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Two upstream elements activate transcription of a major histocompatibility complex class I gene *in vitro*

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ABSTRACT

Expression of major histocompatibility complex (MHC) class I genes exhibits unique tissue and developmental specificity. In an effort to study molecular mechanisms of MHC class I gene regulation, an *in vitro* transcription system has been established. In B cell nuclear extracts a template DNA containing the mouse H-2L^d promoter sequence accurately directed RNA polymerase II-dependent transcription of a G-free cassette. A conserved class I regulatory complex previously shown to moderately enhance promoter activity *in vivo* enhanced transcription *in vitro* by 2–3 fold. Much of this enhancement was accounted for by a 40 bp fragment within the complex, which was capable of activating a basal H-2L^d promoter in either orientation. Farther downstream, another element called site B was identified, which independently activated MHC class I transcription *in vitro* by 2–4 fold. Site B bound a specific nuclear factor(s) through an NF-1 binding site but not through a neighboring CCAAT site. The functional significance of site B *in vivo* was demonstrated in transfection experiments in which site B enhanced MHC class I promoter activity to a degree comparable to that seen *in vitro*. With the identification of the two upstream activators, MHC class I genes may serve as a model to study roles of sequence-specific DNA-binding proteins in transcription *in vitro*.

INTRODUCTION

Major histocompatibilty complex (MHC) class I genes encode a series of membrane glycoproteins that play a central role in immune responses. MHC class I genes are broadly expressed in adult somatic tissues with the exception of the central nervous system (1). MHC class I gene expression is developmentally controlled and is induced in many types of cells by cytokines such as interferons and TNFα. Previous studies of the transcriptional regulation of MHC class I genes have led to the identification of the class I regulatory complex (1–5). This complex, which includes the previously described class I regulatory element (6), resides between −203 and −139 relative to the transcriptional start site, and is conserved among classical MHC class I genes (Fig. 1A, B). The two distal elements in this complex (regions I and II in Fig. 1B) both constitutively enhance transcription of class I genes in various cell types (1–5, 7). Also present in this complex are the interferon consensus sequence (ICS) that is responsible for interferon induction (2, 6, 8) and the negative regulatory element that is required for transcriptional repression of MHC class I genes in embryonal carcinoma cells (5, 9). Studies with transgenic mouse lines indicate that the class I regulatory complex accounts for much of the tissue and developmental specificity of MHC class I gene expression *in vivo* (10). The β-2 microglobulin gene, expressed coordinately with MHC class I genes, also possesses region I and the ICS (3, 4).

Recently, factors that bind to elements in this complex have been cloned (11–18). For example, region I has been shown to bind two distinct series of cloned DNA binding protein families, the c-*rel* oncogene family and a C_2H_2 zinc finger family. Region II binds H-2RIIBP, a member of the nuclear hormone receptor superfamily (13). The ICS binds to factors of the IRF family (16–18). In addition, AP-2 reportedly binds a sequence in this complex (3).

In vitro transcription has served as a valuable means for studying mechanisms of transcriptional regulation of many cellular genes (see for example 20–25). Regulatory elements and binding factors that confer tissue-specific transcription of cellular genes, such as α-globin (25), immunoglobulin (26), and albumin (27) genes, have been studied using *in vitro* transcription assays. Furthermore, purified endogenous and cloned recombinant steroid hormone receptors have been shown to activate transcription of target genes *in vitro* via the respective hormone response elements, establishing *in vitro* models for addressing hormone-mediated gene regulation (28–34).

With the availability of cloned factors that bind to well-defined elements, *in vitro* transcription may also serve as a useful model to analyze the mechanism of transcription of MHC class I genes. With this goal in mind, we have undertaken the initial

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characterization of *cis* elements controlling transcription of MHC class I genes *in vitro*. In this work we have employed the G-free cassette method (35), which has been successfully used for transcription of many cellular and viral genes (see for example 27, 36, 37). This method has proven to be essential for the analysis of MHC class I genes, whose promoter activity is moderate, and which therefore required a sensitive method for detection of transcripts. We show that two conserved upstream regions independently function as transcriptional activators *in vitro*. One region resides within the class I regulatory complex mentioned above. Another region, called site B, is located adjacent to the basal promoter. Further, we present evidence that site B is an NF-1 binding site, and that it is functional *in vivo*.

MATERIALS AND METHODS

DNA Templates

The plasmid pML$(C_2AT)_{19}$ was generously provided by R. Roeder. Various segments of the promoter region of the murine H-2L^d gene were fused to the G-free cassette (35) by overlap amplification as described by Horton et al. (38, Fig. 1). Xba I sites were included in outlying amplification primers to facilitate subcloning into pBS(+) (Stratagene). Amplification reactions were performed for 20 cycles with 100–200 ng substrate DNAs. The nucleotide sequences of all constructs were confirmed by the Sanger dideoxy method. Plasmids pCRC-60GF and p$(CRC)_2$-60GF were constructed by insertion of PCR amplified CRC sequence into the Bgl II site of pL^{d}60GF. The internal control plasmid pML283 was constructed by overlap amplification of the adenovirus 2 major late promoter (Ad2 MLP), and the first 283 bp of the G-free cassette followed by subcloning into pBS(+). All plasmid templates were purified by two steps of sedimentation in cesium chloride density gradients and were greater than 95% supercoiled.

Cell Culture and Nuclear Extract Preparation

The human B-cell line Namalwa was grown in RPMI 1640 with glutamine, gentamicin, and 7.5% fetal calf serum. Exponentially growing cells were harvested, and nuclear extracts were prepared by the Dignam method (39) with the following modifications. Nuclei from lysed cells were pelleted by centrifugation at 10,000 rpm for 30 seconds in a Sorvall HB-4 rotor before extraction. Prior to dialysis extracts were concentrated by precipitation with 0.44 g/ml $(NH_4)_2SO_4$.

