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Purification of KBF1, a common factor binding to both H-2 and β2-microglobulin enhancers

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An enhancer binding factor, designated KBF1, has been purified from the nuclear extract of mouse BW5147 thymoma cells by five column chromatography steps including a sequence-specific DNA affinity column. Gel retardation and footprint analysis have shown that purified KBF1 has a binding activity specific for both H-2 and β2-microglobulin enhancer sequences. After SDS–polyacrylamide gel electrophoresis of the most purified preparation a 48-kd protein showed, after elution and renaturation, a binding activity to both enhancer sequences. These findings suggest that the expression of both H-2 and β2-microglobulin genes utilizes a common regulatory mechanism.

Key words: H-2 gene/enhancer/DNA binding protein/DNA affinity chromatography/gel retardation assay

Introduction

Class I antigens of the major histocompatibility complex (MHC), functioning in the recognition of target cells by cytotoxic T lymphocytes (Zinkernagel and Doherty, 1980), are found on the cell surface as heterodimers composed of non-covalently associated heavy and light polypeptide chains. In the mouse MHC, heavy chains (H-2) are polymorphic 45-kd proteins encoded in K and D regions on chromosome 17. The light chain is β2-microglobulin (β2m), a non-polymorphic 12-kd protein encoded in a region on chromosome 2 (Coligan *et al.*, 1981; Hood *et al.*, 1983; Flavell *et al.*, 1986).

These class I antigens are not found on the cell surface of the egg or early mouse embryo (Buc-Caron *et al.*, 1978), nor on embryonal carcinoma (EC) cells (Morello *et al.*, 1978), while they are expressed on most somatic cells of the adult organism. In EC cells, the lack of class I antigens on the surface is correlated with a very low level of H-2 and β2m steady state mRNA (Croce *et al.*, 1981; Morello *et al.*, 1982). *In vitro* differentiation induced by chemicals (retinoic acid and dibutyryl cAMP) accompanies the appearance of H-2 and β2m molecules at the surface of EC cells (Morello *et al.*, 1983). Additionally, the transcription of H-2 and β2m genes is activated by interferons (Rosa *et al.*, 1983). These experimental results suggest that some common regulatory mechanisms might be involved in the expression of both genes, although it has been reported that the expression of these genes does not always proceed co-ordinately (Ozato *et al.*, 1985; Morello *et al.*, 1985).

Our previous studies have shown that the promoter region of the H-2K^b gene contains two enhancer sequences (enhancers A and B), one of which (enhancer A) overlaps with a consensus interferon response sequence (IRS) (Kimura *et al.*, 1986). Although IRS is essential in the stimulation of the H-2 gene by interferon, this sequence is functional only when it associates with the overlapping enhancer A (Israël *et al.*, 1986). These facts indicate the importance of enhancer A in the regulation of H-2 gene expression. Using a nuclear extract of mouse cultured cells, we have found that a protein factor binds to the overlapping sequence between IRS and enhancer A. It has further been shown that the same or closely related factor also binds to the homologous sequence in the β2m enhancer region. *In vivo* competition experiments suggest that this factor acts positively in the expression of both the H-2 and β2m genes (Israël *et al.*, 1987). An apparently similar factor which binds to the H-2K^b enhancer and 72-bp repeat enhancer element of simian virus 40 has been detected by Baldwin and Sharp (1987) in human HeLa cells.

To demonstrate whether the same protein factor binds to the enhancer regions of both H-2 and β2m gene promoters, it was necessary to isolate it in a highly purified form. Furthermore, an exact understanding of the role of this factor in the expression of both genes depends upon its purification. In this study, we have purified this enhancer binding protein, designated KBF1, by five sequential column chromatography steps including sequence-specific DNA affinity chromatography. In each column chromatography step, DNA binding activities specific for the H-2K^b and β2m enhancer sequences were eluted in the same fractions. Furthermore, we have identified KBF1 as a 48-kd protein by preparative SDS-polyacrylamide gel electrophoresis followed by renaturation with 6 M guanidine HCl treatment. This renatured KBF1 has been shown to have a binding activity specific for both H-2K^b and β2m enhancer sequences.

Results

Preparation of nuclear extract from mouse BW5147 thymoma cells

In a preceding report, we showed that a nuclear extract prepared from mouse 3T6 cells contained a protein factor capable of binding to the enhancer region in the promoters of mouse H-2 and β2m genes (Israël *et al.*, 1987). However, our initial attempts to purify this factor from 3T6 cells indicated that larger amounts of starting material would be essential in further purification. For this reason, we used mouse BW5147 thymoma cells as the starting material in this study. This cell line, derived from a mouse thymoma expressing H-2 antigens (haplotype H-2^k), grows in suspension and contains at least three to four times more KBF1 activity than 3T6 as judged from retardation assays. Recent data have also shown that KBF1-like activity is found in the nuclear extracts of other cell lines: HeLa, BALB/c 3T3 and MEL (Baldwin and Sharp, 1987; our unpublished results).

BW5147 cells were homogenized and centrifuged. The nuclear pellet thus obtained was washed in buffer containing 0.1 M NaCl. To eluate KBF1 activity, the pellet was extracted for 30 min in buffer containing 0.3 M NaCl, then centrifuged. Ammonium sulphate was added to the resulting supernatant up to 45% saturation, and proteins were precipitated by centrifugation as described by Piette *et al.* (1985). Most KBF1 activity was recovered in

