DEVELOPMENTAL AND TISSUE-SPECIFIC EXPRESSION OF NUCLEAR PROTEINS THAT BIND THE REGULATORY ELEMENT OF THE MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I GENE

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MHC class I genes encode transplantation antigens that are essential for recognition of foreign antigens by T cells. Expression of classical MHC class I genes is developmentally controlled. Low levels of class I message and surface antigens become detectable only after the mid-somite stage in mouse embryogenesis (1, 2). MHC class I expression remains very low during gestation, but increases sharply in most organs after birth and during the first 2 wk of postnatal development (3). In adults, the majority of somatic cells express MHC class I genes except for those in the central nervous system (4, 5). Class I genes can be induced by lymphokines such as IFNs (6) and TNF (7) that modulate immune responses.

Transcription of the class I gene is controlled by the conserved *cis*-acting sequence designated class I regulatory element (CRE)[1], which is present in the upstream region of the MHC class I gene (8–10). The CRE is juxtaposed to another regulatory element, the IFN consensus sequence (ICS) (11), which is responsible for IFN-mediated induction of the class I gene (12–14). The CRE elicits an enhancer like function in tissue culture fibroblasts (8–10). In undifferentiated F9 embryonal carcinoma cells, however, the CRE negatively regulates transcription of the class I gene (9). The CRE acts neither negatively nor positively in cells derived from mid- to late-somite stage embryos (15). These findings indicate that the CRE is involved in developmental regulation of the class I gene expression.

By analogy with other systems (16, 17), the CRE is expected to control transcription of the class I gene by interacting with *trans*-acting nuclear proteins. Indeed, the CRE was found to contain three discrete sequences (region I, II, and III; Fig. 3) that bind different nuclear proteins present in a number of cultured cell lines (10, 18, 19). These regions correspond to direct and inverted repeats in the CRE (19).

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[1] *Abbreviations used in this paper:* CAT, chloramphenicol acetyl transferase; CRE, class I regulatory element; LMT II A, human metallothionein II A; ICS, interferon consensus sequence.

The present study was undertaken to delineate the basis of developmental and tissue-specific expression of the class I genes in vivo. We first examined various developing and adult murine tissues for the presence of nuclear proteins that bind the CRE. Then we assessed the functional significance of factor binding to each region of the CRE by site-directed mutagenesis in cultured fibroblasts. Our results show that nuclear factors capable of binding to the CRE are expressed in vivo in correlation with developmental and tissue-specific expression of the MHC class I gene. In addition, we show that both region I and region II of the CRE are capable of positively regulating class I gene transcription.

Materials and Methods

Nuclear Extract Preparation. Nuclear extracts from mouse LH8 T-cells and Ltk$^-$ fibroblasts were prepared according to Dignam et al. (20) with modifications (19). Nuclear extracts from tissues were prepared according to Gorski et al. (21) with minor modifications. Briefly, tissues obtained from BALB/c mice (NIH Small Animal Section) were homogenized in homogenization buffer and centrifuged at 23,000 rpm for 30 min. Crude nuclear pellets were centrifuged again through a 10-ml cushion of a 9:1 mixture of homogenization buffer and glycerol. The nuclear pellets were then lysed in lysing buffer followed by extraction with 4 M ammonium sulfate. Extracted proteins were precipitated by adding solid ammonium sulfate, and the precipitates were dialyzed extensively in dialysis buffer. All the buffers contained the protease inhibitors PMSF (0.1 mM) and aprotinin (0.1%) (Sigma Chemical Co., St. Louis, MO).

Oligonucleotides. All oligonucleotides were synthesized in an automated synthesizer (Coder 300; Vega Biotechnologies, Inc., Tucson, AZ); purified by HPLC, and annealed as described (19). The following duplex oligonucleotides were used as probes or competitors. The CRE spanning from nucleotide position −203 to −161, region I (from −173 to −161), region II (from −203 to −185) (see Fig. 4), and the ICS (from −167 to −139, reference 19) of the H-2L^d gene. Additionally a 70-bp human metallothionein IIA (hMTIIA) sequence containing an AP-1 site (22),

5'GCGCGGCCGGGTGTTTCGCCTGGAGCCGC-
AAGTGACTCAGCGCGGGGCGTGTGCAGGCA 3'

and an AP-1 recognition site of the SV40 gene (23),

5'CAATTAGTCAGCAACCATA 3'

were used.

