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NOTES

Binding of Multiple Nuclear Factors to the 5′ Upstream Regulatory Element of the Murine Major Histocompatibility Class I Gene

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Transcription of mouse major histocompatibility complex class I genes is controlled by the conserved class I regulatory element (CRE) in the 5′ flanking region. The CRE, ~40 base pairs long, acts as a negative control element in undifferentiated F9 embryonal carcinoma cells which do not express the major histocompatibility complex genes. The same element, however, acts as a positive control element in cells expressing the genes at high levels. To investigate the molecular basis of the regulatory role of the CRE, we studied the binding of nuclear proteins to the CRE of the *H-2L*^{d} gene by gel mobility shift and methylation interference experiments. Nuclear extracts from L fibroblasts and LH8 T lymphocytes revealed three distinct factors that bind discrete sequences within the CRE. The three sequences correspond to the inverted and direct repeats within the CRE. In contrast, F9 extracts exhibited factor binding to only two of the three sequences and lack a major factor detected in the above two cell types. Protein-binding sites within each of the three sequences were identified by methylation interference experiments. These data were in full agreement with results obtained by a competition assay performed with a series of mutant oligonucleotides containing a few nucleotide substitutions in each of the three regions. The results illustrate complex DNA-protein interactions in which several independent proteins bind to overlapping sequences in the CRE in a cell type-specific fashion.

Major histocompatibility complex (MHC) class I genes encode highly polymorphic transplantation antigens that play an essential role in T-cell antigen recognition (9). Although the antigens are expressed constitutively in the majority of somatic cells in adults, their expression is developmentally regulated (20). We previously postulated that developmental control of MHC class I gene expression is mediated by the highly conserved class I regulatory element (CRE) and that it involves switching from negative to positive regulation of transcription. The CRE, which resides from approximately −200 to −161 of MHC class I genes, represses transcription of MHC class I genes in undifferentiated F9 embryonal carcinoma (EC) cells that are negative for class I gene expression (18). In contrast, the CRE acts as an enhancer in differentiated fibroblasts which express class I genes at high levels (12, 18). Juxtaposed and overlapped with the CRE, there is another control element, the interferon consensus sequence (ICS), which plays an independent role in the response to interferons (6, 12, 26). Here we describe binding of at least three nuclear proteins to discrete sequences of the CRE of the *H-2L*^{d} gene that occurs in a cell type-specific manner. Additional data on detailed protein-binding sites within each sequence are also presented.

Gel mobility shift analysis of nuclear factors that bind to the CRE. We first examined the interaction of factors to the CRE of the *H-2L*^{d} gene in gel mobility shift analysis (5, 8) using nuclear extracts from L fibroblasts, murine T-cell line LH8, and undifferentiated F9 EC cells (Fig. 1). Nuclear extracts were prepared according to the procedure of Dignam et al. (3), except that 1 mM spermidine and protease inhibitors were added to buffers C and D. Probes used for this work are shown in the lower half of Fig. 1. A 114-base-pair *Pst*I-*Ava*II fragment and a 131-base-pair *Xba*I-*Ava*II fragment were derived from the *H-2L*^{d} 5′ flanking sequence (18), both of which contain the CRE and ICS. The two probes were used interchangeably, since they gave identical patterns in gel mobility shift. Probes were end labeled with [γ-^{32}P]ATP by using polynucleotide kinase. The binding reactions were done in a total volume of 15 to 20 μl containing 20 mM Tris hydrochloride (pH 7.8), 60 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM spermidine, 0.01% Triton X-100, 8% (vol/vol) glycerol, 4 μg of poly(dI-dC) (Pharmacia), 6 to 9 μg of nuclear protein, and 0.1 to 0.2 ng of γ-^{32}P-labeled probe in the presence or absence of duplex oligonucleotide competitor. All oligonucleotides were synthesized in an automated synthesizer, Vega Coder 300, and purified by high-pressure liquid chromatography. Complementary oligonucleotides were annealed before use. The reaction mixtures were incubated for 30 min at 22°C and were then loaded onto a 3.4% polyacrylamide gel (29:1 acrylamide-bis ratio) containing 40 mM Tris, 40 mM boric acid, and 0.4 mM EDTA, which had been prerun for 60 min at 10 V cm^{-1}. After electrophoresis for 1.5 to 2 h at 10 V cm^{-1}, the gels were dried and autoradiographed overnight.