In Vitro Transcription Reactions

Reactions were performed essentially as described by Sawadogo et al. (35). Standard reaction mixes contained 85 mM KCl, 20 mM Hepes (pH 7.9), 6 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0.6 mM ATP, 0.6 mM CTP, 0.25 mM 3'-O -methyl GTP, 0.025 mM UTP, 10 μCi of [α-^{32}P] UTP, 40 U RNasin (Promega), 45 U RNase T1 (BRL), and the appropriate amount of supercoiled plasmid DNA template. Where indicated, α-amanitin (Sigma) was added to the reaction mixture at a final concentration of 0.5 μg/ml. Reactions were carried out at 30°C for 45 min. and terminated by addition of 280 μl of stop mix containing 50 mM Tris-HCl (pH 7.5), 1% SDS, 5 mM EDTA, 20 μg/ml tRNA, and 200 μg/ml protease K and incubation at 37°C for 30 min. The mixture was then extracted with 300 μl of 1:1 phenol-chloroform (equilibrated with 100 mM NaCl, 10 mM sodium acetate pH 5.2, 1 mM EDTA) and precipitated with one-tenth volume 3M sodium acetate (pH 5.2) and 2.5 volumes of ethanol. Pellets were washed with 70% ethanol and dried *in vacuo*. RNA was then resuspended in formamide denaturing dye and heated to 95°C for 5 min. prior to electrophoresis on a 4% polyacrylamide-8M urea gel. Radioactive bands were visualized by autoradiography and counts were determined by scanning on an Ambis Radioanalytic Imaging System. Relative transcriptional activities were determined by dividing test transcripts counts by the corresponding internal control transcript counts. These values

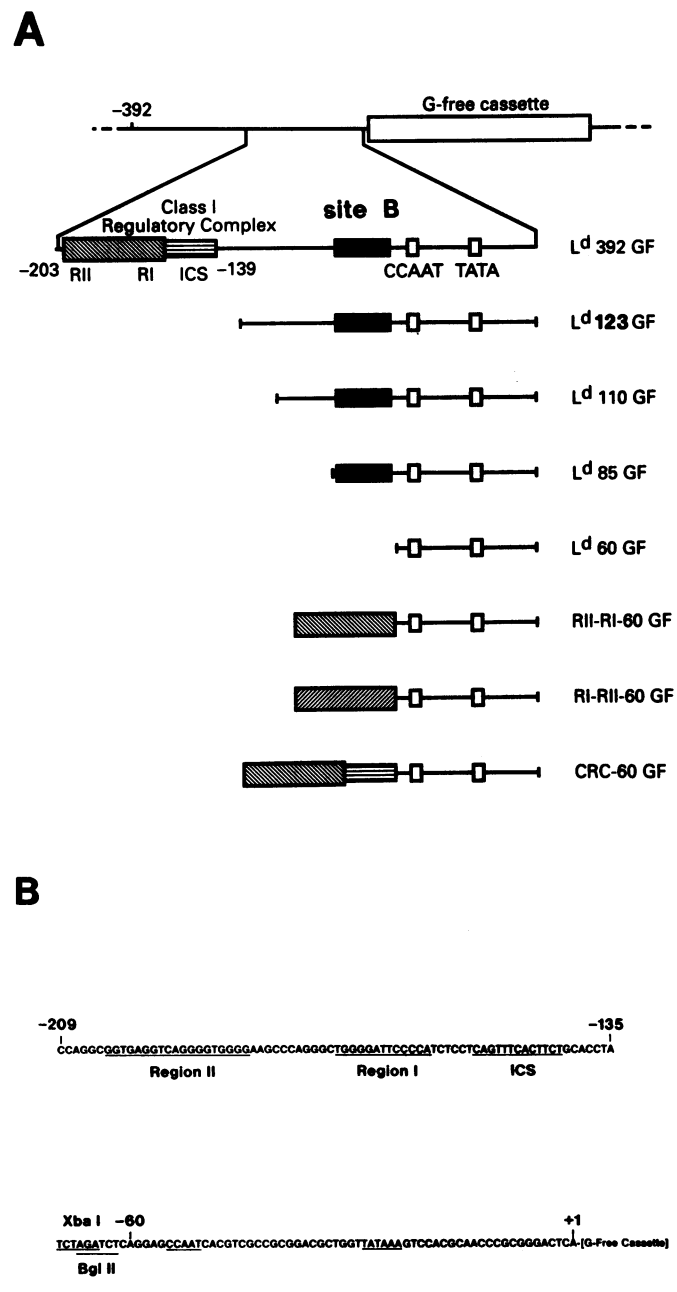

Fig. 1. (A) Schematic representation of H-2L^d-G-free cassette templates. The number indicates nucleotide length of the L^d promoter beginning at +1 fused to a 377 bp G-free cassette. Each construct contains a TATA box at −28 and a CCAAT box at −54. Positions of site B, region I (RI), and region II (RII) are indicated. The class I regulatory complex (CRC), which is present in L^{d}392GF, consists of RII, RI, and the ICS. (B) The nucleotide sequence of the class I regulatory complex (upper). The L^d basal promoter region present in L^{d}60GF (lower). The CCAAT and TATA boxes are underlined.

were then normalized to the value obtained for transcription of the basal promoter template pL^{d}60GF under the same conditions.

