The EMBO Journal vol.11 no.12 pp.4591-4600, 1992

A novel downstream regulatory element of the mouse *H-2K^b* class I major histocompatibility gene

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Communicated by W.F.Bodmer

The *H-2K^b* gene equipped with a minimal promoter (5′ deletion up to −61) was fully expressed in transfected fibroblasts, but inactive in transfected embryonal carcinoma cells. A strong transcriptional regulatory element (H2DRE) was identified when a fragment spanning the second exon and second intron was used to activate transient expression of the reporter chloramphenicol acetyltransferase (CAT) gene in mouse Ltk⁻ or NIH3T3 fibroblasts. Its activity was twice that of a construct where the CAT gene was driven by the *H-2K^b* 5′ enhancer region (H2TF1/KBF1 site) and comparable to that of pRSVCAT construct carrying the strong Rous sarcoma virus LTR enhancer. In accord with regulated transcriptional activity of the intact *H-2K^b* gene, the H2DRE did not activate the CAT expression in P19 mouse embryonal carcinoma cells. The H2DRE did not function as a typical enhancer since its activity was strongly position dependent. Consistent with its anticipated role in transcription regulation, H2DRE displayed more than five target sites for specifically interacting nuclear factors, two of them being present in H-2 positive fibroblasts, but not in H-2 negative teratocarcinoma cells. None of them was cross-competed by sequences of the 5′ enhancer. The results of deletion experiments show that H2DRE is the only regulatory region that can activate transcription from the 5′ enhancerless *H-2K^b* gene in mouse L fibroblasts.

Key words: embryonal carcinoma cells/*H-2K^b* class I MHC gene expression/transcriptional regulation

Introduction

Major histocompatibility complex (MHC) class I genes encode cell surface glycoproteins that function in the presentation of processed antigens to T cells (Townsend *et al.*, 1986; Maryanski *et al.*, 1987) and enable cytotoxic T cells to kill infected or malignant cells (Zinkernagel and Doherty, 1979; Kourilsky *et al.*, 1987). The MHC class I expression is regulated during the development; the mRNA and cell-surface antigens are not detected in early stages of development and in mouse embryonal carcinoma cells (Artzt and Jacob, 1974; Morello *et al.*, 1978, 1982; Croce *et al.*, 1981; Ozato *et al.*, 1985). In the adult organism, high amounts of class I antigens can be detected in spleen, lung and lymph nodes, while very little expression is found in brain, pancreas and testicular germ cells (Klein, 1975; Singer and Maguire, 1990; Chamberlain *et al.*, 1991). The expression is inducible by cytokines such as interferons and tumour necrosis factor (Hood *et al.*, 1983; David-Watine *et al.*, 1990). The suppression of class I gene expression has been repeatedly described in malignant tumour cells (De Baetselier *et al.*, 1980; Travers *et al.*, 1982; Shrier *et al.*, 1983; Goodenow *et al.*, 1985; Bernards, 1987), which may thus escape the immune surveillance by cytotoxic T cells.

Control of transcription initiation of the mouse *H-2K^b* MHC class I gene has been extensively studied and a complex enhancer region, 'enhancer A', centered −166 upstream of the transcription start site has been identified (Kimura *et al.*, 1986; Baldwin and Sharp, 1987; Shirayoshi *et al.*, 1987). Transcription factors KBF1, KBF2, H-2TF1 and NFκB were found to bind to the 5′ enhancer motifs (Yano *et al.*, 1987; Baldwin and Sharp, 1988; Israel *et al.*, 1989a) and complementary DNA for KBF1 has already been cloned (Kieran *et al.*, 1990). The 5′ enhancer confers complex developmental and cell-specific regulation and inducibility to the *H-2K^b* gene. However, data are accumulating on the existence of additional regulatory regions within the MHC class I genes. A distal 5′ element (−1521 to 1837), contributing to positive regulation mediated by the E1A gene product, was defined in the *H-2K^b* gene (Katoh *et al.*, 1990) and partially overlapping sequence of enhancer−silencer complex similar to the yeast α-2 mating type repression system were identified between −771 and −676 bp upstream of the swine MHC class I gene, PD1 (Weissman and Singer, 1991a,b). Furthermore, enhancer-like sequences were observed in introns 3 and 5 of the human HLA-B7 class I gene (Ganguly *et al.*, 1989).

We report here on the localization of a novel, strong downstream regulatory element (abbreviated H2DRE) situated in the second exon and intron of the *H-2K^b* gene. H2DRE functions from a heterologous promoter in a regulated manner, being highly active in fibroblasts but non-functional in embryonal carcinoma cells.

Results

The H-2K^b gene lacking its 5′ enhancer is expressed freely in L fibroblasts

Enhancer motifs at the 5′ end of the *H-2K^b* gene were identified as sequences able to stimulate transcription from the CAT reporter gene and to bind specific nuclear factors (David-Watine *et al.*, 1990). To determine whether their presence is unconditional for the expression of the *H-2K^b* gene, all known 5′ upstream regulatory sequences except the −50 CCAAT and −25 TATA boxes were deleted to produce pΔ1918 construct (Figure 1). The fidelity of the construct (5′ deletion up to position −61) was verified by sequence analysis of the critical region of the gene and by Southern blot comparison with the plasmid p1918 which contains an intact *H-2K^b* gene or with pX1918 which contains the *H-2K^b* gene with a 5′ deletion up to position −367.

The plasmids pΔ1918, pX1918 and p1918 were then transfected into mouse Ltk$^-$ cells to test whether the *H-2K^b* gene could be expressed without its 5′ enhancer. Quantitative radioimmunoassay of 20 independent clones with pΔ1918 and the same number of controls revealed that deletion of the 5′ enhancer did not affect the cell surface expression of the *H-2K^b* gene product (Figure 2). High expression of the 5′ enhancerless *H-2K^b* gene was also detected in transient expression assays in which the effects of integration and neighbouring loci are excluded. These experiments indicate that the presence of 5′ enhancer sequences located in the *Sau*3A fragment (−254 to −61) is not a condition necessary for the expression of the *H-2K^b* gene in L fibroblasts. High level expression of the 5′ enhancerless gene must therefore be controlled by an as yet unidentified regulatory sequence.

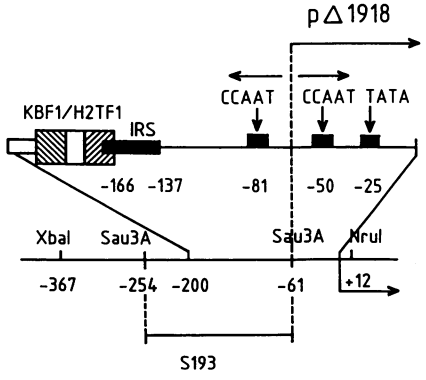


Fig. 1. Regulatory elements in the *H-2K^b* promoter region. Boxes represent *cis*-regulating sequences and binding sites for transcription factors. The numbering is shown relative to the start of transcription (arrow). Position of the 5′ deletion in pΔ1918 construct is indicated. S193, a 193 bp *Sau*3A fragment used as a probe. IRS, interferon response sequence.