Figure 1 shows the mobility shift patterns of nuclear extracts from several cells. The retarded bands seen in lanes 2, 4, and 6 indicate protein-DNA interaction and are caused by specific binding of factor(s) to the CRE, since addition of

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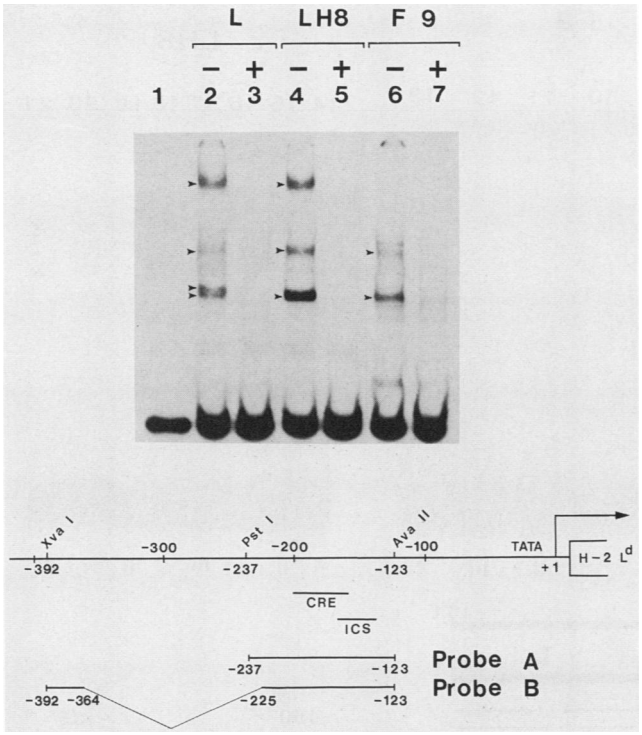

FIG. 1. Binding of nuclear factors to the CRE as detected by gel mobility shift analysis. Nuclear extracts were prepared from L fibroblasts, the LH8 lymphocyte line, and F9 embryonal carcinoma cells, according to Dignam et al. (3) with a small modification. Extracts (6 to 9 μg of protein) were incubated with ~5,000 cpm of γ-^{32}P-labeled probe and 4 μg of poly(dI-dC) in the presence (+) or absence (−) of 2 ng of duplex oligonucleotide competitor corresponding to the CRE (−202 to −159, Fig. 2B) and electrophoresed in 3.4% polyacrylamide gel. Retarded bands representing specific DNA-protein complexes are indicated by arrowheads. Lane 1, Probe only. The probes derived from the 5′ upstream sequence of the *H-2L^d* gene (18) and used in this work are schematically shown in the lower part of the figure. The CRE and ICS are indicated by solid bars. The numbers represent nucleotide positions relative to the transcription start site (+1).

an excess synthetic duplex oligonucleotide corresponding to the CRE (lanes 3, 5, and 7) completely blocked the formation of labeled probe-factor complexes. Neither a duplex oligonucleotide corresponding to the ICS (Fig. 2) nor salmon sperm DNA (data not shown) blocked the formation of retarded bands. With the probes used here, no factor binding to the ICS was detected. However, specific binding was detectable when a probe that encompassed the ICS without the CRE was used, details of which will be presented elsewhere (unpublished data). No obvious retarded bands were found with probes prepared from further upstream sequences from −392 to −238 or from −237 to −191 of the *H-2L^d* gene (data not shown). Pretreatment of the extracts with proteinase or sodium dodecyl sulfate abolished the appearance of the retarded bands (data not shown), indicating that these bands represent DNA-protein complexes. L cells and LH8 T cells exhibited three bands that migrated to the same positions (Fig. 1). L cells had an extra band showing the second-fastest migration in the gel (see arrowheads in Fig. 1). Extracts from NIH 3T3 and SP2/0 B hybridoma cells exhibited patterns similar to those of L cells and LH8 cells, respectively (data not shown). In contrast, nuclear extracts from undifferentiated F9 EC cells did not reveal the uppermost band. The absence of this band in F9 extracts was not attributable to cellular proteases, since prolonged incubation of L-cell extracts with F9 extracts did not eliminate the uppermost band seen with L-cell extracts alone. Nor did F9 nuclear extracts prepared by the Parker and Topol method (21) reveal the uppermost band. It is likely that the factor that forms the uppermost band is absent in F9 nuclear extracts, although it might be sequestered.

