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A Central Role for a Single c-Myb Binding Site in a Thymic Locus Control Region

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Locus control regions (LCRs) are powerful assemblies of *cis* elements that organize the actions of cell-type-specific *trans*-acting factors. A 2.3-kb LCR in the human adenosine deaminase (ADA) gene first intron, which controls expression in thymocytes, is composed of a 200-bp enhancer domain and extended flanking sequences that facilitate activation from within chromatin. Prior analyses have demonstrated that the enhancer contains a 28-bp core region and local adjacent augmentative *cis* elements. We now show that the core contains a single critical c-Myb binding site. In both transiently cotransfected human cells and stable chromatin-integrated yeast cells, c-Myb strongly transactivated reporter constructs that contained polymerized core sequences. c-Myb protein was strongly evident in T lymphoblasts in which the enhancer was active and was localized within discrete nuclear structures. Fetal murine thymus exhibited a striking concordance of endogenous c-*myb* expression with that of mouse ADA and human ADA LCR-directed transgene expression. Point mutation of the c-Myb site within the intact 2.3-kb LCR severely attenuated enhancer activity in transfections and LCR activity in transgenic thymocytes. Within the context of a complex enhancer and LCR, c-Myb can act as an organizer of thymocyte-specific gene expression via a single binding site.

Cellular differentiation requires developmental gene regulation that results from the combined actions of multiple transcriptional factors acting on clustered *cis* elements within enhancers and promoters. The most powerful clusters of *cis* elements are locus control regions (LCRs) that dictate chromatin structure transitions and factor accessibility and organize multiple transcription factor interactions (3, 7, 11, 30). LCRs seem generally composed of classical enhancer segments and additional sequences required for activation in chromatin. Individual factor binding sites of enhancers frequently appear to be dispensable in transfection assays, consistent with a view of enhancers as being composed of a series of redundant binding sites with overlapping functions. Few studies in transgenic mice have addressed the role of individual enhancer elements within the context of intact LCRs. However, available data suggest that individual sites tend not to be required for position-independent transgene expression (7). Consequently, we have limited insight into the process by which individual *cis*-regulatory elements within LCRs participate in chromatin activation and the determination of transcription rates and cell type specificity. LCRs appear to become active in a stepwise fashion (2, 16, 20). A likely early event is a structural transition of chromatin to an accessible state, followed by the formation of a distinct chromatin structure that is hypersensitive to DNase I. As a result of this process, it is a striking feature of cells in which an LCR is active that there is a remarkable cell-to-cell uniformity of mRNA production. This is evident for both endogenous genes and transgenes on a per-copy basis. Most presently characterized LCRs are from genes that express prodigious levels of mRNA in target cells (e.g., β-globin, immunoglobulin heavy-chain, and serum albumin genes). The human adenosine deaminase (ADA) gene, which contains a 2.3-kb thymic LCR, differs from the above-cited examples in that it directs considerably lower levels of mRNA. ADA is expressed in cortical thymocytes, in which T-cell receptor rearrangement, positive selection, and negative selection occur. The vast majority of cortical T cells normally do not mature and undergo programmed cell death (28). When thymocyte DNA is degraded during programmed cell death (34), ADA is essential for purine catabolism, as its genetic absence causes a failure to develop cortical thymocytes.

Deletional analyses in transfected T cells have identified a classical enhancer that encompasses a 200-bp domain corresponding to a thymocyte-specific DNase I-hypersensitive region centered within the 2.3-kb intronic thymic LCR. Flanking the 200-bp enhancer region are extended segments required for insertion site-independent and copy number-proportional transgene expression (2, 3). Using transiently transfected T cells, deletional analyses have indicated that the 200-bp enhancer region is hierarchically structured, composed of a 28-bp core and augmentative flanking elements.

We now report a series of experiments demonstrating that c-Myb protein binds to a site within the core enhancer element, functions as a transcriptional activator within multiple cell types, initiates transcriptional activity from within yeast chromatin, and is present in thymocytic T cells within discrete subnuclear structures. Within the context of the intact LCR, point mutation of the c-Myb site strongly disables enhancer activity in transiently transfected T cells. Thymocytes in five of six transgenic lines with the c-Myb site mutation exhibited very poor LCR function. These data demonstrate a critical role for c-Myb in cortical thymocyte gene expression and suggest that complex regulatory domains such as LCRs can be organized around single sites.

MATERIALS AND METHODS

c-Myb protein. A glutathione *S*-transferase (GST)–truncated c-Myb fusion protein (GSTmyb-0.7) was made to avoid protein degradation observed in bac-

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teria that contained full-length c-Myb expression vectors. To do this, a 721-bp *Nco*I-*Hin*cII fragment of pMbm-1 (37) encoding the c-Myb DNA binding domain was subcloned into pGEX4T-2 (Pharmacia) to produce pGSTmyb-0.7 in host *Escherichia coli* DH5α cells (Gibco/BRL). Midlog cultures were induced with 0.1 mM isopropylthiogalactopyranoside (IPTG) for 3 h and fusion protein was purified according to the manufacturer's recommendations. The DNA binding domain of c-Myb has been shown to retain the specificity of full-length c-Myb for target sequences (29).

Electrophoretic mobility shift assay. Two microliters of eluate from glutathione-Sepharose representing approximately 50 ng of fusion protein (as judged from silver stain intensity) was incubated with labeled and unlabeled oligonucleotides in a total volume of 25 μl that contained 10 mM Tris, 50 mM NaCl, 5 mM dithiothreitol, 1 mM EDTA, 4 mM $MgCl_2$, 50 ng of poly(dI-dC), and 10% glycerol for 30 min at room temperature. The reaction mixture was electrophoresed without the addition of dye on a 5% acrylamide–0.25× Tris-borate-EDTA gel, dried, and exposed to film.

Cell culture, transient transfections, and CAT assay. Molt-4, CCRF-CEM, and Raji lymphoid cells, culture conditions, DEAE-dextran transfections, and chloramphenicol acetyltransferase (CAT) assays have been previously described, as have ADA core element reporter constructs (3). Electroporation of Raji cells was done essentially by the procedure of Bhaumik et al. (5). Briefly, for each transfection, 10^7 cells in exponential growth were washed with 1× phosphate-buffered saline (PBS) and resuspended in 0.3 ml of PBS containing 7.5% fetal bovine serum. Cells were added to 0.4-cm Bio-Rad cuvettes containing transfection DNA, briefly mixed, and electroporated at 260 V and 960 μF in a Bio-Rad Gene Pulser. Cells were immediately plated in 12 ml of RPMI medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U of penicillin-streptomycin (Gibco/BRL) per ml. Cells were harvested 45 to 48 h later for assay of CAT activity.

