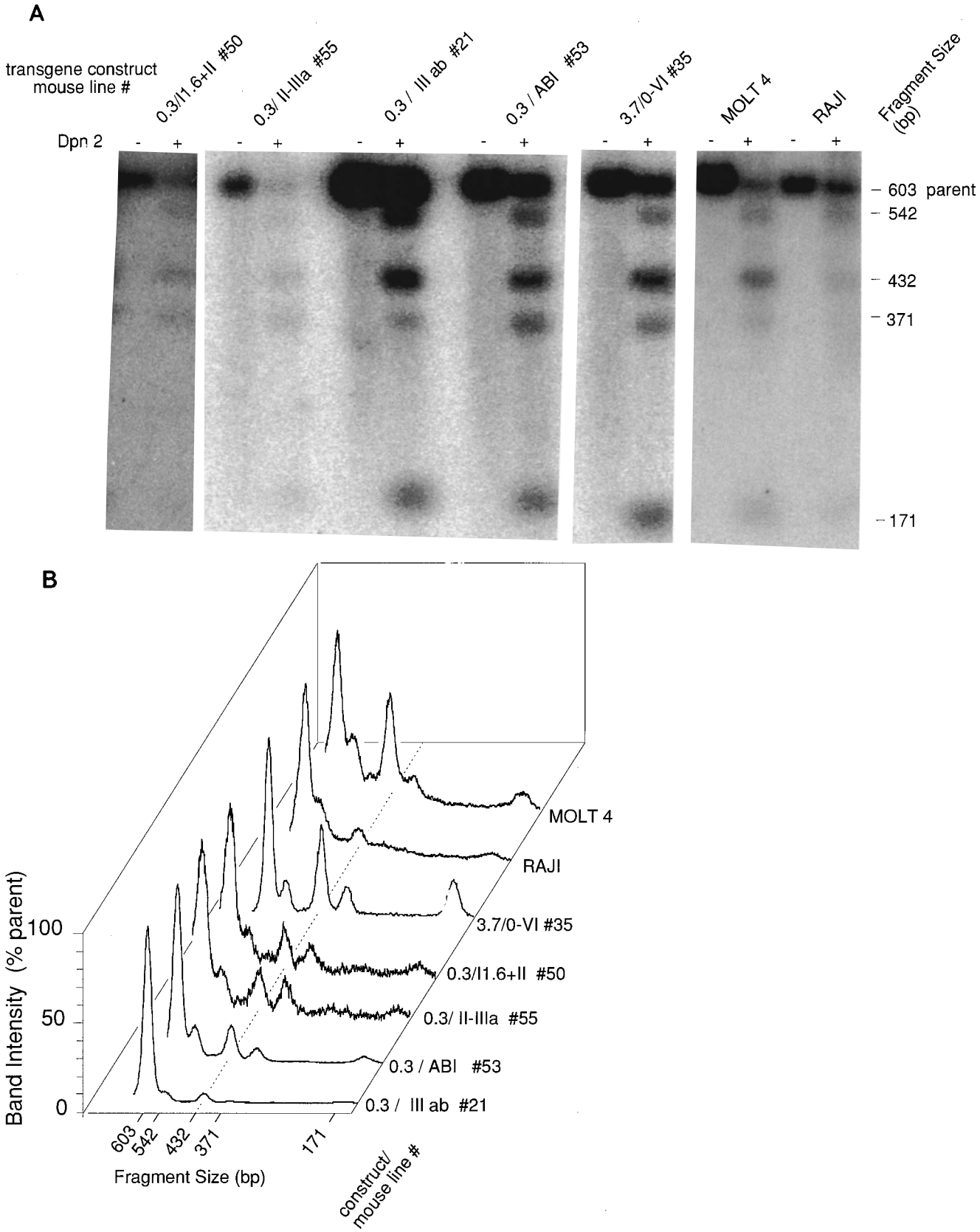


FIG. 7. Facilitator deletion does not abolish enhancer domain sequence accessibility in nuclear chromatin. (A) Autoradiogram depicting *Dpn*II accessibility to facilitated and nonfacilitated transgenes in transgenic thymus nuclei in comparison with the active endogenous gene in human cell lines. (B) PhosphorImager line graph tracings to correct for variations in transgene copy number. Results indicate that poorly expressing lines can exhibit substantial *Dpn*II accessibility, however, with some alterations of the relative cutting efficiencies of the different *Dpn*II sites.

and very poorly expressing lines 50 and 55. This indicates substantial accessibility of nonfacilitated enhancer sequences in thymocytes. Also, in contrast to the facilitated lines, the nonfacilitated lines exhibited increased accessibility of the flanking *Dpn*II sites compared with the central site. These observations indicate that factors present in developing thymocytes cause the regulatory domain to become accessible to factors capable of recognizing specific sequences without the functional facilitators. However, the conformation of the nonfacilitated enhancer domain is altered or disorganized compared with those of the facilitated versions present in human T cells or mouse thymocytes expressing CAT mRNA at high levels.