Competition analysis of subregions involved in protein binding in the CRE. To localize the binding site of each DNA-factor complex seen in Fig. 1, we synthesized various duplex oligonucleotides corresponding to different regions of the CRE and examined them for the ability to compete with the probe (Fig. 2A). Oligonucleotides used corresponded to the subregions I, II, and III shown in Fig. 2B. Results obtained with extracts from L, F9, and LH8 cells are shown in Fig. 2A. All the bands seen in Fig. 1 could be accounted for by binding to three distinct sequences, as oligonucleotides I (−175 to −160), II (−203 to −183), and III (−190 to −160) removed all the bands in a mutually exclusive manner. Thus, band I, the fastest-migrating band in F9 and LH8 cells, could be completely eliminated by oligonucleotide I (lanes 13, 16, and 20). In L cells, addition of oligonucleotide I also removed band I (lane 6), although the removal is less evident, since band I overlaps band II in this case. Addition of oligonucleotide II removed band II, a single band in F9 and LH8 cell extracts (lanes 11, 15, and 19). In L cells, there are several bands that are eliminated by oligonucleotide II (lane 4). A mixture of oligonucleotides I and II removed all the fastest-migrating bands from L-cell extracts (lane 7). Band III, visible in L and LH8 extracts, was completely eliminated by oligonucleotide III (lanes 5 and 17). Addition of this oligonucleotide had no effect on the gel mobility pattern of F9 EC cells (lane 12), demonstrating that the factor(s) that specifically binds to region III is missing in F9 extracts. Oligonucleotide III also eliminated band I in LH8 and F9 cells, as expected, since the two competitors partially overlap. An excess oligonucleotide I also competed for band III, albeit partially (lane 16). We found that the factor that binds to sequence III requires Mg^{2+}. As seen in lanes 18 to 21 in Fig. 2A, the removal of $MgCl_2$ from the binding buffer abrogated appearance of band III in LH8 extracts. This Mg^{2+} dependence is more pronounced when spermidine is not included in the nuclear extract preparation (data not shown). The formation of bands I and II was not dependent on Mg^{2+}, as can be seen in the same lanes. We also tested nuclear extracts from F9 cells treated with retinoic acid, which induces morphological differentiation and concomitant expression of MHC class I genes at low levels (18, 29). No discernible difference was noted in the gel mobility shift pattern of F9 cells treated with retinoic acid for up to 7 days (data not shown).

To map the boundaries of binding domains more precisely, we performed competition experiments with a large number of duplex oligonucleotides corresponding to regions I, II, and III, which were different in length (Table 1). These tests allowed us to delineate DNA sequences necessary for overall protein binding for each of the three regions. Because of its high sensitivity, this general method may be used to approximate protein-binding domains not only for major bands but for minor ones as well, whose binding sites may be difficult to analyze in various footprint techniques (7, 24, 25, 27). The left boundary of region III was assigned to be −189, since truncation of a nucleotide to −188 resulted in a complete loss of binding (Table 1). Similarly, the right boundary was assigned to be −161, because shortening the