The H-2K^b gene lacking its 5′ enhancer is not transcribed in embryonal carcinoma cells

H-2 class I genes are weakly, if at all, transcribed in embryonal carcinoma (EC) cells (Morello *et al.*, 1982). We transfected the pΔ1918 construct into P19 EC cells (McBurney and Rogers, 1982) to assay whether the 5′ enhancerless *H-2K^b* gene would be similarly regulated. Quantitative radioimmunoassay on viable EC cells did not detect cell surface expression of either the endogenous *H-2K^b* gene or the *H-2K^b* transgene (Table I). The results were supported by Northern blot analysis in which the *H-2K* probe (p1954, see Kvist *et al.*, 1983; Forejt *et al.*, 1988) hybridized strongly to mRNA from control L fibroblasts, but not to mRNA from EC cells that have been transfected with pΔ1918 (Figure 3). Other control EC cell lines (F9 and PCC4) did not show a positive signal. The presence of the *H-2K^b* transgene in transfected EC cell lines was confirmed by Southern blot analysis (data not shown). Thus the putative downstream regulatory element(s) of the *H-2K^b* gene is inactive or down regulating in EC cells.

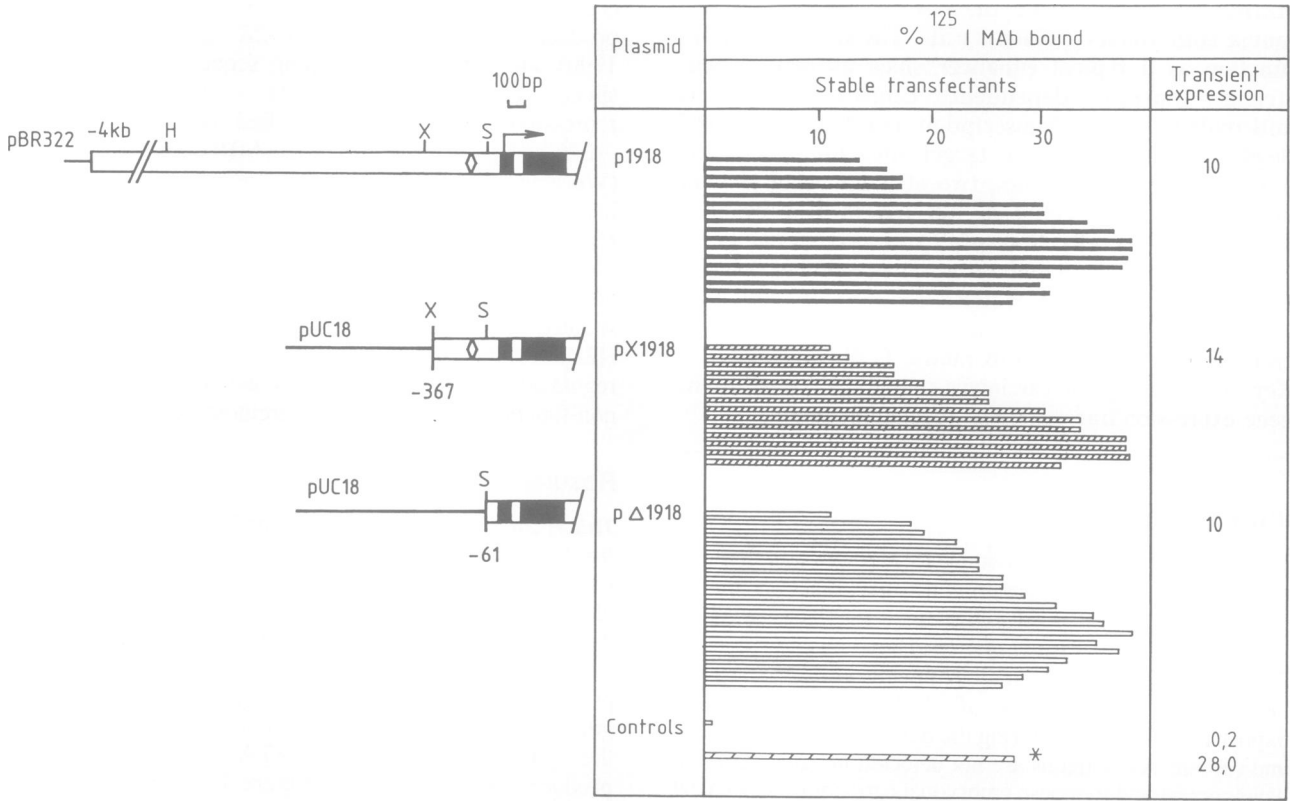


Fig. 2. The cell surface expression of the *H-2K^b* gene. The schemes of the deleted *H-2* genes (pX1918 and pΔ1918) are on the left. The three forms of the gene were transfected into the mouse Ltk$^-$ fibroblasts by a standard calcium phosphate method or by a DEAE-dextran and stable transfectants or transient cultures, respectively, were tested by the direct quantitative radioimmunoassay for the H-2K^b antigen by using 10^5 c.p.m. of anti-H-2K^b antibody labelled with ^{125}I and 2.5×10^5 cells per tube. Each column represents one randomly chosen stable clone in a typical experiment. Each stable clone was tested at least three times. The values of transient expressions are means of four different transfections. H, *Hin*dIII; X, *Xba*I; S, *Sau*3A.

Search for enhancer-like activity in different regions of the H-2K^b *gene*

Various restriction fragments isolated from p1918 were fused to the coding sequence of the CAT gene controlled by the weak conalbumin promoter in the reporter pconCAT vector (Kimura *et al.*, 1986) in order to search for a novel enhancer activity in the *H-2K*^{b} gene. Individual pH-2−conCAT constructs were transfected into mouse L fibroblasts and potential enhancer activity of an *H-2* gene fragment was reflected in an increase of the reporter CAT activity (Figure 4). In agreement with results of others (Kimura *et al.*, 1986; Baldwin and Sharp, 1987) the 193 bp *Sau*3A fragment containing the 5′ upstream enhancer stimulated high CAT expression. The promoter region on the *Sau*3A−*Kpn*I fragment (−61 to +35) cloned in front of the conalbumin promoter of the pconCAT vector, did not promote transcriptional activation of the CAT gene. Furthermore, the same minimal promoter in the context of the *H-2K*^{b} gene proper was unable to initiate an RNA transcription detectable in Northern blots (Figure 12).

Table I. Direct radioimmunobinding assay for H-2K antigens on the surface of control cells and transfected P19 embryonal carcinoma stable clones

Cells	Transfected plasmids	Binding of ^{125}I-labelled Mab (%)	
		anti-H-K^b	anti-H-2K^k
P19	− −	0.19	0.49
P192/6	p1918 + pgkneo	0.23	2.9
P192/8	p1918 + pgkneo	0.12	0.16
P192/15	p1918 + pgkneo	0.42	0.95
P193/3	p1918 + pgkneo	0.65	0.37
P193/4	p1918 + pgkneo	0.65	0.59
P194/4	p1918 + pgkneo	0.15	NT
P194/11	p1918 + pgkneo	0.24	NT
P194/12	p1918 + pgkneo	0.22	0.7
P195/7	p1918 + pgkneo	0.15	NT
P198/2	p1918 + pgkneo	1.2	0.4
P198/6	p1918 + pgkneo	0.46	0.46
Ltk$^-$		0.12	22.0
L23	p1918 + pTK	35.0	6.0

Binding of anti-H-2K^k monoclonal antibody detecting endogenous *H-2K*^{k} gene product and anti-H-2K^b monoclonal antibody detecting *H-2K*^{b} transgene product is expressed as the percentage of total radioactivity bound to cells. Each value represents an average of more than two experiments. Each tube contained 1×10^5 c.p.m. of antibody and 5×10^5 embryonal carcinoma cells or 2.5×10^5 fibroblast cells (Ltk$^-$, L23). NT represents not tested.