The accessibility of the enhancer domain in mouse line 21 thymocyte nuclei was different from that in the three other nonfacilitated mouse lines. While the total intensity of the cleavage bands was similar to that of the cleavage bands from the other lines, given the high gene copy number there was much less cleavage than expected (Fig. 7B). This line is also different from other nonfacilitated lines in that it exhibited a speckled pattern of expression in the thymus. This indicates that in this line occur unique suppressive positional effects that appear to be different from the generalized failure of multicopy expression observed in other lines that lack bilateral facilitators. Unlike those in other nonfacilitated lines that exhibited poor DNase I hypersensitivity and gene expression, most copies of the transgene in line 21 exhibited little accessibility of the core enhancer region. However, despite the fact that most copies of the transgene appeared to be inaccessible to the restriction endonuclease, the ratio of singly cut product (540 bp plus 432 bp) to doubly cut product (371 bp) was similar to those for the lines in which most copies were accessible. This result indicates that there is normal accessibility of some of the gene copies and absent accessibility of most other copies. If each of the copies were only partially accessible, there would be little occurrence of doubly cut product.

DISCUSSION

Our results point to a critical role of nonenhancer sequences in the ability of an enhancer domain to undergo a structural transition that enables it to function in transgenic nuclear chromatin. Despite the tendency of most mammalian transgenes to exhibit line-to-line variable expression, it is a reasonable generalization that large reporter constructs encompassing critical regulatory elements tend to be expressed reliably and in proportion to gene copy number but that smaller constructs with delineated enhancer domains tend to exhibit much less consistency. Our results provide a suggestion as to why this can be the case. We have shown that in order for a segment of the human ADA gene first intron to function as an LCR, its 200-bp enhancer domain must be bilaterally flanked by 1-kb segments that we have termed facilitators. LCRs have been generally defined by their ability to confer integration-site-independent and gene-copy-proportional tissue-specific gene expression upon a transgene, and they frequently appear to be related to DNase I-hypersensitive chromatin domains (27, 41, 54). Some DNase I-hypersensitive sites correspond to the locations of enhancers that are responsible for tissue- and cell-type-specific gene regulation (2, 23, 38, 52), but the molecular significance and mechanism of HS formation in chromatin is unclear. DNase I-hypersensitive sites can represent a structural indicator of active or potentially active *cis* elements (6, 15, 18, 19, 33). Lu et al. (43) have characterized nucleosome-free sequences bound to regulatory factors as HSs whereas McPherson et al. (46) have presented evidence that a hepatocyte-specific enhancer can form a strong DNase I HS with bound transcription factors in a positioned nucleosomal array without the ejection of the underlying nucleosome. This suggests that a variety of mechanisms may underlie regulatory HS formation.

Our results indicate that information necessary to form hypersensitivity and allow activation at the HS III enhancer resides, in part, in sequences considerably removed from the HS, at least 3 to 5 nucleosomal lengths away from the enhancer. This appears to be quite different from the case with beta globin genes, in which delineated DNase I-hypersensitive sites appear to comprise functional LCRs (10, 21, 54, 57). However, other examples of LCRs seem to indicate that globin LCRs are atypical. Like the HS III T-cell enhancer of the ADA gene, the mouse CD2 enhancer functions poorly as an LCR when sequences flanking its major HS are deleted (25, 38, 39). This also appears to be the case for the immunoglobulin heavy chain enhancer, which is flanked by nuclear matrix attachment regions (MARs) (7, 12, 14, 32).