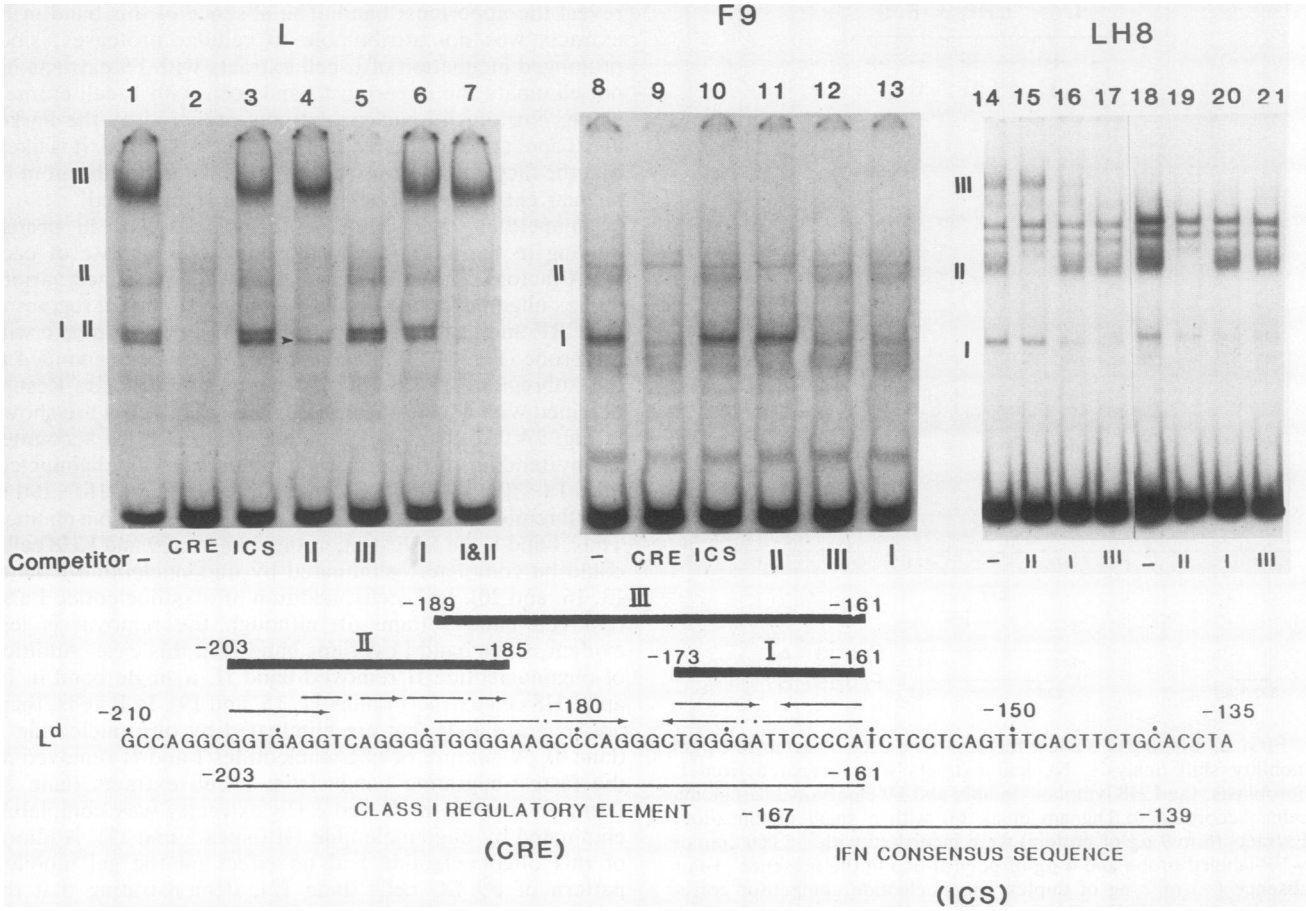

FIG. 2. (A) Identification of protein-binding sequences within the CRE by competition assays. Binding reactions were performed with nuclear extracts from L cells (lanes 1 to 7), F9 (lanes 8 to 13), and LH8 (lanes 14 to 21), as described in the legend to Fig. 1 in the absence (−) or presence of duplex oligonucleotide competitors. Competitor CRE spanned from −203 to −161, and competitor ICS spanned from −167 to −139. Oligonucleotide I used in these experiments extended from −175 to −160, oligonucleotide II extended from −203 to −183, and oligonucleotide III extended from −190 to −160. Oligonucleotides were added at 50- to 100-fold molar excess (~2 ng). In lanes 18 to 21, binding reaction was done without $MgCl_2$. (B) Three protein-binding regions in the CRE of the *H-2L*^{d} gene. Regions I, II, and III were determined by competition assays shown in panel A and Table 1, and are indicated by solid bars. Inverted repeats and tandem repeats are shown by arrows.

oligonucleotide to −162 at the right end lost the competitive activity. Region III as defined represents a single binding unit, since neither the sequence overlapping region I alone (Fig. 2) nor the nonoverlapping sequence from −190 to −173 (Table 1) was effective as a competitor. The boundaries of region I and II binding were assessed in a similar fashion (Table 1). The final assignment of the minimum sequence requirement for each region is illustrated in Fig. 2B.