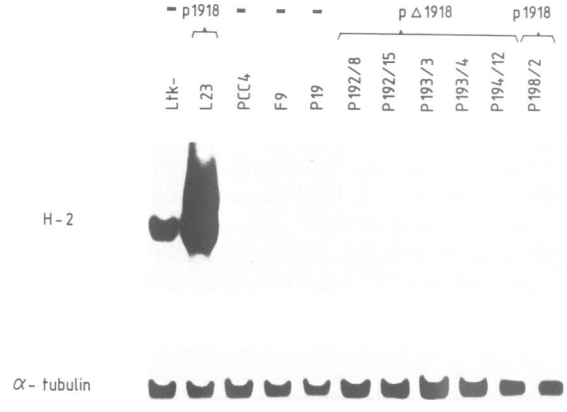


Fig. 3. Levels of H-2 class I mRNA in cell clones transfected with intact (p1918) or 5′ enhancerless (pΔ1918) *H-2K*^{b} gene. 20 μg of total cellular RNA from embryonal carcinoma cell lines PCC4aza, F9, P19 and P19 transfected clones, and from Ltk$^-$ fibroblasts and the L23 (a transfected fibroblast clone) were fractionated on 1% agarose gel, transferred to Zeta Probe membrane and hybridized to labelled probe p1954 (see legend to Figure 10) specific for *H-2* class I genes. To normalize for the amount of RNA, the blots were stripped of probe and rehybridized with a mouse α-tubulin cDNA probe.

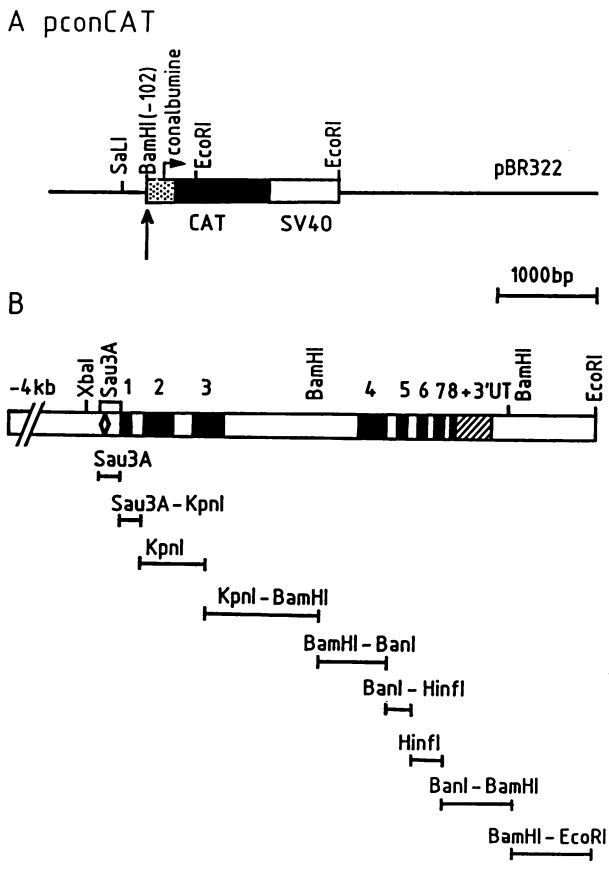
Analysis of the coding region of the *H-2K*^{b} gene revealed strong enhancing activity in the 771 bp *Kpn*I fragment spanning the first and the second exons and corresponding introns (Figures 4 and 5). The striking feature of this novel regulatory region (H2DRE) was that its activity was twice that of the KBF1/H2TF1 enhancer in the upstream 193 *Sau*3A fragment and had 80−120% of the activity of the control pRSVCAT gene, as measured by the CAT assay. Unlike the upstream regulatory sequence, the H2DRE was strongly orientation dependent, functioning only in the inversed position. However, the inverted orientation of the *Kpn*I fragment in an upstream position of pconCAT, can be considered normal because of its natural polarity with respect to the cap site. An alternative explanation could be envisaged if the H2DRE functions over a very small distance. Then the inversion could simply fulfil the role of bringing the H2DRE close enough to the transcription start site. At present we cannot differentiate between these alternatives.

The *Kpn*I fragment with H2DRE when subcloned in front of the bacterial CAT gene (p771conCAT) did not enhance the CAT activity when transfected into undifferentiated P19 EC cells, similar to the construct with the 5′ enhancer on the 193 bp *Sau*3A fragment. In contrast, the CAT activity of the control pRSVCAT construct was strongly enhanced (data not shown).

A possibility that the H2DRE transcriptional activity could be peculiar to the Ltk$^-$ cell line was ruled out in a transfection experiment where the H2DRE activity was tested in CAT assay in the NIH3T3 fibroblasts (Figure 6). The transcriptional activity of H2DRE was comparable to that of the 5′ KBF1/NFκB enhancer or the Rous sarcoma virus LTR enhancer.

*Mapping of the downstream regulatory activity within the Kpn*I *fragment*

The H2DRE was mapped further by assaying a series of smaller fragments derived from the *Kpn*I fragment (Figure 5). Only the *Bss*HII−*Kpn*I fragment (position 272−806) retained full CAT activity and thus functionally defined the 5′ limit of the regulatory element. Splitting of the *Kpn*I fragment at the *Taq*I site, +392, resulted in severe reduction of the CAT activity in both fragments. The transcriptional activity may be expected in the pconCAT construct with the inverted *Taq*I-*Kpn*I fragment, but unfortunately we were unable to recover this type of construct. Further division of the functional *Bss*HII−*Kpn*I fragment (219 bp *Dde*I, 414 bp *Taq*I−*Kpn*I and 289 bp *Dde*I−*Kpn*I fragments in Figure 5) resulted in the disappearance or considerable reduction of transcription enhancing activity. Nevertheless, the *Dde*I−*Kpn*I fragment displayed significant positive transcriptional activity in both directions, representing 20% of that of the non-divided *Kpn*I fragment. These observations point to the



Fragment	Enhancement factor (average) +	−	Range +	−	N +	−
Sau3A 193	112.0	NT	20.0-217.0	NT	3	NT
Sau3A-KpnI 101	1.3	NT	1.2-1.4	NT	2	NT
KpnI 771	220.0	7.3	118.0-354.0	4.4-11.2	5	3
KpnI-BamHI 1300†	1.3	1.8	1.3	1.2-2.6	1	5
BamHI-BanI 700†	2.3	3.2	1.2-3.3	1.8-4.7	2	8
BanI-HinfI 340	1.6	1.6	0.8-2.4	0.8-3.8	2	7
HinfI 263	2.0	NT	1.0-4.3	NT	6	NT
BanI-BamHI 800†	NT	1.4	NT	1.0-2.5	NT	7
BamHI-EcoRI 900†	NT	2.3	NT	1.3-3.9	NT	3

Fig. 4. Search for enhancer-like activities in the *H-2K*^{b} gene. (**A**) Map of the pconCAT enhancer testing vector. The unique *Bam*HI cloning site is arrowed. (**B**) The CAT activities of various *H-2K*^{b} fragments cloned into *Bam*HI site of the pconCAT vector. Exons are shown as black boxes. + or − indicate the natural or inverted orientation of the fragment relative to the transcriptional start site. Thus for all downstream fragments + means inverted position, while with the upstream 193 bp *Sau*3A fragment + means the non-inverted insertion into the upstream *Bam*HI site of pconCAT. Enhancement factor represents a relative value obtained after normalization for transfection efficiency of the tested construct to an arbitrary value of 1 for the pconCAT vector included in each experiment. Sequence information for the third intron and 3′ flank region is incomplete. NT, not tested.

synergistic involvement of more than one component in the functional regulatory element within the *Bss*HII−*Kpn*I fragment.