Gel Mobility Shift Assay. Binding of nuclear proteins to the CRE was studied by a gelmobility shift assay (19). A 114-bp Pst I-Ava II fragment of the mouse H-2L^d gene containing the CRE and ICS as well as oligonucleotides corresponding to region I and region II were end-labeled with γ-[^{32}P]ATP using T4 polynucleotide kinase. The ICS probe was prepared as described (24). These probes (0.1–0.6 ng DNA containing 5,000 to 15,000 cpm) were incubated with 3–5 μg of nuclear extract proteins in the presence of 1.5–4.0 μg poly(dI-dC) (Pharmacia Fine Chemicals, Piscataway, NJ) for 15–30 min at 0°C or room temperature and electrophoresed through a 3.4 or 4% polyacrylamide gel. In some experiments binding reactions were carried out in the presence of additional protease inhibitors (1 μM leupeptin and 1 μM pepstatin). Competition by mutant oligomers was tested by adding 10–40 ng of double-stranded oligomers to the reaction mixture.

Site-directed Mutagenesis. Site-directed mutagenesis was performed as described (25). A 400-bp Xba I-Hind III fragment that contained in 5' upstream region of the H-21^d gene including TATA and CAT boxes and the CRE and ICS was cloned into M13 mp18. 20–21-bp long mutant oligomers, each containing a single 2-bp substitution, were annealed to the tem-

plate, and were extended and ligated by the Klenow fragment of DNA polymerase I and T4 DNA ligase in the presence of a thionucleotide, dCTPαS (Amersham Corp., Arlington Heights, IL). The resultant heteroduplexes were digested with Nci I, followed by exonuclease III treatment, which removes the wild-type template. The remaining sequences were polymerized and ligated in the presence of deoxynucleotides. Mutant phage DNAs were sequenced by the Sanger method to confirm accurate mutagenesis. The efficiency of mutagenesis by this procedure was ~80%. To construct mutant pL^d CAT genes, the mutant Xba I–Hind III fragments were then inserted into the Xba I–Hind III site of pL^d CAT 1.4K (9).

Chloramphenicol Acetyl Transferase (CAT) Assay. Transient CAT assays were performed with Ltk^- fibroblasts using the calcium phosphate precipitation method with BES buffer (26), pH 6.95 (9, 27). Briefly, a mixture of DNA (4 μg of CAT constructs, 2 μg of pCH 110 [28] that contains the *lacZ* gene under the control of the SV40 promoter, and 14 μg of carrier DNA pUC19) was added to 5×10^5 Ltk^- cells and incubated for 16 h. Cells were then washed with PBS and incubated further in medium with or without murine IFN-α/β (800 U/ml) (Lee Biomolecular, San Diego, CA) for an additional 12–14 h. CAT and β-galactosidase activities were measured using equal amounts of extracted protein obtained from each sample. Promoter activity elicited by mutant CRE was assessed after normalizing transfection efficiency of each sample by dividing counts of acetylated chloramphenicol by β-galactosidase activity.

Results

Nuclear Proteins that Bind to the CRE Are Present in Developing and Adult Murine Tissues. The CRE encompasses position −161 to −203 relative to the RNA start site, and elicits positive and negative transcriptional regulation (8, 9). Israel et al. (18), Baldwin and Sharp (10), and Singh et al. (29) showed that specific nuclear proteins interact with this sequence. We showed that there are three independent sequences (region I, II, and III; Fig. 3, diagram) in the CRE that bind distinct nuclear proteins in most tissue culture cells (19).