Yeast strains, reporter genes, and expression plasmids. *Saccharomyces cerevisiae* W303-1A (*MAT***a***can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1*) was used as a host for reporter constructions and subsequently transformed with expression plasmids. Yeast reporter plasmids that integrated into the *URA3* locus were made with ADA core sequences by inserting corresponding oligonucleotides immediately upstream of a *GAL1* TATA yeast promoter and *lacZ* reporter gene designated pLR1delta1delta2μ (36). RC, R1, and R7 oligonucleotides were ligation multimerized into the *Xho*I site of this plasmid, which was then linearized at *Stu*I and transformed into strain W303-1A by the lithium acetate protocol. For each clone, the reporter gene structure was confirmed by Southern blot hybridization. Integrant clones were grown overnight in YEPD medium and transformed with yeast vectors that expressed either wild-type c-Myb (CCC), a C-terminal truncation (CCd), or an N-terminal truncation (dCC). c-Myb truncations correspond closely to those observed in v-Myb. Control yeast reporter strains containing known c-Myb-responsive mim-1A-LacZ reporter genes YSW5(+) (wild type) and YSM5(+) (*myb* site mutated) were generously provided by Rui-Hong Chen and Joseph Lipsick. All expression vectors were provided by Rui-Hong Chen and minimally contained c-Myb DNA binding and transactivation domains in addition to the 2 μm replication origin and *TRP1* selectable marker. Transformants were screened for β-galactosidase activity with the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) filter assay as described by Chen and Lipsick (8). Three representative clones per construct were isolated and subjected to quantitative determination of β-galactosidase activity, using liquid cultures grown to an optical density at 600 nm of 1.0 and the substrate orthonitrophenyl-β-D-galactopyranoside. β-Galactosidase units were calculated by the method of Miller (25). Assays were done in duplicate, with three independent clones assayed per construct. Reproducibility was within 20%.

Northern (RNA) and in situ hybridizations. Total RNA was purified by using Tri-Reagent (9). For Northern analysis, a 0.6-kb *Hin*dIII-*Eco*RI fragment from pRSVmyb (10) was labeled by the random primer method (Bethesda Research Laboratories). For c-*myb* in situ hybridization, the same 0.6-kb fragment subcloned in pBluescript was linearized with *Hin*dIII and labeled with [α-^{35}S]UTP. Mouse ADA and CAT in situ probes as well as mouse embryos and tissues were prepared for cryosectioning and subjected to in situ hybridization analysis as previously described (1, 3).

Western blotting (immunoblotting). A total of 3.3×10^5 cells were centrifuged and resuspended in 2× sodium dodecyl sulfate (SDS) loading buffer. After boiling, samples were electrophoresed on an SDS–7% polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore). An anti-mouse c-Myb type I monoclonal antibody that cross-reacts with human c-Myb (Upstate Biotechnology, Inc., Lake Placid, N.Y.) was incubated 4°C overnight at 0.4 μg/ml. The secondary antibody was an anti-mouse horseradish peroxidase conjugate; bound antibody complexes were visualized with the Amersham enhanced chemiluminescence system.

Immunofluorescence localization of c-Myb. A total of 3×10^5 cells were washed in PBS, cytocentrifuged onto glass slides, and fixed with acetone. Nonspecific binding sites were blocked with PBS that contained 1% bovine serum albumin (BSA), 5% nonfat milk, and 3 drops of normal rabbit serum per 10 ml. An anti-mouse c-Myb type I monoclonal antibody at 50 μg/ml in 1× PBS–1% BSA was allowed to interact with cells for 1 h at 37°C. After a wash in PBS–1% BSA, a rabbit anti-mouse $F(ab')_2$ fluorescein-conjugated secondary antibody was added, and the mixture was incubated for 60 min at 37°C. After three washes, slide covers were affixed with Antifade medium (Oncor) containing propidium iodide for nucleic acid staining. Bound antibody complexes were visualized with an Olympus 100× PlanApo oil objective and photographed with Ektar 100 film.

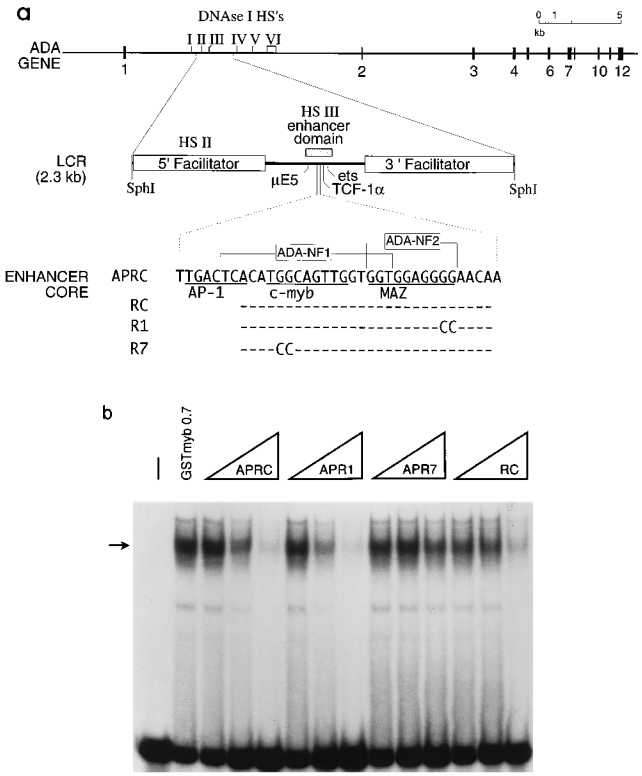

FIG. 1. (a) Structure of the human ADA gene, including a 2.3-kb *Sph*I-*Sph*I fragment LCR, DNase I-hypersensitive sites (HS's) II and III, enhancer core sequences, ADA-NF1 and ADA-NF2 binding sites, and introduced point mutations R1 and R7. The 9-bp c-Myb consensus and adjacent AP-1 sites are underlined. (b) Electrophoretic mobility shift assay of labeled APRC and purified GSTmyb-0.7 fusion protein. The major shifted complex of APRC and GSTmyb-0.7 is indicated (arrow). Competition with unlabeled oligonucleotide, indicated as concentration ramps, was performed with 10-, 40-, and 160-fold molar excesses. Lane 1 is APRC probe only. GST protein alone failed to show any specific binding (data not shown).

Site-directed mutagenesis and transgenic mice. A mutagenic oligonucleotide was used to alter the ADA-NF1 site within the 2.3-kb *Sph*1-*Sph*1 fragment from the first intron of ADA, using the pAlter vector kit (Promega Altered Sites). The mutation was confirmed by double-stranded sequencing and subcloned into the ADA CAT reporter 5′acba (3) to yield plasmid I 2.3 R7 CAT. The transgene fragment was isolated from this plasmid and used to generate independent transgenic mouse lines. F_1 progeny were characterized for transgene integrity, copy number, and CAT expression as previously described (3).

RESULTS

Natural occurrence of an in vitro-identified c-Myb binding site. By the selection of random sequences in vitro, Howe and Watson (19) derived a 9-bp consensus sequence for c-Myb binding, not previously identified in vivo, that is present within the core of the ADA enhancer (Fig. 1a). To determine directly if ADA core element sequences bind to c-Myb, an electrophoretic mobility shift assay was performed with the DNA binding domain of human c-Myb protein. The major complex formed between c-Myb and the APRC extended core fragment (Fig. 1b) was effectively competed for by an unlabeled APRC or RC oligonucleotide. APRC includes the 28-bp RC core and an adjacent AP-1 site, which evidently was not required for c-Myb binding. Within the RC core, two binding sites, ADA-NF1 and ADA-NF2, have been previously defined. Both sites are essential for the ability of the polymerized core element to