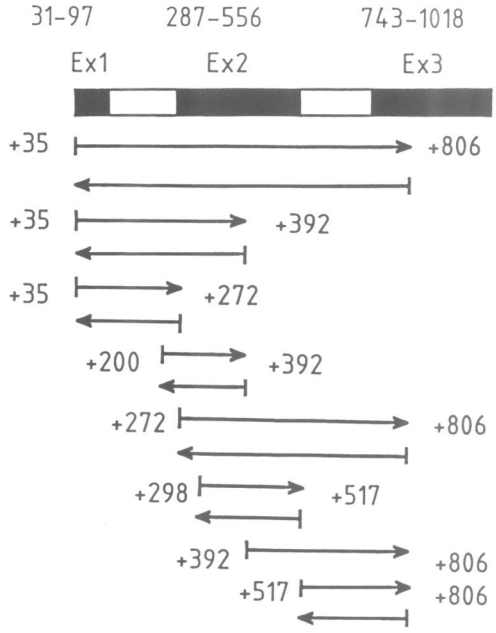
Nuclear factors specifically binding to the H2DRE

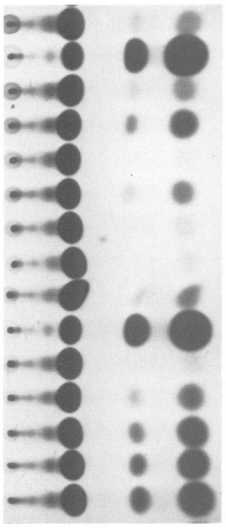
Finding a novel *cis*-regulatory element implies the possible occurrence of specific *trans*-acting factor(s) binding to it (Wingender, 1988). The results of functional CAT assays indicate that the *Bss*HII−*Kpn*I fragment harbours multiple sites that together constitute a functional H2DRE. Thus more than one specific nuclear factor can be expected to bind at different positions within the *Bss*HII−*Kpn*I fragment. The results of gel mobility shift assays indeed point to this conclusion. A set of five end-labelled fragments spanning the *Kpn*I fragment was used to identify more than six sequence-specific binding proteins in Ltk$^-$ nuclear extracts by using the gel mobility shift assay. The sequence specificity of the created complexes was verified in competition experiments with a 50 molar excess of homologous DNA (Figure 7). The ^{32}P end-labelled probe III (298−517 *Dde*I) was further used in competition tests with a series of unlabelled fragments I−IV, fragment carrying the 5′ enhancer of the *H-2K*^{b} gene and with double-stranded oligonucleotides representing the H2TF1/KBF1/NFκB core site and the CP-2 binding site (Figure 8). Fragments II, III and V competed with formation of retarded bands III.1, III.2 and III.3. The relevant factor thus binds in the interval of overlap of all three fragments at position 298−392 at the 5′ end of the second exon. The bands III.2 and III.3 involve apparently the same binding sequences as bands II.2 and II.3 and V.5. This conclusion can be drawn from the competition experiments with fragment II or V used as radiolabelled probes (data not shown).

The first attempt to seek for tissue-specific transcription factors did not reveal any reproducible differences when ^{32}P end-labelled fragments I, II or III were used in gel mobility shift assay to compare P19 and Ltk$^-$ nuclear binding proteins (data not shown). However, with the fragment IV and its subfragment IV^a, we could detect two Ltk$^-$ specific complexes that were missing or greatly reduced in P19 nuclear extracts (Figure 9).

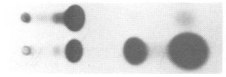
Deletion analysis of the function of H2DRE element within the context of the H-2K^b *gene*

The actual involvement of the downstream regulatory element in the expression of *H-2K*^{b} gene was verified in two deletion experiments. In one experiment, the 771 bp *Kpn*I fragment of the genomic clone of *H-2K*^{b} gene, spanning the first three exons and two introns, was exchanged for a homologous *Kpn*I fragment from a cDNA copy of the same gene. The new constructs pX1918KK-cDNA and pΔ1918KKcDNA differed from their genomic





Fragment	Average enhancement factor	Range	N
KpnI 771	7.3	4.4- 11.2	3
KpnI 771	220.0	118.0-354.0	5
KpnI-TaqI 357	8.2	3.0- 15.8	4
KpnI-TaqI 357	12.3	8.3- 19.0	4
KpnI-BssHII 237	4.0	1.7- 7.7	5
KpnI-BssHII 237	6.9	2.5- 12.0	3
HinfI-TaqI 192	2.3	1.3- 3.2	3
HinfI-TaqI 192	3.2	1.2- 6.0	3
BssHII-KpnI 534	6.1	4.6- 7.7	4
BssHII-KpnI 534	206.3	99.0-316.0	3
DdeI 219	1.9	1.0- 2.4	4
DdeI 219	7.5	3.6- 11.5	2
TaqI-KpnI 414	16.7	7.7- 25.0	4
DdeI-KpnI 289	46.3	27.0- 58.0	3
DdeI-KpnI 289	45.7	27.0- 64.0	3



pconCAT	1.0	-	9
pRSVCAT	226.0	117.0-350.0	9

Fig. 5. Mapping of the downstream regulatory activity within the *Kpn*I fragment of the *H-2K*^{b} gene. Series of smaller fragments were subcloned into the *Bam*HI site of the pconCAT reporter vector. The orientation of inserts with respect to the chicken conalbumin promoter is indicated by an arrow. The enhancement factor was determined as detailed in Figure 4. The autoradiograph shows the chromatographic separation of radiolabelled chloramphenicol and its acetylated derivatives and represents a typical experiment. N, number of determinations.

ancestors in the absence of the first and second introns (Figures 10 and 11). In the pX1918KKcDNA construct initiation of transcription was driven by the H2TF1/KBF1 5′ enhancer and the gene was expressed on the cell surface of stable transfectants in quantity comparable to that of intact *H-2K*^{b} gene transfectants (Figure 11). However, the mean value of expression of the pΔ1918KKcDNA dropped significantly ($P < 0.01$ in the Student−Neumann−Keuls multiple range test) to 38% of that found in cells transfected with pΔ1918 or with pX1918KKcDNA (Figure 11). It can be concluded that deletion of the first and second introns removes a significant portion of the H2DRE activity in the *H-2K*^{b} gene devoid of its 5′ enhancer.