Gel Mobility Shift and Methylation Interference Analysis

Gel mobility-shift analyses were performed essentially as described before (6). Duplex oligonucleotide probes were labeled with [^{32}-P]ATP and T4 polynucleotide kinase. Binding reactions were performed in a volume of 12 μl and contained 20 mM Tris·HCl (pH 7.5), 50 mM KCl, 1 mM EDTA, 1mM DTT, 5% (vol/vol) glycerol, 2 μg poly(dI·dC) (Pharmacia), 5 to 10 μg nuclear protein, and 2 fmol end-labeled probe. Binding was performed for 30 min. at room temperature and protein-DNA complexes were resolved in 5% 1× TBE polyacrylamide gels. Gels were run at 7 V cm^{-1} for 2.5 hours, dried, and autoradiographed overnight. For methylation interference analyses end-labeled duplex oligonucleotides were methylated and bound to nuclear proteins in preparative gel shifts. Radioactive DNA from shifted bands was eluted overnight in TE buffer at 4°C. DNA was precipitated on ice by addition of 4μg poly(dI·dC), 100μM hexadecyltrimethylammonium bromide (C-TAB), and 15mM $(NH_4)_2SO_4$. Precipitated DNA was pelleted by centrifugation, and precipitated counts were recovered by addition of TE buffer and phenol extraction. DNA was precipitated, dried, cleaved with piperidine, and electrophoresed on a 20% polyacrylamide-8 M urea gel.

Transient Transfection Assays

Ltk$^-$ fibroblasts were transfected with 6μg of MHC CAT plasmids and 0.5μg pRSVluc using the calcium phosphate coprecipitation method (41) and cells were harvested 44 hours after transfection (42). CAT activity was normalized for transfection efficiency by luciferase or β-galactosidase activity. For transfection of Namalwa B cells, 1×10^7 cells were incubated with 10μg of MHC class I CAT reporter plasmids and 100μg DEAE dextran in 1 ml serum-free medium for 2 hrs. Four μg of pCMV-lacZ was also added in some transfection experiments. Cells were washed and cultured for an additional 36 hrs. Ltk$^-$ fibroblasts were transfected with the above MHC class I reporters and 0.5μg of pRSVluc (40) using the calcium phosphate co-precipitation method (41), and were harvested 44 hrs after transfection. CAT activity was normalized for transfection efficiency by activities of β-galactosidase for Namalwa or luciferase for fibroblasts.

RESULTS

In Vitro Transcription of a G-Free Cassette Driven by an MHC Class I Promoter

In vitro transcription was carried out using G-free cassettes fused to upstream regions of the mouse H-2L^d gene (Fig. 1A, B). Nuclear extracts prepared from B lymphocytes which express high levels of MHC class I genes were routinely used. The G-free cassette procedure gave lower background levels and higher

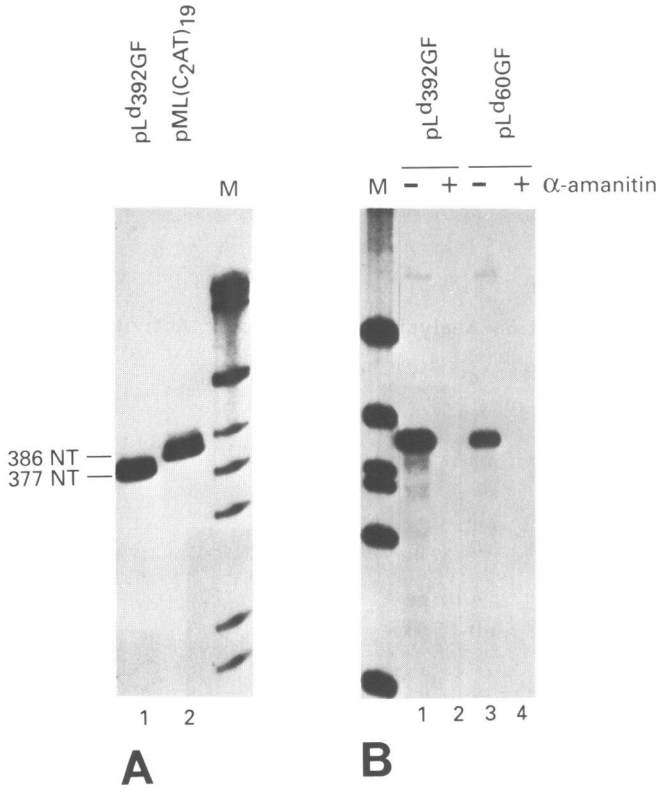

Fig. 2. *In vitro* transcription products of pL^{d}392GF. (A) 2 μg of pL^{d}392GF (lane 1) or the control pML(C$_2$AT)$_{19}$ (lane 2) were transcribed in B cell nuclear extracts under standard reaction conditions (Materials and Methods). Lane M contains labeled DNA size markers. Sizes of markers are (from bottom) 201, 220, 298, 344, 396, 506, 517, 1636, and 2036 nucleotides. (B) α-Amanitin sensitivity of *in vitro* transcription reactions. Reactions were performed with 2 μg of pL^{d}392GF lanes (1 and 2) or pL^{d}60GF (lanes 3 and 4) in the absense (lanes 1 and 3) or presence (lanes 2 and 4) of 0.5 μg/ml α-amanitin.