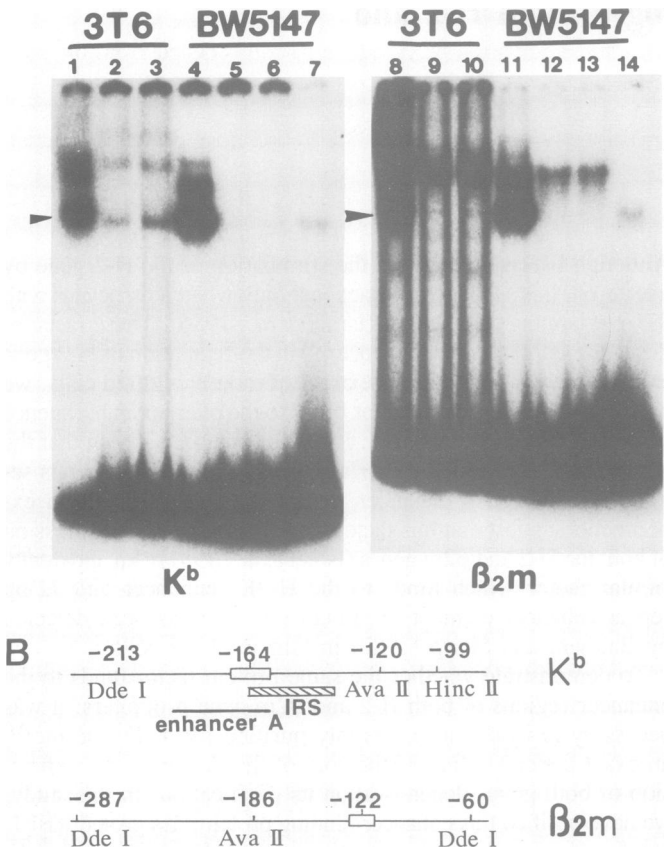

Fig. 1. Comparison of nuclear extract derived from 3T6 and BW5147 cells in gel retardation assay. (**A**) Mouse 3T6 (7 μg) or BW5147 (5 μg) nuclear extracts were incubated with the 5′-labelled *Dde*I/AvaII (−213 to −120) fragment from the H-2K^b enhancer or *Ava*II/*Dde*I (−186 to −60) fragment from the β2m enhancer in the presence of 2 μg poly(dI-dC)(dI-dC), and various oligonucleotide competitors. After incubation, the mixture was subjected to electrophoresis in a 6% non-denaturing polyacrylamide gel as described in Materials and methods. Competitors were as follows: **lanes 1, 4, 8** and **11**, 4 ng of H-2K^b IRS oligonucleotide (31 bp) carrying the entire IRS sequence of the H-2K^b enhancer; **lanes 2, 5, 9** and **12**, 2 ng of H-2K^b oligonucleotide (18 bp) carrying the entire KBF1 binding site in the H-2K^b enhancer A; **lanes 3, 6, 10** and **13**, 2 ng of β2m oligonucleotide (19 bp) carrying the entire KBF1 binding site in the enhancer; **lanes 7** and **14**, the purified KBF1 (0.2 μl) was incubated without competitor. The arrows indicate the retarded bands formed by KBF1. (**B**) The promoter regions of H-2K^b and β2m are shown with relevant restriction sites and regulatory regions. The *Dde*I/*Dde*I (−237 to −60) fragment in the β2m promoter has been shown to exhibit enhancer activity (Kimura *et al.*, 1986). The open boxes indicate the KBF1 binding sites and oligonucleotides used as competitors.

this 0.3 M NaCl eluate. The latter contained only traces of nucleic acid contaminants (2% or less). In gel retardation assay, the retarded bands had the same mobility when 3T6 or BW5147 nuclear extract was incubated with the 5′-labelled *Dde*I/*Ava*II (−213 to −120) fragment from H-2K^b or *Ava*II/*Dde*I (−186 to −60) fragment from the β2m gene, both of which carry the entire KBF1 binding site (Figure 1). Two synthetic double-stranded oligonucleotides carrying the KBF1 binding site derived from the H-2K^b or β2m enhancer sequence competed with both 5′-labelled fragments (Figure 1A, lanes 2, 3, 5, 6, 9, 10, 12 and 13), while the double-stranded IRS oligonucleotide that partially overlaps with the KBF1 binding site in the H-2K^b enhancer sequence (see Figure 1B and Materials and methods), did not compete (Figure 1A, lanes 1, 4, 8 and 11). Indirect footprinting with DNase I showed that this specific retarded band was due to the binding of the factor to the KBF1 binding site

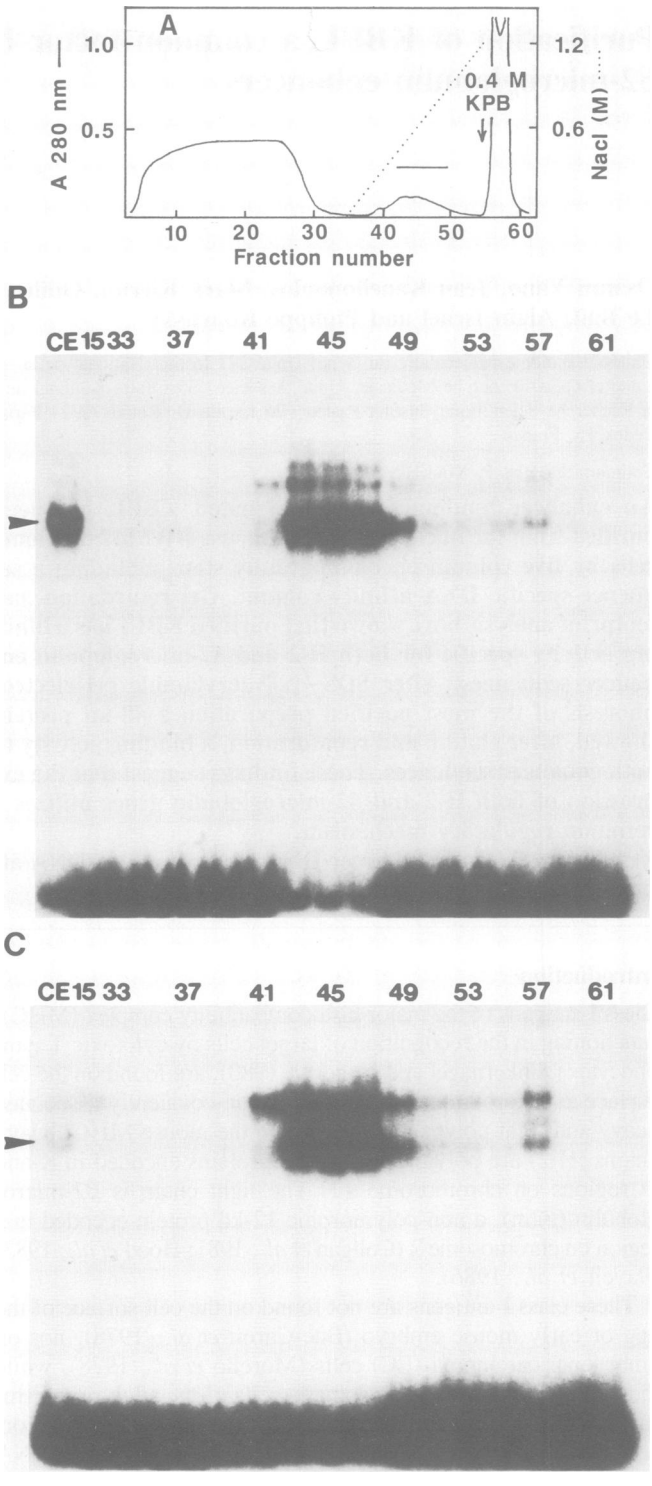

Fig. 2. Hydroxylapatite column chromatography. (**A**) Protein elution and salt gradient profiles. BW5147 nuclear extract dialysed against buffer A was applied to an h.p.l.c. hydroxylapatite column, and eluted with a linear gradient from 0 to 1.2 M NaCl in buffer A, and further with 0.4 M potassium phosphate buffer in buffer A as described in Materials and methods. The pooled active fractions are indicated by a bar. (**B**) Gel retardation assay of hydroxylapatite column chromatography fractions with the 5′-labelled H-2K^b fragment. Each fraction (0.5 μl) was incubated with the 5′-labelled H-2K^b fragment (see Figure 1) in the presence of 0.8 μg of poly(dI-dC)(dI-dC), and subjected to electrophoresis as described in Materials and methods. **Lane CE** indicates a positive control of this assay with 3 μg of BW5147 crude nuclear extract and 2 μg of poly(dI-dC)(dI-dC). The arrow indicates the specific retarded band formed by KBF1. (**C**) Gel retardation assay of hydroxylapatite column chromatography fractions with the 5′-labelled β2m fragment. The experiment was carried out with the 5′-labelled β2m fragment (see Figure 1) as described in (**B**).

