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Identification of sequences responsible for positive and negative regulation by E1A in the promoter of H-2K^{bm1} class I MHC gene

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The mechanism of transcriptional regulation of the H-2K^{bm1} major histocompatibility complex (MHC) class I gene by adenovirus type 12 E1A (Ad12-E1A) was studied in transfected rat embryonal fibroblasts. Results of long-term expression of the chloramphenicol acetyl transferase (CAT) gene placed under the control of the 5′-flanking region of the mouse MHC class I gene, H-2K^{bm1}, and the results of nuclear run-on transcription assays, yield evidence for both positive and negative regulation of H-2K^{bm1} by E1A gene product. Deletion studies in the H-2K^{bm1} promoter region revealed that a proximal 58 bp upstream sequence (−194 to −136, relative to the cap site) and a distal 316 bp sequence (−1837 to −1521) respectively contribute to positive and negative regulation mediated by the E1A gene product. Both regulatory elements of MHC class I gene promoter region are responsible for the differential expression of the H-2K^{bm1} gene in Ad12 transformed cells. A nuclear factor binding to the negative element has been detected only in extracts derived from cells expressing Ad12-E1A.

Key words: Ad12-E1A/class I gene/transcriptional control

Introduction

The major histocompatibility complex (MHC) class I antigens are specified by class I genes. They play key roles in a number of immunological processes, for example in the restricted recognition of foreign antigens by cytotoxic T cells (Zinkernagel and Doherty, 1979). These molecules (H-2K, D and L in the mouse) have been extensively studied at the protein level and at the DNA level (Hood *et al.*, 1982). They are composed of a glycosylated 45 000 heavy chain which spans the plasma membrane, non-covalently associated with a smaller non-glycosylated 12 000 polypeptide, known as β-2-microglobulin (Hood *et al.*, 1982; Yokoyama and Nathenson, 1983).

Human adenoviruses are double-stranded DNA viruses, some of which are capable of inducing neoplasms in rodents. An interesting aspect of adenoviruses is that their oncogenic potential varies: for example, adenovirus 12 (Ad12) is strongly oncogenic and induces tumours at high frequency with a short latency period (1–2 months), while adenovirus 5 (Ad5) is non-oncogenic. Virus or isolated DNA of all serotypes, however, can transform rodent cells *in vitro* (Flint, 1980). The tumorigenicity of the resulting transformed cells in nude mice and syngeneic rats reflects the oncogenic potential of the adenovirus species used for transformation, Ad12-transformed cells being more oncogenic than Ad5-transformed cells (Mak *et al.*, 1979; Gallimore and Panaskeva, 1980; van den Elsen *et al.*, 1982). The transforming activity of the DNA has been strictly correlated with the expression of E1A sequences (Bernards *et al.*, 1983; Schrier *et al.*, 1983). During early times of infection, and also in transformed cells, the E1A region produces two major 12S and 13S mRNAs (Chow *et al.*, 1979). The protein products from these two transcripts are nuclear phosphoproteins of 220 and 266 amino acid residues respectively. Previous studies have demonstrated that primary rat and mouse cells transformed by the oncogenic Ad12 virus express greatly reduced amounts of class I MHC antigens as opposed to cells transformed by the non-oncogenic Ad5 virus. The reduction of class I gene expression is observed at the mRNA level and correlates with the presence of the product of the 13S E1A mRNA of Ad12 (Bernards *et al.*, 1983; Schrier *et al.*, 1983).

On the contrary, during lytic infection of mouse embryonal cells by Ad12 virus, the expression of the H-2K^{b} gene is increased and the E1A gene products are also proposed to cause this increase (Rosenthal *et al.*, 1985). Therefore, the E1A gene product seems to be a key protein which exerts both a negative and a positive effect on MHC class I gene expression. Although the precise mechanism of the gene regulation by E1A is not fully understood as yet, it appears to involve cellular transcription factors (Hen *et al.*, 1985; Reichel *et al.*, 1988).

In order to elucidate the basis for the differential control of the expression of MHC class I genes by E1A, we have examined the promoter activity of the 5′-flanking region of an MHC class I gene in Ad12-transformed cells. For this purpose, we have used a murine H-2K class I gene, H-2K^{bm1} (Schulze *et al.*, 1983; Weiss *et al.*, 1983), as a test gene, because the controlling elements of the promoter region of the H-2K^{b} gene have been recently well documented (Kimura *et al.*, 1986). Since the promotor sequence of the H-2K^{bm1} mutant gene has not been reported, we have sequenced and compared it with the promoter region of the homologous parent H-2K^{b} gene from which it derived.

A series of hybrid genes was constructed in which chloramphenicol acetyl transferase (CAT) gene (Gorman *et al.*, 1982) was placed under the control of various portions of the 5′-flanking region of the H-2K^{bm1} gene. The CAT activity was measured after transfection of the constructs into Ad12-E1A-transformed cells. The data presented here demonstrate the presence of at least two distinct *cis*-acting regulatory elements, both located in the 5′-flanking region,

and which are involved in positive and negative control of the MHC class I promoter in Ad12-E1A-transformed cells. The positive regulatory sequence controlled by E1A appears to be identical to the CRE/ICS (class I regulatory element/ IFN consensus sequence) region of the previously described enhancer of the H-2 gene promoter (Kimura *et al.*, 1986; Korber *et al.*, 1987; Sugita *et al.*, 1987). The negative regulatory sequence identified here for the first time is located far upstream, between −1836 and −1521 (+1 referring to the cap site). This negative element binds a nuclear factor found only in Ad12-E1A-transformed cells.