In an effort to discern functional roles of the CRE for class I gene expression in vivo, we examined various developing and adult tissues for the presence or absence of CRE binding factors in gel mobility shift assays. In the first series of experiments a ^{32}P-labeled probe containing the entire CRE of the $H\text{-}21^d$ gene was used. Results of adult tissue extracts are shown in Fig. 1 *A*. Extracts from liver and spleen revealed a single retarded band, while those of brain had a doublet, all of which migrated essentially the same distance in the gel. The single band and the doublet were competed with an excess of unlabeled oligonucleotide corresponding to the entire 40-bp CRE (Fig. 1 *A*, lanes *3*, *10*, and *17*), but not the ICS (lanes *7*, *14*, and *21*), indicating that the retarded species represents factor binding to the CRE. Protease treatment of nuclear extracts from these tissues eliminated the retarded band (data not shown), indicating that the factor(s) is a protein(s). To analyze binding sites further, competitor oligomers corresponding to region I and region II were added to reaction mixtures. When these competitors were added independently to liver extracts, even at 50 times molar excess, the retarded band was not eliminated (lanes *4*, *5*). However, this band was readily competed when the two oligomers were added together at 10 times molar excess. Hence, it is likely that the band seen in liver extracts consists of two binding activities, one representing binding to region I and the other to region II, both of which migrate the same distance in the gel. The retarded band seen in spleen extracts was almost completely competed by the region I oligomer, leaving only a weak residual band (lane *18*). Addition of the region II competitor increased the intensity of the band (lane *19*), the basis of which has not investigated. However,

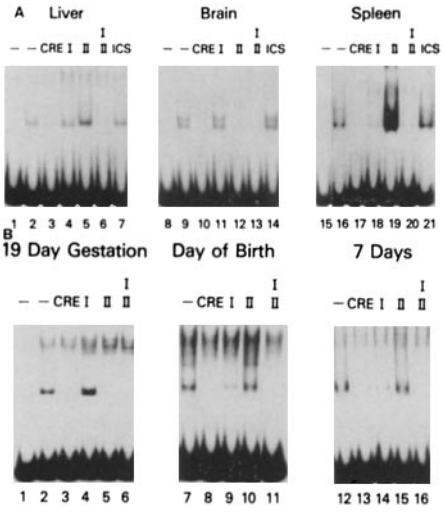

FIGURE 1. Identification of nuclear factors that bind the CRE in adult (*A*) and developing (*B*) tissues in vivo. Gel mobility shift experiments were carried out with a ^{32}P-labeled Pst I-Ava II fragment that contains the CRE and ICS using 3–5 μg of nuclear extract proteins prepared from BALB/c tissues. To determine specificity and subregions of factor binding, double-stranded oligonucleotides corresponding to the CRE, region I and region II, and ICS were added to reaction mixtures at ~20 times molar excess. When competitors for region I and II are added together, ~10 times molar excess of each oligomer was used.

a combination of region I and region II competitors eliminated the band entirely (lane *20*), indicating that spleen extracts have a relatively high region I binding activity and a low region II activity. In contrast, brain extracts generated a doublet that was completely competed by region II oligomer alone (lane *12*). Addition of region I competitor did not remove the doublet. These results indicate that brain extracts do not contain a protein that binds to region I, but contain a region II binding protein(s). The mixing of spleen and brain extracts did not eliminate a band competable by the region I competitor (not shown). Fig. 1 *B* shows gel mobility shift patterns of extracts from developing liver, i.e., from fetus (day 19 of gestation), neonates (day 0), and 7-d-old mice. We tested these tissues to see if developmental regulation of class I genes (1, 3) correlates with CRE binding activities. Extracts from developing liver showed a single retarded band that can be competed by the entire CRE. The upper broad band seen in extracts of fetuses and neonates on the day of birth was nonspecific, since neither CRE nor ICS competitor removed it. The lower band seen with fetal liver extracts was completely eliminated by region II oligomer (Fig. 1 *B*, lane *5*), whereas region I oligomer did not affect the retarded band. In rare cases, the band from fetal liver extracts had a very minor component competed by region I oligomer, suggesting occasional low expression of region I protein during gestation. Extracts from younger fetuses (day 17 and day 18 of gestation) also revealed a band competable only by region II (data not shown). In contrast, the band from neonatal and day 7 mice was not competable by either region I or region II oligomer alone (lanes *9*, *10*, *14*, *15*). As was the case for adult liver and spleen (Fig. 1 *A*), competition was attained after addition of both oligomers (lane *11*, *16*). Hence during gestation region I binding protein appears to be very low or absent, but it increases rapidly following birth.