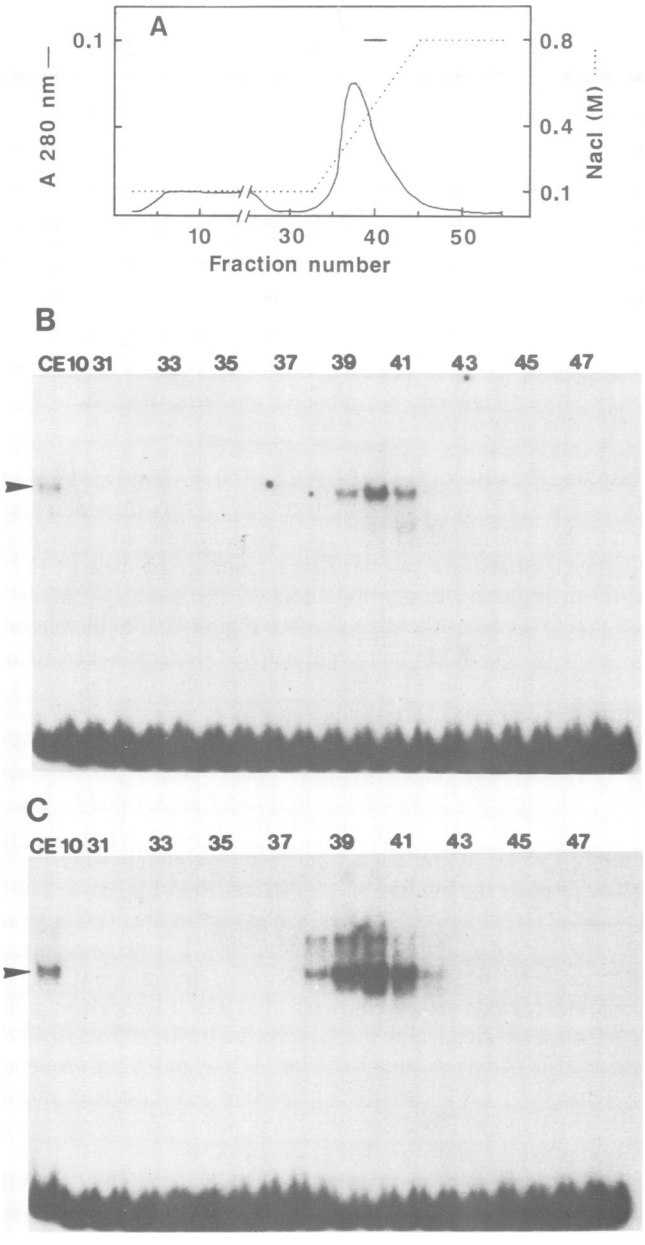

Fig. 3. Heparin–agarose column chromatography. (**A**) Protein elution and salt gradient profiles. The pooled hydroxylapatite fractions were dialysed against buffer B containing 0.1 M NaCl and applied to a heparin–agarose column and eluted with a linear gradient from 0.1 to 0.8 M NaCl in buffer B as described in Materials and methods. The pooled active fractions are indicated by a bar. (**B**) Gel retardation assay of heparin–agarose column chromatography fractions with the 5′-labelled H-2K^b fragment. Each fraction (0.25 μl) was incubated with the 5′-labelled H-2K^b fragment (see Figure 1) in the presence of 0.2 μg of poly(dI-dC)(dI-dC), and subjected to electrophoresis as described in Materials and methods. **Lane CE** indicates a positive control of this assay with 3 μg of BW5147 nuclear extract and 2 μg of poly(dI-dC)(dI-dC). Arrow indicates the specific retarded band formed by KBF1. (**C**) Gel retardation assay of heparin-agarose column chromatography fractions with 5′-labelled β2m fragment. The experiment was carried out with the 5′-labelled β2m fragment (see Figure 1) as described in (**B**).

that we had shown with 3T6 nuclear extract in a previous study (see Figure 8). In addition, the behaviour of KBF1 activity on hydroxylapatite, sulphopropyl ion-exchange and gel filtration columns, supported the view that the KBF1 activity of BW5147 nuclear extract was homologous to that of 3T6 nuclear extract (data not shown).

Purification of KBF1

In each chromatographic step, the fractions eluted from the column were examined for specific DNA binding activity by gel retardation assay (Fried and Crothers, 1981; Garner and Revzin, 1981) with 5′-labelled H-2K^b or β2m fragments described above, and the specificity of the retarded band was tested by competition experiments with the plasmids or synthetic oligonucleotides carrying the KBF1 binding site from the H-2K^b or β2m gene. Optimal amounts of poly-(dI-dC)(dI-dC) were added as a non-specific competitor in these assays. The DNA binding activity was estimated from the intensity of the retarded band in autoradiography. Protein elution from the columns was monitored by continuous u.v. (280 nm) absorption.

In preliminary experiments with ion-exchange and gel filtration columns, KBF1 activity eluted with the contaminating nucleic acid, although the latter composed only a minor component of the nuclear extract. Since hydroxylapatite chromatography is effective in removing nucleic acid from nuclear protein preparation (Simon and Felsenfeld, 1979), and furthermore, can also be applied to purify proteins under adequate conditions, we employed an hydroxylapatite column as a first step. BW5147 nuclear extract was dialysed again buffer A (see Materials and methods), and applied to an h.p.l.c. hydroxylapatite column equilibrated with the same buffer. KBF1 activity was eluted by a linear NaCl gradient, while the contaminating nucleic acid was retained in the column. As shown in Figure 2 (A, B and C), KBF1 activity specific for H-2K^b and β2m enhancer was eluted between 0.5 and 0.8 M NaCl, while the remaining nucleic acids and proteins were eluted with buffer A containing 0.4 M phosphate buffer, as judged from u.v. absorption at 260 nm (data not shown). From these results, we felt that most of the nucleic acid contaminants in the nuclear extract were removed by this method. This chromatographic step resulted in an ~100% recovery of binding activity and 11-fold purification (Table I).

Active fractions from the hydroxylapatite column were dialysed against buffer B containing 0.1 M NaCl, and applied to a heparin–agarose column (3 ml). Most of the protein bound to this column and was eluted as a broad peak by a linear NaCl gradient. KBF1 activity was eluted between 0.5 and 0.6 M NaCl (towards the far shoulder of the peak) (Figure 3A) and concentrated. The retarded bands were observed in the same fractions using either 5′-labelled H-2K^b or β2m fragments (Figure 3B and C). Heparin–agarose column chromatography provided a 2.5-fold increase in specific activity with a 40% recovery of the activity (Table I). While the recovery of this step was low, it was found to be essential for the separation from other K^b enhancer DNA binding proteins. The active fractions were pooled and concentrated by ammonium sulphate precipitation so that the sample could be injected into a small h.p.l.c. gel filtration column (TSK G3000 SW). In preliminary experiments we found that the main KBF1 activity was eluted as a broad peak in the 40- to 100-kd range in a buffer (buffer C, cf. Materials and methods) containing 0.1 M NaCl. Furthermore, a small amount of activity was observed in most fractions resulting in rather poor resolution. While 0.1% BSA had little effect, we found that the addition of 1 M NaCl in the eluting buffer significantly improved the resolution. Most of KBF1 activity appeared between 40 and 85 kd (Figure 4A). The H-2K^b and β2m enhancer binding activities were both observed in the same fractions (Figure 4B and C), suggesting that both factors have identical or similar size (Figure 4A). Gel filtration resulted in a 4-fold purification with 95% recovery of the activity (Table I). Although 105-fold overall purification was achieved with these three columns, SDS-

Table I. Purification of KBF1

Fraction	Volume (ml)	Total protein (mg)	Total binding activity[a] (ng)	Yield (%)	Sp. act.[b] (ng/μg)	Purification (fold)
Nuclear extract	22	67.3	5380	100	0.08	1
Hydroxylapatite	18	6.3	5360	100	0.85	11
Heparin–agarose	3	1.0	2130	40	2.13	27
G3000 SW	1.7	0.24	2020	38	8.43	105
First DNA affinity	4.4	5.7 μg	1700	32	299	3740
Second DNA affinity	2.0	1.3 μg	810	15	623	7790

[a]The binding activity was obtained by multiplying the sp. act. by the total amount of protein (in μg).
[b]Specific activity is defined as the nanogram quantity of labelled DNA (H-2K^b, 5′-*DdeI*/*Ava*II fragment) bound per microgram of protein, which was determined by counting radioactivity contained in the specific retarded band excised after gel retardation (see Materials and methods).

polyacrylamide gel electrophoresis analysis showed that the preparation still contained many proteins and that no dominant band could be assigned to KBF1 activity.