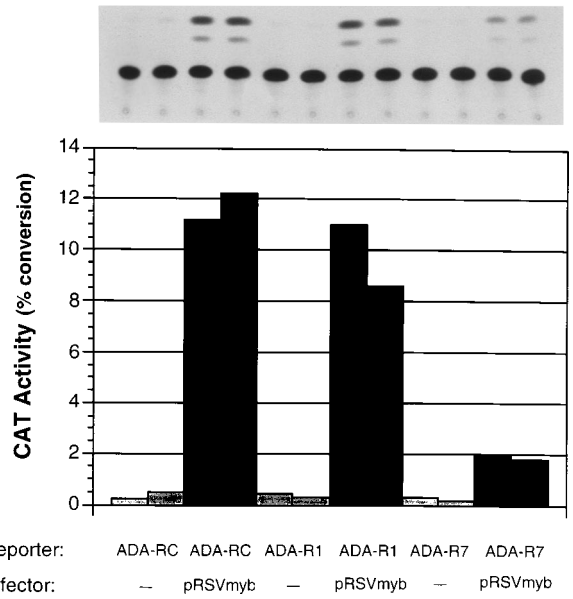

FIG. 2. c-Myb transactivates ADA core element CAT reporters in transiently cotransfected Raji B cells. A total of 10^7 cells were cotransfected by electroporation with 15 μg of expression vector pRSVmyb and 7.5 μg of ADA CAT reporter plasmid that contained four copies of the RC, R1, or R7 element. Duplicate datum points are shown from one representative experiment; very similar results have been obtained in at least five independent experiments. Conversion of [^{14}C]chloramphenicol to monoacetylated product was quantitated on a PhosphorImager by using ImageQuant software (Molecular Dynamics).

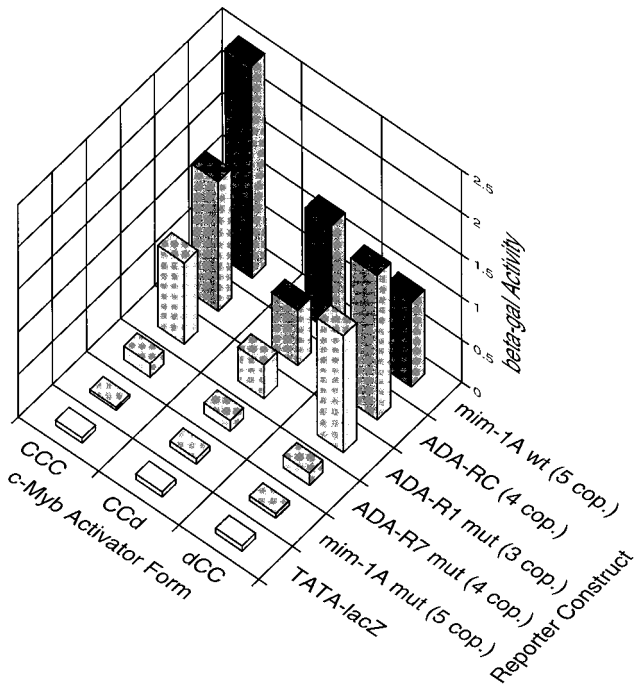

FIG. 3. c-Myb transactivates chromatin-integrated ADA core elements in yeast cells. c-Myb expression vectors CCC, CCd, and dCC were introduced as 2 μm plasmids (*trp*) in yeast strains containing *lacZ* reporter constructs mim-1A wt, mim-1A mut, ADA-RC, ADA-R1, and ADA-R7 integrated at the *URA3* locus. For all constructions, the putative *myb* response elements were placed immediately 5′ of the yeast TATA element. The parental TATA-*lacZ* reporter contains promoter-only sequences and had very low activity under all conditions. β-Galactosidase (β-gal) units were determined by the method of Miller (25). Values represent the averages of three independent transformant clones. Three independent experiments showed highly reproducible activation profiles. cop., copies.

act as an enhancer in transfected Molt-4 cells. To test for the site specificity of c-Myb interaction, two oligonucleotides with point mutations in either the ADA-NF1 or ADA-NF2 site were used as competitors. Mutation at the ADA-NF1 site (APR7) prevented competition for c-Myb protein binding. However, mutation at the ADA-NF2 site (APR1) allowed competition indistinguishable from that of wild-type APRC. Thus, these results identify c-Myb as a protein which binds in vitro to the ADA-NF1 site.

c-Myb transactivates ADA enhancer sequences in lymphoid cells. The in vitro analysis described above indicates that c-Myb protein can bind to the ADA-NF1 site but does not address its ability to bind and transactivate within a cell. Raji B cells were cotransfected with pRSVmyb and CAT reporter construct ADA-RC, which contained four tandem copies of the RC core oligonucleotide placed downstream of a 4-kb human ADA promoter and a CAT reporter gene. Raji cells express minimal ADA and exhibit no transactivation from the thymic enhancer or polymerized core element (3). c-Myb reproducibly transactivated ADA-RC approximately 30-fold over the value for the reporter alone (Fig. 2). Control transfections using of a version of c-Myb with the transactivation domain deleted (10) nearly eliminated transactivation of ADA-RC (results not shown). Cotransfection was also performed with pRSVmyb and ADA CAT reporter plasmids that contained polymerized oligonucleotides with mutated sites for ADA-NF1 (ADA-R7) or ADA-NF2 (ADA-R1) (3). ADA-NF1 site mutation strongly diminished c-Myb transactivation, but mutation of the ADA-NF2 site had no effect on c-Myb transactivation. Also, c-Myb failed to show any transactivation of the 4-kb ADA promoter alone (data not shown). These results demonstrate that c-Myb is competent to transactivate promoter activity via a minimal enhancer site.

c-Myb transactivates ADA enhancer sequences in yeast chromatin. Endogenous enhancers and LCRs function within the context of chromatin, which may be considerably less permissive for transactivation than transiently transfected plasmids. However, we have shown that the function of the ADA LCR in chromatin is dependent on a series of architecturally constrained elements (2). To test c-Myb's ability to transactivate from the isolated enhancer core in chromatin, ADA-RC *lacZ* reporter genes were constructed and integrated into the yeast genome. We compared the ability of c-Myb to transactivate reporter strains containing wild-type and mutant ADA core elements with the known c-Myb-responsive element, mim-1A, as shown by Chen and Lipsick (8). We also evaluated N- and C-terminal truncated forms of c-Myb (dCC and CCd, respectively) to compare the relative roles of c-Myb terminal domains that may modulate transactivation from the ADA core element. As shown in Fig. 3, c-Myb strongly transactivated the four-copy ADA-RC element (60%) as well as the five-copy mim-1A reporter. The three-copy ADA-R1 mutation was transactivated nearly as well as the ADA-RC mutation, but the ADA-R7 mutation caused a greater than 90% loss of activity. The mutated mim-1A site was apparently even more detrimental than the ADA-R7 mutation. Overall, these results are corroborative of those observed in transient cotransfection assays and additionally indicate that c-Myb can recognize and transactivate from the ADA-NF1 site within the context of yeast chromatin.