Results

Correlation of class I gene expression with expression of E1A mRNA

3Y-1 cells (Kimura *et al.*, 1975) isolated from Fisher rat embryo, have been transfected by the Ad12-E1A recombinant vector pgE1A (Shiroki *et al.*, 1984) to establish the clone CYpAdC3, which contains a single copy of the E1A gene. This clone was found phenotypically transformed, as forming large colonies in soft agar, and inducing tumors in syngeneic animals (Shiroki *et al.*, 1984; Yamashita *et al.*, 1982). We also prepared Ad12-E1A transfectants by co-transfection with pE1A12.13S (13S cDNA) and pRSVneo plasmids. These transfectants were maintained in DME medium containing 1 mg/ml of G418. The steady state level of specific mRNA produced by these transformants was examined by Northern blots using class I and E1A probes (Figure 1A). A lower expression of the class I mRNA (1.6 kb) was observed in E1A-containing cells (lanes 1 and 2) as compared with 3Y-1 cells (lane 4) and antisense E1A transformants (lane 3). Confirming the results of earlier studies (Schrier *et al.*, 1983), the reduction of all endogenous class I mRNAs (lanes 1 and 2) was associated with the increase of the cytoplasmic E1A mRNA both in Ad12-transformed clone CYpAdC3 (selected on the basis of its transformed phenotype: lane 2) and in cells transfected by pE1A-12.13S (and selected with G418: lane 1). The extent of repression of class I mRNA expression is well correlated with the amount of E1A mRNA. A similar reduction of the expression of transfected H-2K^{bm1} gene was also observed in 3Y-1 transfectant with pgE1A (data not shown). Previous studies have shown that introduction of Ad12-E1 gene into established cell lines including 3Y-1, as opposed to culture of primary or secondary cells, did not result in reduction of class I gene expression (Vaessen *et al.*, 1986). The reason for this discrepancy is not readily apparent. A decreased class I gene expression was observed in other Ad12-E1A-transformed cones CYpAdC1, CYpAdC4, CYpAdC5 (Yamashita *et al.*, 1982) and E1A-Y (Shiroki *et al.*, 1984) in which the copy numbers of E1A were estimated as 3, 5, 10 and 1 respectively. These clones can induce tumors in syngeneic animals, although the extent of class I suppression is variable. In these clones, the amount of E1A mRNA correlates well with the degree of suppression of class I expression (not shown). On the other hand, the expression of class I genes is not reduced in Ad5-E1A transfectants obtained by the same co-transfection procedure (pE1A5.13S + pRSVneo). We also did not observe a significant suppression of class I expression in cells transfected with pE1A12.12S and pRSVneo (data not shown, see also Table I).

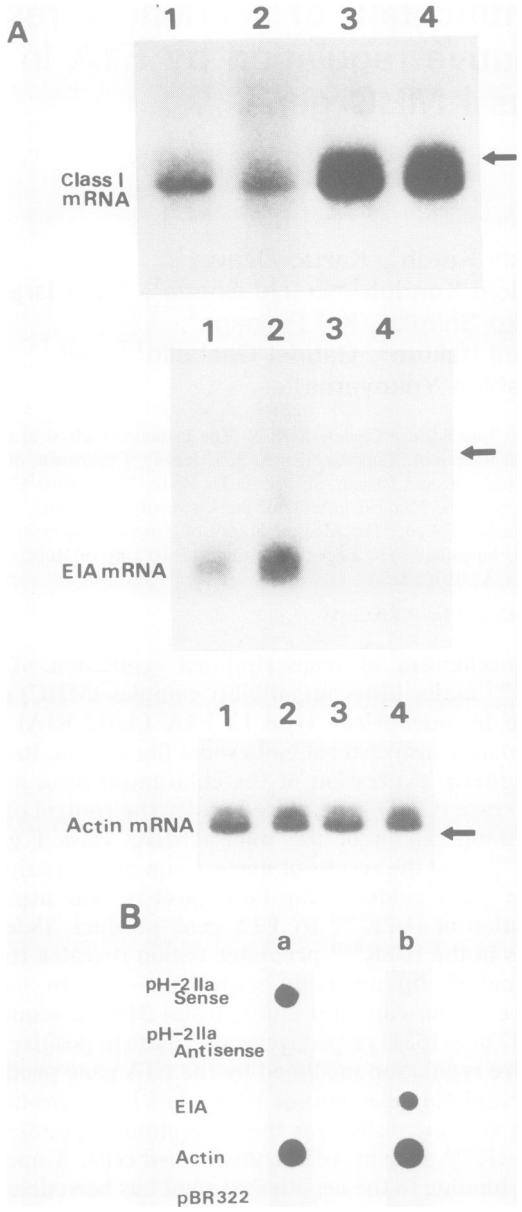


Fig. 1. (**A**) Level of class I MHC and E1A mRNAs in various cell lines and (**B**) *in vitro* run-on transcription assays using isolated nuclei. (**A**) 10 μg of total cytoplasmic RNA from the following cell lines were examined by Northern blot analysis using riboprobes (Melton *et al.*, 1984); **lane 1**, rat 3Y-1 cells transfected by pE1A12.13S and pRSV-neo; **lane 2**, rat Ad12-E1A-transformed clone, CYpAdC3; **lane 3**, rat 3Y-1 cells transfected by antisense pE1A; **lane 4**, rat parental fibroblast cell line 3Y-1. The arrow is 18S rRNA marker. Upper panel, filter probed with an homologous MHC class I heavy chain gene probe (pH-2IIa). Middle panel, filter probed with an E1A probe. Lower panel, filter probed with a β-actin probe. (**B**) Nuclei were isolated from Ad12-E1A-transformed and non-transformed 3Y-1 cells. RNA synthesized by 2 × 10^7 isolated nuclei was labeled for 20 min with 200 μCi [^{32}P]UTP in a 300 μl reaction volume as described in Materials and methods. The single strand DNAs containing homologous MHC class I probe (pH-2IIa), E1A, β-actin and pBR322 fragments were hybridized to labeled RNA from 3Y-1 (**a**) and CYpAdC3 (**b**) cells.

Transcriptional regulation of class I genes

In order to determine whether the reduction in the amount of class I mRNA is caused by a decreased rate of transcrip-

Table I.

[b]Plasmids	[a]Co-transfection with pE1A expression plasmid			
	(−)	Type 12		Type 5
		13S cDNA	12S cDNA	13S cDNA
pH-2K^{bm1}CAT61(58)	3.8	8.4	4.0	4.6
pH-2K^{bm1}CAT61(316)	1.0	0.4	1.0	0.8
pH-2K^{bm1}CAT61	1.0[c]	1.3	1.1	1.2
pH-2K^{bm1}CAT2014	4.5	0.2	4.1	3.6

[a]CAT activity was measured in 3Y-1 cells in the presence or absence (−) of various E1A expression vectors.
[b]Positive or negative elements of H-2K^{bm1} were cloned upstream of the H-2 promoter. Construction of the hybrid genes and transfection into 3Y-1 with or without pEA1 (type 12.13S cDNA or 12S cDNA or type 5 13S cDNA) are described in Materials and methods.
[c]Normalized CAT activity of pH-2K^{bm1}CAT61 in 3Y-1 (E1A) (−) is arbitrarily taken as 1.0.

tion, we measured the initiation rate of transcription of class I genes by nuclear run-on assays using nuclei isolated from Ad12-E1A-transformed cells (Figure 1B). The probe used was a single-stranded pH-2IIa cDNA. This probe corresponds to the 4th exon of mouse MHC class I genes, the sequence of which is well conserved throughout mammalian species. The transcription of class I genes was significantly decreased in the Ad12-E1A-transformed clone CYpAdC3 (lane b) as compared with untransformed 3Y-1 cells (lane a). The β-actin gene was used as a control since its expression is known to be unaffected in the presence of E1A (Stein and Ziff, 1984). Indeed, the level of β-actin mRNA was found to be identical in both cell types. The level of antisense class I mRNA remained undetectable (lanes a and b). Thus, the difference in cytoplasmic class I mRNA concentration between 3Y-1 and CYpAdC3 is most probably caused by a different initiation rate of the transcription of class I genes. These results are consistent with recent reports (Friedman and Ricciardi, 1988; Ackrill and Blair, 1988).