In recent years, several sequence-specific DNA binding factors have been highly purified by DNA affinity columns carrying their specific binding sites (Camier *et al.*, 1985; Rosenfeld and Kelly, 1986; Briggs *et al.*, 1986; Jones *et al.*, 1987; Wiederrecht *et al.*, 1987). We have also employed this technique to purify KBF1. Double-stranded oligonucleotides (18 bp) containing the KBF1 binding site of the H-2K^b promoter were polymerized and utilized as a specific ligand. Polymerized oligonucleotides were coupled to Affi-Gel 15 (which contains 15-atom spacer arms). The partially purified pooled gel filtration fractions were dialysed against buffer C containing 0.1 M NaCl, and added to this affinity column. After washing with buffer C containing 0.1 M and 0.25 M NaCl, KBF1 was eluted with a linear NaCl gradient. In retardation assays, binding activity specific for both 5′-labelled H-2K^b and β2m fragments co-eluted around 0.6 M NaCl (fractions 28–31) (Figure 5A, B and C). Each fraction from this column was analysed by SDS-polyacrylamide gel electrophoresis under non-reducing conditions. As shown in Figure 6A, several major bands were detectable in the active fractions. Within the limit of sensitivity of this analysis, only one band, corresponding to a 48-kd protein paralleled the appearance of the retarded band. This purification step provided a 35-fold purification with 84% recovery (Table I). Active fractions from the first affinity chromatography were combined and rechromatographed on the same column. Elution of the specific DNA binding activity was similar to that found in the first affinity chromatography. As was expected, the 48-kd protein co-eluted with the specific DNA binding activity. The second affinity chromatography resulted in a 2.1-fold purification (Table I). Two bands, 58 and 48 kd, were observed in the purified KBF1 fraction after SDS–polyacrylamide gel electrophoresis and silver staining (Figure 6B). Table I summarizes the purification of KBF1 from BW5147 nuclear extract. The overall purification and the yield of the specific DNA binding activity were ~8000-fold and 15% respectively.

DNA binding properties of the purified KBF1

To determine whether purified KBF1 (second affinity chromatography fraction) could bind to both H-2K^b and β2m enhancer sequences specifically, we carried out competition experiments using the gel retardation assay. The retarded band could be detected in the presence of non-specific competitor plasmid (pconaCAT) with 5′-labelled H-2K^b or β2m fragments (Figure 7A, lanes 1 and 4). These retarded bands were competed away by the addition of each homologous sequence present in two plasmids, Dd-cIIconaCAT (lanes 2 and 5) or β2m DdeconaCAT (lanes 3 and 6). We then examined whether the retarded bands could be competed away by the addition of two short oligonucleotides carrying the KBF1 binding sequence of H-2K^b or β2m, because the plasmids contain extraneous sequence in addition to the KBF1 binding site (see Figure 1B). In the presence of either the H-2K^b or β2m oligonucleotide (see Materials and methods), the retarded band, formed by the binding between purified KBF1 and each 5′-labelled probe, was efficiently competed away as shown in Figure 7B (lanes 2, 3, 6 and 7). In contrast, H-2K^b IRS oligonucleotide that overlaps partially with KBF1 binding site competed little if any (lanes 4 and 8). These results indicate that the purified KBF1 fraction shows a DNA binding specificity similar to the factor originally described.

To delineate the binding sites of the purified KBF1, we subsequently performed DNase I footprinting experiments (Galas and Schmitz, 1978). Figure 8 shows the DNase I protection patterns obtained with the 5′-labelled H-2K^b or β2m fragment. In both cases, the regions protected by purified KBF1 (lanes 4 in panels A and B) were identical to those protected by the starting material (lanes 2 in panels A and B). The H-2K^b and β2m sequences recognized by KBF1 purified from BW 5147 nuclear extract, were the same as those demonstrated with 3T6 nuclear extract in our previous study (Israël *et al.*, 1987).

These results indicate that the DNA binding activities, specific for the H-2K^b and β2m enhancers, have copurified through five column chromatographic steps, including two sequential sequence-specific DNA affinity columns.

Identification of the KBF1 binding protein by SDS–polyacrylamide gel electrophoresis and renaturation

From the above experiments, it was not possible to exclude the possibility that our purified KBF1 activity originated from multiple factors contained in the purified KBF1 preparation. To provide conclusive evidence, preparative SDS–polyacrylamide gel electrophoresis was carried out. As shown in Figure 6B, only two bands (58 and 48 kd) were detected in the purified KBF1 preparation. The 48-kd protein band co-eluted with the specific KBF1 DNA binding activity as described above and shown in Figure 6A. Therefore, this 48-kd protein is likely to be responsible for the KBF1 activity of the purified fraction.

To confirm this, we isolated the 58- and 48-kd proteins by preparative SDS–polyacrylamide gel electrophoresis followed by renaturation as described before (Hager and Burgess, 1980). After staining with 0.25 M KCl, each region was excised from the gel under the guidance of known mol. wt markers. The proteins were electroeluted, precipitated by acetone and dissolved in 6 M guanidine HCl. Each protein was then dialysed to remove

