The EMBO Journal vol.8 no.13 pp.4215-4227, 1989

The SV40 TC-II(κB) enhanson binds ubiquitous and cell type specifically inducible nuclear proteins from lymphoid and non-lymphoid cell lines

Mary Macchi, Jean-Marc Bornert, Irwin Davidson, Masamoto Kanno, Ricardo Rosales, Marc Vigneron, Jia-Hao Xiao, Catherine Fromental and Pierre Chambon

Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Unité 184 de Biologie Moléculaire et de Génie Génétique de l'INSERM, Institut de Chimie Biologique, Faculté de Médecine, 11, rue Humann, 67085 Strasbourg Cédex, France

Communicated by P.Chambon

We have characterized the complexes resulting from the specific binding *in vitro* of proteins present in nuclear extracts of several lymphoid and non-lymphoid cell lines to the TC-I and TC-II sequences of the simian virus 40 (SV40) enhancer. No proteins could be detected, binding selectively to the TC-I sequence, but two proteins TC-IIA and TC-IIB were identified interacting specifically with both the TC-II/κB enhanson, 5'-GGAAAGTCCCC-3' (important for the activity of the SV40 enhancer *in vivo*), and with the related H-2K^b enhanson, 5'-TGGGGA-TTCCCCA-3'. The binding of these two proteins to mutated TC-II enhansons correlates with the effect of these mutations *in vivo*, suggesting that both proteins may be important for SV40 enhancer activity. The TC-IIA binding activity was present in nuclear extracts of mature lymphoid B cells and was increased in pre-B cell nuclear extracts by lipopolysaccharide (LPS) and cycloheximide treatment. Futhermore, complex formation between the TC-IIA protein and the TC-II enhanson was efficiently competed by the κB motif from the κ chain enhancer, indicating that TC-IIA is the NF-κB factor or a closely related protein. However, in contrast to previous reports, a TC-IIA/NF-κB-like protein whose properties could not be distinguished from those of the TC-IIA protein present in lymphoid B cells, was found in nuclear extracts of several untreated non-lymphoid cell lines, notably of HeLa cells, but not of undifferentiated F9 embryonal carcinoma (EC) cells [F9(ND)]. The TC-IIA binding activity which was moderately increased in HeLa cell nuclear extracts by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and/or cycloheximide treatment could be induced in nuclear extracts of F9(ND) cells by cycloheximide, but not by TPA. Moreover, the TC-IIA binding activity could be induced in cytosolic fractions from F9(ND) cells by treatment with deoxycholate, indicating that these cells contain an inhibitor protein similar to the previously described NF-κB inhibitor, IκB. The second TC-II enhanson binding protein, TC-IIB, which could be clearly distinguished from the TC-IIA/NF-κB-like protein, by a number of differential properties, resembles the previously described KBF1/H2TF1 protein as it binds with a higher affinity to the H-2K^b enhanson than to the TC-II/κB enhanson, and its pattern of methylation interference on the H-2K^b and TC-II/κB enhansons is identical to that reported for the KBF1/H2TF1 protein. Proteins TC-IIA and TC-IIB also bind with different affinities to a TC-II-related sequence in the interleukin-2 promoter. A third DNA-protein complex, which was also formed within the region of the SV40 enhancer containing the TC sequences most probably, corresponds to the binding of the previously described AP-2 factor. However, its binding site is not indispensable for SV40 enhancer activity *in vivo*.

Key words: DNA binding *in vitro*/NF-κB/H2TF1/KBF1-like factors/SV40 enhancer/TC motifs/undifferentiated and differentiated F9 cells

Introduction

Enhancer elements are *cis*-acting DNA sequences which stimulate transcription from RNA polymerase B (II) promoters in an orientation and to some extent distance independent manner (Banerji *et al.*, 1981; Moreau *et al.*, 1981; Chambon *et al.*, 1984; Serfling *et al.*, 1985; Sassone-Corsi and Borrelli, 1986; Maniatis *et al.*, 1987; Atchison, 1988; Hatzopoulos *et al.*, 1988; Jones *et al.*, 1988; Müller *et al.*, 1988; Wasylyk, 1988; and references therein). The simian virus 40 (SV40) enhancer (see Figure 1) is composed of a mosaic of functional elements (enhansons) whose synergism is necessary for normal enhancer activity (Herr and Gluzman, 1985; Herr and Clarke, 1986; Zenke *et al.*, 1986; Nomiyama *et al.*, 1987; Schirm *et al.*, 1987; Fromental *et al.*, 1988; Ondek *et al.*, 1988; and references therein). Analysis of the proteins binding *in vitro* to the SV40 enhansons (Davidson *et al.*, 1986; Landolfi *et al.*, 1986; Singh *et al.*, 1986; Staudt *et al.*,1986; Wildeman *et al.*, 1986; Fletcher *et al.*, 1987; Rosales *et al.*, 1987; Scheidereit *et al.*, 1987; Sturm *et al.*, 1987; Xiao *et al.*, 1987a,b; Davidson *et al.*, 1988) has indicated an unexpectedly complex pattern due to the binding of both ubiquitous and cell specific proteins. Two HeLa cell proteins TEF-1 and TEF-2, whose cell specificity correlates with that of their cognate enhansons which are inactive in lymphoid B cells, have been purified. TEF-1, a 53 kd protein, binds to two class A enhansons, GT-IIC and Sph, of unrelated sequence (Xiao *et al.*,1987a; Davidson *et al.*, 1988; Fromental *et al.*, 1988; see Figure 1 and also Introduction of Kanno *et al.*, 1989). TEF-1 binds cooperatively *in vitro* to tandem repeats of either of these enhansons which correlates with the proto-enhancer activity *in vivo* of these tandem repeats, whereas non-cooperative binding is observed with spaced or inverted repeats which have weaker proto-enhancer activity *in vivo*. The GT-IIC or Sph enhansons cooperate *in vivo* with the GT-I class B enhanson to form proto-enhancers, but in contrast with the tandem repeats of the GT-IIC or Sph enhansons, this cooperativity does not reflect a cooperative binding *in vitro* between TEF-1 and the TEF-2 (GT-IC)

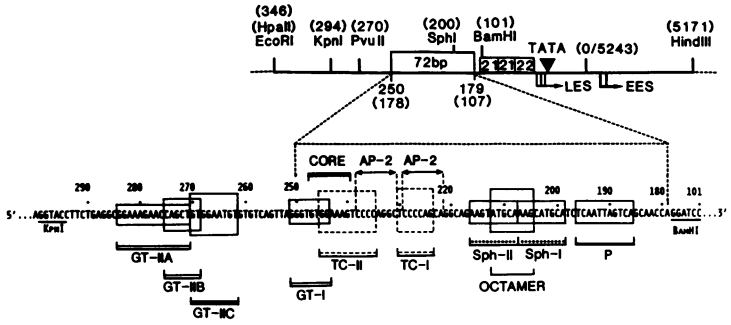

Fig. 1. Organization of the SV40 early promoter. The top line shows the structure of the SV40 early promoter in plasmid pAO, containing a single copy of the 72 bp sequence (Zenke *et al.*, 1986). The position of the essential elements of the promoter, the TATA box, the 21 bp repeat element (22, 21,21) and 72 bp enhancer element are indicated along with the coordinates (BBB system; Tooze, 1982) of several natural or engineered restriction enzyme recognition sites. EES and LES indicate the position of the early-early and late-early mRNA start sites respectively. The second line shows the sequence of the late coding strand of the 72 bp element (indicated by the vertical broken lines) and the 5′-flanking sequences. The locations of the sequence motifs which have been identified in the enhancer region (see text) are indicated by the boxes. In addition the 'core' sequence and AP-2 binding sites are indicated above the sequence.

protein which specifically interacts with the GT-I enhanson (Xiao *et al.*, 1987b; Davidson *et al.*, 1988). In addition to these proteins several other HeLa cell proteins bind to the SV40 enhancer: AP-1, AP-3 and AP-4 (Angel *et al.*, 1987; Chiu *et al.*, 1987; Imagawa *et al.*, 1987; Lee *et al.*, 1987; Mitchell *et al.*, 1987; Mermod *et al.*, 1988; Mercurio and Karin, 1989). However the role of these latter proteins in SV40 enhancer activity *in vivo* is not clearly established (see Kanno *et al.*, 1989).