Preliminary analysis of the 5′ regulatory region of H-2K^{bm1} gene

In a preliminary attempt to delineate which DNA sequences in the H-2K^{bm1} gene are responsible for an alteration of transcriptional rate, we performed a 5′ end deletion study. Two portions of the 5′-flanking region of H-2K^{bm1} gene (from −2014 to +12 and −367 to +12 respectively) were fused to the coding sequence of the CAT gene to generate pH-2K^{bm1}CAT2014 and pH-2K^{bm1}CAT367 respectively.

We transfected CAT hybrid constructs into rat 3Y-1 cells with or without the pE1A expression vector (pE1A12.13S), and compared the CAT activity in the extracts of long-term cultured transfectants. The most striking feature was the effect of the E1A gene product on the promoter activity of the H-2K^{bm1} gene. In the presence of pE1A, the CAT activity of pH-2K^{bm1}CAT2014 construct was repressed >10-fold (Figure 2, panel A, lanes 1 and 2). In contrast, the CAT activity of pH-2K^{bm1}CAT367 construct was enhanced ~2.6−4 times by co-transfection with pE1A (lanes 3 and 4). The copy numbers of the H-2−CAT hybrid genes (panel B) are approximately two copies per cell in both cases. The E1A mRNA was detected in E1A mediated transformants (panel C, lanes 2, 4 and 6). Therefore, the negative regulatory element sensitive to E1A gene products

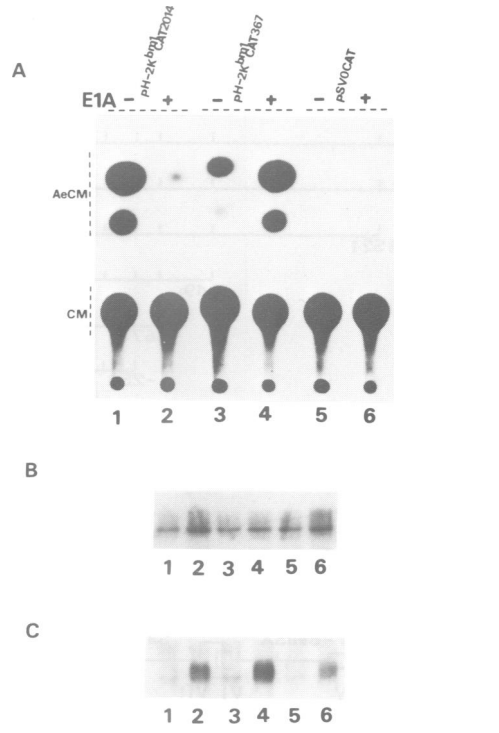


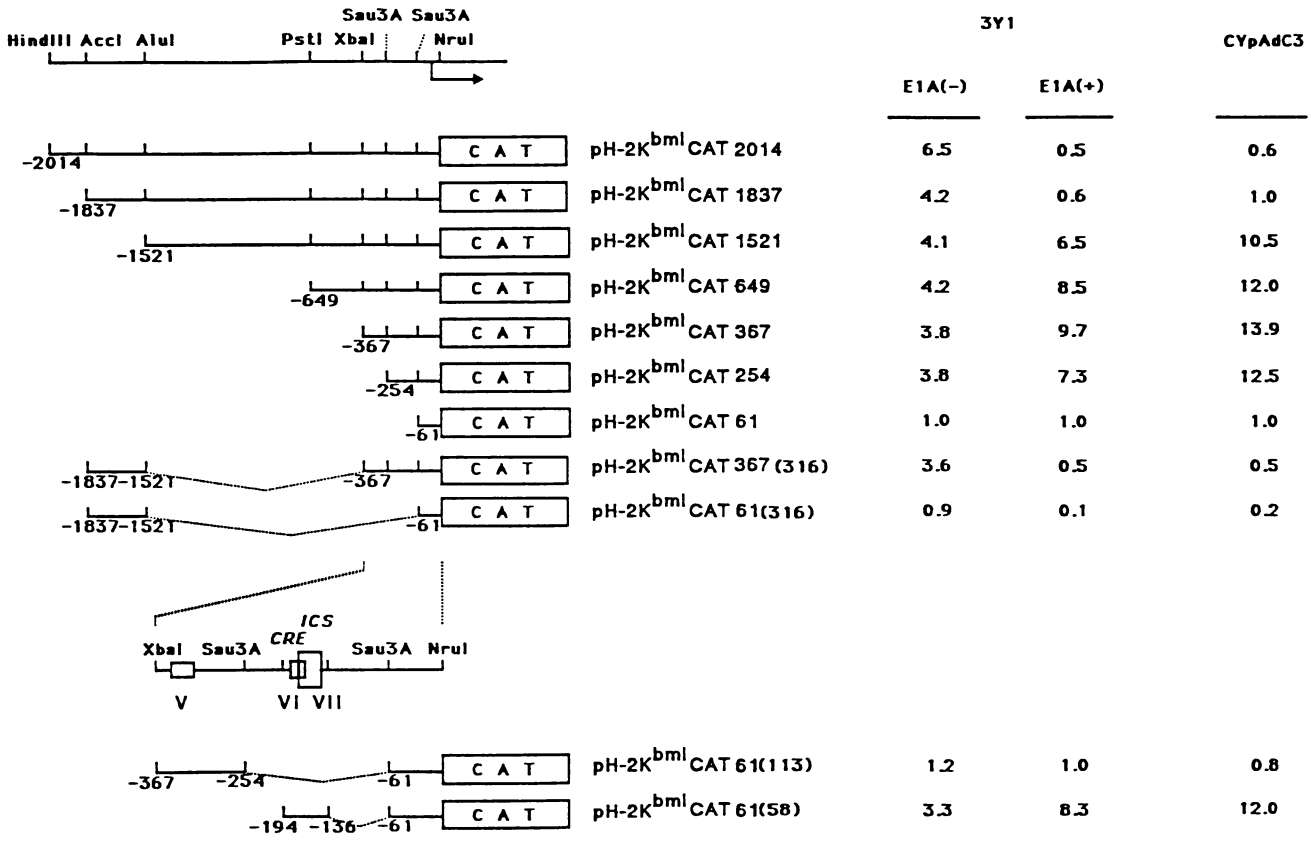
Fig. 2. Effect of Ad12-E1A on the level of CAT expression directed by pH-2K^{bm1}CAT2014 and pH-2K^{bm1}CAT367. (**A**) 3Y-1 cells were co-transfected with pRSVneo and 10 μg of pH-2K^{bm1}CAT2014 (**lanes 1, 2**), pH-2K^{bm1}CAT367 (**lanes 3, 4**) or pSV0CAT (**lanes 5, 6**) with (**lanes 2, 4, 6**) or without pE1A (13S cDNA) (**lanes 1, 3, 5**) as described in Materials and methods. Cell extracts were prepared 2 weeks after transfection and selection with G418 and assayed for CAT activity by incubating with [^{14}C]chloramphenicol for 1 h at 37°C. The reaction products were separated by TLC. The autoradiograph of the TLC plate is shown with the positions of unacetylated chloramphenicol (CM) and acetylated products (AeCM) indicated. Spots were cut from the plate and radioactivity was determined by scintillation counting to determine the percentage conversion of chloramphenicol to its acetylated derivatives. (**B**) Level of integrated CAT DNA in the transformants. Southern blot hybridization was carried out using CAT fragment (*Hin*dIII−*Bam*HI) as a probe. (**C**) Level of the E1A mRNA in the transformants. Northern blot hybridization was carried out using the E1A fragment as a probe.