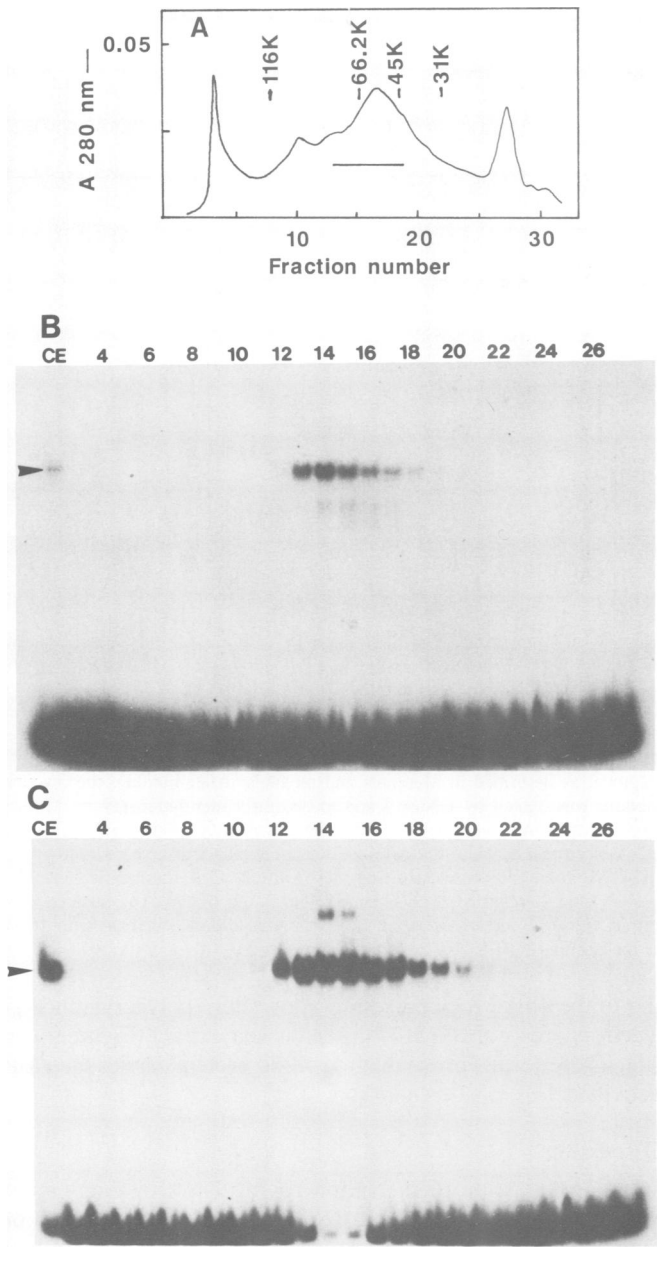

Fig. 4. G3000 SW gel filtration. (**A**) Protein elution profile. The pooled heparin–agarose fractions were concentrated by ammonium sulphate precipitation and chromatographed on a TSK G3000 SW column with buffer C containing 1 M NaCl as described in Materials and methods. The pooled active fractions are indicated by a bar. (**B**) Gel retardation assay of gel filtration fractions with the 5′-labelled H-2K^b fragment. Each fraction (0.25 μl) was incubated with the 5′-labelled H-2K^b fragment (see Figure 1) in the presence of 50 ng of poly(dI-dC)(dI-dC), and subjected to electrophoresis as described in Materials and methods. **Lane CE** indicates a positive control of this assay with 3 μg of BW5147 nuclear extract and 2 μg of poly(dI-dC)(dI-dC). The arrow indicates the specific retarded band formed by KBF1. (**C**) Gel retardation assay of gel filtration fractions with the 5′-labelled β2m fragment. The experiment was carried out with the 5′-labelled β2m fragment (see Figure 1) as described in (**B**).

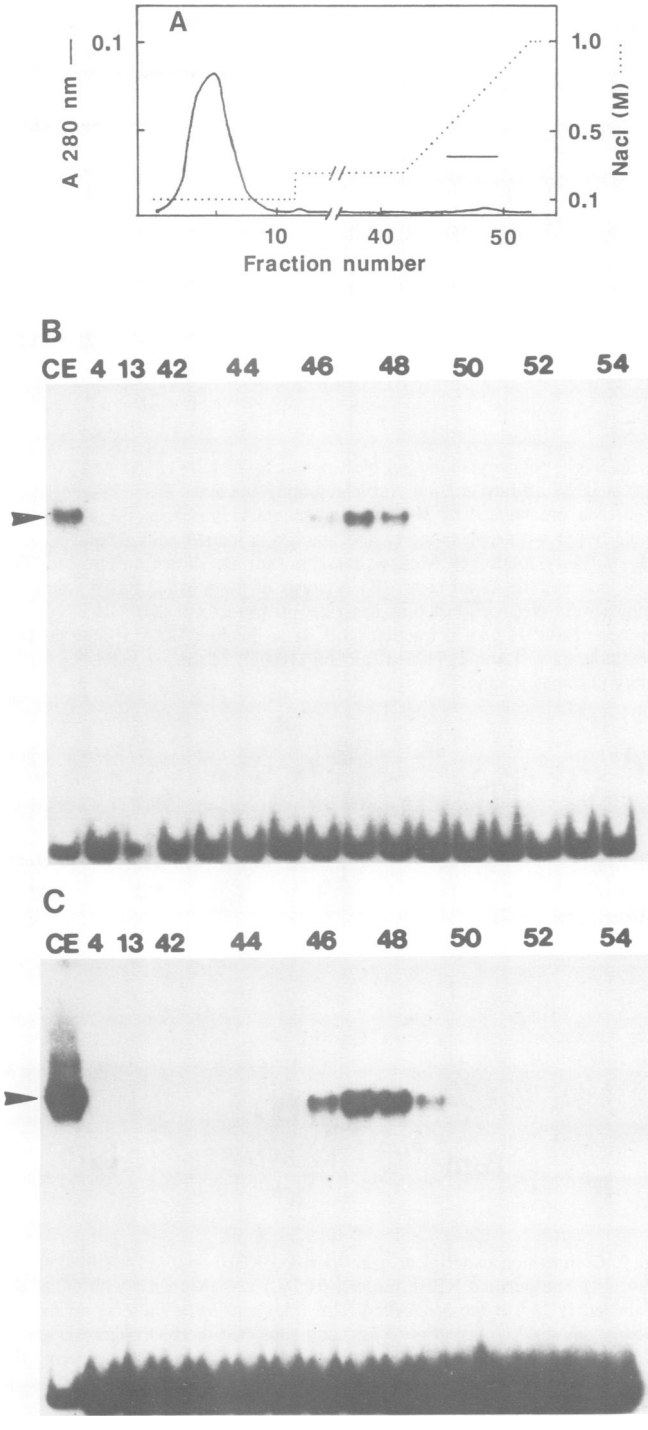

Fig. 5. DNA affinity column chromatography. (**A**) Protein elution and salt gradient profiles. The pooled gel filtration fractions dialysed against buffer C containing 0.1 M NaCl were applied to a sequence-specific DNA affinity column containing the H-2K^b derived KBF1 binding sequence, and eluted with a linear gradient from 0.25 to 1.0 M NaCl as described in Materials and methods. The pooled active fractions are indicated by a bar. (**B**) Gel retardation assay of DNA affinity column chromatography fractions with the 5′-labelled H-2K^b fragment. Each fraction (0.25 μl) was incubated with the 5′-labelled H-2K^b fragment (see Figure 1) in the absence of poly(dI-dC)(dI-dC), and subjected to electrophoresis as described in Materials and methods. **Lane CE** indicates a positive control of this assay with 3 μg of BW5147 nuclear extract and 2 μg of poly(dI-dC)(dI-dC). Arrow indicates the specific retarded band formed by KBF1. (**C**) Gel retardation assay of DNA affinity column chromatography fractions with the 5′-labelled β2m fragment. The experiment was carried out with the 5′-labelled β2m fragment (see Figure 1) as described in (**B**).

the guanidine hydrochloride and to facilitate renaturation (Johnson *et al.*, 1987; Wiederrecht *et al.*, 1987). These renatured proteins were re-examined for specific DNA binding activity by gel retardation assay. The renatured 48-kd protein exhibited a binding activity to the 5′-labelled H-2K^b fragment (Figure 9, lane 3), while no retarded band was observed with the renatured 58-kd protein (lane 7) or a renatured fraction from a 20-kd region of