In the second experiment the whole 771 *Kpn*I fragment was deleted from the *H-2K*^{b} gene and the shortened gene was transfected into Ltk$^-$ fibroblasts as pX1918ΔK (with 5′ enhancer) or as pΔ1918ΔK (without 5′ enhancer). A product of the shortened gene, lacking its second exon and parts of its first and third exons, was not detectable on the cell surface of the transfected cells (data not shown). However, Northern blot analysis revealed the expected faster mRNA band in the clones transfected by the pX1918ΔK, but not in cells transfected by pΔ1918ΔK (Figure 12). Thus the results clearly show that the H2DRE is a regulatory sequence necessary and sufficient for transcription initiation in the *H-2K*^{b} gene with deleted 5′ enhancer. Furthermore, it seems obvious that the 5′ enhancerless *H-2K*^{b} gene does not possess any other regulatory sequence than H2DRE that would be able to activate its transcription in Ltk$^-$ fibroblasts.

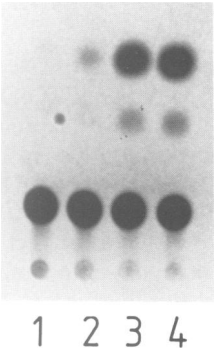


Fig. 6. Verification of the H2DRE regulatory activity in the NIH3T3 mouse fibroblast cell line. The following constructs were tested in the CAT assay after calcium phosphate transfection: lane 1, pconCAT, a negative control without an insert; lane 2, pconCAT193, a positive control which carries the *Sau*3A fragment (−244 to −61) with the KBF1/NFκB 5′ enhancer; lane 3, pconCAT771, carrying the H2DRE on the *Kpn*I fragment (35−806) cloned in the opposite direction into the *Bam*HI site of pconCAT; lane 4, pRSVCAT, another positive control.

Discussion

Our search for a novel regulatory element in the *H-2K*^{b} gene was initiated by an unexpected observation of the cell surface expression of the 5′ deletion gene construct lacking the 5′ enhancer. The quantity of the *H-2K*^{b} antigens, as measured in 20 clones of stably-transfected L fibroblasts by

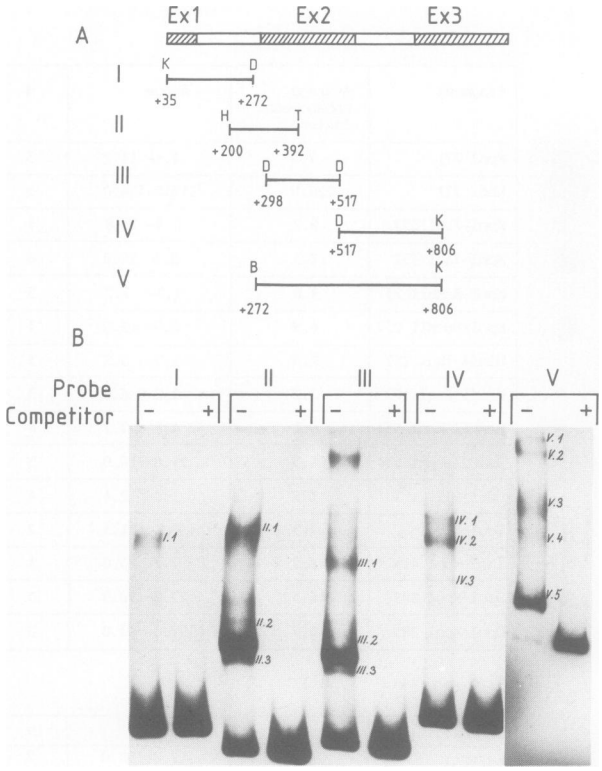


Fig. 7. Binding of nuclear factors to the H2DRE by gel mobility shift assay. (**A**) Set of ^{32}P-end labelled fragments spanning the *Kpn*I region of the *H-2K*^{b} gene were used as probes I–IV in the binding reactions with Ltk^{-} nuclear extracts. (**B**) Specificity of binding of nuclear proteins to the individual probes was determined by *in vitro* competition, using a 50 molar excess of homologous DNA. Retarded bands representing specific DNA–protein complexes are indicated by numbers 1–5.

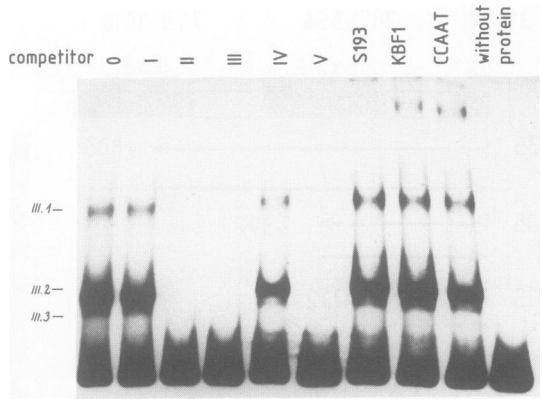


Fig. 8. Gel mobility shift competition assay. The end-labelled fragment III was used as a probe in the binding reaction with the Ltk^{-} nuclear extracts and with 50 molar excess of the following competitors: fragments I–IV (spanning the *Kpn*I region, see Figure 7A for details); *Sau*3A (−264 to −61) fragment carrying the 5′ enhancer of the *H-2K*^{b} gene (S193); the KBF1 double-stranded oligonucleotide (−171 to −158) representing the core site of the 5′ enhancer (KBF1); and the CCAAT oligonucleotide (−91 to −63) representing the CP2 binding site in the *H-2K*^{b} gene (CCAAT).

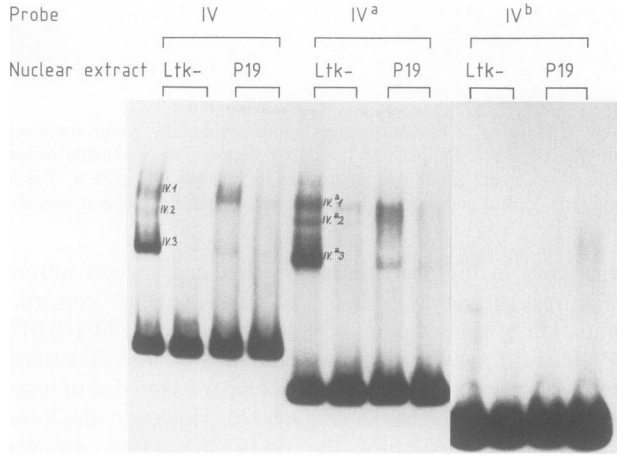


Fig. 9. Differential binding of nuclear proteins from Ltk^{-} fibroblasts and P19 teratocarcinoma cells to H2DRE. Fragment IV (*Dde*I–*Kpn*I 517–806) and its *Hin*fI digestion products, fragments IV^{a} (*Dde*I–*Hin*fI, 517–684) and IV^{b} (*Hin*fI–*Kpn*I, 684–806) were end-labelled and used as probes in gel mobility shift assay. As a control of the specificity of created complexes, the 50 molar excess of non-labelled homologous fragment was included in tubes run in even lanes. The binding reaction was modified by adding $MgCl_2$ to 10 mM. Band IV.2, IV.3 and bands IV.^{a}2 and IV.^{a}3 are diminished or absent in reactions with P19 nuclear extracts.