lies between −2014 and −367 bp upstream of the cap site of the H-2K^{bm1} gene. Similar results were obtained in experiments using different cell lines such as mouse NIH3T3, African monkey kidney CV-1 cells, human HeLa cells and HL 60 cells (data not shown).

A similar analysis of the H-2K^{bm1} gene promoter was carried out using the Ad12-E1A-transformed clone, CYpAdC3 and identified the region (−2014 to −367) as being a negative element (data not shown).

Further analysis of the 5′ regulatory regions

In order to determine the precise nucleotide sequences required for this dual regulation we constructed the various deletion mutants described in Figure 3. These constructs were transfected into 3Y-1, with or without pE1A12.13S or into CYpAdC3 and relative CAT activities were measured. The normalized CAT activity of pH-2K^{bm1}CAT61 in 3Y-1 or CYpAdC3 cells is arbitrarily taken as 1.0. Results shown in Figure 3 indicate that the *cis*-acting negative regulatory sequence is localized within a distal 316 bp fragment (−1837 to −1521) of the H-2K^{bm1} promoter region. The internal



	3Y1		CYpAdC3
	E1A(−)	E1A(+)	
pH-2K^{bml}CAT 2014	6.5	0.5	0.6
pH-2K^{bml}CAT 1837	4.2	0.6	1.0
pH-2K^{bml}CAT 1521	4.1	6.5	10.5
pH-2K^{bml}CAT 649	4.2	8.5	12.0
pH-2K^{bml}CAT 367	3.8	9.7	13.9
pH-2K^{bml}CAT 254	3.8	7.3	12.5
pH-2K^{bml}CAT 61	1.0	1.0	1.0
pH-2K^{bml}CAT 367(316)	3.6	0.5	0.5
pH-2K^{bml}CAT 61(316)	0.9	0.1	0.2
pH-2K^{bml}CAT 61(113)	1.2	1.0	0.8
pH-2K^{bml}CAT 61(58)	3.3	8.3	12.0

Fig. 3. Schematic representation and deletion study of the H-2K^{bml} promoter region and of various hybrid H-2K^{bml}CAT constructs. The H-2K^{bml} promoter was fused with the CAT gene to generate various deletion mutants. +1 represents the cap site. V, VI (CRE element) and VII (ICS element) denote class I regulatory elements as described (Kimura *et al.*, 1986; Miyazaki *et al.*, 1986). The dotted line represents the internal deletions. Deletion endpoints are shown as the nucleotide position relative to the cap site. Relative CAT activity of mutants was measured in the presence (+) or absence (−) of the pE1A12.13S expression vector (pE1A) in 3Y-1 cells. Normalized CAT activity of pH-2K^{bml}CAT61 in 3Y-1 (E1A) (−) or CYpAdC3 is taken arbitrarily as 1.0. Relative CAT activities of pSV0CAT were 0.3 [E1A (−)], 0.3 [E1A (+)] and 0.6 (CYpAdC3) and CAT activities of pSV2CAT were 17.5 [E1A (−)], 6.8 [E1A (+)] and 7.5 (CYpAdC3).

deletion mutants deleted between −1521 and −61 and −1521 and −367 display the same E1A-induced suppression of CAT activity. The mutants whose deletion endpoints are located between −254 and −1521 showed stimulation by E1A (6- to 14-fold). However, this stimulating activity is lost when the 153 bp (−254 to −61) of the promoter region was deleted (pH-2K^{bml}CAT61). These experiments suggest the presence of an enhancing element located between −254 and −61, in addition to the TATA and CAAT boxes. A 58 bp fragment (−194 to −136) covering the CRE/ICS region was attached to pH-2K^{bml}CAT61 to generate pH-2K^{bml}CAT61(58), which still showed stimulation by E1A (Table I). Therefore the positive regulatory sequence seems to be localized within the CRE/ICS element. Thus differential effects on the promoter activity were observed with a 58 bp proximal fragment (positive) and a 316 bp distal fragment (negative). When both pH-2K^{bml}CAT367(316) and pH-2K^{bml}CAT61(58) plasmids were tested in other Ad12-E1A-transformed clones, CYpAdC1, CYpAdC4, CYpAd5 and E1A-Y, the same positive and negative regulation of the H-2K^{bml} promoter was observed (data not shown). Thus it can be concluded that the 58 bp sequence behaves as an E1A inducible enhancer and the 316 bp fragment is responsible for the negative regulation by E1A. We also analyzed the effect of both elements in the presence or absence of either pE1A12.12S, pE1A12.13S or pE1A5.13S (Table I). The 12S mRNA product has no significant effect on the activity driven by any of the two elements. Thus, positive and negative regulations observed are specific for the 13S E1A gene product. The positive and negative effects of Ad5-E1A on H-2K^{bml} promoter was marginal, and quite weaker than that of Ad12-E1A (Table I).

Regulatory elements of H-2K^{bm1} gene can act on an heterologous promoter

We examined the effect of the negative and positive regulatory sequences of H-2K^{bml} gene in either orientation on the activity of an heterologous promoter. The results are shown in Table II.