Methylation interference determination of protein-binding sites. While the above assignment provides information on overall binding domains, it does not reveal actual protein contact points. To study the site of protein binding in more detail, methylation interference experiments were performed. In this method, binding sites are detectable because methylation of guanine residues interferes with protein binding (22, 24). The DNA probe was labeled with γ-^{32}P at either 5′ end with a kinase reaction and partially methylated with dimethyl sulfate (17, 24). The labeled and methylated probe was incubated with LH8 nuclear extracts, and probes complexed with each of the three factors were separated from the free probe in gel mobility shift as described above. For regions I and III, a probe with radioactivity of ~3.5 × 10^4 cpm was reacted with 220 μg of nuclear extracts. For region II, a probe with ~4 × 10^5 cpm was mixed with 80 μg of extract protein prepared by heparin-Sepharose column fractionations. Free and complexed probes were cut out of the gel, transferred onto DEAE-paper, and eluted. The probes were cleaved with piperidine (17, 24), and equal amounts of radioactive materials were resolved in a sequencing gel containing 8% acrylamide and 7.5 M urea. Results are shown in Fig. 3. For region I, four consecutive G residues from positions −172 to −169 in the coding sequence and those from −165 to −162 of the noncoding region showed clear interference (arrowheads), indicating that these residues are involved in the binding of protein(s). The results are consistent with those reported by Baldwin and Sharp (1) obtained from human HeLa cell extracts as well as those by Israel et al. (11), who tested murine 3T6 cells. In region II, the G residues from −200 to −191 of either the coding or noncoding sequence showed interference. No interference was seen in G residues present in the distal parts of region II. These results suggest that protein binding occurs at the central part of region II. As for region III, no interference was observed in the central part of the sequence where the

TABLE 1. Assessment of left and right boundaries of factor binding[a]

Length of duplex oligonucleotide		Competition in region:		
From	To	I	II	III
−175	−163	−		
−175	−162	+/−		
−175	−161	+		
−172	−159	−		
−173	−161	+		
−203	−185		+	
−203	−186		−	
−203	−187		−	
−202	−183		+/−	
−200	−183		−	
−189	−161			+
−189	−160			+
−189	−162			−
−188	−161			−
−190	−173			−

[a] Competition experiments were performed in a gel mobility shift assay by using various lengths of duplex oligonucleotides corresponding to the three regions seen in Fig. 2A. Competitors were added at approximately 20-fold molar excess (0.5 to 1 ng). For region I competition, extracts from F9 EC and LH8 cells were used. Competition for region II and region III binding was tested with extracts from L and LH8 cells. The competition patterns were the same with extracts from different sources of cells.

large inverted repeat meets (from −177 to −173). Rather, strong interference was detected from −189 to −179 and from −173 to −162, which are both within the inverted repeat. A very dense band was observed at residue −178 of the coding and noncoding strands (A and T). A few additional dense bands were observed in protein-bound DNA of region III, the significance of which is not clear (Fig. 3). The methylation interference pattern from positions −173 to −162, which overlaps region I, was identical to that found with region I binding itself. A summary of these experiments is illustrated in Table 2.

Competition study with mutant oligonucleotides. To further assess the binding sites, mutant duplex oligonucleotides corresponding to each region were prepared which differ by two residues from the native sequences (Table 2). They were tested for the ability to compete with the native probe in a gel mobility shift assay (Table 2). Approximately 50- to 100-fold molar excess of competitors was added to the reaction mixture. For region II, mutants 1 and 2 (M1 and M2), which have nucleotide substitutions in the central portion, failed to compete for the binding, whereas changes in the distal part of region II (M3) retained competition activity. These results point to the importance of the central part of the region II sequence, in agreement with data obtained by methylation interference tests. Competition for region I and III binding was tested with six mutant duplex oligonucleotides, M4 through M9 (Table 2). Binding of a protein(s) to region III was highly dependent on the upstream half of the sequence; M4 and M6, which have alterations in this half, failed to compete for binding. M5 also exhibited only partial competition (Table 2), whereas alterations in the downstream half of the region III that overlaps region I were more capable of retaining competitive activity (M8 and M9), as binding was either unaffected or only partially affected. At higher concentrations, M8 was able to compete completely (Fig. 2). The alterations in the central portion of region III (M7) had no effect on competition activity. These results are fully consistent with the results obtained with methylation interference and suggest a critical role of the upstream half of region III for protein binding. They also suggest a relatively low contribution of the central portion of the region III sequence for protein binding.