Defining the effects and constraints of the facilitators. The surprising aspect of our present characterization is that the flanking facilitator sequences fail to act on the enhancer unless both are present and placed in specific positions relative to the enhancer. The failure to generate a facilitated regulatory domain by moving the 5′ facilitator downstream of the 3′ facilitator indicates that the physical linkage to the required sequences is insufficient, perhaps indicating that the facilitator and enhancer sequences are involved in a large stereospecific structure. If binding to matrix components were the sole explanation of the requirement for 5′ and 3′ segments, facilitator function would be accomplished by the misarranged versions. Alternatively, the requirement for bilateral binding to a rigid matrix may allow the formation of torsional stress within the enhancer domain. However, the failure of a construction that reverses the orientation of the 3′ facilitator construct indicates an additional constraint on the interaction between the facilitators and the enhancer. Several of the lines containing this construction, however, exhibited the highest levels of expression by a "nonfacilitated" construction that we have seen. Interestingly, one of these lines also exhibited the highest level of fractional cleavage of its thymic nuclear chromatin by DNase I, suggesting that its partially facilitated function was a result of better DNase I-hypersensitive site generation. However, most lines with inversion of the 3′ facilitator show typical unfacilitated function. This indicates that most integration sites do not permit the crippled facilitator to function as if it were structurally normal.

Unfacilitated transgenic constructions exhibit two forms of failed expression. The first represents a failure of multiple gene copies to be expressed but a tendency for single, likely end copies to be expressed. This indicates that the absence of the facilitators could not be compensated by random transgene insertion sites and that the "compensation effect" of insertion sites is only able to act on immediately adjacent transgenes. The other component of attenuated nonfacilitated expression was an apparent susceptibility to positional effects. Overall, about one-quarter of the nonfacilitated mouse lines exhibited considerably less expression than that expected of even a single copy, and we surmise that this represents positional repression due to the insertion site. Interestingly, in situ hybridization analysis indicated that in some of both the 5′- and the 3′-facilitator-deleted constructs, there was significant medullary thymocyte expression. This indicates that there are additional positional influences to which nonfacilitated transgenes are susceptible that are not observable by total CAT activity assays. An additional line exhibited a speckled pattern of heterogeneous expression in the thymus, despite exhibiting single-copy total expression. Thus, in addition to crippling the quantitative