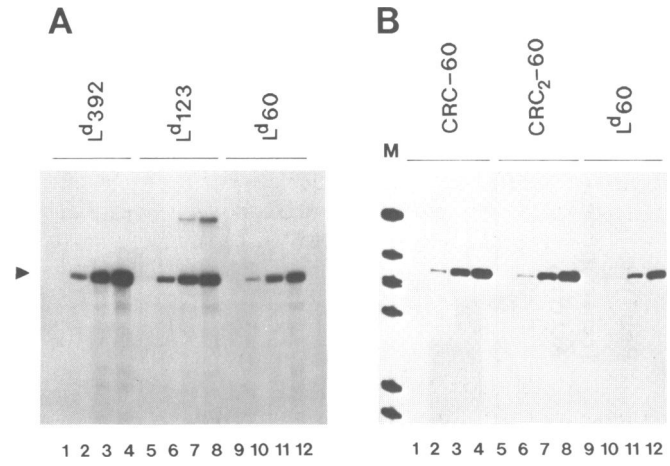

Fig. 3. The class I regulatory complex activates transcription *in vitro*. (A) *In vitro* transcription reactions were carried out with pL^{d}392GF (lanes 1–4), pL^{d}123GF (lanes 5–8), and pL^{d}60GF (lanes 9–12) using increasing template concentrations; 25 μg/ml (lanes 1, 5, and 9), 50 μg/ml (lanes 2, 6, and 10), 75 μg/ml (lanes 3, 7, and 11), or 100 μg/ml (lanes 4, 8, and 12). Quantitation of radioactivity in bands was performed on an Ambis radioanalytic imaging system. Levels of transcripts from pL^{d}392GF were from 2.8- to 4.1-fold greater than those from pL^{d}60GF, while pL^{d}123GF gave 1.4- to 3.0-fold greater levels than pL^{d}60GF. (B) Reactions were carried out as above with pCRC-60GF (lanes 1–4), p(CRC)$_2$-60GF (lanes 5–8), and pL^{d}60GF (lanes 9–12); using increasing template concentrations of 25 μg/ml (lanes 1, 5, and 9), 50 μg/ml (lanes 2, 6, and 10), 75 μg/ml (lanes 3, 7, and 11), or 100 μg/ml (lanes 4, 8, and 12). pCRC-60GF gave 1.8- to 3.7-fold higher levels of transcripts than pL^{d}60GF, while p(CRC)$_2$-60GF gave 1.5- to 3.5-fold higher levels of transcripts than pL^{d}60GF.

sensitivity than run-off transcription and RNase protection assays (not shown). The plasmid pL^{d}392GF contains the entire class I regulatory complex as well as site B, a CCAAT element at position −54, and a TATA box at −28 (Fig 1A). When pL^{d}392GF was added to a reaction mixture a correctly initiated transcript of 377 nt was observed (Fig. 2A, lane 1) This band was distinct from the 386 nt transcript (lane 2) produced from the control plasmid pML$(C_2AT)_{19}$ in which the G-free cassette is fused to the Ad 2 MLP (35). As expected, pL^{d}60GF also produced a 377 nt transcript. Transcription was mediated by RNA polymerase II since addition of α-amanitin (0.5 μg/ml) abolished transcription from both pL^{d}392GF and pL^{d}60GF (Fig. 2B). In these experiments the level of transcription was higher with pL^{d}392GF than with the basal promoter template, pL^{d}60GF, indicating that transcription enhancing elements are present between −392 and −60 (see below). We have reproducibly observed this enhancing effect at protein concentrations ranging from 2.0−10.0 μg/ml.

The region between −139 to −203, the class I regulatory complex, contains two moderately active enhancers, regions I and II (see Fig. 1B). To test whether this region acts as a transcriptional activator *in vitro* and to determine whether a less well characterized downstream region (−123 through −60) is functional in the *in vitro* transcription system we examined three successively truncated promoter constructs. Results obtained with increasing concentrations of template DNA are shown in Fig. 3. Transcription of pL^{d}392GF was 2- to 3-fold higher than that of pL^{d}123GF at higher template concentrations (compare lanes 1−4 with lanes 5−8). Further, transcription from pL^{d}123GF was 2-fold higher than that from the basal promoter construct pL^{d}60GF at the same template concentrations (compare lanes 5−8 with lanes 9−12). These results indicate that the promoter containing the class I regulatory complex enhances transcription *in vitro* as observed *in vivo* (1, 2, 4, 5). The data also show that there is an additional moderate transcriptional activator located between −123 and −60. The level of activation conferred by