Recently, a novel SV40 class C enhanson, TC-II (5′-GGAAAGTCCCC-3′; see Figures 1, 2 and 7B) has been characterized and shown to be active in both lymphoid and non-lymphoid cells (Kanno *et al.*, 1989). This enhanson is homologous to the κB motif (KAP-1 in Figure 2) of the immunoglobulin κ chain enhancer (Sen and Baltimore, 1986a; Atchison and Perry, 1987; Lenardo *et al.*, 1987), and closely related to an upstream regulatory element of the MHC class I H-2K^b and β2-microglobulin genes, as well as to motifs present in several other viral and cellular promoters [see Figure 7C and also Kanno *et al.* (1989) and referencess therein]. The TC-II/κB sequence has been shown to interact with a B cell specific DNA binding activity NF-κB (Sen and Baltimore, 1986a; Atchison and Perry, 1987; Kawakami *et al.*, 1988; Lenardo *et al.*, 1988), which can be induced in pre-B, lymphoid T, and non-lymphoid cells by treatment with lipopolysaccharide (LPS) and tumor promoters [12-*O*-tetradecanoylphorbol-13-acetate (TPA)] (Sen and Baltimore, 1986b; Baeuerle and Baltimore, 1988a,b; Shirakawa and Mizel, 1989; Shirakawa *et al.*, 1989), tumor necrosis factor α and interleukin-1 (IL-1) (Osborn *et al.*, 1989) by virus infection (Lenardo *et al.*, 1989; Visvanathan and Goodbourn, 1989) or by the *tat* I gene product of human lymphotrophic virus I (Leung and Nabel, 1988). A second inducible DNA binding activity, HIVEN86A, has also been described (Franza *et al.*, 1987; Ballard *et al.*, 1988; Böhnlein *et al.*, 1988). In contrast, KBF1/H2TF1 is a DNA binding activity, present in a variety of untreated lymphoid and non-lymphoid cells, which binds to the TC-II/κB and H-2K^b motifs (Baldwin and Sharp, 1987, 1988; Israël *et al.*, 1987; Yano *et al.*, 1987; Singh *et al.*, 1988). In addition, a 57 kd HeLa cell protein, EBP-1, has been identified and affinity purified using the region of the SV40 enhancer containing the TC-II motif (Clark *et al.*, 1988, 1989; Clark and Hay, 1989). At present, the relationship between each of these proteins and their potential role in SV40 enhancer activity *in vivo* has not been established.

Two binding sites for the HeLa cell AP-2 protein (Imagawa *et al.*, 1987; Mitchell *et al.*, 1987) are also present in the region of the SV40 enhancer containing the TC motifs. These sites overlap with, but are not identical to the TC motifs or the TC-II enhanson (see Figures 1 and 2). Mutations which are specific to the AP-2 sites have no deleterious effect on enhancer activity in any of the cell types tested [mutations pA16 to pA19 in Zenke *et al.*, (1986) and Nomiyama *et al.* (1987); see Figure 5 in Kanno *et al.* (1989)] and the SV40 AP-2 binding sites appear not to be functional enhansons (Kanno *et al.*, 1989). Thus, the possible role of the AP-2 binding sites in SV40 enhancer activity *in vivo* also remains unclear.

The observation that the SV40 TC-II enhanson is active in both lymphoid and non-lymphoid cells (Kanno *et al.*, 1989) and the reports that both cell-specific and ubiquitous proteins bind to this enhanson (see above) prompted us to analyse the proteins binding to this enhanson in the variety of cell lines in which we have analysed the TC-II enhanson activity. Our results show the existence of at least three distinct DNA binding activities. Surprisingly, one of these activities, TC-IIA which is indistinguishable from the NF-κB factor, was present in nuclear extracts not only from lymphoid B cells, but also from untreated human HeLa cells, mouse fibroblast, LMTK$^-$ cells and retinoic acid-differentiated EC F9 cells [F9(D)]. While already present in nuclear extracts from untreated HeLa cells, the TC-IIA binding activity could be further increased by treatment with TPA and cycloheximide, whereas its presence in nuclear extracts of undifferentiated F9 EC cells [F9(ND)] was detectable only following cycloheximide treatment. The other two DNA binding activities exhibit properties analogous to the KBF1/H2TF1 (the TC-IIB protein) and AP-2 proteins. The efficiency of binding of both the TC-IIA/NF-κB-like and TC-IIB/KBF1/H2TF1-like proteins to a series of mutated TC-II enhansons correlates wth the effect of these mutations *in vivo* (Kanno *et al.*, 1989), suggesting an important role for these proteins in SV40 enhancer activity.

Results

Multiple protein DNA complexes are formed in vitro with the SV40 TC-II enhanson

To examine the interaction of nuclear proteins with the TC motifs, complementary oligonucleotides were synthesized