Involvement of nearly the entire region I sequence is

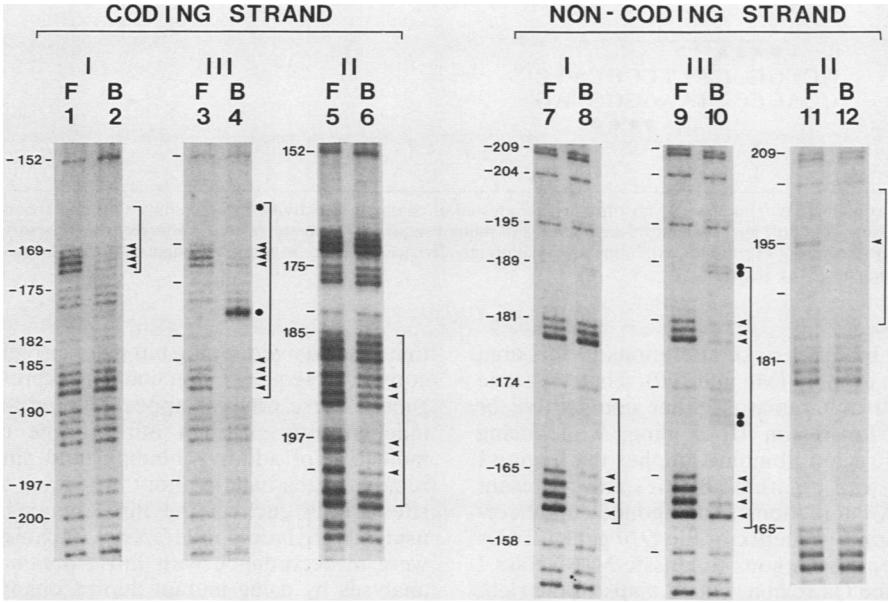

FIG. 3. Assessment of protein-binding sites by methylation interference tests. Coding (left panel) and noncoding (right panel) strands of the probe were end labeled with γ-^{32}P and partially methylated and mixed with LH8 nuclear extracts (for the region II, partially fractionated extracts were used). Probes complexed with proteins were separated from free probes by gel mobility shift. Each band was eluted from the gel and cleaved by piperidine. Methylated guanine residues were visualized in a sequencing gel. F and B stand for free and bound probe, respectively. Positions of methylation interference are indicated by arrowheads. Closed circles indicate dense bands on protein-bound strands.

TABLE 2. Assessment of protein-binding sites by competition analysis using mutant deplex oligonucleotides[a]

Region and mutant	Competitor oligonucleotides	Competition in region: I	II	III
Region II				
	−203 −185			
	AGGCGGTGAGGTCAGGGGTGGGGAA			
Wild type	TCCGCCACTCCAGTCCCCACCCCTT	−	+	−
M1	----------TT----------	−	−	−
	----------AA----------			
M2	----------TT----------	−	−	−
	----------AA----------			
M3	----------TT---	−	+	−
	----------AA---			
Region I and III				
	−189 −161			
	GGTGGGGAAGCCCAGGGCTGGGGATTCCCCAT			
Wild type	CCACCCCTTCGGGTCCCGACCCCTAAGGGGTA	+	−	+
	−173 −161			
M4	----TT----------	+	−	−
	----AA----------			
M5	------TC----------	+	−	+/−
	------AG----------			
M6	----------TT----------	+	−	−
	----------AA----------			
M7	----------TT----------	+	−	+
	----------CC----------			
M8	----------GG----------	−	−	+/−
	----------GG--			
M9	----------CC--	−	−	+
Region I				
	−173 −161			
	GCTGGGGATTCCCCATC			
Wild type	CGACCCCTAAGGGGTAG	+	−	−
	----A---C---------			
Q10	----T---G---------	−	−	−

[a] Mutant oligonucleotides containing two nucleotide substitutions were tested or competition activity in gel mobility shift experiments by using LH8 extracts. About 20- to 100-fold molar excess (0.5 to 2 ng) competitors were used. For mutant sequences, only the residues different from the native ones are shown. Closed circles on the native sequences represent G residues exhibiting methylation interference. A large excess of M6 oligonucleotide did not change the outcome, but excess M9 showed partial competition (Fig. 2A).