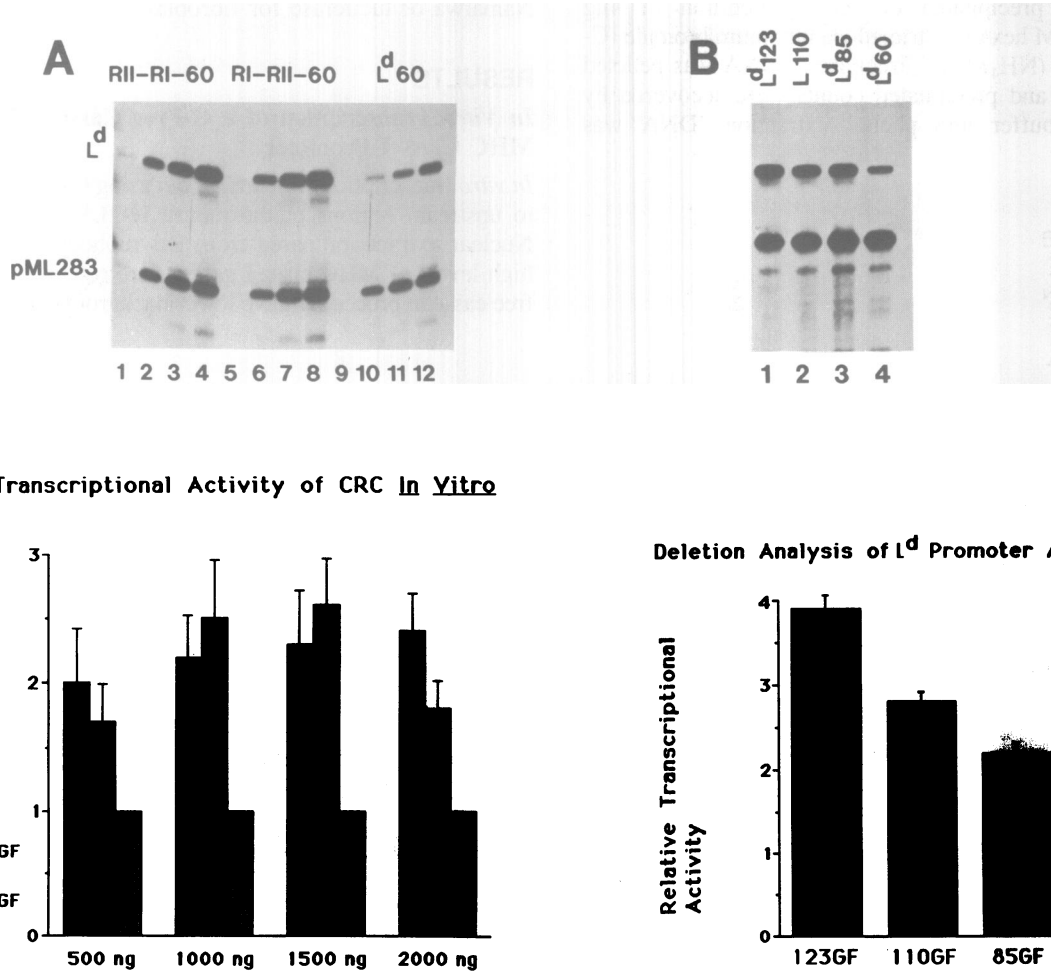

Fig. 4. (A) Mapping of transcription activating elements in the L^d promoter. In the upper panel transcription reactions were performed with pRII-RI-60GF (lanes 1−4), pRI-RII-60GF (lanes 5−8), and pL^{d}60GF (lanes 9−12), using increasing amounts of template ; 500 ng (lanes 1, 5, and 9), 1000 ng (lanes 2, 6, and 10), 1500 ng (lanes 3, 7, and 11), and 2000 ng (lanes 4, 8, and 12). Each transcription reaction also contained 300 ng of the internal control plasmid pML283GF. Quantitation was performed on an Ambis radioanalytic imaging system (lower panel). Bars represent relative transcriptional activities obtained for the indicated amounts (ng) of each test template ± standard errors. Transcriptional activities were normalized relative to the level of transcription obtained with pL^{d}60GF. Values represent averages of three independent experiments. (B) The upper panel shows mapping of transcription activating elements present between −123 and −60 in the L^d promoter. 1 μg of test templates pL^{d}123GF (lane 1), pL^{d}110GF (lane 2), pL^{d}85GF (lane 3), or pL^{d}60GF (lane 4) was transcribed under standard conditions. Each reaction contained 400 ng of the internal control plasmid pML283GF. The lower panel shows the relative transcriptional activities of each template. Values were normalized relative to the transcription level obtained with pL^{d}60GF. Bars represent means of three independent experiments ± standard error.

the class I regulatory complex was somewhat variable from extract to extract. Transcription reactions performed at relatively high protein concentrations (>6 μg/ml) gave greater enhancement. The enhancement produced by pL^{d}123GF was seen over a wide range of protein and template concentrations (not shown). The above results demonstrate that at least two distinct activating elements are present in pL^{d}392GF which are active in the *in vitro* transcription system.

The conserved upstream Class I Regulatory Complex acts as an enhancer during transcription *in vitro*

The region from −203 through −139 is well conserved in MHC class I genes; most classical MHC class I alleles in the mouse have essentially identical nucleotide sequences in this region. In order to determine whether this region enhances transcription *in vitro* in the absence of site B, we constructed plasmid templates in which the class I regulatory complex is fused to pL^{d}60GF. Fig. 3, panel B shows that over a range of four different template concentrations transcription of pCRC-60GF containing the class I regulatory complex was approximately 2-fold higher than that of pL^{d}60GF (compare lanes 1−4 and lanes 9−12). A construct containing a CRC dimer, p(CRC)$_2$-60GF, gave essentially the same level of transcription as the monomeric pCRC-60GF (compare lanes 1−4 with lanes 5−8). We also tested constructs containing only regions II and I of the CRC to determine whether these elements alone are sufficient for enhancement of transcription *in vitro* (see fig. 4A). As seen in Fig. 4A (lower panel) the RII-RI sequence enhanced transcription *in vitro* in either orientation. These results suggest that the RII-RI sequence accounts for most, if not all, of the constitutive activity seen for the class I regulatory complex.

Identification of site B that also activates the MHC class I promoter *in vitro*

In order to precisely map the transcription activating element located between −123 and -60 we tested *in vitro* transcription of templates with successive additions of promoter sequence starting from the basal pL^{d}60GF (Fig. 4B). Plasmid pL^{d}85GF, which contains an additional 25 bp gave a greater than 2-fold increase in transcriptional activity over the basal construct. Plasmids pL^{d}110GF and pL^{d}123GF gave additional small increases over pL^{d}85GF, although the relative increases were less than that for pL^{d}85GF over pL^{d}60GF. These results indicate that a sequence located between −85 and −60, which we call site B, functions as an activator *in vitro*. The results also suggest the presence of an additional activator element located between −85 and −123. Because the largest transcriptional effect was seen for site B, and because this region contains an inverse CCAAT sequence (table 1), we further investigated this region. The sequence from −85 to −60 of the H-2L^d gene differs from

Table 1. Summary of Competition Analysis for Site B Binding

Gene	Oligonucleotide Sequence	Competition	Ref.
H-2L^d	-87 TGTCACTATCATTGGG TGGCGAGATTCCA -56 GGA ACAGTGATAGTAACCC ACCGCTCTAAGGT CCT	+++	4
M1	-------CGAC----------	+++	
M2	----------GGTT----------	+++	
M3	----------TGTT----------	+	
H-2K^b	-85 TCTTACCCCCATTGGGTGGCGCGA -62	±	4
HLA-A2	TCTCACTCCCATTGGGTGTCGGGT	-	64
L^d -54 CCAAT	(-39) CCGGCGACGTGATTGGCTCCTGGAG (-63)	+	
DRα Y Box	(-81) TATTTTTCTGATTGGCCAAAGAGTA (-57)	+	65
hu α-globin	(-62) CGCTCATTGGCTGGCGCGGAGCC (-84)	+	66
Ad2 MLP	(-85) AGGTGATTGGTTTATAGGTGTAG (-63)	-	67
HSV tk	(-96) GCGTCTTGTCATTGGCGAATTCGG (-73)	++	68
NF-kB	CTCAACAGAGGGGACTTTCCGAGAGGCCAT	-	69
NF-1	(+22) TTTTGGCTTGAAGCCAATATG (+42)	+++	70

Numbers refer to nucleotide positions relative to transcriptional start sites. +++, competition observed at fold molar excess, ++, competition at 1000-fold molar excess, +, competition at 1000-fold molar excess, partial competition at 1000-fold molar excess, −, no competition at 1000-fold molar excess. CCAAT sequences are underlined and the NF-1 site is boxed.