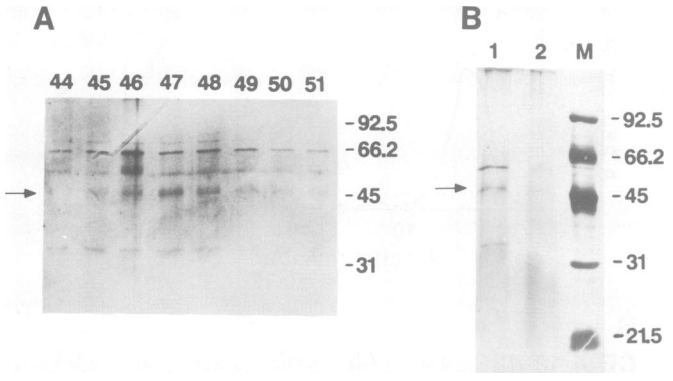

Fig. 6. SDS−polyacrylamide gel electrophoresis of KBF1. (**A**) Analysis of the first DNA affinity column chromatography fractions. Each sample (50 μl) was precipitated by 10% trichloracetic acid, resuspended in SDS sample buffer without beta-mercaptoethanol, and loaded onto a 10% SDS−polyacrylamide gel. Mol. wt markers (kd) are shown on the side. The arrow indicates the 48-kd band. (**B**) Analysis of the purified KBF1 fraction. Samples were electrophoresed as described in (**A**) except that a 12% gel was used. **Lane 1**, pooled fraction (100 μl) of the first DNA affinity column chromatography. **Lane 2**, pooled fraction (100 μl) of the second DNA affinity chromatography.

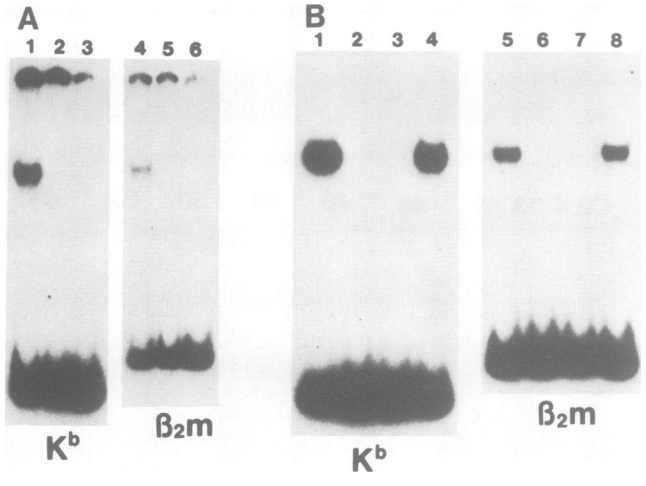

Fig. 7. Competition experiment using purified KBF1 in a gel retardation assay. (**A**) The purified KBF1 fraction (0.5 μl) was incubated with either the 5′-labelled H-2K^b or the 5′-labelled β2m fragment in the presence of the following plasmid competitors (1 μg), and subjected to electrophoresis as described in Materials and methods. **Lanes 1** and **4**, pconaCAT carrying the conalbumin promoter; **lanes 2** and **5**, Dd-cIIconaCAT carrying the *DdeI*/*Hin*cII fragment (−213 to −99) from the H-2K^b promoter; **lanes 3** and **6**, β2m DdeconaCAT carrying the *DdeI*/*DdeI* fragment (−287 to −60) from the β2m promoter. (**B**) The purified KBF1 fraction (0.5 μl) was incubated with either the 5′-labelled H-2K^b or the 5′-labelled β2m fragment in the presence of an oligonucleotide competitor (see Figure 1) and subjected to electrophoresis as described in Materials and methods. **Lanes 1** and **5**, no oligonucleotide; **lanes 2** and **6**, 2 ng of the oligonucleotide carrying the KBF1 site of H-2K^b; **lanes 3** and **7**, 2 ng of the β2m oligonucleotide carrying the KBF1 site of β2m; **lanes 4** and **8**, 4 ng of the H-2K^b IRS oligonucleotide.

the gel (lane 2). The retarded band formed from the renatured 48-kd protein was specifically competed by the addition of H-2K^b or β2m oligonucleotide, and not by the IRS oligonucleotide (compare lanes 4, 5 and 6). These results correspond exactly to those observed with the native KBF1 fraction (Figure 7B). Additionally, we detected KBF1 activity in a gel slice corresponding to the 48-kd region excised after SDS−polyacrylamide gel electrophoresis of BW5147 crude nuclear extract (Figure 9, lane 11). These results prove that the 48-kd protein carries the KBF1 activity.

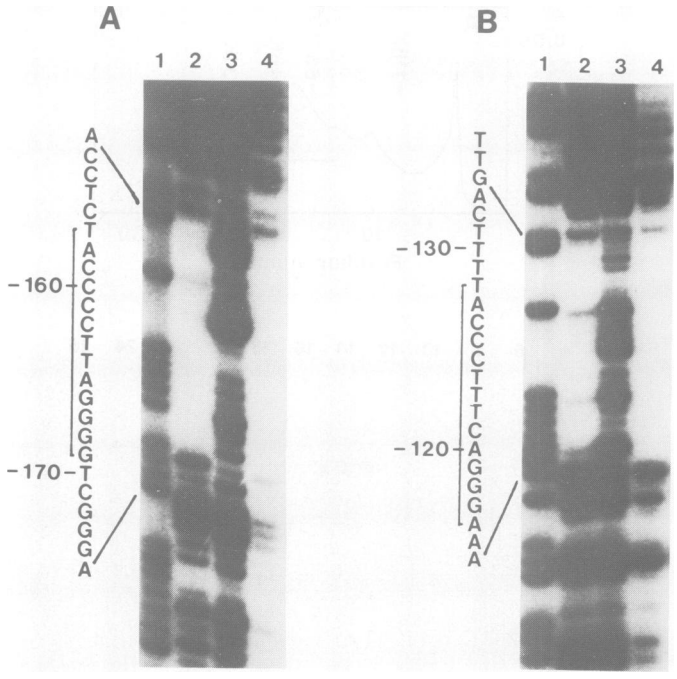

Fig. 8. DNase I footprinting analysis of purified KBF1. Footprintings were performed with the 5′-labelled H-2K^b (**A**) or the 5′-labelled β2m (**B**) fragment as described in Materials and methods. After binding, the reaction mixture was treated by DNase I and loaded on a retardation gel. Appropriate bands were excised from the gel after autoradiography. The DNA electroeluted from the gel slice was precipitated by ethanol and analysed on an 8% urea sequencing gel. **Lane 1**, G + A specific sequence ladder; **lane 2**, retarded band obtained with 22 μg of BW5147 nuclear extract; **lane 3**, unretarded band; **lane 4**, retarded band obtained with 16 ng of the purified KBF1.