a highly sensitive radioimmunoassay, was not significantly changed after the 5′ flanking region of the gene was deleted, leaving only 61 bp with a minimal promoter. Moreover, the truncated gene was not transcribed in embryonal carcinoma cells, in spite of the fact that the 5′ silencing element (Miyazaki *et al.*, 1986; Flanagan *et al.*, 1991) was deleted. These findings allowed us to predict the occurrence of a strong developmentally-specific transcriptional element downstream to the cap site of the *H-2K*^{b} gene. Until now, most examples of transcriptional regulation of *H-2* class I genes have been explicable in terms of the 5′ *cis*-acting elements scattered in the region of −80 to −1837 from the transcription start site: (i) the constitutive expression was found to be under the control of the A and B enhancer regions (Kimura *et al.*, 1986; Israel *et al.*, 1989a) overlapping with CRE regions as defined by Miyazaki *et al.* (1986); (ii) the tissue and developmental specificity of the expression corresponded with the presence or absence of the KBF1/H2TF1 factors binding to the 5′ enhancers (Burke *et al.*, 1989; Leonardo *et al.*, 1989); (iii) pathological down-regulation of MHC class I genes in malignant tumour cells (Bernards, 1987; Henseling *et al.*, 1990), as well as in virus infected cells (Katoh *et al.*, 1990), were mediated by upstream sequences; (iv) the inducibility of the *H-2* class I gene expression was found to operate from the 5′ *cis*-responsive elements and to include the effects of interferons (IFN) (Israel *et al.*, 1986; Korber *et al.*, 1987; Sugita *et al.*, 1987), tumour necrosis factor, phorbol esters (Israel *et al.*, 1989b) and retionoic acid-mediated activation of MHC class I transcription in embryonal carcinoma cells (Nagata *et al.*, 1992). The concentration of cAMP was reported either to increase (Israel *et al.*, 1989b) or decrease (Saji *et al.*, 1992) the MHC class I mRNA levels, and both effects were attributed to different parts of the 5′-flanking sequences.

All these types of transcriptional regulations may now need to be reexamined for the possible involvement of the 3′ regulatory sequences. The first indication for the existence of such sequences has been provided by estimation that 40% of the inducibility of the *H-2* class I gene by INF could be attributed to the upstream response element (Korber *et al.*, 1988). Another clear-cut evidence for a downstream

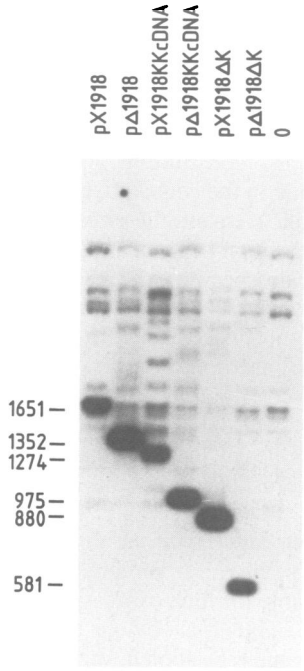


Fig. 10. Southern blot analysis of truncated forms of the *H-2K*^{b} gene transfected into the Ltk$^-$ fibroblasts. 7 μg of genomic DNA were digested with *Pst*I, electrophoresed in a 0.8% agarose gel and transferred to a nylon membrane. The blot was probed with *H-2K* probe, p1954 (a 1.8 kb *Pst*I fragment of the *H-2K*^{d} gene including its 5′ non-coding region and the first two exons). The structure of the truncated *H-2K*^{b} constructs is shown in Figure 9. The calculated size of diagnostic *Pst*I fragments (in bp, shown at the left) corresponds to their actual mobility. The 5′ end of each diagnostic *Pst*I fragment is in the polylinker of pUC18 vector and 3′ end is in the third intron of the *H-2K*^{b} gene, +1273 bp from the RNA startsite in the intact gene. Control lane with DNA from non-transfected Ltk$^-$ fibroblasts is designated 0.

regulatory element(s) responding to IFN type I treatment was provided for human MHC class I genes HLA-B7 and HLA-B27 (Yoshie *et al.*, 1984; Schmidt *et al.*, 1990). Moreover, the truncated forms of both genes lacking their 5′ enhancers were still compatible with the cell surface expression of the respective antigens even in the absence of IFN induction, thus pointing to the existence of a constitutive downstream regulatory activity.

The screening of the body of the *H-2K*^{b} gene and its 3′-flanking region for enhancer-like activities revealed the 538 bp long *Bss*HII−*Kpn*I fragment encompassing most of the second exon, second intron and 67 bp of the third exon as the most potent source of positive transcriptional activity monitored by the CAT reporter gene in fibroblasts (Figure 5) and by its effect *in situ* on transcription in deletion mutants of the *H-2K*^{b} gene (Figures 11 and 12). The lack of CAT activation in the same construct transfected into embryonal carcinoma cells indicated a developmentally regulated activity of the new regulatory element. The actual size and complexity of this regulatory element will be established by DNA footprint analysis and other techniques. Nevertheless, the inactivation of the transcriptional enhancement by splitting the 538 bp fragment into two parts and the presence of more than six specific nuclear protein binding sites within the region point to a possible complex structure of this downstream regulatory element. In a similarly designed experiment, Ganguly *et al.* (1989) reported multiple enhancer-like sequences in the third and fifth introns of the human HLA-B7 class I gene. An activity similar to that of the H2DRE was not detected probably because none of the HLA-B7 fragments contained the second exon with the second intron.

The results of deletion experiments presented here indicate that the H2DRE is the only regulatory region downstream