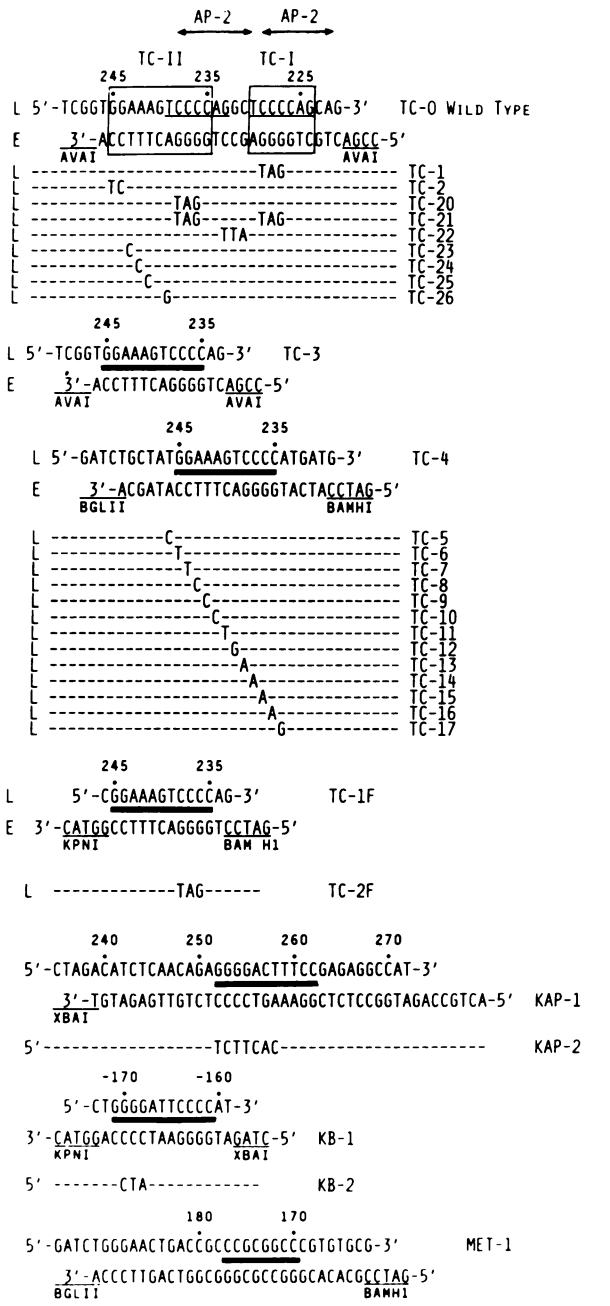

Fig. 2. Nucleotide sequence of templates containing the TC-II enhanson and related sequences. The top line shows the wild type sequence from coordinates 221 to 247 of the SV40 early promoter, which spans the TC-II and TC-I sequences (E is early coding strand and L is late coding strand). The sequences underlined in TC-0 show the TC sequences as originally defined by Zenke *et al.* (1986), while the boxed TC-II sequence is the TC-II enhanson. The positions and L strand sequence of the mutations in templates TC-1, TC-2 and TC-20 to TC-26 are indicated below TC-0. Templates TC-3 and TC-4 contain the wild type TC-II enhanson (indicated by a bar). The positions and L strand sequence of the mutations present in TC-5 to TC-17 are shown below TC-4. TC-1F and TC-2F show the wild type or mutated TC-II enhanson which has been subcloned into the *Kpn*I–*Bam*HI site of a modified pUC18 vector (see Materials and methods and Rosales *et al.*, 1987). KAP-1 corresponds to the nucleotide sequence of the kappa light chain enhancer (the κB enhanson is indicated by a bar) from positions 240 to 270 (numbering system of Picard and Schaffner, 1984) and KAP-2 to its mutant. KB-1 (the H-2K^b enhanson is indicated by a bar) and KB-2 refer to the wild type and mutated sequences respectively of the H-2K^b promoter region spanning nucleotides 159–173 (Kimura *et al.*, 1986), while MET-1 (the AP-2 binding site is indicated by a bar) corresponds to the metallothionein enhancer region from coordinates 164 to 191 (Imagawa *et al.*, 1987).

spanning the wild type SV40 enhancer from coordinates 221–247 (BBB numbering system; Tooze, 1982) (TC-O in Figure 2). Mutations were introduced at nucleotides 227–229 to destroy the TC-I motif (TC-1), and at positions 244–245 (TC-2) as the latter nucleotides had been previously shown to be essential for the *in vivo* activity of the TC-II enhanson within either the whole SV40 enhancer (Zenke *et al.*, 1986; Nomiyama *et al.*, 1987) or an oligomer containing the GT-I and TC-II motifs (Ondek *et al.*, 1987).

The ^{32}P-5'-end-labelled oligonucleotides TC-0, TC-1 and TC-2 were incubated with six different nuclear extracts of both lymphoid and non-lymphoid cell origin and the DNA–protein complexes formed were separated by electrophoresis on a 6% polyacrylamide gel (Fried and Crothers, 1981; Garner and Revzin, 1981; Strauss and Varshavsky, 1984) (Figure 3). In all cases the same specific complexes were observed with either TC-0 (not shown) or TC-1 templates, However, many non-specific interactions were detected using the TC-0 template, and thus only data using the TC-1 template will be shown hereafter. Incubation of template TC-1 with HeLa cell nuclear extract yielded three complexes, A, B and C (Figure 3A). The mutations present in TC-2 reduced binding to complexes A and B, but not to complex C, and an additional band migrating between complex A and B appeared. Note that the remaining TC-2 complex at the position of complex A corresponds to that formed by the binding of the AP-2 like factor, whereas the nature of the intermediate band which could not be competed using the metallothionein AP-2 binding site is unknown (see below and Figure 10A). Mutations at positions 236–238 (CCC to TAG, specific to motif TC-II) were also deleterious to formation of complexes A and B only (TC-20 in Figure 2, data not shown). A fourth complex, D, was detectable in varying intensity in all extracts, but neither of the aforementioned mutations were detrimental to the formation of this complex (Figure 3A and B and data not shown). Thus, complexes C and D were considered to be non-specific.

A complex corresponding to HeLa cell complex A was detected with nuclear extracts derived from the mouse myeloma B cell line MPC11, the pre-B cell line 70Z/3 (Paige *et al.*, 1978; Sakaguchi *et al.*, 1980), 70Z/3 cells treated with LPS [70Z/3(I)], 70Z/3 cells treated with LPS and cycloheximide [70Z/3(I + CYC)] (Figure 3A), as well as in mouse L cell fibroblasts (LMTK$^-$) (Figure 3B, lane 13), and retinoic acid-differentiated F9 cells [F9(D)] (data not shown). The amounts of complex A varied in the different extracts, with a marked 'induction' in 70Z/3(I) and a 'superinduction' in 70Z/3(I + CYC) (see lanes 7–16 in Figure 3A). Note that complex A was undetectable only in the non-differentiated F9 cells [F9(ND) in Figure 3; see also Figure 9B]. On the other hand, complexes analogous to band B were found in all cell lines tested (Figure 3 and data not shown).

Complex B corresponds to the binding of a KBF1/H2TF1-like protein

Factors binding to the upstream regulatory region of the mouse MHC class I gene H-2K^b (underlined sequence in the KB-1 oligonucleotide in Figure 2, see also Figure 7B) have been reported in extracts of murine 3T6 cells (factor KBF1; Israël *et al.*, 1987) and HeLa, BALB/c3T3 and mouse erythroleukemia (MEL) cells (factor H2TF1; Baldwin and Sharp, 1987). In both cases identical methylation

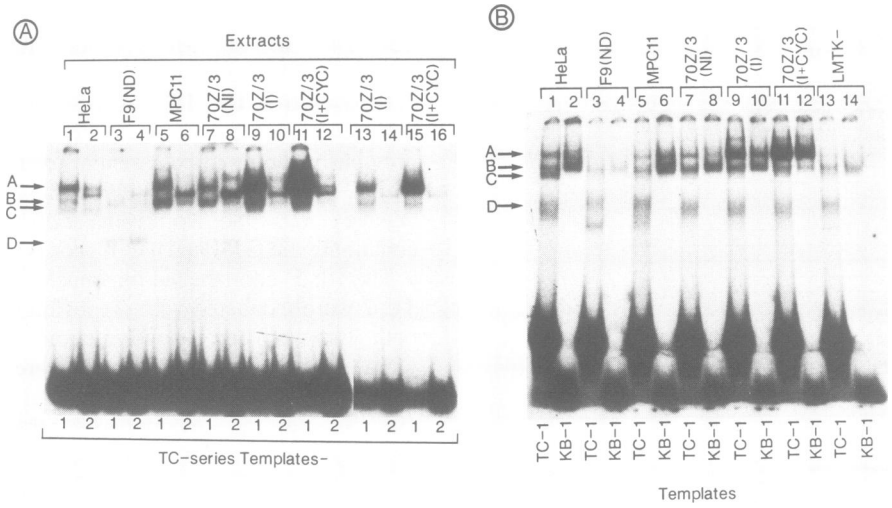

Fig. 3. Formation of DNA–protein complexes using the TC-II or H-2K^b enhansons. (**A**) The ^{32}P-5′-end-labelled TC-series templates used are indicated below the figure and the nuclear extracts and lane numbers at the top. The arrows to the left indicate the positions of complexes A–D. Gel retardation was performed with 5 μg of each cell extract and 2 μg of poly(dI-dC) as described in Materials and methods. **Lanes 13–16** show a shorter exposure of **lanes 9–12**. (**B**) Comparison of the complexes formed using the SV40 TC-II and the H-2K^b enhansons. The nomenclature is as in (**A**). Note that in (**A**) and (**B**) similar results were obtained with at least two independently prepared extracts from each cell type.

interference patterns were found and KBF1/H2TF1 has been shown to bind to the TC-II motif (Baldwin and Sharp, 1987; Israël *et al.*, 1987). We thus investigated if this protein was involved in the formation of one of the complexes obtained with the TC-1 template. Using the KB-1 oligonucleotide (Figures 3B and 9) and its mutant KB-2 (Figure 2, Figure 9 and data not shown), we observed specific complexes similar to those obtained with the TC-1 template (Figure 3B). Complex A was detected in all extracts except F9(ND), and in increased amounts in 70Z/3(I) and 70Z/3(I + CYC) extracts. Complex B, however, was stronger with the KB-1 than with the TC-1 template, suggesting that the protein responsible for the formation of complex B has a higher affinity for this motif, and thus could be the KBF1/H2TF1 protein. This suggestion was further supported by the results of the competition experiments shown in Figure 8 (see below), which indicate clearly that the protein forming complex B exhibits a higher affinity for the KB-1 than for the TC-1 template.