Discussion

In this study, a purified fraction having DNA binding activity specific for both H-2K^b and β2m enhancer sequences was obtained from BW5147 nuclear extract. This KBF1 activity is attributable to a single protein factor contained in the purified fraction. The following results support this viewpoint. First, the behaviour of DNA binding activity specific for H-2K^b enhancer sequence corresponded exactly with that for the β2m enhancer sequence through five column chromatographic procedures including sequence-specific DNA affinity columns (Figures 2−5). Second, in gel retardation assays, the purified KBF1 fraction showed a DNA binding activity specific for both enhancer sequences, and this specificity was the same as that found in nuclear extract (Figure 1 and 7). Third, in footprint experiments, the purified fraction formed the same footprints as those formed with nuclear extract, and the binding sequences were the same as those demonstrated in our previous study (Figure 8; Israël *et al.*, 1987). Finally, after further purification on preparative SDS−polyacrylamide gel and renaturation, only the 48-kd protein exhibited the same binding property as that found in the native purified KBF1 fraction (Figure 9). From these experiments, we conclude that this 48-kd protein is a single factor (KBF1) which specifically binds to both the H-2K^b and β2m enhancer sequences. This conclusion can be interpreted as meaning that a common *trans*-acting

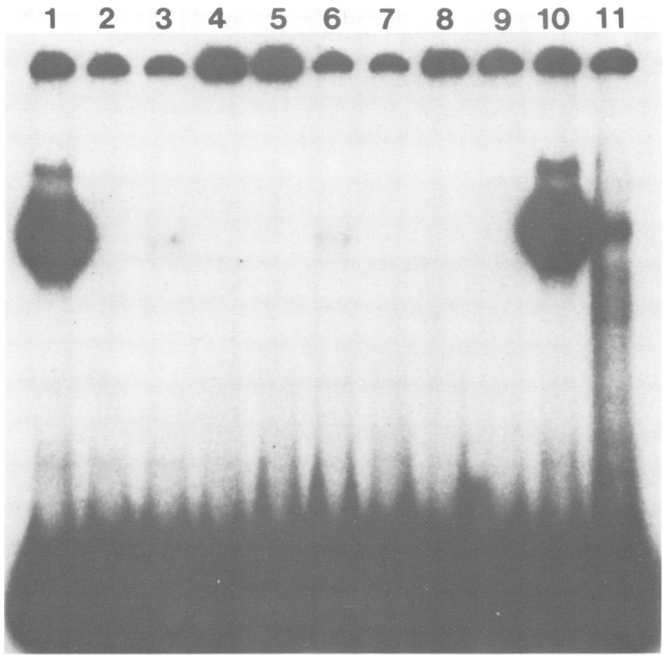

Fig. 9. Competition experiment using gel-purified and renatured KBF1. The native purified KBF1 preparation was subjected to SDS−polyacrylamide gel electrophoresis and the regions corresponding to the 58- and 48-kd bands were excised. After electroelution, the proteins were precipitated by acetone, and renatured with 6 M guanidine−HCl treatment as described in Materials and methods. The renatured protein fractions were incubated with the 5′-labelled H-2K^b fragment in the presence of competitor oligonucleotides (see Figure 1), and subjected to electrophoresis as described in Materials and methods. **Lanes 1** and **10**, native KBF1 fraction (0.5 μl) with no competitor added; **lane 2**, renatured fraction from a 20-kd region of the gel (5 μl) with no competitor added; **lane 3**, renatured 48-kd protein fraction (5 μl) with no competitor added; **lane 4**, renatured 48-kd protein fraction (5 μl) with 2 ng of H-2K^b oligonucleotide; **lane 5**, renatured 48-kd protein fraction (5 μl) with 2 ng of β2m oligonucleotide; **lane 6**, renatured 48-kd protein fraction (5 μl) with 4 ng of IRS oligonucleotide; **lane 7**, renatured 58-kd protein fraction (5 μl) with no competitor added; **lane 8**, renatured 58-kd protein fraction (5 μl) with 2ng of H-2K^b oligonucleotide; **lane 9**, renatured 58-kd protein fraction with 4 ng of IRS oligonucleotide; **lane 11**, renatured 48-kd region from the lane loaded with BW5147 crude nuclear extract with no competitor added. The bands showing a lower mobility in **lanes 1** and **10** are due to non-specific interaction only detectable after prolonged exposures (10 days).

factor binds to a *cis*-regulatory element in both genes. Consequently, this suggests that the expression of both genes comprises a common regulatory mechanism.

We have already shown that this enhancer binding activity represents a positive regulatory factor for both the H-2K^b and β2m genes by *in vivo* competition experiments (Israël *et al.*, 1987). H2TF1, a similar factor that recognizes the same sequence in the H-2K^b enhancer (as shown by DNase I footprinting and methylation interference experiments), has also been reported to stimulate class I H-2K^b gene expression using similar techniques (Baldwin and Sharp, 1987), though its purification has not yet been reported.

Only two bands could be seen in the purified KBF1 fraction with SDS−polyacrylamide gel electrophoresis followed by silver staining, of which only one band (48 kd) showed KBF1 activity. Based on the estimation of specific DNA binding activity of the purified KBF1 fraction, this 48-kd protein alone seems to be present in sufficient quantities to occupy the KBF1 binding site of the DNA probe used in the gel retardation assay. Hence, it is unlikely that any minor species (if they exist in the purified fraction) possess the KBF1 activity.

It has been shown that KBF1 recognizes a palindromic structure in the H-2K^b promoter. This observation suggests that, like several prokaryotic DNA binding proteins (Pabo and Sauer, 1984), KBF1 binds to DNA as a dimer (Baldwin and Sharp, 1987; Israël *et al.*, 1987). Could dimeric KBF1 exist in solution? In gel filtration, KBF1 activity was eluted in a broad range of mol. wts extending from 40 to 100 kd with buffer containing 0.1 M NaCl. Thus part of KBF1 could exist in solution as a dimer (96 kd). While this hypothesis fits the gel filtration data, further work is required to confirm it.

KBF1 seems to be a minor constituent of cell nuclei, since starting from a 0.1−0.3 M NaCl nuclear extract, ~8000-fold purification was required to identify it. If KBF1 binds to its recognition site as a dimer, as discussed above, and we assume that the loss of activity during the purification is only due to physical ones, the nuclear extract contains 8 μg of KBF1 as based on the results shown in Table I. This amount of KBF1 corresponds to 1×10^{14} molecules of monomer KBF1. Thus, if we assume that the recovery of KBF1 is 100%, each cell nucleus contains ~10 000 KBF1 molecules. This concentration is similar to that of MLTF (10 000 molecules per HeLa cell) (Chodosh *et al.*, 1986) and one-tenth of EBP20 (100 000 molecules per rat liver nucleus) (Johnson *et al.*, 1987), though we have not determined the KBF1 activity in BW5147 whole cell extract.

The purification and identification of KBF1 make it possible to prepare antibodies against KBF1 and determine a partial amino acid sequence which will facilitate the cloning and characterization of the KBF1 gene. This and other investigations currently being performed in our laboratory will hopefully help elucidate the role which KBF1 plays in gene regulation.

Materials and methods

Preparation of oligonucleotides and affinity column

Chemically synthetized oligonucleotides, A (5′-GATCTGGGGATTCCCCAT-3′) and B (5′-GATCATGGGGAATCCCCA-3′), that form the recognition site for KBF1 in the H-2K^b enhancer, were purified on a denaturing 20% polyacrylamide sequencing gel, phosphorylated, annealed and ligated as described by Kadonaga and Tjian (1986). The average length of ligated DNA corresponds to ~15 polymerized oligonucleotides. The DNA oligomers were passed through a PD10 column (Sephadex G25M, Pharmacia) equilibrated with 0.1 M Hepes buffer (pH 7.5) and coupled to Affi-gel 15 (BioRad) in the same buffer overnight at room temperature. Approximately 25 μg of DNA was coupled per ml of resin. We also used two β2m enhancer derived complementary oligonucleotides:

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5′ GATCAAGGGACTTTCCCAT      3′
5′      TTCCCTGAAAGGGTACTAG 5′
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and two derived from the H-2K^b interferon response sequence (IRS):

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5′ GATTCCCCATCTCCACAGTTTCACTTCTGCA      3′
3′   AAGGGGTAGAGGTGTCAAAGTGAAGACGTCT   5′
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Purification of KBF1

Nuclear extracts of mouse cells were prepared as described by Piette *et al.* (1985) and kept at −80°C until use. All procedures were performed below 4°C except the chromatography steps which were done at room temperature. Nuclear extract (22 ml) prepared from 1×10^{10} BW5147 cells was dialyzed overnight against 500 ml of buffer A (50 mM potassium phosphate buffer, pH 7.5; 1 mM DTT; 0.01% NP4O; 0.1 mM PMSF; 0.01 mM $CaCl_2$; 10% glycerol) and centrifuged at 9000 *g* for 20 min. The resulting supernatant was applied to an h.p.l.c. hydroxylapatite column (BioRad, HPHT column) equilibrated with the same buffer. The column was washed with buffer A. Elution with a linear gradient from 0 to 1.2 M NaCl in buffer A (20 ml) was applied, followed by stepwise elution with buffer A containing 0.4 M potassium phosphate buffer. Fractions of 1 ml were collected at a flow rate of 0.5 ml/min, and active fractions (nos 42−49) were pooled.