The Use of Region-specific Probes Confirms the Correlation between Region I Binding and Class I Gene Regulation In Vivo. To extend the observations described above we used two probes that separate regions I and II of the CRE. These probes were used to test whether there are additional binding activities that are not detected with the

probe covering the entire CRE used in the preceding section. In some cases probes encompassing shorter sequences can reveal binding activities not readily found with longer probes (24, 30). In addition, these probes allowed us to examine whether binding of region I and region II occur independently of each other. Results of extracts obtained with adult tissues (*A*) and developing liver (*B*) are shown in Fig. 2. The region I probe encompassing the nucleotide stretch from −173 to −161 of the CRE (see Fig. 4) elicited a broad single band or doublet in adult spleen and liver extracts. These retarded species were competed by the region I competitor but not by the region II competitor. In contrast, in brain extracts region I probe did not generate a detectable band. These data indicate that spleen and liver, but not brain extracts have a region I binding activity, and confirm the results seen with the CRE probe in Fig. 1 *A*. Further, these data show that the region I binding activity can be detected independently of region II. The region II probe encompassing nucleotides −203 to −185 of the CRE (see Fig. 4) generated two closely migrating bands in adult liver and in brain extracts, which were specifically competed by the region II oligomer. The region II probe did not reveal an appreciable binding activity with spleen extracts. These data indicate that region II binding activity is present abundantly in liver and brain, but at a very reduced level in spleen, in agreement with the results presented in Fig. 1 *A*. Thus, binding activities to region I and region II are independent of each other, and the lack of region I binding activity seen in

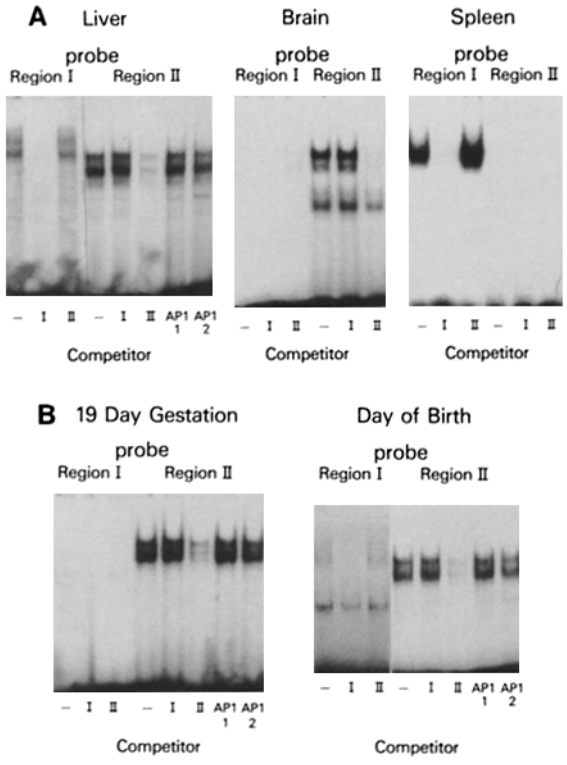

FIGURE 2. Binding of factors to region I and region II of the CRE. Gel mobility shift experiments were carried out with ^{32}P-labeled oligonucleotide probes corresponding to region I or region II of the CRE (see the top of the figure). Unlabeled competitor oligomers were added to reaction mixture at 20–50 times molar excess (see the bottom of figure). (*A*) Adult tissue extracts, (*B*) fetal (day 19 of gestation) and neonatal liver extracts. AP-1-1; hMTIIA competitor (22); AP-1-2; the AP-1 sequence in the SV40 promoter (23).

brain extracts was not caused by region II binding. We observed the formation of doublets with some probes (see Fig. 1 *A*, brain; Fig. 2 *A*, liver and brain), the basis of which is not entirely clear. It may reflect the heterogeneity of binding proteins possibly caused by post-translational modifications, such as phosphorylation (31).