Positive enhancer activity. The proximal 367 bp fragment of the H-2K^{bml} promoter was divided into three short fragments: 193 bp *Sau*3A fragment (−254 to −61), 113 bp fragment between −367 and −254, and 73 bp between −61 and +12. These fragments were subcloned at the *Bgl*II site of the pA10CAT2 vector which contains the SV40 early promoter without any enhancer sequence (Gorman *et al.*, 1982). Of several fragments tested, the 193 bp *Sau*3A fragment (−254 −61), encompassing the CRE/ICS region was shown to be responsible for the stimulatory effect (K.Yokoyama, unpublished result). This result is consistent with a previous report (Kimura *et al.*, 1986). The 58

Table II.

[a]Plasmids	[b]Co-transfected with pE1A expression plasmid	
	(−)	(+)
Positive effect		
pH-2K^{bm1}CAT61(58) (F)	5.4	14.3
pH-2K^{bm1}CAT61(58) (R)	5.2	14.0
pA10CAT58 (F)	3.8	5.2
pA10CAT58 (R)	3.6	5.0
Negative effect		
pH-2K^{bm1}CAT61(316) (F)	2.0	0.3
pH-2K^{bm1}CAT61(316) (R)	1.8	0.3
pSV2CAT316 (F)	15.8	4.9
pSV2CAT316 (R)	15.2	6.1
Control		
pH-2K^{bm1}CAT61	1.8	2.0
pSV2CAT	17.2	8.2
pA10CAT	1.0[c]	1.0[c]

[a]Positive or negative elements of H-2K^{bm1} were cloned upstream of the homologous promoter or upstream of a heterologous promoter. The orientation of the inserts is denoted by F (forward) or R (reverse). Construction of the hybrid genes and transfection into 3Y-1 cells with or without pE1A (13S cDNA) are described in Materials and methods.
[b]CAT activity was measured in 3Y-1 cells in the presence (+) or absence (−) of pE1A-12.13S.
[c]Normalized CAT activity of pA10CAT in 3Y-1 [E1A (−)] is arbitrarily taken as 1.0.

nucleotides which cover the CRE/ICS were inserted into either pA10CAT2 or pH-2K^{bm1}CAT61 vectors which contained only TATA and CAAT box sequences. The homologous construct showed a 3-fold enhancement of CAT activity by E1A while the heterologous construct showed only a 1.4-fold stimulation (Table II). This element can function in either orientation in association with the homologous or heterologous promoter (Table II). The CRE/ICS sequence has been characterized previously as an enhancer element involved in both constitutive and IFNs induced class I expressions (Kimura *et al.*, 1986; Sugita *et al.*, 1987).

Negative activity. The 316 bp sequence (*Acc*I−*Alu*I) was inserted at the *Bam*HI site of the pSV2CAT to create the pSV2CAT316 vector. We compared the repression by E1A of this construct to that of pH-2K^{bm1}CAT61(316) (Table II). It is also evident that the CAT activity under the control of the H-2K^{bm1} promoter is much more repressed by E1A than when the promoter is derived from SV40 (6.7- to 6.0- versus 3.3- to 2.5-fold) especially since the SV40 early promoter also shows repression by E1A, in accordance with the data of Velcich and Ziff (1985) (construct pSV2CAT in Table II). The negative regulatory element can function in either orientation in association with the homologous promoter.

CAT mRNA is initiated at the correct transcription start site

To confirm that the CAT activities measured in the above experiments reflect the initiation of the CAT gene transcripts at the correct sites, we performed primer extension experiments with total RNA prepared from CYpAdC3 cells, using a primer complementary to the 5′ end of the CAT coding region (Figure 4). All H-2K^{bm1}CAT gene transcripts tested gave an extension product of 50 nucleotides. Thus,

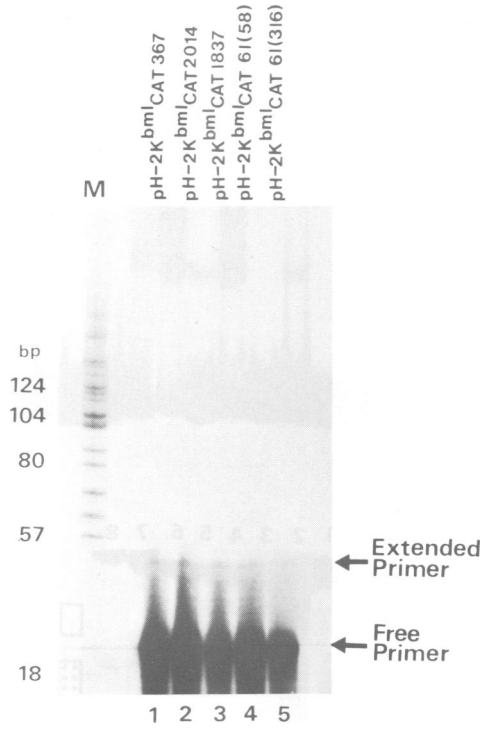


Fig. 4. Analysis of transcription start site by primer extension. Total RNA was prepared after stable transfection of CYpAdC3 cells. Primer extension was carried out as described in the text. The left lane represents the size marker. The arrowheads on the right indicate the extended product and the free primers. Primer extension experiments using the 3Y-1 cells, with or without E1A, showed the same results (not shown).

transcription is initiated at 24 bp downstream the TATA box of the H-2K^{bm1} promoter, a value consistent with the localization of the cap site in the H-2K^{b} gene (Kimura *et al.*, 1986). In all cases, E1A does not seem to modify the transcription start sites in the H-2K^{bm1} promoter.

Specific factors in nuclear extracts from CYpAdC3 cells

To investigate whether specific factors in nuclear extracts derived from 3Y-1 or CYpAdC3 were capable of binding to the 58 bp CRE/ICS or the 316 bp *Acc*I−*Alu*I fragments, gel shift assay was carried out. We used an end-labeled 58 bp CRE/ICS (−194 to −136) and a 316 bp probe that spans positions −1837 to −1521. In a gel shift assay using the 58 bp CRE/ICS, similar patterns were obtained using extracts derived from either cell line (data not shown). Using the 316 bp negative regulatory sequence as a probe (Figure 5A), we detected two retarded bands (B1 and B2) with extracts derived from Ad12-E1A-transformed clones (CYpAdC3, CYpAdC4, CYpAdC5 and E1A-Y) (lanes 6, 8, 10) but no corresponding band was apparent using nuclear extracts from normal 3Y-1, baby rat kidney cells and Ad5-E1A-transformed cells (lanes 2, 4, 12).