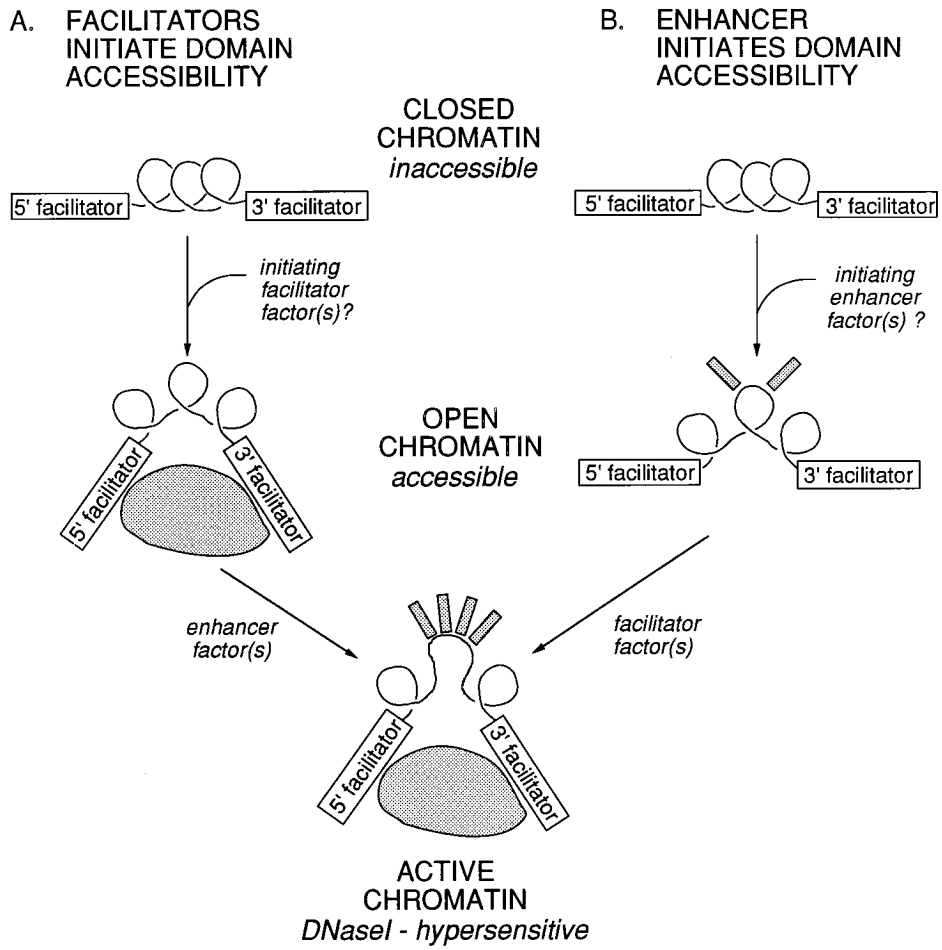


FIG. 8. Alternative models for the relative role of facilitators in the activation of a chromatin domain at an enhancer. (Scheme A) Facilitators initiate a structural transition that subsequently allows *trans*-acting factors to make productive stable interactions with the HS III enhancer. (Scheme B) HS III enhancer element recognition by initiating cell-type-specific transactivators induces accessibility that allows additional enhancer transactivators and facilitator factors to induce a secondary structural transition that converts the accessible domain into a hypersensitive domain that is capable of distal promoter activation. Scheme B is favored by two lines of evidence. The first is that tissue-specific hypersensitivity and gene expression can occur for one or two transgene copies in the absence of 5′ or 3′ facilitator sequences. This implies that the facilitators are not absolutely required and that the enhancer domain itself has chromatin structure organizing and activating potential in vivo. The second line is that factor accessibility occurs in nonfacilitated lines without DNase I hypersensitivity or gene expression. However, at least one mouse line did not exhibit *Dpn*II accessibility that was proportional to its gene copy number. Thus, although the enhancer domain can determine local accessibility without HS formation, even this process can be affected in part by the facilitators.

function of the enhancer, the absence of facilitation also allows for at least some degree of misregulated expression.

At what stage do the facilitators act on the enhancer? Do the facilitators initiate a structural transition that subsequently allows *trans*-acting factors to make productive stable interactions with the HS III enhancer? Conversely, domain activation could first involve HS III enhancer element recognition by transactivators, followed by a structural transition that requires the presence of the facilitators. These possibilities are distinguished in Fig. 8. To approach this question, we used a restriction endonuclease as a probe of enhancer sequence accessibility in nuclear chromatin. The ability of the endonuclease to recognize and cleave enhancer sequences varied by cell type and tissue, with B-cell and liver nuclei exhibiting little or no access to the enhancer domain. In contrast, T cells and facilitated transgenic thymocytes afforded easy endonuclease access to sequences both within and straddling the DNase I-hypersensitive domain. This indicates that factors present in MOLT 4 cells and developing thymocytes are capable of converting the enhancer domain and adjacent sequences into a conformation that is accessible to the restriction enzyme. Surprisingly, in contrast to their DNase I hypersensitivity and CAT expression, nonfacilitated transgenic lines exhibited an impressive accessibility to the restriction enzyme. The simplest interpretation of these results is that a facilitator-dependent structural transition necessary for both DNase I hypersensitivity and enhancer function occurs after limited enhancer element recognition by thymocyte-specific factors independent of facilitators, shown in pathway B in Fig. 8. However, none of the unfacilitated lines were quite as accessible as the facilitated versions. This may relate in part to conformational differences between the accessible and hypersensitive chromatin configurations (see Results), but line 21 exhibited virtually no accessibility for the great majority of its 120 copies. Thus, in the absence of the facilitators, some transgenic lines could package the regulatory domain into virtually inaccessible chromatin, just as occurred for the endogenous gene in human B-cell nuclei and for the facilitated transgene in mouse liver nuclei. This indicates that in the absence of the facilitators, the en-

hancer binding factors are not the sole determinants that initiate enhancer domain accessibility.

Recent work by Jenuwein et al. (32) strongly supports the idea that developmental activation of an enhancer domain is a multistep process. In their analyses of virus-transformed transgenic pre-B cells, core elements of the immunoglobulin μ enhancer were capable of mediating a chromatin structure transition that allowed adjacent sequences of the region to become accessible to T7 RNA polymerase in nuclear chromatin. This accessibility occurred without DNase I-hypersensitive site formation or the productive stimulation of high-level RNA polymerase II activity. Their results indicate that in vivo chromatin structure transitions leading to initial factor accessibility can be mediated by enhancer core elements but that these structure transitions are distinct from (or likely precede) the remodeling of an enhancer domain into a DNase I-hypersensitive structure. It is unclear whether accessibility without hypersensitivity leads to the occupancy of critical enhancer elements with stage-specific binding proteins and to what extent the accessibility that was observed was cell type specific or reflective of domain activation in nonvirally transformed B cells. Regardless, their studies indicate that additional *cis* information is necessary for the transition to a DNase I-hypersensitive structure. This information has been ascribed to 5′ and 3′ matrix attachment elements of the immunoglobulin enhancer region and other genes (7, 8, 12–14, 47), but the effects of these elements when added to other promoters or enhancers in transgenes has been less than would be expected for independently active elements (45). Our results may offer an explanation for this discrepancy if the ability of the heterologous MAR-containing sequences to function is constrained to specific positions and orientations with respect to the enhancer or *cis*-regulatory domain.