Further support for the similarity between the protein forming complex B and KBF1/H2TF1 was provided by methylation interference assays of complex B. The interference pattern obtained using HeLa, F9(ND), LMTK$^-$ and 70Z/3(I) cell extracts was identical (data not shown; summarized in Figure 7B) to that observed with the KBF1/H2TF1 factor (Israël *et al.*, 1987; Baldwin and Sharp, 1987, 1988).

Complex A corresponds to the binding of a NF-κB-like protein

The protein NF-κB (see Introduction) has been shown to bind to the κB motif located within the kappa light chain enhancer which is identical over 11 bp to the region containing the TC-II motif of the SV40 enhancer (see KAP-1 in Figure 2). NF-κB, which has been implicated in the activation of kappa light chain gene transcription appears to be B cell specific, although it could also be detected in HeLa and lymphoid T cell nuclear exracts following phorbol ester (TPA) treatment and in both lymphoid and non-lymphoid cells following virus infection (see Introduction

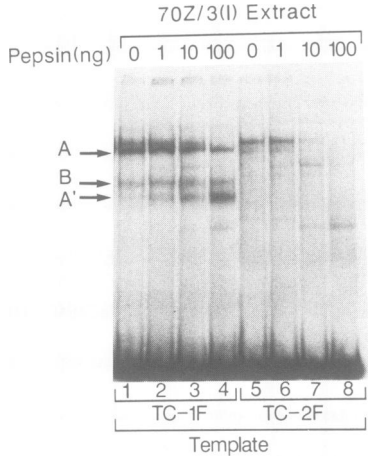

Fig. 4. Effect of partial proteolysis on complexes TC-IIA and TC-IIB. The ^{32}P-5′-end-labelled templates and lane numbers are indicated at the bottom, and the quantity (in ng) of pepsin added following complex formation at the top along with the type of extract. The arrows to the left indicate the positions of the A, B and A′ complexes. Retardation and proteolysis were performed as described in Materials and methods with 2 μg of extract, 4 μg poly(dI-dC) and the cloned TC-1F and TC-2F templates.

for references). In addition, the NF-κB activity could be induced in 70Z/3 cells treated with LPS, and superinduced with LPS plus cycloheximide (Sen and Baltimore, 1986b).

Although the protein involved in complex A formation mimicked the general behaviour described for NF-κB in 70Z/3 cells (Figure 3A), a band of similar electrophoretic mobility was observed in all extracts tested except those from F9(ND). Moreover, using either template TC-1 (Figure 3A) or KAP-1 (data not shown), the same complex A was observed in each extract, and in all cases cold TC-1 or KAP-1 competed the formation of complex A with similar efficiency (see below in Figure 8, and data not shown). We thus asked whether the protein responsible for the formation of complex A in non-lymphoid cell extracts could be distinguished from the NF-κB protein previously detected in B cell lines.

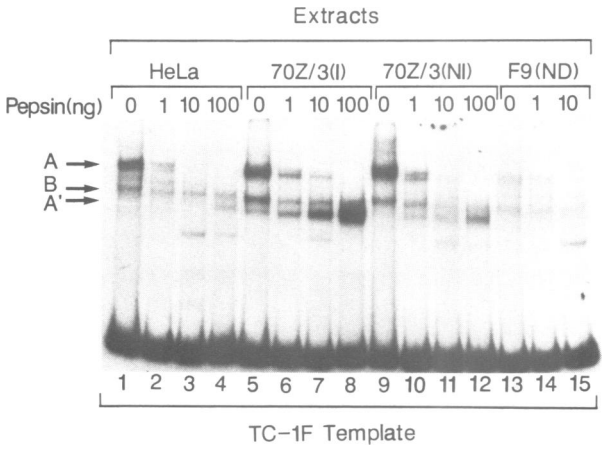

Fig. 5. Proteolysis of the TC-IIA complex formed with lymphoid and non-lymphoid cell extracts. The ^{35}P-5′-end-labelled TC-1F template was used in each lane with 5 μg of the nuclear extracts indicated above the lanes. The quantity of pepsin is also indicated above each lane and the lane numbers indicated at the bottom of the figure. The positions of the A, B and A′ complexes are indicated by the arrows to the left.

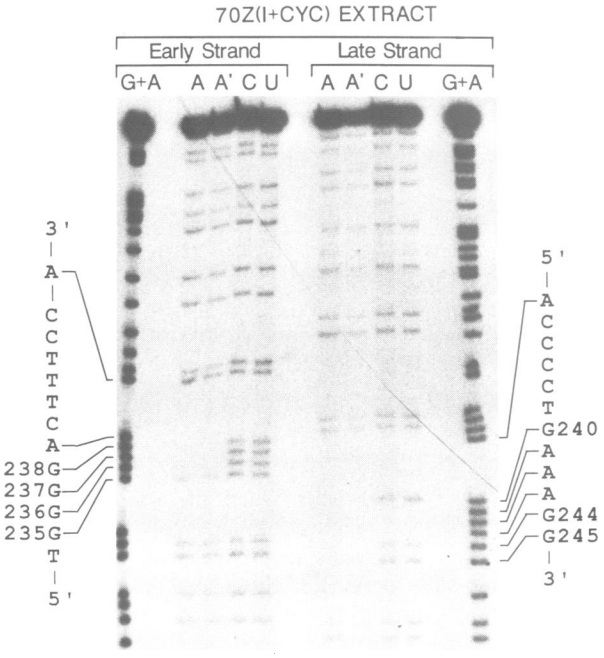

Fig. 6. DMS methylation interference of the A and A′ complexes formed with the SV40 TC-II enhanson in the TC-1F template. Methylation of the TC-1F template, ^{32}P-5′-end-labelled on either the early or late coding strands, with DMS, and gel retardation were performed as described in Materials and methods. The figure shows the methlation interference performd on the A complex and its proteolytic product A′. The lanes containing the SV40 early or late coding strands are indicated above the figure, and G+A shows the ladder of G and A residues of each strand obtained by depurination. The nucleotide sequence of the appropriate strand is shown to the left and to the right of the figure along with the coordinates of the interfering G residues. C is a control lane with the original methylated DNA cleaved with piperidine. U is the unbound DNA and A and A′ the DNA form the A and A′ complexes respectively. The residues which interfere with complex formation when methylated are summarized in Figure 7B.