To investigate the nature of the E1A-mediated negative factor binding to the H-2K^{bm1} gene regulatory elements, we performed DNase I footprint experiments. The negative regulatory sequence (−1836 to −1521) was digested with *Dde*I (−1689) and terminally labeled by 'fill in' with the Klenow fragment of DNA polymerase I. Both fragments were gel purified and used as probes. When the upstream

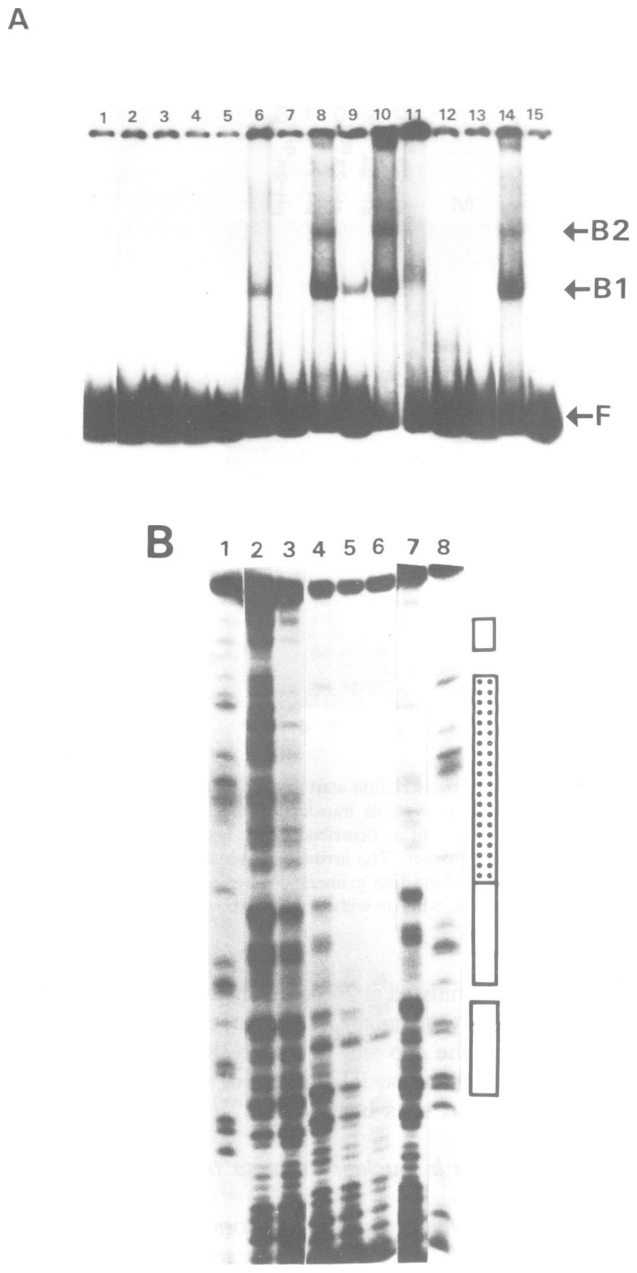


Fig. 5. Specific binding of a nuclear factor to the negative regulatory sequence (316 bp). (**A**) The gel mobility assay was performed as described in Materials and methods with 1.0 ng of ^{32}P-labeled probe, 2 μg of poly(dI:dC), 5 μg of 3Y-1 or CYpAdC3 cell nuclear extract, and with or without 500 ng of fragment competitor. The probe spanned region −1983 to −1521 of the H-2K^{bm1} promoter. The positions of specific bands (B1, B2) and the free migrating probe (F) are indicated; **lane 1**: ^{32}P-labeled probe; **lanes 2** and **3**: 3Y-1 nuclear extract; **lanes 6** and **7**: E1A-Y nuclear extract, **lanes 8** and **9**: CYpAdC4 nuclear extract, **lanes 10** and **11**: CYpAdC5 nuclear extract, **lanes 12** and **13**: Ad5-E1A transformed cells nuclear extract, **lanes 14** and **15**: CYpAdC3 nuclear extract. **Lanes 3, 5, 7, 9, 11, 13** and **15**: an excess of cold competitor was added. (**B**) DNase I experiments using the negative regulatory element. Nuclear extracts were prepared from CYpAdC3 cells as described in Materials and methods, and the probe was the *Acc*I−*Dde*I fragment (1836 to −1689). G cleavage sequencing pattern is shown in **lanes 1** and **8**. **Lane 2** is a competition with 5 μg of the *Acc*I−*Alu*I fragment. The footprinting reactions contained no protein (**lanes 3, 7**), nuclear extract from CYpAdC3 (**lanes 4, 5, 6**): (13 μg of protein: **lane 4**; 26 μg of protein: **lane 5**; 86 μg of protein: **lane 6**). The strong protection is indicated by the dotted rectangle and the marginal protection by the open rectangles.

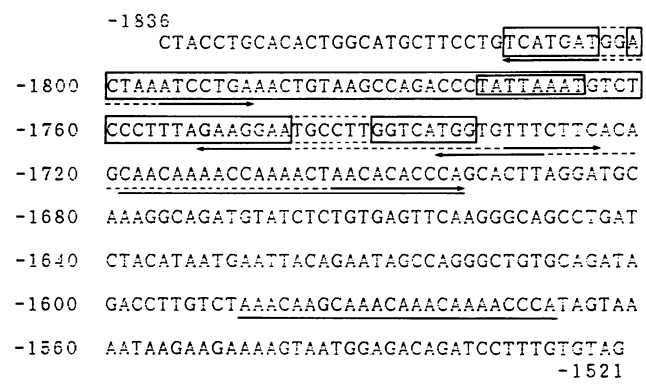


Fig. 6. Nucleotide sequence of the E1A responsive negative element of the H-2K^{bm1} promoter. The nucleotide is numbered as relative to the cap site. The TATA-like sequence is shown in the small open box. The closed and broken arrow lines show the imperfect overlapping dyad symmetry structure. The AAAC repeat is underlined. The protected sequences shown in Figure 5B are enclosed in the large open box.

fragment was used, nucleotides between −1801 and −1760 were protected by nuclear extracts derived from CYpAdC3 (Figure 5B, lanes 4−6). Increasing the amount of nuclear extract resulted in an extended footprint (−1801 to −1747) as well as the appearance of two new protected areas (−1740 to −1733 and −1811 to −1804). As expected, the addition of an excess of the *Acc*I−*Alu*I fragment (−1836 to −1521) abolished the footprinting completely (lane 2). When the *Dde*I−*Alu*I fragment was used as a probe, however, no specifically protected region could be seen (not shown). When using extracts from 3Y-1 cells no protection could be detected on either fragment (data not shown).

Sequence of the H-2K^{bm1} promoter region

Finally, we determined the nucleotide sequence of the H-2K^{bm1} promoter delineated by the *Acc*I and *Alu*I restriction sites (−1836 to −1521) (Figure 6). Three imperfect 7 bp inverted repeats, starting at position −1810 and −1694 are present. AAAC repeats are present at −1599 to −1567 and −1719 to −1694. The TATTAAA sequence localized between −1771 and −1765 shows homology with a putative TATA box. The sequences protected from DNase I by the E1A-transformed cell nuclear extracts are shown in the large open boxes.