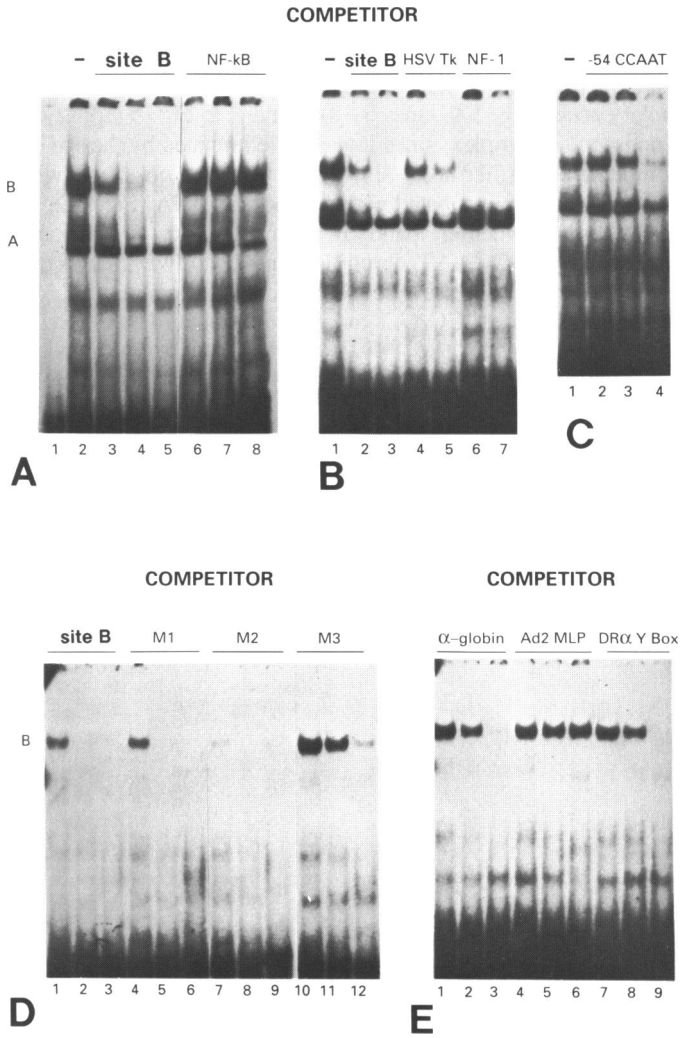

Fig. 5. Competition analysis of site B factor binding. Gel mobility shift experiments were performed with 2 fmol of ^{32}P-labeled site B oligomers and Namalwa B cell nuclear extracts. Competitor oligomers were added together with the labeled probe in A, B, and C, whereas competitor oligomers were added 15 min prior to the addition of the labeled probe in D and E. (A) Molar ratios of competitor to probe were 10:1 (lanes 3 and 6), 50:1 (lanes 4 and 7), or 100:1 (lanes 5 and 8). (B) Molar ratios were 10:1 (lanes 2, 4, and 6) or 100:1 (lanes 3, 5, and 7). (C) Molar ratios were 10:1 (lane 2), 100:1 (lane 3), or 1000:1 (lane 4). (D) Molar ratios of competitor to probe were 10:1 (lanes 1, 4, 7, and 10), 100:1 (lanes 2, 5, 8, and 11), or 1000:1 (lanes 3, 6, 9, and 12). (E) Molar ratios were 10:1 (lanes 1, 4, and 7), 100:1 (lanes 2, 5, and 8), or 1000:1 (lanes 3, 6, and 9).

that of the H-2K^b and other MHC class I genes such as HLA-A2: the sequence immediately upstream from the inverse CCAAT box shows allelic variability (see Table 1 for the K^b and HLA-A2 sequences). To assess whether this region is transcriptionally active regardless of allelic differences, the corresponding sequence in the K^b gene was fused to the basal pL^{d}60GF construct and its activity in *in vitro* transcription was assessed. Similar to pL^{d}85GF, transcriptional activity seen for the K^b construct was about twice that of pL^{d}60GF, indicating that the activating function of this region is not limited to a sequence specific for the L^d gene (not shown).