Fig. 2 *B* depicts results with fetal and neonatal liver extracts. Consistent with the data in Fig. 1 *B*, the region I probe did not generate a band from fetal liver extracts, while the region II probe elicited two closely spaced bands competed by the region II competitor, similar to those seen with adult tissues (Fig. 2 *A*). Neonatal extracts, on the other hand, showed a weak but reproducible band with the region I probe. This band was specifically competed by the region I oligomer. These results are in agreement with the data in Fig. 1 *B* and indicate that the region I binding activity becomes detectable in correlation with the increase in class I expression during development.

The AP-1 protein, a *trans*-acting factor for the SV40 control region as well as other genes (22, 23), has been shown to bind to region II of mouse MHC class I genes (23, 32). We tested whether the region II binding activity seen in our work represents binding of AP-1. In Hela cell extracts, a 70-bp hMTIIA promoter containing an AP-1 recognition site (22) generated several retarded bands that were competed by the probe sequence itself and by the SV40 AP-1 site (see Materials and Methods). We tested these sequences for their ability to compete for region II binding. As seen in Fig. 2 *B* no competition was observed with either sequence. Competition was not detected with adult tissues either (Fig. 2 *A*). In addition, using mouse extracts we did not observe a specific band generated by hMTIIA, indicating that AP-1 binding activity is low or absent in extracts we tested (not shown). Thus, it is highly unlikely that the region II binding activity detected in this study represents AP-1 binding.

The absence (or very low levels) of the region I binding activity in brain and fetal liver extracts and of the region II binding activity in adult spleen was not due to differential degradation of nuclear proteins: With the probe covering the ICS (19), all tissues shown in Figs. 1 and 2 generated a single retarded band of the same mobility, which was competed by the ICS oligomer, but not by the CRE oligomer (not shown). Furthermore, the c-*fos* enhancer probe generated the identical mobility shift patterns in fetal and adult liver extracts (33). These results lend further credence to the proposed correlation between region I binding and class I gene regulation in vivo.

We did not observe unequivocal binding of a factor to region III in any of extracts prepared from tissues, even though extracts from most tissue culture cell lines showed a binding activity to region III (19; Hirschfeld, S., unpublished data). The basis of this observation is not clear. Differences in the procedures of extract preparation probably do not account for it, as spleen extracts prepared according to Dignam et al. (20) showed no specific binding activity to region III either.

Functional Significance of Protein Binding to Subregions of the CRE. The above study suggested that protein binding to region I plays a role in positive regulation of the class I gene. Therefore, we studied relative contributions of region I, II, and III sequences to the enhancer-like activity in tissue culture cells (8–10). We used pL^d CAT 1.4K (9) in which the CAT reporter gene is under the control of a 1.4-kb upstream region of the H-$2L^d$ gene. This upstream region contained the CRE, ICS, and the basic promoter region, and exerts positive regulation of class I gene tran-

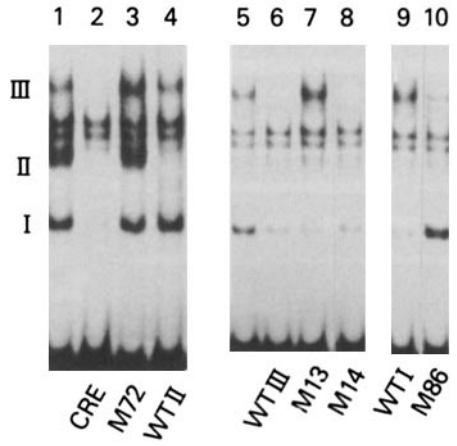

FIGURE 3. Competition by mutant CRE. Gel mobility shift experiments were carried out with 8 μg of LH8 T cell nuclear extract proteins as described (19). Wild-type or mutant oligomers, containing 2 bp substitutions, M72, M13, M14, M86 (Fig. 4), were added at 20 times molar excess as shown at the bottom of each lane. Reactions in lanes *5–10* were carried out in the presence of region II oligomer at ~10 times molar excess.

scription in fibroblasts (9, 15). By site-directed mutagenesis single 2-bp substitutions were introduced independently into regions I, II, and III (Fig. 4).