Discussion

The expression of class I genes encoding MHC antigens is known to be suppressed in cells transformed by the oncogenic adenovirus 12 (Ad12). It has been proposed that the suppression of the expression of class I genes enables transformed cells to escape the immune response and thus form tumors efficiently *in vivo* (Bernards *et al.*, 1983; Schrier *et al.*, 1983). It is assumed that this reduction is mediated by the E1A protein of Ad12. On the other hand, it has been reported that the infection of mouse embryo cells with AD12 transiently increases the amount of transcripts from the H-2K^{b} gene (Rosenthal *et al.*, 1985). In order to explain this contradiction in the effects of Ad12-E1A gene products, we analyzed the promoter activity of a class I MHC gene

in transfection experiments in the presence or absence of E1A.

The present study demonstrates that upstream elements of the 5′-flanking region of H-2K^{bm1} gene are responsible for the differential effect of the E1A gene product. A distal 316 bp sequence (−1837 to −1521) mediates the repression of the promoter activity by E1A, whereas the proximal 58 bp CRE/ICS element is responsible for the positive effect. The effects of both elements were shown to be mediated by the product of the 13S cDNA and not of the 12S, as shown in Table I. These data are consistent with a previous report (Schrier *et al.*, 1983). The proximal 58 bp CRE/ICS element has been previously identified as an enhancer-like element (Kimura *et al.*, 1986; Israël *et al.*, 1986; Korber *et al.*, 1987; Sugita *et al.*, 1987). We now identified this 58 bp CRE/ICS as being the target sequence for the positive regulation by E1A gene product. In terms of the negative regulation of H-2K class I genes, our results are consistent with the conclusions of Kimura *et al.* who reported that CRE is not the target of the AD12-mediated inhibition of H-2 expression (Kimura *et al.*, 1986). The negative controlling sequence is localized between −1837 and −1521. The positive and negative regulatory sequences described above show a much stronger effect when linked to the homologous H-2K^{bm1} promoter than to the SV40 promoter in either orientation. We have also examined other vectors where CAT is driven by the conalbumin promoter or by the human β-actin promoter and showed that the homologous promoter−enhancer (silencer) combination always displays a stronger positive and negative effect than the heterologous association (K.Yokoyama, unpublished data). These results suggest that some interaction between the promoter and the enhancer (silencer) is probably necessary in order to obtain an optimum positive or negative effect. Recently, Ehrlich *et al.* (1988) identified both positive and negative regulatory elements in a porcine class I antigen gene, which were mapped within 1.1 kb upstream of exon I. The negative regulatory element reduced the activity of both the class I promoter and an SV40 promoter. The nucleotide sequence of this porcine negative region is not identical with the sequence described here.

The regulation of class I gene expression at the level of transcription has been studied in adenovirus-transformed cells by DNA-mediated gene transfer, although conflicting results were obtained (Kimura *et al.*, 1986; Vogel *et al.*, 1986). Transfection of H-2K^{b}-derived CAT constructs yields a 10-fold greater activity in Ad5 compared with Ad12-transformed cells (Kimura *et al.*, 1986) whereas the other study detected no suppression of CAT expression in Ad12-transformed cells (Vogel *et al.*, 1986). Our conclusion, based on CAT assays, supports the notion that the transcription of class I gene is reduced in Ad12-E1A-transformed rat cells while the effect of Ad12-E1A is marginal. This result is in good agreement with the results obtained by Northern analysis of the endogenous class I genes (Figure 1A).

It is well known that H-2 antigens are not expressed in early embryonic cells (Morello *et al.*, 1978, 1982). Recently, it has been reported that the nucleotide sequence controlling negative expression of class I genes in undifferentiated embyronal carcinoma cells (EC) lies between positions −95 and −161 relative to the cap site of the H-2L^{d} gene; this region covers the CRE/ICS (Miyazaki *et al.*, 1986). This result suggests the existence of a different type of negative regulation in EC cells. The CRE/ICS behaves as a positive or negative *cis*-acting element depending on the host cell. In our study, the CRE/ICS element behaves only as a positive element in the E1A-mediated H-2 gene regulation.

One of the possible explanations to account for these different effects is that modification of the promoter region of the H-2K^{bm1} gene, for example by methylation, might vary during embyronic development (Croce *et al.*, 1981; Silver *et al.*, 1983). Also transcriptional regulatory proteins may be different in EC cells and 3Y-1 fibroblasts.

Recently it has been reported that the decreased level of class I mRNA in the nucleus and in the cytoplasm of Ad12-transformed cells was caused by post-transcriptional degradation of the mRNA, a process taking place during the maturation of pre-mRNA (Vaessen *et al.*, 1987). Although we cannot rule out such a possibility, our results clearly show that Ad12-E1A does suppress class I MHC gene expression in Ad12-transformed cells by reducing the rate of transcription initiation, as assayed by nuclear run-on using a single-stranded H-2 cDNA as a probe (Figure 1B). This conclusion is in agreement with other reports (Friedman and Ricciardi, 1988; Ackrill and Blair, 1988). It has been shown that there is no difference in class I H-2 expression between 3Y-1 cells transfected with Ad5-E1A or Ad12-E1A (Vaessen *et al.*, 1986). However, all 3Y-1 transformants used in this study showed the same repression of H-2 class I gene expression although the reason for this discrepancy is not known.

In our studies, positive or negative regulation by E1A involves different sequences in the H-2K^{bm1} promoter. It is conceivable that the positive CRE/ICS element (−194 to −136) and negative control region (316 bp, −1837 to −1521) can regulate MHC gene expression by interacting with respective positive and negative *trans*-acting factors.

A gel shift assay using a probe that covered the CRE/ICS region revealed identical specific bands in extracts derived from control or E1A-transformed cells. On the contrary, we have detected proteins which bind specifically to the negative regulatory element (−1836 to −1521) in extracts derived from Ad12-E1A-transformed clones (CYpAdC3, CYpAdC4, CYpAdC5, E1A-Y) but not from 3Y-1 or Ad5-E1A-transformed cells (Figure 5A). *In vitro* DNase I footprinting experiments, using the same fragment, revealed protected regions only with CYpAdC3 extracts: −1811 to −1804, −1801 to −1747 and −1740 to −1733. These regions contain an overlapping repeat sequence including a putative TATAA box (−1771 to −1765) (Figure 6). The proteins responsible for this protection are possible candidates for negative regulation mediated by E1A. The identification and subsequent purification of the proteins which bind to these sequences are currently under way and will hopefully help to elucidate the functional role that these DNA binding proteins play in gene regulation.