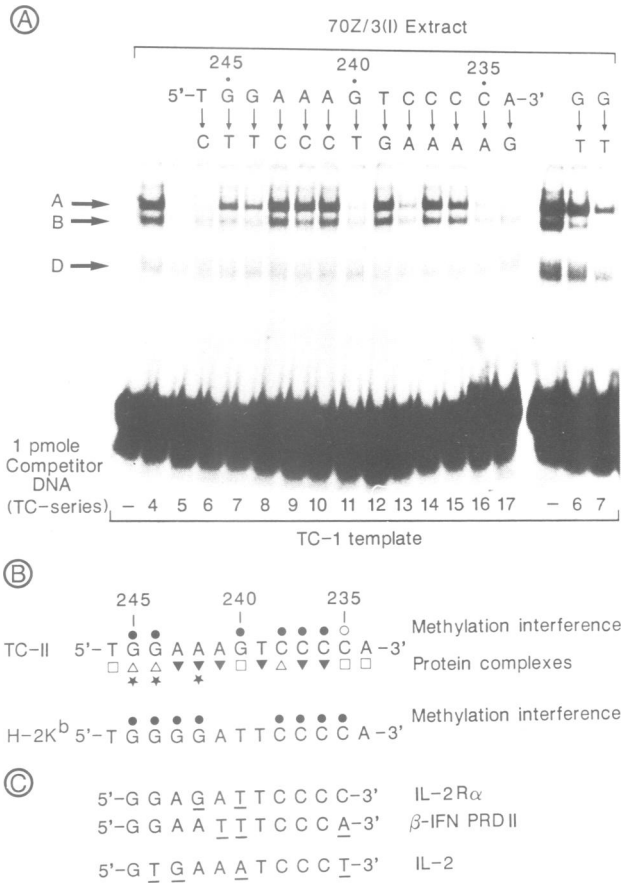

Fig. 7. (**A**) Effect of single base pair changes in the SV40 TC-II enhanson on the formation of the complexes TC-IIA and TC-IIB. Each lane contains the ^{32}P-5′-end-labelled TC-1 template with 5 μg of 70Z/3 (I) extract, 2 μg poly(dI-dC) and 1 pmol of the cold competitor template indicated below each lane (– is no added competitor). The base changes in each competitor template are shown above each lane, where the WT sequence is on the top, along with the SV40 coordinates 235, 240 and 245. The arrows to the left indicate the positions of the specific A and B, and non-specific D complexes. TC-4 is a control using the wild type competitor. The lanes –, 6 and 7 to the right of the panel show independent duplicate competitions with (–) no competitor or the TC-6 and 7 templates. (**B**) The positions of full or partial methylation interference of the TC-IIA complex are shown above the SV40 TC-II sequence by the filled or open circles respectively. For the TC-IIB complex full interference of G235 was seen. The effect of the single base changes from (**A**) are summarized below the sequence. The open squares indicate no significant effect on A and B complex formation, the filled triangles a fully deleterious effect and the open triangles a partially deleterious effect. The stars indicate those mutations which had a more deleterious effect on formation of complex A than on formation of complex B. The H-2K^b sequence is aligned to have maximum homology with the SV40 TC-II sequence. The filled circles show those G residues whose methylation fully interfered with complex formation in Israël *et al.* (1987), Baldwin and Sharp (1987) and our unpublished results. (**C**) Comaprison of the sequences present in the IL-2 receptor α (IL-2Rα), the β-interferon PRDII site (β-IFN-PRDII) or the IL-2 promoters, with the SV40 TC-II enhanson (i.e. each case, see text for references). Each motif has been aligned to have maximum homology with the SV40 TC-II sequence and the differences from the SV40 sequence are underlined. The IL-2 sequence is separated from those of IL-2Rα and β-IFN-PRDII to indicate that in competition retardation experiments this template behaves more like the H-2K^b enhanson than the TC-II/xB enhanson.

We first undertook a series of proteolysis experiments. As it was difficult to achieve a good resolution of the proteolysed complexes formed with template TC-1, we used a cloned 123 bp template fragment containing the wt TC-II motif, TC-1F, or its corresponding mutant, TC-2F (Figure 2 and Materials and methods) and 70Z/3(I) (Figure 4) and other (Figure 5, and data not shown) nuclear extracts. TC-1F

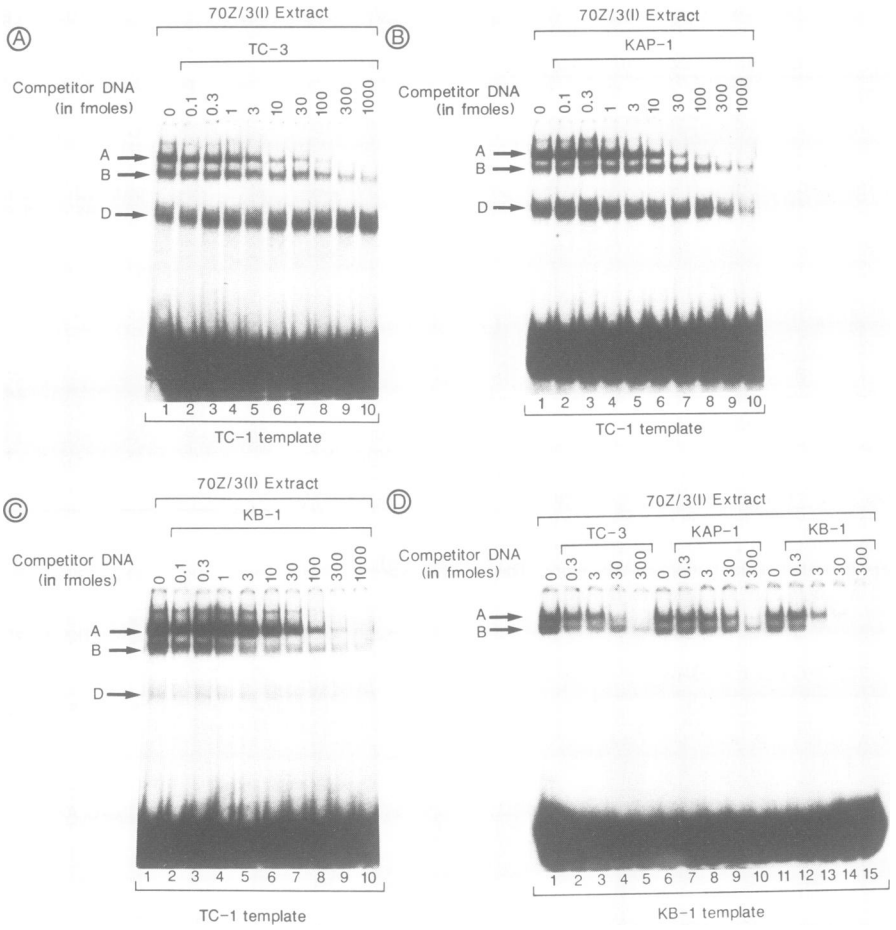

Fig. 8. Relative affinities of the SV40 TC-II, κB and H-2K^b enhansons or the proteins TC-IIA and TC-IIB. Each lane in each panel contains 5 μg of 70Z/3(1) nuclear extract with 2 μg of poly(dI-dC), the ^{32}P-5′-end-labelled template indicated at the bottom of the panel and the cold competitor indicated above. The lane numbers are below each lane and the quantity (in pmoles) of the cold competitor above each lane. The positions of the A, B and D complexes are indicated by the arrows to the left of each panel. Note that with the TC-1 template in (**A**), (**B**) and (**C**) there is a non-specific complex comigrating with complex B which is not competed for even at the highest concentrations of the TC-3 and KAP-1 competitors or with the KB-1 competitor which competes efficiently for formation of the B complex (see also text).

gave specific complexes analogous to the A and B complexes obtained with the oligonucleotide template TC-1 (Figure 3A). Notably, complex A formed with the TC-1F fragment was also 'induced' in 70Z/3(I) and 'superinduced' with 70Z/3(I + CYC) extracts (data not shown). Using the mutated template TC-2F, non-specific complexes were also seen running at the same position as complex A (Figure 4, lane 5), which accounts for a fraction of the A complex observed with 70Z/3 extracts (Figure 5, lane 9). Following formation of the complexes with 70Z/3(I) extracts, increasing amounts of pepsin (1, 10 or 100 ng) were added, resulting in the gradual appearance of a new complex, A′, whereas complex A was reduced and complex B appeared to be unaltered (Figure 4, lanes 1–4). Neither complexes A, B nor A′ could be detected after similar proteolytic treatment using the mutated template, TC-2F (Figure 4, lanes 5–8). We then performed similar proteolytic digestions with nuclear extracts from HeLa, 70Z/3(NI) and F9(ND) cells (Figure 5), as well as with extracts from F9(D), MPC11 and 70Z/3(I + CYC) cells, HeLa cells treated with TPA, and LMTK$^-$ cells (data not shown). In every case, except F9(ND) cells, identical patterns were observed, with an increase of band A′ while complex B remained unaltered. The absence of complex A′ formation in F9(ND) cells correlates with the absence of complex A in F9(ND) cells when the oligonucleotide template TC-1 is used (Figure 3A). Proteolytic digestions performed with all extracts using the mutated template TC-2F, gave results identical to those seen in lanes 5–8 of Figure 4 (data not shown). Note that complex A′ was weakly present in some of the extracts, even in the absence of pepsin, suggesting that some proteolysis occurred during extract preparation. These results indicate that the proteins forming the complexes A detected in 70Z/3(I) and (I + CYC) cell and in HeLa cell extracts, all contained similar pepsin sensitive sites, thus suggesting that in each case they were very similar or identical.