N-terminal and C-terminal truncated c-Myb forms have been shown to transactivate from the mim-1A element as much as 60 to 70% less than full-length c-Myb in yeast cells (8). We reproduced these results for the mim-1A element and

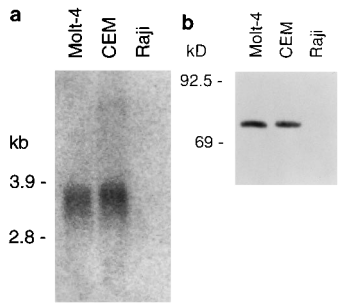

FIG. 4. Expression of c-myb in human lymphoid cell lines. (a) Northern blot analysis. Total cellular RNA (10 μg) from Molt-4, CEM, and Raji cells was electrophoresed, transferred, and probed with a human c-*myb* fragment. The approximately 3.8-kb band appears equivalently expressed by Molt-4 and CEM cells but is absent in Raji cells. Equal loading of RNA was evident by ethidium bromide staining intensity of rRNA bands. (b) Western blot analysis. Whole cell extracts of Molt, CEM, and Raji cell lines were electrophoresed through an SDS-polyacrylamide gel, transferred to Immobilon-P, and incubated with a c-Myb monoclonal antibody (type I; Upstate Biotechnology) followed by a secondary antibody conjugated to peroxidase. Complexes were visualized by chemiluminescence. A single band, of approximately 75 kDa, corresponding to c-Myb protein can be detected in Molt-4 and CEM cells but not Raji cells.

showed for the ADA core element that C-terminal truncation of c-Myb (CCd) also caused reduced transactivation. In contrast to mim-1A, the N-terminal truncated form of c-Myb (dCC) did not cause a decreased activation of the ADA-RC element in that it showed strong transactivation comparable to that of CCC. The difference between the RC and mim-1A elements cannot be due to the adjacent ADA-NF2 site because its mutation (ADA-R1) also showed similar strong activation by both dCC and CCC. These results imply that c-Myb interacts with the ADA core element differently than the mim-1A element.

Expression of c-*myb* mRNA and protein in lymphoid cell lines. For c-Myb to activate the ADA gene in developing thymocytes, it must be present in the appropriate cells. Molt-4 is an immature $CD4^+$ $CD8^+$ T-cell lymphoblastoid line that produces high amounts of ADA and strongly transactivates from the ADA core element. CEM is a $CD4^+$ $CD8^-$ cell line that expresses one-fifth as much ADA mRNA as Molt-4 and demonstrates a reduced response to the RC core (3). Raji B cells have less than 1% as much ADA as Molt-4 cells and completely fail to transactivate the core element. As shown by Northern and Western blot analyses (Fig. 4), Molt-4 and CEM lines both exhibit strong expression of c-Myb as a single band corresponding to 3.8-kb mRNA and a protein of 75 kDa, whereas Raji B-cells express no detectable c-Myb mRNA or protein. Thus, equally high levels of c-Myb are present in the T-cell lines, suggesting that factors in addition to c-Myb are likely to determine the difference in core element enhancer strength in Molt-4 and CEM T cells.

c-Myb is localized in discrete subnuclear structures in lymphoid cells. As c-Myb appears to be equivalently expressed in Molt-4 and CEM T cells, we sought to determine if a difference in the function of c-Myb between the two cell types might be the result of alternative interactions or its distribution within the cell. When the c-Myb monoclonal antibody and a secondary fluorescein-conjugated antibody were used, c-Myb protein was largely localized to discrete, intensely labeled speckles present throughout the nuclei of Molt-4 and CEM cells (Fig. 5). For most cells, from 15 to more than 50 speckles were evident. However, occasional cells failed to show any c-Myb within their nuclei. The nuclear morphology of these cells suggests that they are in M phase, consistent with the short half-life and cell cycle regulation of c-Myb (23, 35). CEM and Molt-4 cells did not appear to differ in their nuclear patterns, but some CEM cells appeared to have additional cytoplasmic signal, also present as discrete particles (Fig. 5b). Cytoplasmic signal in both Molt-4 and CEM cells is likely to be real because Raji cells, which lack c-Myb by both Northern or Western analyses, failed to show any background immunofluorescence staining (results not shown). Interestingly, a cytoplasmic c-Myb signal could be observed in a small fraction of Molt-4 or CEM cells that lacked a nuclear signal. Also, there appeared to be some heterogeneity of signal in the cytoplasm, with both a finely dispersed fluorescence and the definite presence of speckles that appeared to be similar in size to those in the majority of the cell's nuclei. These observations are consistent with a cycle of c-Myb synthesis, assembly, trafficking, and degradation within T cells.

The intact enhancer requires the core c-Myb site for activity in transfected T cells. Polymerization of individual *cis* elements often exaggerates their relative contribution and may not reflect their role or requirement within complex enhancers. We therefore made reporter constructs that contained the 2.3-kb extended ADA enhancer with and without the 2-bp ADA-NF1 mutation. In transiently transfected Molt-4 cells, I 2.3 R7 CAT had approximately 4% as much CAT activity as the unmutated parental LCR reporter (Fig. 6). Thus, the function of the intact enhancer was strongly dependent on the single c-Myb binding site. This result supports previous hypotheses that elements within the central core region are essential, even when both flanking elements are intact.

LCR activity requires a c-Myb site for activity in transgenic mice. Transient transfection analyses can exaggerate the importance of single elements and be insensitive to additional sites and factors responsible for activating gene expression in dynamic lineages of an intact animal. We therefore sought to observe the importance of the ADA-NF1 c-Myb site in the context of the ADA LCR in transgenic mice. The ADA LCR has been defined previously on the basis of its ability to generate position-independent transgene expression that is proportional to gene copy number (2, 3). Six independent lines of I 2.3 R7 CAT mice were derived and analyzed for CAT reporter activity and gene copy number. In all lines, CAT activity was consistently and considerably higher in the thymus than it was in the spleen, bone marrow, or liver (data not shown). Within the thymus, three lines exhibited less than 1% of the expected CAT activity per gene copy, two lines exhibited approximately 10% of the expected expression, and interestingly, one line exhibited essentially 100% of the expected activity (Table 1). The presence of the c-Myb mutation was confirmed in each of these lines, but in the high-expressing line 2, Southern blot analysis indicated the presence of a nonconventional concatemeric array in which there were variable-size genomic insertions in half of the 20 transgene copies. Overall, the data show that among independent transgenic lines containing the c-Myb site mutation, there is poor and inconsistent LCR function that is independent of gene copy number. Thus, the mutation of the c-Myb site cripples the entire ADA LCR.

In situ hybridization analysis of c-*myb*, ADA, and CAT transgene expression. To evaluate the potential of c-Myb to regulate the ADA LCR in vivo, we sought to determine and correlate the patterns of mouse c-Myb, mouse ADA, and human ADA CAT transgene expression. Published analyses of the in vivo expression pattern of c-Myb in the mouse have been based only on Northern blot analyses and suggest that c-Myb is present at a high level in cortical thymocytes and bone marrow (32) but at a much lower level in most other tissues. In situ hybridization analysis (Fig. 7a) of a gestational day 16 mouse