Materials and methods

Plasmids and plasmid constructions

A 15.0 kb long genomic clone containing the entire H-2K^{bm1} gene (Schulze *et al.*, 1983) was kindly provided by B.R.Wallace. H-2 cDNA clones, pH-2IIa and pH-2III cDNA (Schulze *et al.*, 1983) were gifts of S.G.Nathenson. The following constructs, pConaH-2K^{b}CAT (*Sau*3A) and pH-2CAT (*Hin*dIII−*Nru*I) are described in Kimura *et al.* (1986). Chimeric 5′ end promoter deletion of CAT constructs, described in Figure 3, were prepared by isolating the respective fragments digested with the appropriate enzymes, filling in the ends, adding *Hin*dIII linkers, and inserting it in place

of the *Acc*I–*Hin*dIII SV40 promoter region of the pSV0CAT plasmid (Gorman *et al.*, 1982). Inernal deletion mutants were prepared by ligating the appropriate fragments to pH-2K^{bm1}CAT61, pH-2K^{bm1}CAT367 and pSV2CAT to generate pH-2K^{bm1}CAT61(113), pH-2K^{bm1}CAT61(316), pH-2K^{bm1}CAT367(316) and pSV2CAT316, respectively. Double-stranded oligodeoxynucleotide 59mers (−194 to −136) were synthesized on an Applied Biosystem model 308 synthetizer. This enhancer-containing element was inserted at either the *Bgl*II site of pA10CAT2 or at the *Hin*dIII site of pH-2K^{bm1}CAT61 by adding *Bgl*II or *Hin*dIII linkers respectively to generate pA10CAT58 and pH-2K^{bm1}CAT61(58). Plasmids pgE1A (Shiroki *et al.*, 1984), pE1A12.12S and pE1A12.13S (12S cDNA or 13S cDNA expression vector) (Little *et al.*, 1986; Shiroki *et al.*, in preparation) derived from Ad12, pE1A5.13S derived from Ad5 (Zerler *et al.*, 1986) and pCH110 (Hall *et al.*, 1983) have been described previously.

DNA transfection, chloramphenicol acetyl transferase (CAT) and β-galactosidase assays

Rat 3Y-1 and Ad12-E1A transformants CYpAdC3, CYpAdC4, CYpAdC5 and E1A-Y cells were cultured as described (Kimura *et al.*, 1975; Shiroki *et al.*, 1984; Yamashita *et al.*, 1982). Transfections for long-term expression were carried out as follows (Wiegler *et al.*, 1979): briefly, 10 μg of the CAT plasmid, 2 μg of pCH110, 2 μg of pRSVneo and 6 μg of pBR322 were added to 4×10^5 cells in a 100 mm plate as a calcium phosphate co-precipitate. After 5 h, the cells were exposed to 15% glycerol for 30 s at room temperature and cultured for a further 36 h in non-selective medium. Selection medium containing G418 (Gibco) at a final concentration of 1 mg/ml was refed every 3 days. Several thousand colonies could be scored after 2 weeks. They were spread onto six plates at 4×10^5 cells per plate and cultured for 2 days. Each plate was harvested for assay of CAT activity, β-galactosidase activity (Kimura *et al.*, 1986) and Southern blot hybridization to estimate the number of copies of integrated DNA. The relative ratio of CAT to β-galactosidase activities was used as a normalized measure. These relative values were normalized according to the number of copies integrated into genomic DNA.

RNA analysis and nuclear run-on assay

Total cellular RNA was isolated by the guanidium thiocyanate procedure (Chirgwin *et al.*, 1979), followed by sedimentation through a CsCl layer. Total RNA (10 μg) was denatured and run on a 1% formaldehyde/formamide agarose gel, blotted onto nitrocellulose, and hybridized with nick-translated DNAs (Maniatis *et al.*, 1982) or RNA riboprobes, pH-2IIa and Ad12-E1A *Hin*dIII fragments driven by the SP6 promoter (Melton *et al.*, 1984). Nuclear run-on assays were performed as previously described (Greenberg and Ziff, 1984; Yokoyama and Imamoto, 1987) using 2×10^7 nuclei in each experiment. Approximately 10^7 c.p.m. of each [^{32}P]RNA run-off product was hybridized to DNA (2 μg each) applied onto nitrocellulose filters. DNA probes were pBR322, probes for H-2 (a single-stranded sense or antisense M13 DNA of pH-2IIa), probe for E1A (a single-stranded *Hin*dIII fragment of E1A) and probe for human β-actin cDNA clone (Stein and Ziff, 1984).

Nuclear extract preparation, gel retardation assay and DNase I footprinting

Nuclear extracts were prepared essentially as described by Dignam *et al.* (1983). The salt concentration in extracts were adjusted to 50 mM either by dilution or dialysis against TM buffer [50 mM Tris–HCl (pH 7.9), 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 20% glycerol]. Final protein concentrations were typically 1–2 mg/ml. Assay conditions were similar to those previously described (Fried and Crothers, 1981; Carthew *et al.*, 1985). Binding reactions (15 μl) contained up to 16 μg of protein extract, 0.3 ng ^{32}P-end-labeled probe ($1–4 \times 10^4$ c.p.m.), 2 μg of poly(dI:dC) (Pharmacia Inc.), 12% glycerol, 12 mM HEPES (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 4 mM Tris–HCl (pH 7.4), 0.6 mM EDTA, 0.6 mM dithiothreitol (DTT), and competitor DNA where indicated. After a 20 min incubation at 30°C, reaction mixtures were loaded on 4% polyacrylamide gel (39:1 acrylamide to bisacrylamide) in 0.25 × TBE [1 × TBE is 89 mM Tris-borate (pH 8.3), 2.5 mM EDTA] which was run for ~2 h at 10 V/cm. The gel was then dried and autoradiographed with intensifying screens at −80°C. DNase I footprinting reactions were carried out as described previously (Lefevre *et al.*, 1987).

Primer extension

Fifty μg of total RNA was hybridized with 3 ng of the synthetic 5′ end-labeled 26mer, 5′-CTCCATTTTAGCTTCCTTAGCTCCTG-3′, which is complementary to the 5′ end of the coding sequence of the CAT gene transcript. Primer extension was carried out as described (Maniatis *et al.*, 1982). The final product was analyzed on a 8% polyacrylamide–urea sequencing gel.

DNA sequencing

The nucleotide sequence of *Acc*I–*Alu*I fragment of H-2K^{bm1} promoter region (−1837 to −1521) was determined by M13 dideoxy sequencing (Sanger *et al.*, 1977).

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