These mutants were first tested for their ability to bind the respective protein in competition assays (Fig. 3). Extracts from LH8 T-cells showed multiple retarded bands I, II, and III. These bands represented protein binding to region I, II, and III, respectively (19), and were competed by excess unlabeled CRE (lane *2*). Band II, readily eliminated by the wild-type region II competitor oligomer, was not eliminated by mutant M72 oligomer (lanes *3* and *4*), indicating that M72 mutation abrogates protein binding to region II. Lanes *5–10* show competition by region III and region I mutants, which were tested in the presence of unlabeled wild-type region II oligomer. As expected, the wild-type region III oligomer (lane *6*) removed band III completely, and reduced the intensity of band I. M13 oligomer did not compete for region III binding, but did compete for region I binding (lane *7*). M14 oligomer on the other hand, retained the ability to compete for region III binding (lane *8*), consistent with the previous methylation interference data, which indicated that protein binding occurs in the right and left halves but not in the center of region III (19). As seen in lane *9*, the wild-type region I oligomer removed band I without significantly affecting band III binding activity. Addition of increased amounts of region I competitor, however, did eliminate region III binding (data not shown). In contrast, the M86 mutant, which disrupts the inverted repeat of region I, failed to compete for band I (lane *10*). These results indicate the mutations M86, M72, and M13 selectively abolish protein binding to region I, II, and III of the CRE, respectively. Extracts from Ltk$^-$ cells gave similar results (data not shown).

The ability of these mutant CRE to enhance transcription of the class I gene was then tested with mutant pL^d/CAT 1.4K constructs (Fig. 4) in a transient CAT assay with Ltk$^-$ fibroblasts. Results are shown in Table I. Control plasmid pL^d CAT 123, which does not have the CRE, yielded ~10 times lower CAT activity than the wild-type pL^d CAT 1.4K. The higher CAT activity seen by pL^d CAT 1.4K is attributable to the enhancer-like activity elicited by the CRE (8–10). Mutant pL^d CAT 1.4K M86, which failed to bind region I in vitro, did not manifest enhancer-like activity

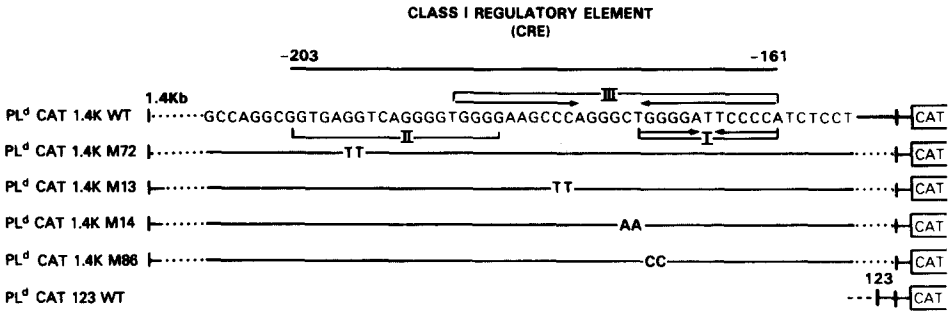

FIGURE 4. Mutant CAT constructs used for evaluation of the enhancer-like activity. The nucleotide sequence of the CRE and its subregions is shown on top of the wild-type pL^d CAT 1.4K construct (9, 24) that had the entire 1.4-kb upstream region of the H-2L^d gene connected to the reporter CAT gene. In mutants only mutated nucleotides are shown. Solid line represents nucleotides identical to the wild-type CRE. pL^d CAT 123 had the promoter region containing TATA and CAT boxes but lacked both the CRE and ICS.

fully. CAT activity by this mutant was consistently about half that of the wild-type pL^d CAT 1.4K. M72 mutation, which precluded protein binding to region II in vitro also reduced the enhancer-like activity, but to a lesser extent (~30%). The reductions in transcriptional activity seen by these two mutants were statistically significant (Table I). In contrast, M13 mutation, which abrogated protein binding to region III (but not regions I and II), maintained full enhancer function. The same was true for M14, which does not affect factor binding to any of the three regions. This indicates that region I and II are capable of positively regulating class I genes, presumably through the binding of specific proteins. In contrast, region III appears to have no major role in exerting enhancer-like activity in Ltk$^-$ cells.