An appealing model for MAR function suggests that loop anchorage provides insulating effects that allow the true potential of *cis* elements within the construct to function without interference from the surrounding genes or regulatory elements. However, attachment regions that immediately flank *cis*-regulatory domains (7, 8, 13, 14, 37) may be functionally distinct from MAR sequences occurring at the boundaries of genes or loci (22, 47, 48, 51), and they may influence chromatin structure at a different level. It is possible that certain classes of chromatin-organizing sequences function as insulators (34) and that others act as agents that enable enhancer function. Our suggestion is that the ADA HS III facilitators participate in a structure transition that occurs after accessibility is established at the enhancer domain by initiating transactivators. However, as yet we do not know whether the facilitators surrounding the ADA gene HS III contain MAR activity.

Our results are inconsistent with a simple model in which attachment to a nuclear scaffold is sufficient for locus-activating ability of a regulatory region, and they suggest that the activation of the enhancer domain requires oriented DNA interactions with constrained nuclear structures that extend well beyond the enhancer domain. Identification of individual elements within the facilitator segments may point us towards novel and crucial nuclear factors responsible for organizing chromatin structure during developmental differentiation. Given the constraints for facilitator function, it is interesting to speculate that the activation of the ADA thymic regulatory region occurs in the context of a large nuclear structure, such as that which has been implicated in active transcription and processing centers in nuclei by confocal immunofluorescence (9, 64). Since the absence of facilitation can lead to a virtually complete extinction of enhancer function within cells that contain all of the *trans*-acting factors responsible for generating enhancer activity, it is tempting to speculate that mechanisms for processes such as allelic exclusion or selective expression or inactivation of arrayed genes could work at a similar level as do facilitators. Beyond the fundamental questions of how complex genomes selectively and efficiently utilize *cis*-regulatory elements, an understanding of how chromatin structure is regulated may be of practical significance. In the emerging field of human gene therapy, stem cell-derived gene expression in particular may be greatly augmented by assemblies of gene regulatory elements that include those responsible for governing chromatin structure.