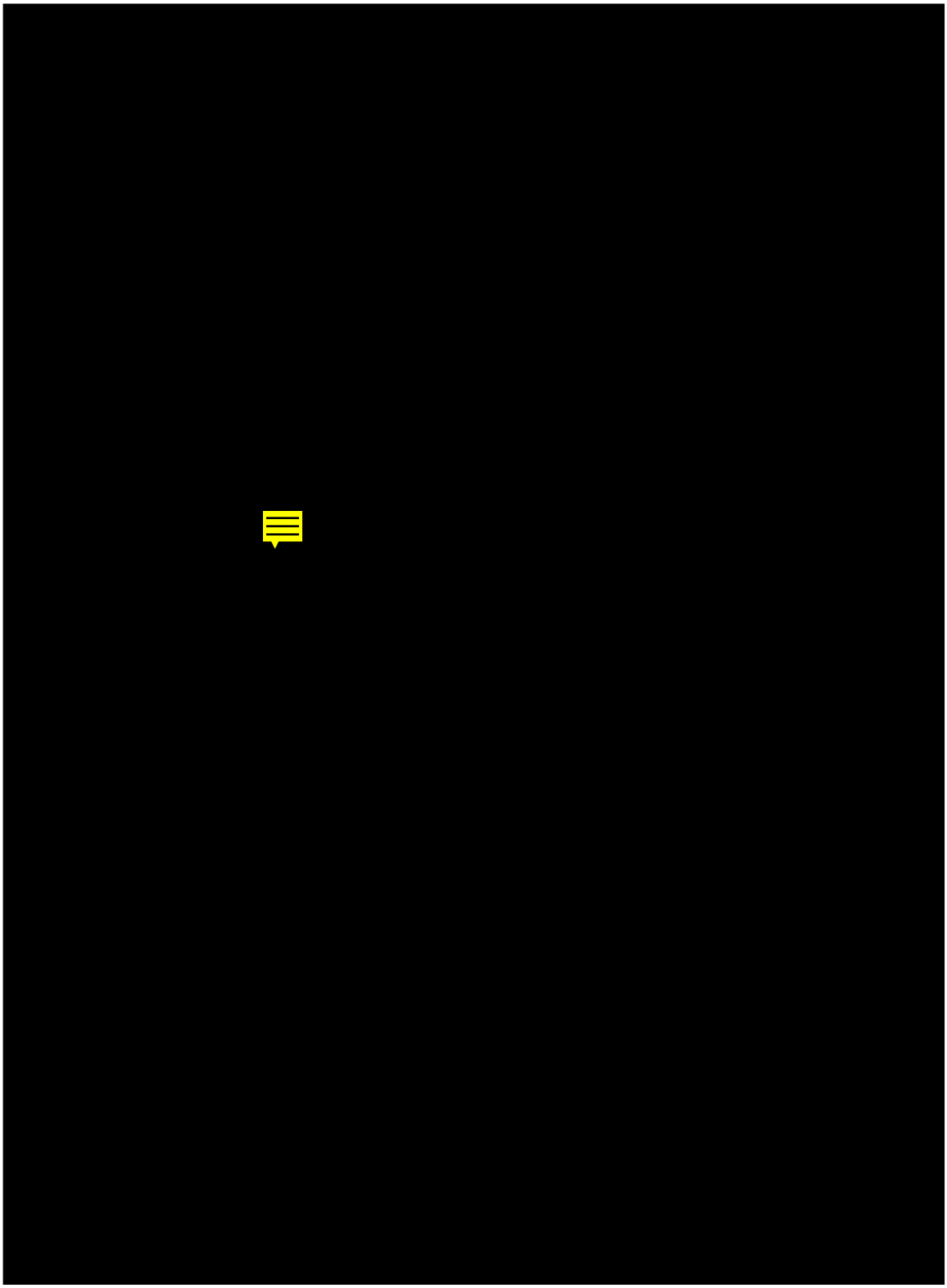

FIG. 5. Immunofluorescence localization of c-Myb in discrete subnuclear structures. (a) Molt-4 cells. (b) CEM cells. Cells were cytospun onto slides, fixed for 10 min in acetone, air dried, and incubated with a c-Myb monoclonal antibody followed with a fluorescein-conjugated secondary antibody and Antifade mounting medium with propidium iodide. Nuclear DNA appears red as a result of propidium iodide counterstaining, and fluorescein fluorescence appears green over the cytoplasm and yellow over the nuclear background. Omission of the primary monoclonal antibody abolished immunostaining. Raji cells exhibited no signal (not shown).

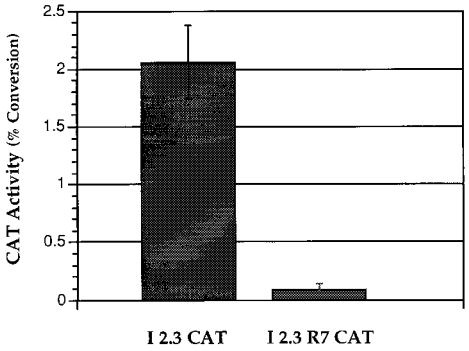

FIG. 6. The 2.3-kb ADA enhancer domain requires a functional c-Myb site in transfected cells. Molt-4 cells were transiently transfected with 5 μg of the I 2.3 CAT or I 2.3 R7 CAT reporter construct. Data represent mean percent conversion of [^{14}C]chloramphenicol (± standard deviation) from five experiments using two different preparations of DNA. Percent conversion was determined with a PhosphorImager.

fetus reveals very strong c-*myb* expression in the cortical region of the thymus as well as its expected high expression in hematopoietic cells of the fetal liver. Interestingly, there is also strong expression of c-*myb* in both tracheal and bronchial epithelial cells of the developing lung. In contrast, there was very little expression of c-*myb* in nearly all other cell types, including medullary thymocytes. In older animals, the thymic medulla becomes more prominent and c-*myb* expression remains confined entirely to the cortical cells (results not shown). This cortical-medullary distribution of c-Myb is identical to that of human and mouse ADA in the mature thymus (1).

Expression of endogenous mouse ADA and ADA CAT transgene mRNAs was also analyzed in fetal mouse tissues by in situ hybridization. Strikingly, CAT mRNA expressed by the wild-type (4/12 ADA CAT) LCR transgene in day 16 fetuses was intensely expressed and localized to the thymus (Fig. 7b), as was endogenous mouse ADA mRNA (Fig. 7c). Compared with the expression pattern of c-Myb, ADA and CAT mRNAs were much more restricted. No detectable expression of either occurred in the fetal liver or in the upper airway epithelium of the developing lung.

Since occasional transgenic lines that contained the c-Myb site mutation retained some, or in one case considerable, total thymic CAT activity, we examined this effect using in situ hybridization. Among independent transgenic lines that contained the mutant I 2.3 R7 transgene, there was no detectable expression in lines 3, 4, and 6 in any of the tissues examined, including thymus, liver, and spleen. Not even occasional cells were expressing reporter gene. This finding indicates that diminished expression was affecting all of the cells uniformly, rather than causing an alteration in the percentage of cells within the tissue that activate the locus. However, we find it interesting that the higher-expressing lines 2 and 5 exhibited strong and uniform CAT mRNA signal over the cortical thymocytes, with appropriate lack of expression over the medullary thymocytes. In the limited other tissues analyzed from these lines, no ectopic expression of CAT mRNA was observed. This indicates that factor binding sites in addition to that of c-Myb are capable of specifying thymocyte-specific gene expression and that occasional integration sites are capable of overcoming the absence of a c-Myb binding site from the ADA LCR.

TABLE 1. Mutation of a single c-Myb site disables the ADA LCR in transgenic thymocytes[a]

Construct	Mouse line	Transgene copy no.	Thymic CAT activity/copy
I 2.3 CAT	1	5	29,000
	22	3	26,000
	23	8	63,000
	24	6	55,000
	25	2	52,000
	41	3	38,000
	42	45	24,000
I 2.3 R7 CAT	2	20[b]	20,500
	3	1	311
	4	1	393
	5	10	4,388
	6	2	38
	7	2	4,500

[a] All mice analyzed were 5- to 10-week-old F_1 transgenic I 2.3 CAT mice as previously described (for descriptions of lines 22 to 25, 41 and 42, see Fig. 8 of reference 3; data for line 1 are from Haynes and Wiginton [17a]). Transgene copy numbers were determined by quantitative Southern blotting using a PhosphorImager. CAT activity was determined as picomoles of acetylated chloramphenicol per 100 μg of protein per hour.