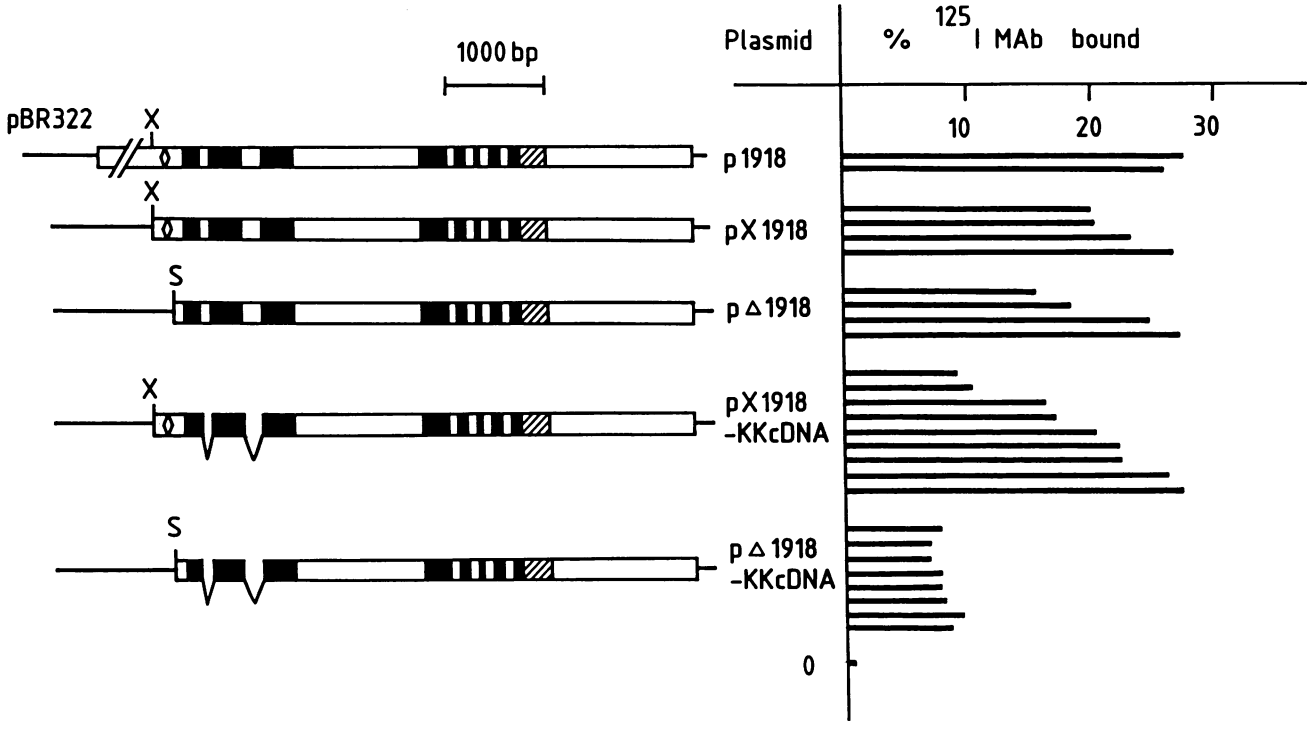


Fig. 11. The quantitative radioimmunoassay of cell surface expression of the truncated *H-2K*^{b} genes. The schemes of truncated genes (pΔ1918, *H-2K*^{b} without 5′ enhancer; pX1918KKcDNA, without second and third intron; pΔ1918KKcDNA, without 5′ enhancer and without second and third intron) are on the left. Each column represents the mean relative quantity of *H-2K*^{b} molecules in one stably transfected clone based on three independent measurements. The conditions of assay are the same as described in Figure 2.

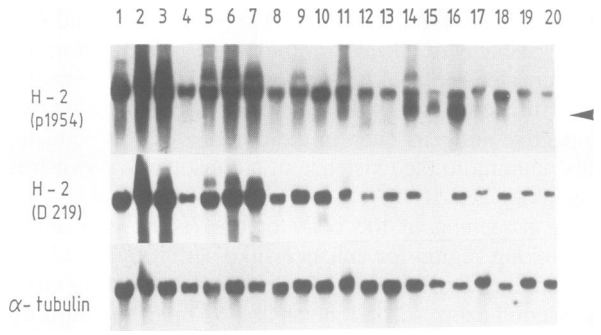


Fig. 12. Northern blot analysis of *H-2* class I mRNA levels in cell clones stably transfected with truncated *H-2K^b* genes. Total cellular RNA (20 μg per lane) was fractionated by electrophoresis in 0.8% agarose–formaldehyde gel, transferred to Hybond N$^+$ and hybridized consecutively to ^{32}P-labelled probes; *H-2* class I probes (p1954, see legend to Figure 8; D219, a 219 bp *Dde*I fragment from within the second exon of the *H-2K^b* gene) and to a mouse α-tubulin probe. The analysed clones were transfected with: p1918 (lane 1), pX1918 (lanes 2–4), pΔ1918 (lanes 5–7), pX1918KKcDNA (lanes 8–10), pΔ1918KKcDNA (lanes 11–13), pX1918ΔK (lanes 14–16), pΔ1918ΔK (lanes 17–19) and the non-transfected control Ltk$^-$ (lane 20). The arrowhead points to the truncated transcripts from *H-2K^b* genes with deleted 771 *Kpn*I fragment.

of the transcription start site capable of transcriptional activation of the 5′ enhancer-less *H-2K^b* gene. This points to possible species-specific differences in the regulation of MHC class I genes. Another significant difference in regulation of MHC class I genes found between the mouse and human MHC class I downstream regulatory activity was in their strength and enhancer-like properties. The H2DRE appeared to be twice as strong as the 5′ enhancer (H2TF1/KBF1/NFκB site) and comparable in its activity to the Rous sarcoma virus LTR enhancer when tested in the reporter pconCAT vector, while the intron enhancers of the HLA-B7 gene did not reach the activity of their 5′ counterpart. In contrast to the HLA-B7 intronic enhancers, the H2DRE was strongly position dependent. It functioned only in the inverted position from the 5′ *Bam*HI insertion site of pconCAT (Figure 5), thus indicating that the correct polarity of the H2DRE element may be necessary with respect to the promoter.

Data are accumulating on the regulatory elements located 3′ to the transcription start site in various genes transcribed by RNA polymerase II. They include genes coding for immunoglobulins (Atchinson, 1988), β-globin (Behringer *et al.*, 1987; Kolias *et al.*, 1987), collagens (Horton *et al.*, 1987; Rippe *et al.*, 1989), human class II MHC antigens (Wang *et al.*, 1987), 4F2HC (Karpinski *et al.*, 1989), ADA (Aronow *et al.*, 1989), keratin 18 (Oshima *et al.*, 1990), rpL32 (Chung *et al.*, 1989), human β-actin (Ng *et al.*, 1989), Thy-1 (Vidal *et al.*, 1990) or TIMP (Coulombe *et al.*, 1988). Very often these elements were found in the first intron where they behaved as real enhancers (Kawamoto *et al.*, 1988; Karpinski *et al.*, 1989) or they functioned from a heterologous promoter, but in an orientation- and position-dependent manner (Bornstein *et al.*, 1988; Chung *et al.*, 1989; Collis *et al.*, 1989; Oshima *et al.*, 1990; Franklin *et al.*, 1991). It is the latter type of regulatory element for which we propose to coin the term DRE, Downstream Regulatory Element. The H2DRE could be a member of this tentative class of *cis*-acting elements in spite of its location in the region of the second intron and second exon because the distance from the cap site is comparable to that of many genes with the DRE in the first intron.

It should be noted that at least two observations point against the DRE as a general promoter subunit. First, apparently the same DRE of the first intron of the TIMP gene governs its constitutive expression and virus inducibility. While in the constitutive expression it behaves as an enhancer-like element, the virus inducibility is strictly orientation- and position-dependent (Coulombe *et al.*, 1988). Secondly, the brain-specific expression of the mouse *Thy-1* gene is conferred by a DRE in the first intron, but the expression in thymus is controlled from an enhancer in the third intron. The insertion of an irrelevant DNA sequence between the promoter and the DRE resulted in abolition of brain expression, but did not harm the thymus expression of the gene. This clearly indicates that the Thy-1DRE is not essential for general function of the *Thy-1* promoter (Vidal *et al.*, 1990).

Many questions arose with the discovery of H2DRE. It has been proved that H2DRE represents a new regulatory element responsible for high expression of the 5′ enhancer-less *H-2K^b* gene. Thus it is obvious that our understanding of MHC class I gene regulation in various immune responses including the immune surveillance of malignant tumour cells will not be complete without detailed analysis of these MHC downstream regulatory sequences.

Materials and methods

Genes and plasmid constructions

The genomic *H-2K^b* gene situated on a 10.5 kb long *Eco*RI fragment cloned in pBR322 (p1918) (Daniel-Vedele *et al.*, 1983) was a kind gift from G.Gachelin. The full length cDNA clone of the same gene (1600 bp clone in pUC19, Schonrich *et al.*, 1991) was kindly provided by B.Arnold. Plasmid pTK with a cloned thymidine kinase gene from Herpes simplex virus was obtained from P.N.Goodfellow. Plasmid pCH110 contains the *LacZ* gene from *Escherichia coli* under the control of SV40 early promoter (Hall *et al.*, 1983). In pCMVb the same gene is under the control of cytomegalovirus promoter. Plasmid pgkneo was obtained from M.McBurney and contains the *Neo^r* gene driven by the phosphoglycerate kinase-1 promoter-enhancer region. Plasmid pconCAT, in which the bacterial CAT gene is under the control of a weak chicken conalbumin promoter (Kimura *et al.*, 1986), was a gift from A.Israel.