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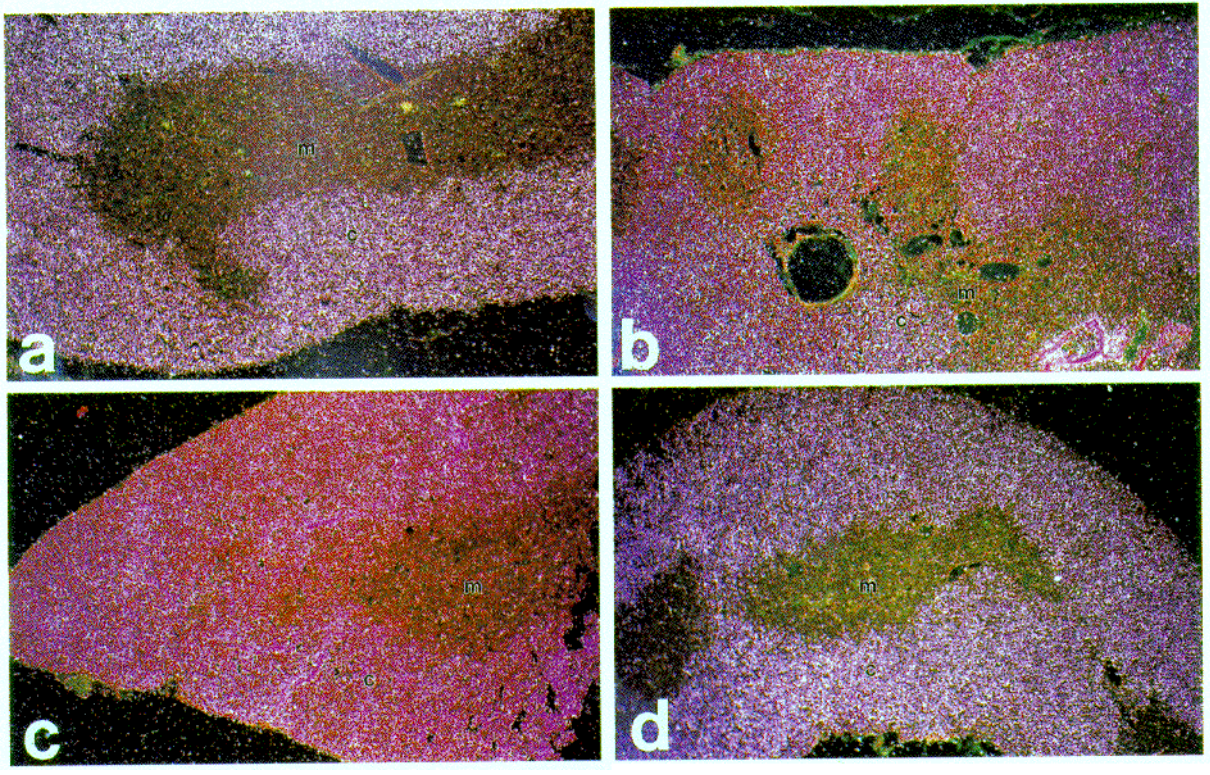
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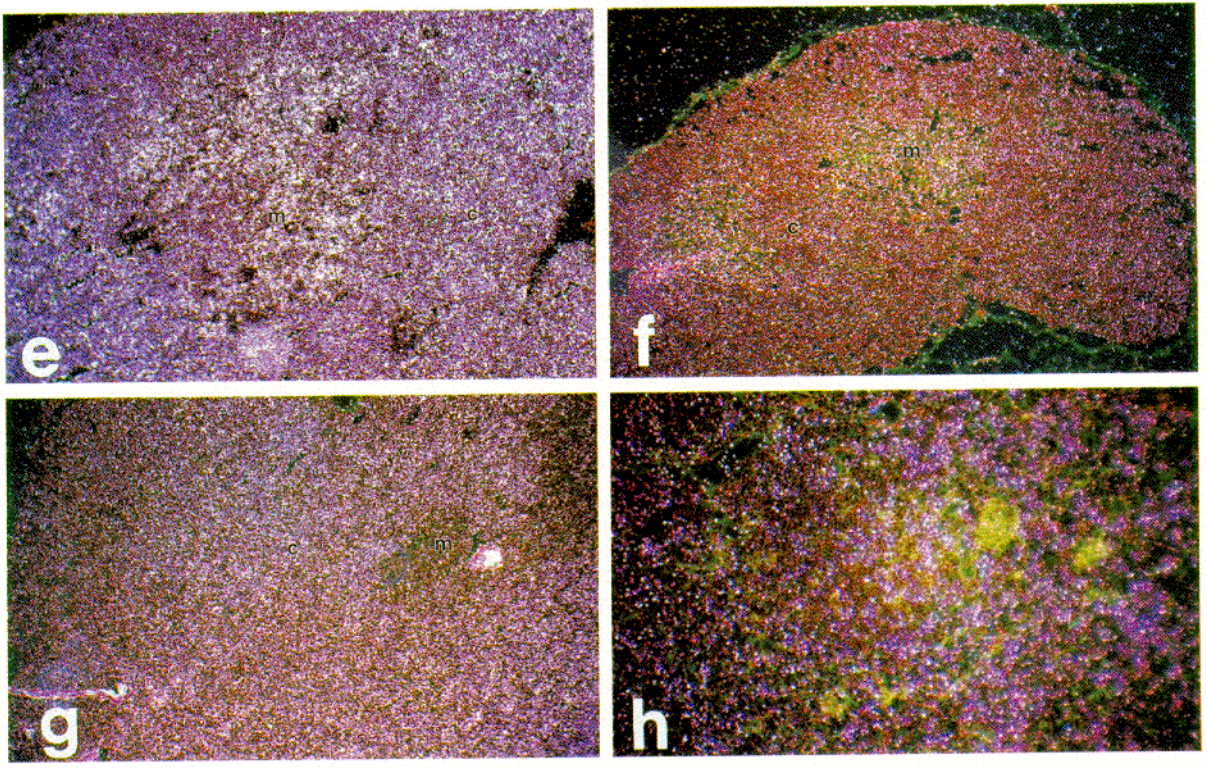
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