[b] Southern analysis of line 2 indicates numerous additional sequences present within the transgene concatemer (data not shown).

DISCUSSION

Deletional analyses of regulatory sequences in the ADA gene first intron indicate a complex hierarchy of *cis* elements that combine to form a thymocyte-specific enhancer and LCR (2, 3). Chromatin structure studies indicate that activation appears to be a stepwise process in which factor accessibility to regulatory sequences precedes the formation of DNase I hypersensitivity. The enhancer is able to generate initial accessibility, but the distal flanking facilitators participate in the formation of the DNase I-hypersensitive LCR that is essential for in vivo enhancer function. We have now shown that c-Myb binds to and transactivates from a single element within the enhancer core. c-Myb is present within T-lymphoblast cells that support the function of the enhancer and is also strongly expressed in cortical thymocytes in which the LCR is strongly active. In the cortex of fetal thymus, there is coincident high-level expression of c-*myb* and endogenous mouse ADA as well as ADA LCR-directed CAT gene expression. Within the context of the intact LCR, mutation of the c-Myb site caused a severe loss of enhancer activity in both T-cell transfection assays and the majority of transgenic mice. Taken together, these data demonstrate that within the highly organized ADA thymic regulatory region, there is a central role for a single c-Myb binding site. Since c-Myb can perform a dominant functional role in the ADA LCR and is present at a very high level in the thymus, we hypothesize that c-Myb performs a similarly dominating role in the regulation of multiple genes necessary for T-cell lymphogenesis.

c-Myb is a nonredundant determinant of early-stage hematopoiesis, as shown by severe multilineage anemia in embryonic day 14 mice with a targeted deletion of the c-*myb* gene (26). Both immature myeloid and erythroid cell lines express c-Myb (17, 24), and their induced differentiation requires down-regulation of c-Myb expression. In addition, c-*myb* antisense oligonucleotides can inhibit the progression of human chronic myeloid leukemic cells in a *scid* mouse (31). These results have suggested a critical role for c-Myb in the differentiation of erythroid and myeloid lineages, but a similar critical role for c-Myb in T cells has been more difficult to delineate.

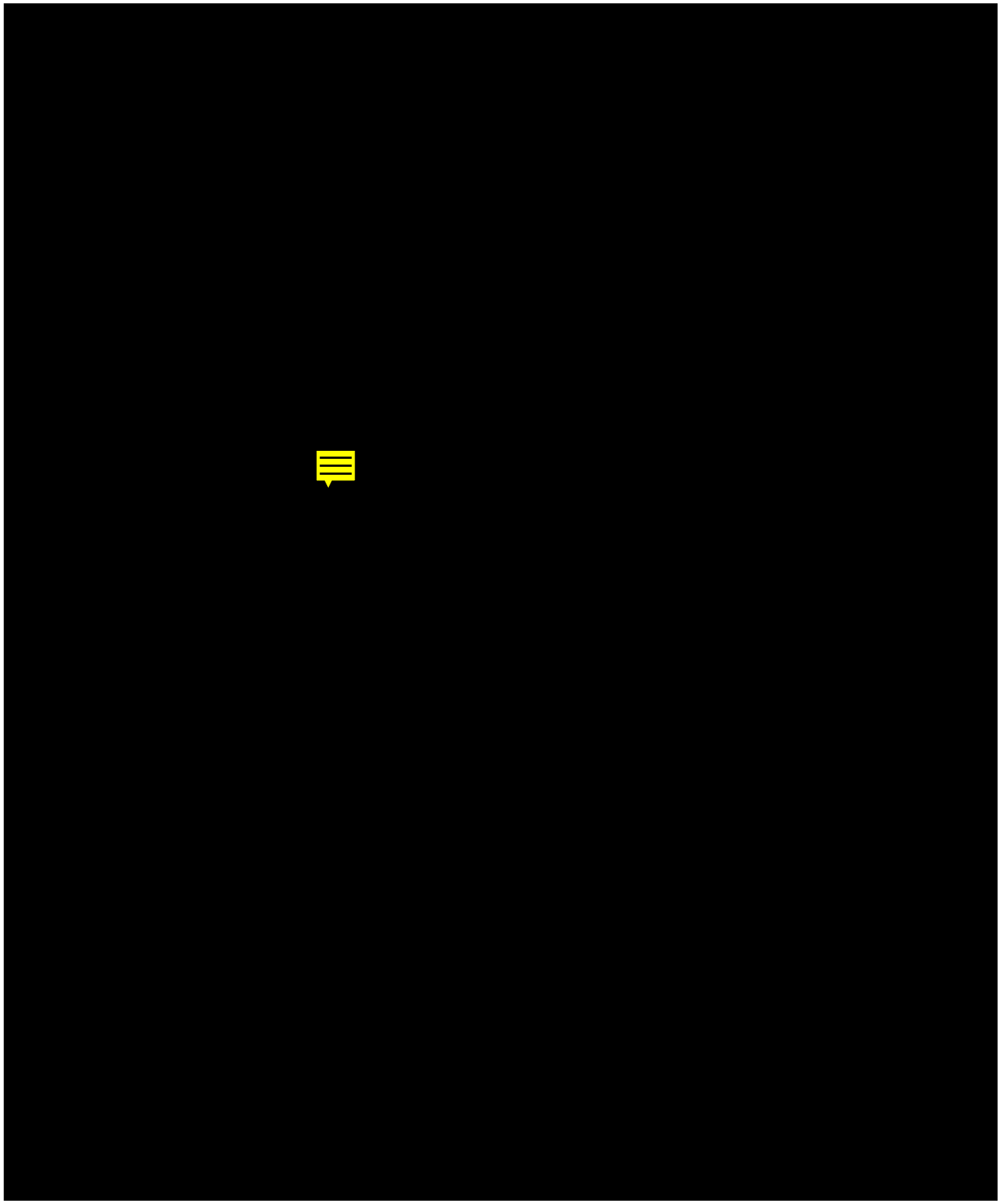

FIG. 7. In situ hybridization analysis for the detection of c-*myb*, endogenous ADA, and CAT mRNAs. (a) c-*myb* expression in an embryonic day 16.5 mouse. The immature thymus (thy) shows very high expression in cortical thymocytes. The liver (li) shows c-*myb* expression exclusively in hematopoietic cells. Additional expression is seen in epithelial cells of developing lung (lu). c-*myb* expression is also high in day 15 thymus (not shown). Final magnification, × 13. (b) CAT expression in transgenic 4/12 ADA CAT fetal day 15 cells. The thymus shows a strong signal, but there is no detectable expression in hematopoietic or lung cells (not shown). Final magnification, × 26. (c) Endogenous ADA expression in transgenic 4/12 ADA CAT fetal day 15 cells. The thymus again shows a strong signal, but there is no detectable expression in hematopoietic or lung cells (not shown). Final magnification, ×26. (d) CAT expression in transgenic 4/12 ADA CAT adult thymus. Cortical thymocytes (C) are exclusively labeled; medullary thymocytes (M) are negative. Final magnification, × 26. (e) CAT expression is absent in adult thymus from transgenic I 2.3 R7 line 6. Final magnification, ×65. (f) CAT expression in adult thymus transgenic I 2.3 R7 line 2 is comparable to that of a normal LCR CAT transgene with an appropriate cortical-medullary expression pattern. Final magnification, ×26.