The 5′ enhancerless *H-2K^b* gene, called here pΔ1918, was constructed in three steps. First, the 5′ *Xba*I–*Kpn*I fragment of the *H-2K^b* gene (−367 to +35) was cloned into pUC18. The plasmid obtained was used for isolation and subcloning of subfragments *Sau*3A–*Sau*3A (−254 to −61) and *Sau*3A–*Kpn*I (−61 to +35) to generate plasmids pS193 and pS101. The final construct, pΔ1918, containing the *H-2K^b* gene with upstream sequences only up to −61 was prepared by inserting a 5.4 kb *Nru*I–*Eco*RI fragment into unique *Nru*I and *Eco*RI sites of pS101. The fidelity of the construct was verified by extensive restriction mapping, Southern blot analysis and sequencing the critical region of the gene. To construct a series of pH-2–conCAT hybrid plasmids, various restriction fragments were isolated from the 5′ end of the *H-2K^b* gene, from its coding and 3′-flanking regions, adapted by adding *Bam*HI linkers and subcloned into the *Bam*HI site of the pconCAT vector.

Cell culture and transfection experiments

Mouse Ltk$^-$ fibroblasts, P19, F9 and PCC4aza embryonal carcinoma cell lines were grown in Dulbecco's modification of Eagle's medium (DMEM, Flow Laboratories) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

For stable transfections the calcium phosphate co-precipitation technique (Weber *et al.*, 1984) was used. For one 60 mm dish containing Ltk$^-$ fibroblasts, 10 μg of plasmid DNA was co-precipitated with 1 μg of pTK plasmid and 9 μg of carrier DNA from host Ltk$^-$ or P19 cells. The cells were exposed to DNA for 4 h and then shocked by 15% glycerol in buffered saline for 4 min at room temperature. HAT selective medium was refed every 3 days. Plasmid pgkneo and selectable medium containing geneticin (G418, Gibco) at a final concentration of 400 μg/ml were used for dominant

selection of transfected EC cells. To achieve transient expression, the cells were transfected with DNA using the DEAE-dextran procedure enhanced by chloroquine treatment (Sambrook *et al.*, 1989). Briefly, 2×10^6 cells were plated into a 60 mm dish on day 1, then on day 2 the cells were washed three times in serum free medium and exposed to transfection cocktail of 10 μg of appropriate plasmid purified on CsCl gradient, 2 μg pCH110 to monitor the efficiency of transfection, 500 μg/ml DEAE dextran and 170 μM chloroquine in 2 ml of serum free medium per plate. After 3 h incubation at 37°C the transfection cocktail was removed, cells were exposed for 2 min to 15% DMSO in Tris-buffered saline, washed with complete DMEM medium and subsequently cultured for 2 days.

Enzymatic assays

Cell extracts were prepared from washed cells by three cycles of freeze–thaw in 60 μl of 0.25 M Tris–HCl (pH 7.8) and nuclei were spun down. 10 μl of supernatant were assayed for β-galactosidase activity by using standard technique (Amin, 1985). CAT assays were performed on cell extracts normalized for equal β-galactosidase activity. Proteases in cell extracts were destroyed by heating to 65°C for 10 min. Then CAT activity was assayed by incubation with [^{14}C]chloramphenicol and acetyl CoA for 16 h as described (Sambrook *et al.*, 1989).

Quantitative radioimmunobinding assay

The quantity of the H-2K antigens on the surface of viable cells was determined by direct binding of ^{125}I-labelled monoclonal antibodies specific for H-2K^b (B8-24-3, Kohler *et al.*, 1981) and for H-2K^k (11.4.1, Oi *et al.*, 1978) as described (Draber and Stanley, 1984; Mosinger and Forejt, 1989).

RNA and DNA analysis

Total cellular RNA was isolated by the lithium chloride–urea procedure (Auffray and Rougen, 1980) or by acid phenol–quanidine thiocyanate method (Chomczynski and Sacchi, 1987) and 20 μg of RNA per lane was separated in 1% agarose gels containing 1.1 M formaldehyde (Maniatis *et al.*, 1982). To normalize for the amount of RNA in each lane, blots were stripped from the probe after autoradiography and rehybridized with a 1.7 kb *Pst*I fragment of the mouse α-tubulin cDNA probe (Cleveland *et al.*, 1980). Genomic DNA was isolated by the method of Fasano *et al.* (1984), 10 μg aliquots were digested with an appropriate restriction enzyme, fractionated by electrophoresis in 0.8% agarose gels and blotted to Zeta probe nylon membranes. Hybridizations were carried out with DNA probes ^{32}P-labelled by the oligonucleotide random priming method (Feinberg and Vogelstein, 1983) in the presence of 50% formamide and 10% dextran sulfate at 42°C for 20 h, washed under stringent conditions (60°C, 0.1 × SSC, 0.1% SDS) and the filters were exposed to X-ray film for 3–6 days.

Nuclear extracts

Nuclear extracts were prepared essentially according to Fujimura (1986). In brief, $2-5 \times 10^7$ Ltk$^-$ cells washed in phosphate-buffered saline were resuspended in 2 ml of lysis buffer (10 mM HEPES, pH 8.0, 0.5 mM spermidine, 0.15 mM spermine, 50 mM NaCl, 1.0 mM EDTA, 7 mM 2-mercaptoethanol, 0.5 mM PMSF, 0.5 M saccharose and 0.5% NP40) and the nuclei were pelleted by centrifugation. The pellet was resuspended in extraction buffer identical with lysis buffer, but with 350 mM NaCl and 10% glycerol instead of saccharose and the nuclei were extracted for 30 min on ice, extensively dialysed against binding buffer (see below). Protein concentration was determined by the method of Bradford (1976).

Electrophoresis mobility shift assays

The fragments used as probes were end-labelled with [α-^{32}P]dCTP or [α-^{32}P]dATP (Amersham) by the Klenow fragment of DNA polymerase I. Binding reactions were carried out in a final volume of 20 μl of the binding buffer (20 mM HEPES, pH 8.0, 70 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF and 15% glycerol). A typical reaction mixture included 1 ng of ^{32}P end-labelled probe ($1-2 \times 10^4$ c.p.m.), 5 μg of nuclear protein, 0.5–2 μg of poly(dI–dC):poly(dI–dC). In competition experiments the specific competitor was added 5 min prior to addition of the labelled probe. The binding mix was incubated for 25 min at room temperature and then resolved in a 4% polyacrylamide gel in 0.25 × TBE buffer at a constant current of 25 mA. The gel was then dried and autoradiographed.

DNA sequencing

The DNA sequence of the deleted 5′ region of the *H-2K*^{b} gene in pΔ1918 was established, starting from the pUC18 polylinker region to the first *Kpn*I site (+35) by the method of Maxam and Gilbert (1980). The sequencing reactions were run on 8% denaturing polyacrylamide gels.

Acknowledgements

We are grateful to F.Smrt and J.Sedlacek for oligonucleotides and D.Stursova and M.Landikova for expert assistance. The work of J.F. was supported in part by the Imperial Cancer Research Fund (London, UK).

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Received on 11 May, 1992; revised on 10 August, 1992