TABLE I

Mutations in the CRE Reduce the Enhance Activity

Constructs	Enhancer activity (*P*)	IFN response + IFN/ - IFN (*P**)
pL^d CAT 1.4 K	9.8 (-)	3.9 (>0.06)
pL^d CAT 1.4 K M72	6.8 (>0.06)	2.8 (>0.06)
pL^d CAT 1.4 K M13	11.2 (NS)	4.4 (>0.06)
pL^d CAT 1.4 K M14	8.9 (NS)	4.3 (>0.06)
pL^d CAT 1.4 K M86	5.1 (>0.06)	2.9 (>0.06)
pL^d CAT 123	1.0 (>0.06)	1.1 (-)

Transient CAT activity was measured in Ltk$^-$ cells treated with or without IFN-α/β (800 U/ml) for 12-14 h. To adjust for variability of transfection efficiency, CAT activity in each sample was normalized using β-galactosidase activity elicited by the cotransfected pCH110; this variability did not exceed 13%. Each value represents a mean of four tests. Enhancer activity is the activity of pL^d CAT 1.4K (and its mutants) divided by that of pL^d CAT 123. *P* value was derived from a one-tail signs test using four independent experiments. NS, not significantly different from pL^d CAT 1.4K. IFN response is estimated as CAT activity with IFN divided by CAT activity without IFN for each construct. P*, IFN response by all mutants were significantly higher than pL^d CAT 123.

We also tested whether these mutations affect the ability to enhance class I gene transcription in response to IFN. IFN induces class I gene transcription through the conserved ICS (12–14), which partially overlaps the CRE. We found that the ICS binds nuclear factors inducible by IFN, and that the protein binding to the ICS is necessary for IFN-mediated transcriptional induction of the class I gene (30) in fibroblasts. Additional evidence indicates that the CRE also participates in responding to IFN (12–14). Israel et al. (12) and Korber et al. (13) reported that in addition to the ICS, the CRE is necessary for responding to IFN. We found, on the other hand, that the CRE provides a synergistic but auxiliary effect (14). Thus, it was of interest to study the role of each region of the CRE for responsiveness to IFN. As seen in Table I, IFN treatment enhanced CAT activity in all CAT constructs except for pL^d CAT 123. This construct does not have the ICS and is known to be incapable of responding to IFN (14). The levels of CAT induction by IFN among the mutants were, however, somewhat variable. pL^d CAT 1.4K M72 and pL^d CAT 1.4K M86 gave lower induction than the wild-type pL^d CAT 1.4K, whereas the remaining two mutants, M13 and M14, were induced to a level comparable to the wild type. It should be noted that pL^d CAT 1.4K and pL^d CAT 123 both initiated CAT mRNA synthesis at the expected RNA initiation site both before and after IFN treatment (14; data not shown). These results suggest that region I and II are involved in responding to IFN in an auxiliary role.

Discussion

The present study shows that nuclei in murine tissues contain proteins that bind to discrete subregions of the CRE. By using probes corresponding to the entire CRE as well as region I and region II separately, we have shown that while binding activity to region II occurs in tissues, irrespective of class I gene expression, protein binding to region I is detected only in tissues that express class I genes at relatively high levels. Region I binding activity was essentially absent in nuclear extracts from fetal tissues and adult brain where class I gene expression is low or absent. This correlation suggests that the region I binding activity plays a role in controlling developmental and tissue-specific expression of class I gene in vivo.

The biochemical nature of region I binding proteins in tissue extracts has not been studied in detail. Yano et al. (34) isolated from a murine thymoma line a 48-kD nuclear factor, KBF1, that binds region I. In addition to this protein, there appears to be another region I binding factor in cultured cell extracts (Israel, A., personal communication). More recently, Singh et al. (29) cloned a cDNA that encodes a protein called H-2TF1, capable of binding region I, which is yet distinct from KBF1 in size and binding properties. NFkB that binds to the Igk gene enhancer also binds to region I (35). NKkB binding activity is constitutively expressed in a B cell–specific fashion. Thus, region I is capable of binding multiple proteins. It remains to be determined whether any of the above reported factors represents the region I binding seen in this work. Factors that bind region II have not been as extensively studied as region I binding factors. Although the purified AP-1 protein is reported to bind to region II (23, 24), our results show that the region II binding activity seen in these extracts does not represent AP-1. Further, we failed to detect binding activity to AP-1 sequences with extracts prepared from various murine cells cultured in vitro. Thus, the region II binding activity detected in mouse extracts appears to be relatively specific for the CRE.