A role for c-Myb in developing T cells was suspected from observations of its expression in thymocytes (32, 35), but the phenotype of the c-*myb* knockout mouse was not revealing, presumably because hematopoietic failure precedes thymic development. Distinguishing the role of c-Myb in hematopoietic multilineage progenitors from an additional role as a T-cell developmental factor requires cell-type-specific targeting. Recently, Badiani et al. (4) produced transgenic mice with dominant-negative c-*myb* transgenes under the control of CD2 regulatory elements. Thymocyte expression of dominant-negative c-Myb derivatives caused thymocyte number reduction, apparently due to a failure to form double-positive thymocytes. These results are suggestive of a role for c-Myb in T-cell ontogeny. However, it remains possible that dominant-negative c-Myb forms represent broadly acting effectors, able to distort gene expression and differentiation in lineages that are not necessarily dependent on the native protein. In fact, the selective disruption of the thymic c-*myb* gene could be more destructive to T-cell development than expression of a dominant-negative form.

Other evidence that c-Myb regulates T-cell-specific gene expression includes the identification of c-Myb-responsive elements in the T-cell receptor δ gene (18), the CD4 gene (33), and the c-*myb* gene itself (27). Interestingly, in the T-cell receptor δ gene, mutation of a c-Myb binding site, or an adjacent core factor site, crippled the activity of a 370-bp enhancer in transfected T cells. For the other thymocyte-expressed genes, the relative contribution of the c-Myb sites to their regulation is unclear, as the hierarchical organization of their promoters or enhancer(s) has not yet been defined. Our data extend the suggestion that c-Myb is a powerful effector for some T-cell enhancers and additionally demonstrate that a single site for its binding can act as an organizer for LCR function.

There is a marked discrepancy between the activational potential of endogenously expressed c-Myb and that of cotransfected overexpressed c-Myb. When polymerized core element reporter is transfected into Molt-4 T cells, there is a strict requirement for both ADA-NF1 and ADA-NF2 binding sites. The introduction of exogenous c-Myb cannot circumvent this requirement for both binding sites in Molt-4 cells (13a). In contrast, only an intact ADA-NF1 site was required when c-Myb was added exogenously by cotransfection in Raji (Fig. 2), CEM (13a), and yeast (Fig. 3) cells. Given that Molt-4 and CEM T cells appear to express comparable amounts of c-Myb, variations of some additional factor(s) modulating endogenous c-Myb function is the likely explanation for why the polymerized core is a stronger element in Molt-4 than CEM cells. An additional hypothesis is that there are distinct forms of c-Myb present in some T cells (33), perhaps as a result of alternative mRNA splicing (37). However, if present, these forms could not be detected by Western analysis with the type I Upstate Biotechnology monoclonal antibody.

Deletional analyses of the ADA enhancer provide additional supportive evidence for the hypothesis that endogenously expressed c-Myb acts in partnership with other factors. In transfected Molt-4 cells, strong enhancer activity required the enhancer core to be adjoined to elements present in sequences located up to 100 to 200 bp 5′ or 3′ of the core (3). The presence of both flanking regions conferred neither greater activation nor alteration in cell type specificity than was evident with only a single flanking segment. In contrast, mutation of the single c-Myb site abolished enhancer activity with both flanking elements present. Finally, ADA LCR expression occurred only in the context of the regulatory factors expressed in thymocytes, not in the other c-*myb*-expressing fetal hepatic hematopoietic or lung epithelial cells. These results provide additional support for the hypothesis that c-Myb-directed gene expression requires additional factors for target gene specificity in disparate cell types such as thymocytes, pre-erythroid or myeloid cells, or even lung epithelium. Thus, the central role of c-Myb in the ADA LCR stands in contrast to its requirement for additional factors within different cellular compartments.

S. cerevisiae represents an evolutionarily divergent cell type within which c-Myb can be tested for its ability to overcome the structural constraints imposed by a chromatin environment. In transgenic animals, the dependence of the intact ADA LCR on a large number of heterologous elements could mask the capability of c-Myb to act in chromatin. Thus, in yeast cells, the function of the core element itself could be evaluated in a chromatin environment without the need for an extended complex regulatory region as required in transgenic mouse thymocytes. Expression vectors encoding wild-type and truncated forms of c-Myb allowed us to evaluate domains implicated to modify the actions or interactions of c-Myb (8). An interesting difference was evident between the divergent ADA and mim-1A c-Myb binding sites in which only the C-terminal c-Myb truncation decreased transactivation of the ADA enhancer core. That there is a difference between c-Myb domain requirements for activation of an ADA response element and the mim-1A myeloid response element provides an additional suggestion as to how c-Myb can act on distinct target sequences in combination with other factors found in different cellular contexts. The N-terminal deletion effect on the ADA core element is consistent with a model of c-Myb DNA interaction in which the amino-terminal helix 1 serves only to modify the recognition of the target binding site (29).

The potential for organized regulatory factor interactions that involve c-Myb is also suggested by its immunofluorescence observations in Molt-4 and CEM cells. c-Myb was evident in large intranuclear speckled structures, broadly similar to those that have been observed for splicing and polyadenylation processes and factors (6, 13). We hypothesize that these c-Myb-containing structures represent a direct visualization of T-cell transcriptional complexes. The requirement of the ADA LCR (2) and other cell-specific LCRs (20) for extended *cis*-regulatory regions may reflect what is required for assembly into large intranuclear structures such as those implied by the "mybosome." Further studies are required to determine the identity of component subunits and if they contain factors that directly interact with c-Myb (12, 14).

One transgenic line, I 2.3 CAT R7-2, exhibited essentially wild-type expression, suggesting a position-dependent escape mechanism. The simplest explanation for the activity of this line is that its transgene has integrated into an active chromatin region that provides a compensatory effect for the lack of a c-Myb binding site. Because all of the other sites are present, the architecture of a multifactor complex could be preserved. This complex could even include c-Myb, perhaps by its interaction with multiple other factors through its C-terminal leucine zipper region (14, 21). In some contexts, c-Myb has been shown to activate gene expression without the presence of a c-Myb binding site or even its DNA binding domain as long as other specific factor binding sites are present (15, 22). Regardless of the mechanism by which the c-Myb site-mutated transgene can acquire function, most transgenic lines do not allow the thymocyte-specific transcriptional regulators to activate the enhancer region. This observation indicates that the single c-Myb site plays a central role in the activation of the LCR. We hypothesize that the localization of c-Myb in discrete subnuclear regions represents its concentration within transcription centers. In the developing thymus, multiple enabling and restricting factors may interact with c-Myb to create a

powerful deterministic complex that selectively activates the target genes required for T-cell lymphogenesis.