These pooled fractions were dialysed against 500 ml of buffer B (20 mM potassium phosphate buffer, pH 7.5; 10 mM $MgCl_2$; 0.1 mM EDTA; 1 mM

DTT; 0.01% NP40; 0.1 mM PMSF; 10% glycerol) containing 0.1 M NaCl, and centrifuged at 9000 *g* for 20 min. The supernatant was applied to a 3 ml heparin–agarose (Heparine-Ultrogel A4R, LKB) column equilibrated with buffer B containing 0.1 M NaCl. After washing with the same buffer, KBF1 was eluted with a linear gradient from 0.1 to 0.8 M NaCl in buffer B (15 ml). Fractions of 1 ml were collected at a flow rate of 0.5 ml/min, and active fractions (nos 39–41) were pooled and precipitated with 2 vol saturated ammonium sulphate in buffer B. After centrifugation at 12 000 *g* for 60 min, the precipitate was dissolved in 120 μl of buffer C (20 mM potassium phosphate buffer pH 6.0; 10 mM $MgCl_2$; 0.1 mM EDTA; 1 mM DTT; 0.01% NP40; 0.1 mM PMSF; 10% glycerol) containing 1 M NaCl and centrifuged. The supernatant was applied to an h.p.l.c. gel filtration column (TSK G3000 SW, Toyo-Soda) equilibrated with the above buffer, and eluted with the same buffer. Fractions of 0.25 ml were collected at a flow rate of 0.5 ml/min and the active fractions (nos 13–18) were pooled.

The pooled fractions were dialyzed against 300 ml of buffer C containing 0.1 M NaCl. To this fraction, 80 μg of poly(dI-dC)(dI-dC) (Pharmacia) was added, and allowed to stand on ice for 10 min. Then, this mixture was applied to a 1 ml DNA affinity column equilibrated with the above buffer at a flow rate of 0.2 ml/min. After washing with 10 column volumes of buffer C containing 0.1 M NaCl, and further with 30 column volumes of buffer C containing 0.25 M NaCl, KBF1 was eluted with a linear gradient from 0.25 to 1 M NaCl in buffer C. Fractions of 1 ml were collected at a flow rate of 0.25 ml/min and active fractions (nos 46–49) were pooled, dialysed and rechromatographed on another DNA affinity column under the same conditions. Active fractions were pooled, and frozen at −80°C until used.

Gel retardation and footprinting assay

Binding reactions were performed in 20 μl of 20 mM phosphate buffer, pH 6; 10 mM $MgCl_2$; 0.1 mM EDTA; 2 mM DTT; 0.01% NP40; 0.1 mM PMSF; 50–100 mM NaCl; 15% glycerol; 100 μg/ml BSA (binding buffer). Protein fractions were added to binding buffer containing 5′-labelled DNA fragment (1000–3000 c.p.m.) and poly(dI-dC)(dI-dC) as a non-specific competitor, as indicated in the legends, incubated for 30 min at room temperature, and loaded onto a 6% polyacrylamide gel in Tris/borate/EDTA electrophoresis buffer. Electrophoresis was performed at 200 V for 2–2.5 h. The gel was dried and autoradiographed with an intensifying screen (Dupont Cronex Lightning plus).

For the determination of specific activity, a gel retardation assay was employed as described previously (Cohen *et al.*, 1986) and carried out in the presence of various amounts of poly(dI-dC)(dI-dC). After autoradiography of the gels, the retarded bands were excised and counted by liquid scintillation. As a control, unretarded DNA bands (5′-labelled *DdeI*/*AvaII* fragment of H-$2K^b$, 7000 c.p.m./ng) from lanes containing no extract, were excised and counted in the same manner. The amount of specific retarded DNA was calculated from the condition giving maximal binding. The specific activity is expressed as the nanogram quantity of the DNA probe bound per microgram of protein.

DNase I footprinting was performed as described (Arcangioli and Lescure, 1985) with slight modifications. The initial binding reaction was performed in 50 μl of binding buffer (see above) containing 10 mM $CaCl_2$ instead of $MgCl_2$. Protein fractions were added to binding buffer containing 5′-labelled DNA fragment (25 000 c.p.m.), and incubated as described above with poly(dI-dC)(dI-dC). After binding, DNase I (10 ng) was added for 1 min at room temperature. The reaction mixture was loaded directly on a 5% polyacrylamide gel (which stops the reaction), and the retarded and unretarded bands were excised from the gel after autoradiography. The DNAs electroeluted from the gel were precipitated by ethanol and then subjected to electrophoresis in an 8% urea-sequencing gel. G+A chemical degradation of the DNA probe was carried out as described by Maxam and Gilbert (1980).

Preparative SDS–polyacrylamide gel electrophoresis and renaturation

Gel purification and renaturation was carried out according to Hager and Burgess (1980). The BW5147 nuclear extract (30 μl) and purified KBF1 fraction (200 μl) were precipitated by the addition of 4 vol cold acetone, and dried. Each precipitate was dissolved in SDS sample buffer without beta-mercaptoethanol, heated to 70°C for 5 min and subjected to electrophoresis on a 10% SDS–polyacrylamide gel. After electrophoresis, gel slices were excised under the guidance of known mol. wt markers (BioRad) stained by 0.25 M KCl. Proteins were electroeluted from the gel slices (Wiederrecht *et al.*, 1987) and 10 μg of carrier BSA was added to each eluate. After precipitation by cold acetone and two washes with 80% cold acetone, each precipitate was dried and dissolved in 50 μl of buffer D (30 mM Hepes, pH 7.5; 10 mM $MgCl_2$; 0.1 mM EDTA; 0.01% NP40; 0.1 M NaCl; 10% glycerol) containing 6 M guanidine HCl. Then, an equal volume of buffer D was added, and the sample was dialysed against buffer D containing 1 mM DTT at 4°C overnight (Johnson *et al.*, 1987; Wiederrecht *et al.*, 1987).

Other methods

The h.p.l.c. used was the LKB GTI system. Protein concentration was determined with the BioRad protein assay kit and BSA was used as a standard. SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) was carried out without beta-mercaptoethanol and gels were stained by the BioRad silver staining kit. Proteins (mol. wts are shown in parenthesis) phosphorylase *b* (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), lysozyme (14 400) were used as mol. wt markers (low mol. wt marker kit from BioRad).

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