indicated for region I binding, since alterations in this area abolished competition activity (M8 and M9). That the same amount of M8 or M9 mutants was either competitive or partially competitive for region III binding, while being unable to compete for region I binding, implies that region I and III proteins bind with different affinities and represent complex protein-DNA interactions. An oligonucleotide corresponding to the region I sequence of the *Q10* gene (2) was also tested. This gene, homologous to classic MHC class I genes, is encoded in the Qa region, which maps to the right of the MHC and is expressed only in liver (2, 14). The *Q10* gene has two nucleotide substitutions at positions −167 to −171 and does not elicit an enhancer activity in fibroblasts (12). The corresponding oligonucleotides (Table 2) did not have competitive activity at all for region I binding.

We identified at least three independent nuclear factors that bind to overlapping but distinct regions of the CRE, the conserved sequence that controls expression of MHC class I genes. These proteins appear to bind to discrete sequences independently of each other, since there are no bands indicative of additive binding, and since each competitor removes each band without producing a new band. Binding sites within each of the three regions were examined by using methylation interference techniques. Data obtained were in accordance with those obtained with competition analyses by using mutant duplex oligonucleotides. Binding of a protein(s) to the regions I in L, LH8, and F9 EC cell extracts was identical in competition assays and most likely represents the same factor binding as that reported by Baldwin and Sharp (1) and Israel et al. (11). Those researchers studied factor binding with the *H-2K*^{b} gene (the CRE of the *H-2L*^{d} and *H-2K*^{b} are identical in sequence, except for

one nucleotide at position −202). The methylation interference pattern for region I revealed by using LH8 extracts was also the same as that reported by these researchers, who used HeLa cells and 3T3 cells. This factor is present not only in fibroblasts and lymphocytes but in F9 EC cells in which the CRE acts as a negative element (18). Thus, it seems unlikely that protein binding to region I by itself exerts an enhancer activity (11, 12). It is, however, possible that factor binding to region I by itself has some relevance to the negative regulation of MHC class I genes; very recently Le Bouteiller et al. found that human cells negative for the expression of HLA class I genes contain a nuclear factor capable of binding to a CRE homolog of the HLA-A2 sequence and that the amounts of the binding factor are greater in these cells than in cells expressing high levels of HLA class I genes (personal communication).

We describe two additional factors that bind to regions II and III. To our knowledge, factor binding to these regions has not been described before (1, 11). The previous failure to detect these factors is presumably caused by differences in binding conditions and the use of different probes.

Region II binding most likely occurs through the core sequence −200 GAGGTCAGGG −190, as judged from the methylation interference data, and competition results with mutant sequences (Table 2). It is clear, however, that overall protein binding requires an additional few nucleotides on both ends (Table 1). Region II elicits multiple bands with L-cell extracts but a single band in LH8 and F9 EC cells. Thus, region II binding may involve multiple factors, or it may undergo secondary modifications. In preliminary experiments, we found that deletion of a sequence encompassing region II results in partial reduction of both positive and negative effects of the CRE (data not shown), suggesting a functional role for region II.

Region III represents a relatively long stretch (29 base pairs) of DNA for protein binding (Table 1, sequence requirement). This length is, however, not unusual for protein-DNA interactions; regulatory sequences that bind proteins range from an octamer to sequences longer than 40 base pairs (4, 13, 15, 19, 22, 23, 27). It is clear, though, that the central portion of region III, from −178 to −174, is not essential for protein binding, as evidenced by the lack of methylation interference and the retention of competition activity after nucleotide substitution in this area. It may be envisaged that the left and the right halves of region III are independently involved in the binding of a protein(s). The proteins for each half may be distinct, since the symmetry represented by the large inverted repeat is not a prerequisite for region III binding (see competition by M9). Alternatively, each half of region III may bind the same protein(s). The hypothetical proteins for the left and right halves are expected to interact with each other and are dependent on each other for binding, since neither the left nor the right half of region III itself is sufficient for protein binding (Table 1 and 2).

It is tempting to postulate that region III binding is relevant to the functional elicitation of the CRE, since this is the only binding that shows cell type specificity. Functional significance of the protein binding to region III regions defined in this work is being assessed by site-directed mutagenesis using mutant oligonucleotides in Table 2.

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