To analyse further the relationship between complexes A and A′, we performed DMS methylation interference assays. Following pepsin treatment and electrophoresis, complexes A and A′ were excised from the gel, eluted, and the DNA cleaved at the modified guanine residues by piperidine (Maxam and Gilbert, 1980). The interference patterns obtained with 70Z/3(I + CYC) extracts are shown in Figure 6 (see also Figure 7B). Identical patterns were obtained with 70Z/3(I) extracts (data not shown). Lanes labelled A and A′ show on both the SV40 early and late coding strands, the guanine (G) residues whose methylation interfered with the formation of their respective complexes. Identical

interference patterns of G residues at positions 240, 244 and 245 on the late strand and 235, 236, 237 and 238 on the early strand were observed for complexes A and A′, indicating that the DNA binding domain was essentially unchanged following proteolytic treatment. The interference patterns for complexes A and A′ correlated well with those obtained for NF-κB (Sen and Baltimore, 1986a), except that in our case there was a partial interference at G235. Taken together these results show that proteins with similar DNA binding domains and pepsin sensitive sites must be present in each of the nuclear extracts, including those from non-lymphoid cells, with the exception of F9(ND) cells.

The same SV40 sequence is required for the formation of complexes A and B

As we were unable to differentiate the proteins forming complex A in lymphoid and non-lymphoid cell extracts by methylation interference and proteolytic digestion, we attempted to define the nucleotides required for the formation of the A complexes in each extract, and their relationship to those required for formation of complex B. Formation of complexes A and B with TC-1 in 70Z/3(I) nuclear extracts was competed using a series of oligonucleotides containing single point mutations throughout the TC-II motif (TC-5 to TC-17 in Figure 2). Mutations at positions 236, 237, 239, 241, 242 and 243 (TC-series 15, 14, 12, 10, 9 and 8 respectively) were all highly deleterious to the formation of both complexes as these oligonucleotides were unable to compete efficiently for the binding of proteins A and B (Figure 7A, cf. with wild type TC-4 competition; see also Figure 7B). Note, however, that the mutation present in TC-9 at position 242 may be more detrimental to the formation of complex A than to that of complex B, and also that A to G transitions at positions 242 and 243 were not deleterious to formation of complexes A and B (data not shown). In contrast mutations at positions 234, 235, 240 and 246 (TC-competitor series 17, 16, 11 and 5 respectively) had little or no effect on the formation of complexes A and B. Templates with mutations at positions 244 and 245 (TC-series 6 and 7) competed efficiently for the formation of complex B, and only partially for that of complex A. The template mutated at 238 (TC-13) competed more efficiently than TC-6 and 7 (mutated at positions 245 and 244) for formation of complexes A and B, but still to a lesser extent than the wild type template (TC-4). It is noteworthy that while methylation of G238 and G240 resulted in full interference (see above), the replacement of these G by T residues still allows protein binding. A summary of the bases that appear to be important for the binding of both KBF1/H2TF1 and NF-κB-like proteins is shown in Figure 7B. Similar results were obtained using either HeLa or MPC11 nuclear extracts (data not shown), indicating that the proteins forming complex A in each cell line recognize the same sequence and thus cannot be distinguished in this way. It is also evident that the sequences recognized by the proteins forming complexes A and B are similar, but not identical.

Relative affinity of the NF-κB and KBF1/H2TF1-like proteins for the TC-II and H-2K^b motifs

As similar sequences appear to be involved in formation of complexes A and B, we compared the relative affinity of the corresponding proteins for templates containing either the TC-II/κB or H-2K^b sequences in competition retardation assays. Increasing quantities (in fmoles) of either the TC-II motif (TC-3 in Figures 2 and 8A), the κB motif (KAP-1 in Figures 2 and 8B), or the H-2K^b motif (KB-1 in Figures 2 and 8C), were used to compete complex formation on the TC-1 template in 70Z/3(I) extracts. Virtually identical competition for formation of complex A was observed using either the TC-II motif (TC-3) or the κB motif (KAP-1) as a competitor. Moreover, formation of both A and B complexes was similarly competed by the TC-II (TC-3) and κB (KAP-1) motifs (compare Figure 8, A and B, taking into account the presence of a non-competable non-specific band migrating at the position of the B complex), which suggests that proteins A and B have a similar affinity for the TC-II/κB motif. The H-2K^b motif, KB-1 (Figures 2 and 8C), competed nearly as efficiently as TC-3 or KAP-1 for the formation of complex A (cf. competition of complex A by TC-3, KAP-1 and KB-1 in Figures 8A, B and C) which indicates that protein A has similar affinities for all three motifs. However, the H-2K^b motif competed much more efficiently for the formation of complex B than for that of complex A (Figure 8C), indicating that protein B has a higher affinity for this motif than for the TC-II/κB motif. As expected, using the H-2K^b motif as a template (KB-1, Figure 8D), the TC-II/κB motif (TC-3 and KAP-1) competed more efficiently for formation of complex A than for that of complex B, while the H-2K^b motif was more efficient at competing the formation of complex B. Similar patterns were also observed using either HeLa or MPC11 nuclear extracts (data not shown).

Effect of tumor promoter (TPA) and cycloheximide treatments of HeLa and F9(ND) cells on formation of complexes A and B

It has been reported that the activity *in vivo* of a number of enhancer elements is induced by treatment with phorbol esters, which results in the activation of protein kinase C (Nishizuka, 1988; and references therein). These elements include the sequences binding the proteins AP-1 (Angel *et al.*, 1987; Lee *et al.*, 1987), AP-2 (Imagawa *et al.*, 1987) and NF-κB (Sen and Baltimore, 1986b; Kaufman *et al.*, 1987; Lenardo *et al.*, 1987); Nabel and Baltimore, 1987; Nelsen *et al.*, 1988; Pierce *et al.*, 1988). Induction by TPA of the *in vivo* activity of the element binding the NF-κB protein (Nabel and Baltimore, 1987; Nelson *et al.*, 1988; Pierce *et al.*, 1988) has been correlated with an increase of this protein in nuclear extracts prepared from TPA-treated Jurkat T and HeLa cells (Sen and Baltimore, 1986b; Nabel and Baltimore, 1987; Baeurele and Baltimore, 1988a). Moreover, Shirakawa and Mizel (1989) have shown that NF-κB can be 'activated' by protein kinases in cytosolic extracts of untreated pre-B cells. As both the NF-κB and KBF1/H2TF1 proteins can bind to each others cognate sequence, we wished to determine whether the KBF1/H2TF1-like protein could also respond to TPA in the presence or absence of the protein synthesis inhibitor cycloheximide (CYC).

Figure 9A shows the results of TPA/CYC treatments of HeLa cells on the formation of NF-κB or KBF1/H2TF1-like complexes using either the TC-1 (lanes 1, 3, 7 and 11) or KB-1 (lanes 2, 5, 9 and 13) templates (lanes 4, 8, and 12 and 6, 10 and 14, depict the results using the mutated templates TC-2 and KB-2 respectively). Densitometric scanning indicated that the amount of the NF-κB-like TC-IIA

protein in both HeLa/TPA and HeLa/CYC extracts doubled, with a 4-fold increase noted in extracts treated with both TPA and cycloheximide. These increases in the level of the TC-IIA protein following TPA and/or cycloheximide treatments correlate well with the transcriptional stimulation brought about by the TC-II proto-enhancer *in vivo* following similar treatments (Kanno *et al.*, 1989). On the other hand, densitometry of the KBF1/H2TF1 complex B using either the TC-1 or KB-1 templates indicated no significant changes following TPA/CYC treatments. Thus, although the NF-κB and KBF1/H2TF1-like proteins have similar DNA binding sequence requirements, they exhibit different responses to either TPA or cycloheximide treatments in HeLa cells.