Results of CAT assays with mutant CRE constructs show that the mutation M86 in region I has a detrimental effect. Mutation M72 in region II also reduced the enhancer-like activity, albeit to a lesser extent. The results indicate that both region I and region II are capable of enhancing transcription of class I genes, but neither region alone appears to exert a full effect. Thus, it may be postulated that the low level of class I gene expression in fetal tissues is mediated primarily by a factor binding region II of the CRE. At birth there is the additional appearance of a factor that binds to region I and a concomitant rise in class I mRNA. However, tissue-specific in vivo regulation appears to be rather complex, and cannot be explained by the presence or absence of CRE binding factors alone. Adult spleen extracts show very low levels of region II binding activity, despite having high levels of class I gene expression. Adult brain extracts show a region II binding activity, but have no detectable class I gene expression. Possible explanations include the inhibition of binding to region II DNA in vivo by the chromatin structure (reviewed in reference 36) found in brain or a different DNA methylation pattern. Alternatively, transcription of class I gene may be repressed in the brain by a negative *trans*-acting factor. Repression of class I gene transcription mediated by the CRE has been noted in undifferentiated F9 embryonal carcinoma cells (9). To date, a *trans*-acting factor responsible for the negative regulation of the class I gene in F9 cells has not been identified.

Extracts from tissues consistently failed to reveal a separate binding activity for region III, even though region III binding was detected in a majority of tissue culture cells. This discrepancy is not explained by differences in the procedures for extract preparation. Protein binding to region III is unlikely to be an artifact of tissue culture, since the region III sequence is well conserved in the murine CRE and in the human counterpart (37). It is possible that factor binding to region III is usually absent in tissues and occurs only transiently under specific physiological conditions or in particular developmental stages. This may account for the apparent lack of the enhancer-like activity by region III as detected by mutant pL^d CAT 1.4K M13 (Table I).

In conclusion, nuclear proteins are present in vivo that bind to discrete regions of the CRE. Further, their binding to the CRE enhances transcriptions of the class I gene, providing evidence that binding of nuclear proteins to the CRE regulates class I gene expression in vivo.

Summary

Expression of MHC class I genes varies according to developmental stage and type of tissues. To study the basis of class I gene regulation in tissues in vivo, we examined binding of nuclear proteins to the conserved *cis* sequence of the murine H-2 gene, class I regulatory element (CRE), which contains two independent factor-binding sites, region I and region II. In gel mobility shift analyses we found that extracts from adult tissues that express class I genes, such as spleen and liver, had binding activity to region I. In contrast, extracts from brain, which does not express class I genes, did not show region I binding activity. In addition, fetal tissues that express class I gene at very low levels, also did not reveal region I binding activity. Binding activity to region I became detectable during the neonatal period when class I gene expression sharply increases. Most of these tissues showed binding activity to region II, irrespective of class I gene expression. Although region II con-

tained a sequence similar to the AP-1 recognition site, AP-1 was not responsible for the region II binding activity detected in this work. These results illustrate a correlation between region I binding activity and developmental and tissue-specific expression of MHC class I genes.

The CRE exerts an enhancer-like activity in cultured fibroblasts. We evaluated the significance of each factor binding to CRE. Single 2-bp mutations were introduced into the CRE by site-directed mutagenesis and the ability of each mutant to elicit the enhancer activity was tested in transient CAT assays. A mutation that eliminated region I protein binding greatly impaired enhancer activity. A mutation that eliminated region II binding also caused a lesser but measurable effect. We conclude that region I and region II are both capable of enhancing transcription of the class I gene. These results indicate that in vivo regulation of MHC class I gene expression is mediated by binding of *trans*-acting factors to the CRE.

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