Nuclear factor binding to site B

We then examined nuclear factor binding to site B by gel mobility shift experiments. A labeled probe corresponding to site B produced two prominent bands, one of which (B in Fig. 5) was competed by addition of unlabeled site B oligonucleotides at 10- to 50-fold molar excess (Fig. 5A, lanes 2–5). Band B was not competed by an unrelated sequence, NF-*k*B, even at a 100-fold molar excess (Fig. 5A, lanes 6–8). Band A in Fig. 5 represented a nonspecific complex, since it was not eliminated by the specific competitor. Furthermore, this band was not observed when extracts were pre-incubated with nonspecific DNA (Fig. 5D, E). To examine whether band B is related to a previously characterized CCAAT binding activity, we performed competition analyses using sequences known to contain CCAAT factor binding sites. CCAAT motifs from human HLA-DRα Y-Box, human α-globin, Ad2 MLP, and HSVtk all competed poorly (see Table 1 for a summary of the binding data). With the HSVtk sequence competition was observed at molar ratios greater than 100-fold (Fig. 5B; lanes 4 and 5), while the HLA-DRα, human α-globin, and L^d-54 CCAAT sequences competed only at a 1000-fold molar ratio (Fig. 5E; lanes 7–9 and 1–3, and Fig. 5C; lanes 2–4, respectively). The Ad2 MLP sequence failed to compete even at a 1000-fold molar ratio (Fig. 5E; lanes 4–6). The H-2K^b and HLA-A2 sequences, both of which contain inverted CCAAT sequences but are devoid of sequences from −63 to −56, failed to compete for the specific factor even when present at a 1000-fold molar excess (data not shown). To further assess the sequence responsible for complex formation, three mutant constructs were examined (Fig. 5, panel D). Changing nucleotides −80 through −77 (M1; lanes 4–6) or nucleotides −76 through −73 (M2; lanes 7–9) did not affect the ability to compete for the specific factor. However, the mutations introduced from −72 through −69 (M3; lanes 10–12) resulted in an approximately 100-fold reduction in its ability to compete for complex formation (compare in Fig. 5D, lanes 1 and 12). Because the mutant competitor M2 which lacks the CCAA of the CCAAT motif competed efficiently, and since various CCAAT-containing promoter sequences were unable to compete for complex formation, we conclude that the CCAAT element is not directly involved in factor binding. In contrast, a high affinity NF-1 binding site competed for complex formation most efficiently. Competition by the NF-1 site was more efficient than the L^d sequence (Fig. 5B; compare lanes 2 and 3 with lanes 6 and 7).

Figure 6 shows results of methylation interference experiments. Strong interference was detected that centered over a TGG palindrome both in the coding and noncoding strands. However, no interference was observed near or over the inverse CCAAT box. The lack of interference over the CCAAT sequence was consistent with results of competition analyses as shown in Fig. 5, and indicated that the critical site for this binding activity is outside CCAAT. This interference pattern is similar to the patterns reported for sites to which the NF-1 family of transcription factors bind (43, 44). Taken together these results suggest that site B of MHC class I genes binds a member of the NF-1 family or a similar factor.

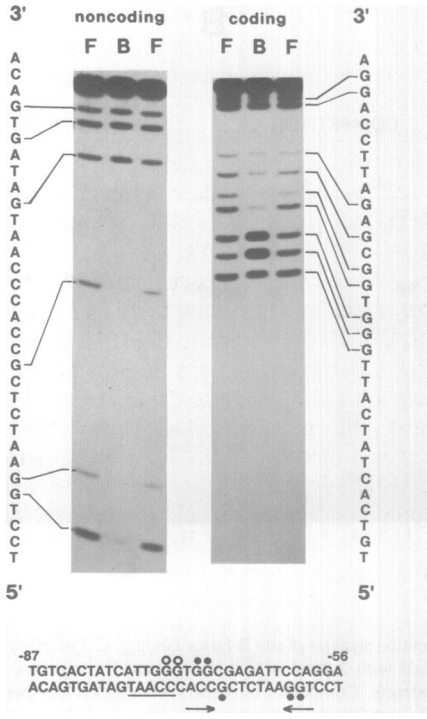

Fig. 6. Methylation interference analysis of factor binding to site B. F and B represent free and bound probes. Filled circles indicate G residues the methylation of which interfered with complex formation. Open circles indicate G residues which when methylated gave increased binding. The inverse CCAAT element present in site B is underlined. Arrows indicate position of the TGG palindrome in the NF-1 binding site.

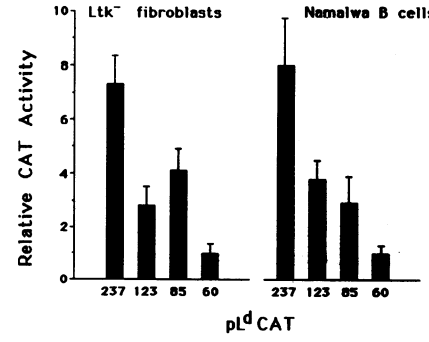

Fig. 7. Site B enhances MHC class I promoter activity *in vivo*. CAT constructs were transfected into Ltk$^-$ cells or Namalwa B cells by the calcium phosphate precipitation method, or DEAE dextran, respectively. Cells were harvested 44 hours after transfection. The right panel represents the results of three independent experiments each of which consisted of three sets of transfections ± standard deviation. A representative autoradiograph is shown in the left panel. Namalwa B cells (1×10^7 cells) or Ltk$^-$ fibroblasts (10^6 cells) were transfected with 6 or 10μg of MHC class I reporter constructs together with pCMV-lacZ or pRSVluc used to adjust for transfection efficiency. CAT activities were expressed relative to the activity produced by the minimal reporter, pL^{d}60CAT. Data represent the average of three independent experiments ± standard deviation.

Site B enhances MHC class I promoter activity *in vivo*

To test whether site B is also active *in vivo*, transfection assays were performed using four reporter constructs in which the CAT gene was fused to 237, 123, 85, or 60 bp upstream of the L^d transcriptional start site. These plasmids were transiently transfected into either Namalwa cells or mouse Ltk^- fibroblasts. Promoter activities of these constructs were normalized relative to the CAT activities produced by the minimal promoter, pL^d 60CAT. Results are summarized in figure 7. In agreement with *in vitro* transcription data (fig. 4), activities produced by pL^d85CAT were about 3- to 4-fold higher in both Namalwa and Ltk^- fibroblasts. In Namalwa cells the activity of pL^d123CAT was slightly higher than that of pL^d85CAT, again consistent with *in vitro* transcription data. In Ltk^- fibroblasts pL^d123CAT exhibited lower CAT activity than pL^d85CAT. There may be an additional element between −123 and −85 that acts in a cell type dependent fashion. Evidence supporting this idea has also been obtained by *in vivo* footprinting of MHC class I genes, and will be presented elsewhere (Dey et al., submitted). As expected, pL^d237CAT which contains the class I regulatory complex exhibited the highest CAT activity in both Namalwa B cells and fibroblasts. These results demonstrate that site B, identified in this work, is functional and enhances MHC class I promoter activity *in vivo*.