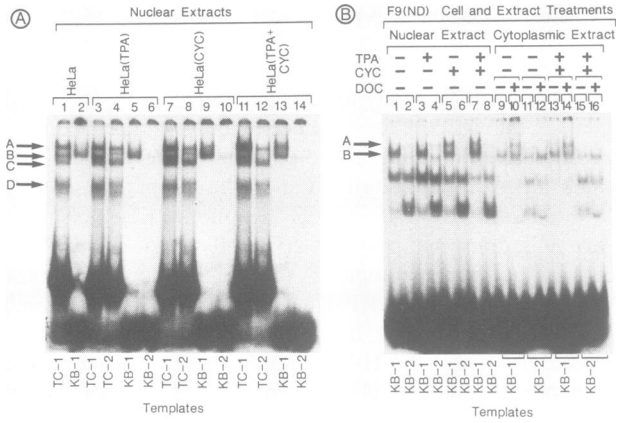

Fig. 9. Effect of TPA and/or CYC treatment on the proteins TC-IIA and TC-IIB present in extracts of HeLa and F9(ND) cells. Above each lane are the lane numbers, and the positions of the A, B, C and D complexes are indicated by the arrows to the left of the figure. (A) Each lane contains 5 μg of HeLa nuclear extract with the *in vivo* treatments indicated above each lane along with 2 μg of poly(dI-dC) and the ^{32}P-5′-end-labelled template indicated below the lane. The same results were obtained with two independently prepared nuclear extracts. The induction of the A and B complexes was determined by densitometric scanning of suitable exposures of the autoradiograms and subtraction of the background observed using the mutated TC-2 template. These results show that while complex B remained constant, complex A was induced 1.6-fold by TPA, 2.3-fold by cycloheximide and 4.1-fold by TPA and CYC. (**B**) Each lane contains 5 μg of either nuclear or cytoplasmic F9(ND) extract as indicated above each lane along with 0.8 μg of poly(dI-dC) and the ^{32}P-5′-end-labelled template indicated below the lane. + and − above each lane show the presence or absence of the TPA, CYC or DOC treatments. DOC treatment consisted in the addition of 0.2% DOC and 0.2% NP40 to the cytoplasmic extract (Materials and methods) after addition of the ^{32}P-5′-end-labelled KB-1 or mutated KB-2 templates, as indicated below each lane. Note that with the cytoplasmic extracts the complex formed at the position of complex B is a non-specific complex formed by a cytoplasmic protein as its formation is unaffected by the mutations present in the KB-2 template.

Kanno *et al.* (1989) found that the activity of both the TC-II/κB and H-2K^b proto-enhancers could be increased *in vivo* following treatment of F9(ND) cells with cycloheximide, but not with TPA. Since the TC-IIA complex could not be detected in F9(ND) cells (see above), we studied the formation of complexes A and B in nuclear extracts from F9(ND) cells treated with TPA and/or cycloheximide. Although neither TPA nor cycloheximide treatments affected the formation of complex B, the TC-IIA activity which was undetectable in untreated F9(ND) nuclear extracts was clearly 'induced' by cycloheximide, but not by TPA treatment (Figure 9B, lanes 1−8; the KB-2 mutant template is used as a control). Thus, as in HeLa cells, the KBF1/H2TF1-like protein TC-IIB did not respond to TPA/CYC treatment, whereas the TC-IIA/NF-κB-like protein did. However, in contrast to the HeLa cell case, only cycloheximide, but not TPA, could induce the TC-IIA binding activity in F9(ND) cells.

Baeuerle and Baltimore (1988a,b) have reported that the NF-κB binding activity is sequestered in the cytoplasm of untreated non-lymphoid cells by the inhibitor protein IκB from which it can be released by deoxycholate (DOC) treatment. Such an inhibitor also seems to be present in the cytoplasm of untreated F9(ND) cells as the TC-IIA activity could be induced by DOC treatment (Figure 9B, lanes 9−12). Moreover, it appears that some TC-IIA/NF-κB-like activity remained sequestered in the cytoplasm even after TPA and cycloheximide treatment of these cells (Figure 9B, lanes 13−16).

Sequence requirement for binding of the AP-2 protein to the TC region

A 52 kd HeLa cell protein, AP-2, which binds to sequences located within the human metallothionein IIA enhancer

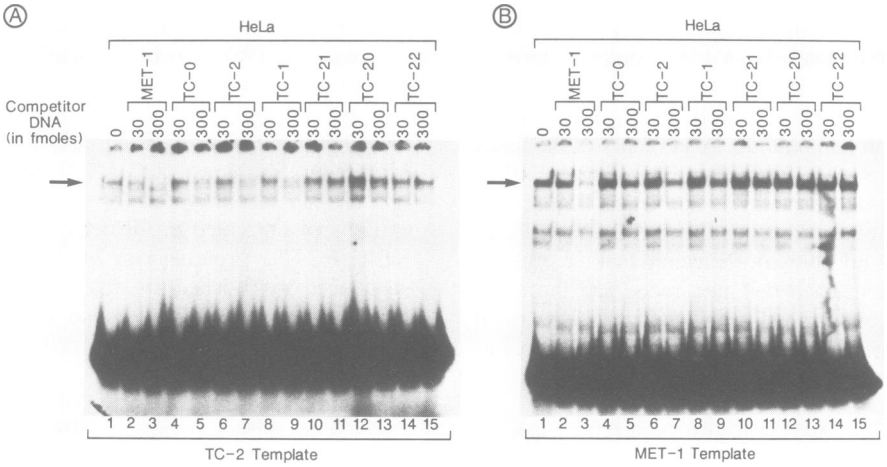

Fig. 10. The hMTIIA AP-2 binding site competes for complex formation with the TC-2 template which contains intact AP-2 binding sites (see text). Each lane contains 5 μg of nuclear extract from untreated HeLa cells with the ^{32}P-5′-end-labelled TC-2 template (**A**) or hMTIIA MET-1 (**B**) template. The quantity of the cold competitor templates are indicated above each lane, and the lane numbers at the bottom. The arrow to the left of each panel shows the position of the complex formed with the AP-2-like protein. Note that this complex comigrates with complex A formed with the wild type TC-II motif in template TC-1.

(MET-1 in Figure 2) and the SV40 enhancer region which overlaps the TC motifs (Figures 1 and 2) has been purified (Imagawa *et al.*, 1987; Mitchell *et al.*, 1987), and its corresponding cDNA cloned (Williams *et al.*, 1988). Methidiumpropyl-EDTA-FE(II) footprinting (Imagawa *et al.*, 1987) and binding to mutated templates (Mitchell *et al.*, 1987) have indicated that protection of the SV40 enhancer sequence, 5′-AAAGTCCCCAGGCTC-3′, by AP-2 did not require the presence of either the guanines at positions 244 and 245, nor the intact TC-I motif. However, while both the TC-0 and TC-1 templates contain the putative AP-2 binding site, we were unable under our conditions to detect the formation of a protein complex that did not correspond to either the NF-κB or KBF1/H2TF1-like complexes (Figure 3A, and data not shown).