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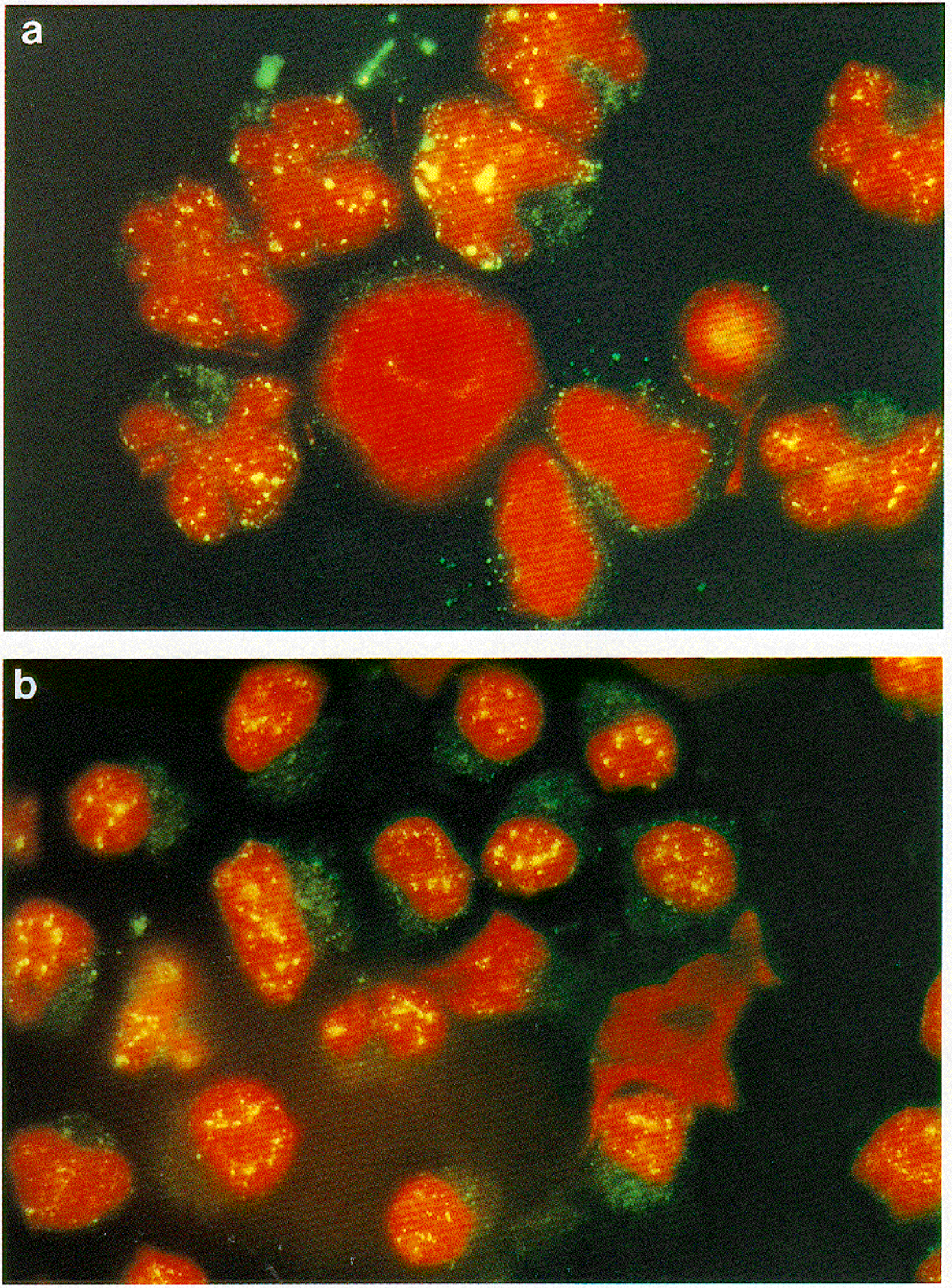
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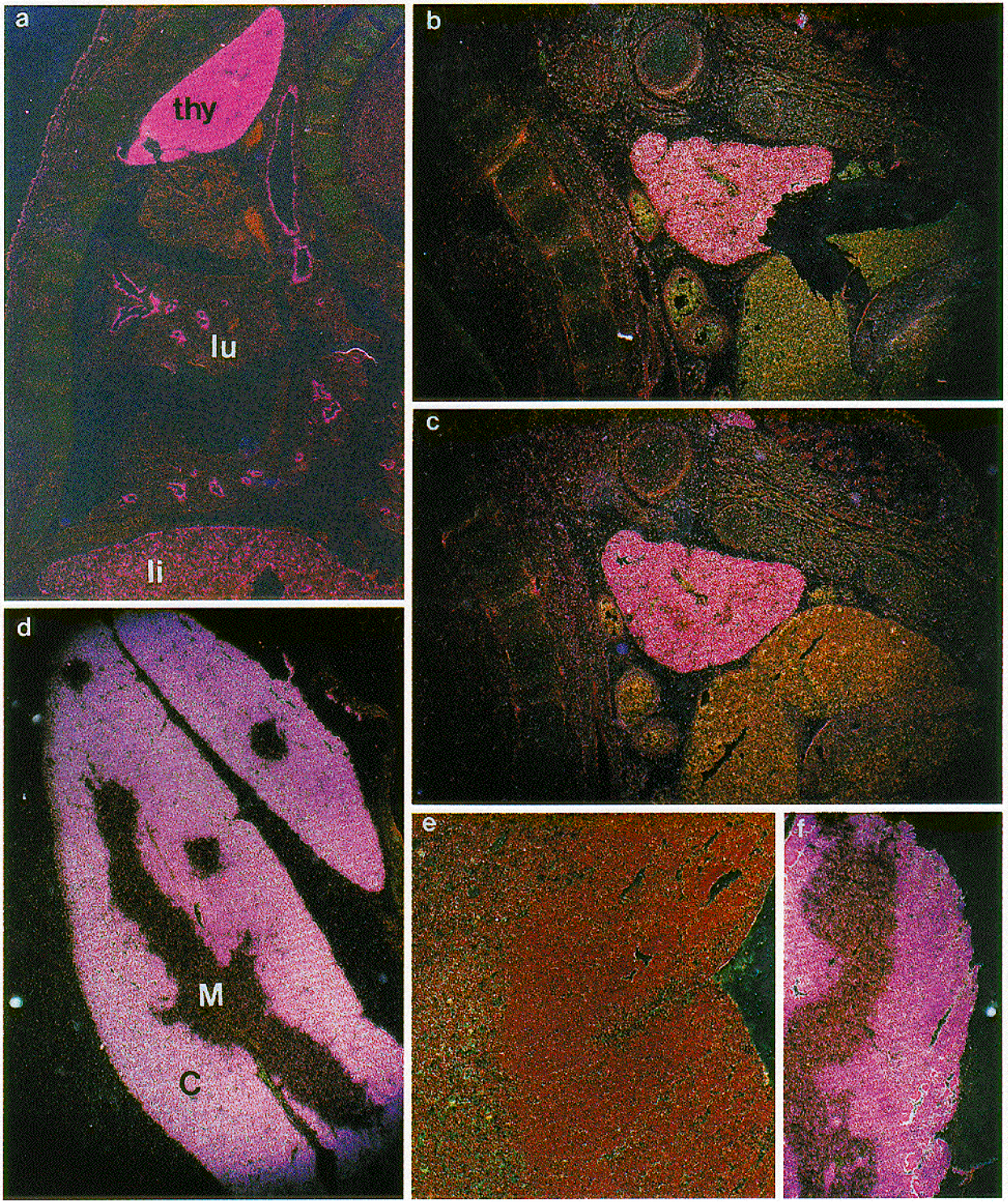
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