DISCUSSION

We show that a MHC class I promoter can accurately direct transcription of a G-free cassette *in vitro*. Under the conditions employed here, approximately 0.02% of pL^d392GF template molecules are transcribed, assuming that all template molecules in the reaction mixture are equally functional. This rate of transcription is comparable to the rates reported for other genes (39, 46, 47). We have identified two separate upstream elements that are involved in the enhancement of transcription *in vitro*. One of them is located from −209 to −160. This region corresponds to regions I and II of the class I regulatory complex that had been shown to elevate MHC class I promoter activity *in vivo* (1, 2, 4, 5). Another sequence, called site B resides within −85 to −60. Site B bound a specific nuclear factor through an NF-1 site. These two regions independently increased the level of transcription by 2−4 fold. The relatively small increase seen with the two regions is consistent with the modest level of enhancer activity noted for MHC class I genes in vivo (1−5). To our knowledge *cis* elements controlling transcription of MHC class I genes *in vitro* have not been reported so far.

In vitro transcription has been a valuable tool for studying the mechanisms and molecular components involved in basal promoter activity (reviewed in 48). With the advent of cloned factors that bind to upstream elements, *in vitro* transcription has also become advantageous for studying roles of sequence-specific DNA binding proteins and upstream activators that confer specificity to transcription of cellular genes (30, 31, 33, 34, 49). In some cases physical contact between a factor binding to an upstream activator and a general transcription factor may be required for transcription activation (50, 51). A similar mechanism may be anticipated for factors binding to upstream regions of MHC class I genes. Such interactions may exhibit promoter specificity since some general transcription factors appear to be specific for some but not all promoters (52). Thus it is important to study the roles of MHC class I upstream elements in the context of their natural promoter. Below we discuss possible roles of factors that bind to the two activator sequences present in MHC class I genes.

The RII-RI fragment from −209 to −160 that confers transcriptional enhancement *in vitro* contains two elements, region I and region II, to which distinct factors bind (2, 6). The region I binding factors are broadly expressed in many cells, but not in cells negative for MHC class I expression (1). The region I sequence is similar to the NF-*k*B binding site of Ig genes. Members of the c-*rel* oncogene family are known to bind region I and NF-*k*B motifs (12, 14). Further, a series of factors that belong to the C_2H_2 zinc finger protein family bind to region I and to the NF-*k*B site (11, 15, 53, 54). Thus, a complex interplay of these molecules may be responsible for enhancer activity elicited by region I. Region II binds H-2RIIBP, a member of the nuclear hormone receptor superfamily, through the GGT-CA motif (13). H-2RIIBP has been shown to be capable of *trans*-activating MHC class I promoters *in vivo* (7).

Another transcriptional activator, site B, is located from −85 to −60. We have observed that site B of the L^d and K^b genes are equally capable of enhancing transcription *in vitro* although the site B sequence in the L^d gene differs slightly from that of many other class I genes including K^b and HLA-A2 (table 1). Thus, the enhancing activity of site B is likely to be common among MHC class I genes. We have also shown that site B is functional *in vivo*, since addition of the site B sequence to the basal MHC class I promoter led to enhanced CAT activity following transfection into both B cells and fibroblasts. Site B may represent part of the previously reported enhancer mapped between −120 and −60, that is reported to be weakly active in fibroblasts (4). An inverse CCAAT sequence is present within site B that was previously shown to bind a CCAAT-binding protein from HeLa cells (45). Gel mobility shift and methylation interference analyses indicated that a nuclear factor(s) specific for site B bound to a palindromic NF-1 site rather than the inverse CCAAT element. Competition data obtained by mutant site B oligomers and various promoter sequences containing CCAAT elements were consistent with these results. Thus, it is unlikely that the factor(s) that binds site B belongs to the CCAAT binding factor families NF-Y (55) or YB-1 (56), which have strong requirements for the CCAAT sequence for binding. The site B binding factor is unlikely to belong to another CCAAT binding factor family, C/EBP (57), because C/EBP requires an enhancer core motif that is not present in site B. Because the site B factor binding occurs at an NF-1 site and the consensus NF-1 oligomer competes most efficiently for site B, it is likely that the site B binding factor is a member of the CTF/NF-1 family. Multiple forms of CTF/NF-1 proteins are known to exist, and are presumably produced by alternatively spliced mRNAs which may be transcribed from multiple genes (43, 58). Further, NF-1 binding activity may represent a heterogeneous set of proteins (43, 45). CTF/NF-1 proteins appear to be involved in diverse functions such as adenovirus replication and activation of basal promoter activity of many cellular genes. In MHC class I genes, the NF-1 binding site apparently can function as a transcriptional activator. Consistent with a role for NF-1 as an upstream activator, tissue-specific enhancers of the adipose P2 gene and the β-globin gene have been shown to bind NF-1-like factors (59, 60). NF-1 sites can also interact with other *cis* elements and have been reported to synergize with glucocorticoid, progesterone, and estrogen response elements (28, 61−63). The NF-1 site may also functionally interact with other *cis* elements in the negative

regulation of MHC class I genes in cells in which these genes are not transcribed.

In conclusion, a MHC class I promoter can direct transcription of a reporter *in vitro* via two independent upstream activator regions, both of which are functional *in vivo*. This system may be used to study mechanisms of transcriptional regulation by upstream activator proteins.

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