The TC-2 template, in which the guanines at positions 244 and 245 had been mutated, was therefore used to prevent possible interference of AP-2 binding by the NF-κB or KBF1/H2TF1-like proteins. To determine the specificity of protein binding to the TC-2 template, competitions were performed in HeLa cell nuclear extracts using varying quantities of either the TC-0, TC-1, TC-2, TC-20, TC-21, TC-22 or MET-1 templates (see Figure 2). The MET-1 template competed efficiently for the formation of the complex indicated by the arrow (Figure 10A), supporting the proposal that we could not detect the binding of AP-2 using the TC-1 template, because the weaker AP-2 complex was masked by complex A migrating at the same position (see also TC-2 template in Figure 3A). A minimum 5-fold less efficient competition for this AP-2-like complex was noted using either the TC-0 (TCII + I wild type), TC-1 (TC-I mutated) or TC-2 templates. No competition for this complex was observed using similar quantities of either TC-20 (TC-II mutated), TC-21 (TC-II + TC-I mutated) or TC-22 (GGC at positions 231−233 mutated) templates, indicating that efficient binding of the AP-2-like factor required the presence of sequences contained within the intact TC-II motif plus adjacent 3′ sequence (mutated in TC-22), but not the TC-I motif. In addition the indicated complex was formed with, and competed out, by either TC-23, 24, 25 or 26 templates (Figure 2, data not shown). Non-specific competitor DNAs containing the GT-I (Xiao *et al.*, 1987b), TC-3 or KB-1 sequences failed to compete (data not shown) [the second faster migrating complex formed using the TC-2 template (Figure 10A) was considered non-specific as all competitor DNAs failed to compete efficiently]. Similar results were obtained using the MET-1 template (Figure 10B) in which the complex indicated by an arrow was competed in a manner similar to that observed in panel A. The second and third complexes were not competed by any of the TC or MET-1 templates, while the fourth complex was competed by all, regardless of the mutations present. Thus, each of these three bands was considered to correspond to non-specific complexes.

Discussion

Several proteins interact specifically with the region of the SV40 enhancer containing the TC-II enhanson, but none recognize the enhancer 'core' sequence per se

This study shows that at least three nuclear proteins exhibiting different properties interact specifically with the region of the SV40 enhancer containing the TC-II motif. One of these proteins, TC-IIA, was found in nuclear extracts from each of the differentiated cell types studied here, but was absent from those of untreated F9(ND) cells, while the second protein TC-IIB appears to be ubiquitous. Both proteins TC-IIA and TC-IIB bind to the TC-II enhanson 5′-(245)GGAAAGTCCCC(235)-3′ (Kanno *et al.*, 1989). Note that while a mutation at position 235 has no effect on protein binding *in vitro* (see Figure 7A and B) or on the enhancer activity *in vivo* (Kanno *et al.*, 1989), this residue has been included in the enhanson since G235 methylation interferes with protein binding either partially (TC-IIA) or fully (TC-IIB). The third, AP-2-like, protein interacts with a sequence which partially overlaps the TC-II enhanson, but requires additional 3′ sequences. In contrast no proteins appear to interact selectively with the TC-I motif which correlates with the 'inactivity' of this motif *in vivo*, both in the whole SV40 enhancer and when it is oligomerized either alone or in association with the GT-I enhanson (Zenke *et al.*, 1986; Nomiyama *et al.*, 1987; Kanno *et al.*, 1989).

Competition and direct binding experiments show that the protein forming complex A in each cell type binds with similar affinity to the SV40 TC-II, immunoglobulin κB and H-2K^b enhansons, while the protein forming complex B has a much higher (~10 times) affinity for the H-2K^b enhanson than for the TC-II and κB enhansons. However, both proteins appear to exhibit similar, although not fully identical, sequence requirements for binding to the TC-II enhanson. Analysis of the binding of each protein to a series of templates mutated in the TC-II enhanson indicates that several mutations were more detrimental to the formation of complex A than to that of complex B (see below).

The binding site for the TC-II and IIB proteins overlaps by two nucleotides, at positions 244 and 246 (see Figure 1) with the SV40 enhanson GT-I, 5′-(251)GGGTGTGG (244)-3′, which binds the protein TEF-2 (Xiao *et al.*, 1987b; Davidson *et al.*, 1988). Both the GT-I and TC-II enhansons, also overlap with the enhancer 'core' sequence [5′-(247)GTGG$^{AAA}_{TTT}$G(240)-3′] (Laimins *et al.*, 1982; Weiher *et al.*, 1983). However, in the case of the proteins TC-IIA and TC-IIB, sequences 3′ to the 'core' are indispensable for binding, whereas for the TEF-2 protein sequences 5′ to the 'core' are absolutely required (Xiao *et al.*, 1987b), establishing clearly that the proteins TC-IIA and B are different from the AP-3 protein which has been reported to footprint on the SV40 enhancer 'core' sequence and was purified using an affinity column containing this SV40 sequence (Chiu *et al.*, 1987; Mitchell *et al.*, 1987; Mercurio and Karin, 1989). Moreover, in agreement with our previous gel retardation results (Xiao *et al.*, 1987a,b), no protein binding specifically to the 'core' sequence could be detected in the present study, as evidenced by the absence of a complex which would be formed with the TC-20 template, but not with the TC-2 template (Figure 2A and data not shown). These *in vitro* results correlate well with the *in vivo* observations showing that the 'core' motif has no detectable enhanson activity in any of the cell types which have been tested up to now (Fromental *et al.*, 1988; Ondek *et al.*, 1988; Kanno *et al.*, 1989).

TC-IIA is an NF-κB-like protein present in nuclear extracts of both lymphoid and non-lymphoid cells with the exception of F9(ND) cells where its activity can be induced by cycloheximide, but not TPA

The protein NF-κB, binds to the κB enhanson of the κ chain

enhancer (Sen and Baltimore, 1986a; Lenardo *et al.*, 1987). The NF-κB activity, which is present in lymphoid B cells producing κ chains, is induced in the pre-B cell line 70Z/3 by LPS or TPA and a superinduction is observed by associating LPS or TPA with cycloheximide (Sen and Baltimore, 1986b). We have observed here that the TC-IIA activity, which is present in MPC11 lymphoid B cells, is LPS-induced in 70Z/3 cells and is superinduced in the latter cells in the presence of LPS and cycloheximide. A complex, with an electrophoretic mobility identical to that of complex A formed with the TC-II enhanson, was also observed with the κB enhanson in nuclear extracts from the various cell lines except in untreated F9(ND), and in each case the κB enhanson competed as efficiently as the TC-II enhanson for formation of complex A. In addition the DMS methylation interference patterns obtained with complex A (summarized in Figure 7B) were identical to those obtained for NF-κB (Sen and Baltimore, 1986a). Taken together, all of these observations strongly suggest that the protein forming complex A in the different nuclear extracts is a NF-κB-like protein(s), if not NF-κB itself. The activity found in the non-lymphoid cells was indeed indistinguishable from that found in the induced 70Z/3 cells [70Z/3(I)] by several criteria: electrophoretic mobility, methylation interference, binding affinity to the TC-II, κB and H-2K^b enhansons, nucleotide requirements for binding to the TC-II enhanson, and sensitivity to proteolytic digeston. In particular, the fact that the products of proteolytic digestion are identical in lymphoid and non-lymphoid cells indicates that in each case the same or very similar proteins are involved.

It is noteworthy that we detected the presence of an NF-κB-like activity in nuclear extracts of untreated, HeLa, F9(ND) and LMTK$^-$ non-lymphoid cells, whereas previous observations indicated that NF-κB was totally sequestered in the cytoplasm of HeLa cells by an inhibitor protein, IκB, and could be found in nuclear extracts only following TPA treatment (Baeuerle and Baltimore, 1988a,b). Baeuerle and Baltimore (1988a,b) showed also that NF-κB could be released from its IκB inhibitor by exposing the cytoplasm of untreated HeLa cells to deoxycholate. We have, however, detected a moderate (maximum 4-fold) induction of the TC-IIA/NF-κB-like activity in nuclear extracts from HeLa cells treated with TPA and/or cycloheximide. On the other hand, no TC-IIA/NF-κB-like activity could be detected in the nuclear extracts of untreated F9(ND) cells (Figures 3 and 9B). Interestingly, this activity was induced in the nuclear extracts of these cells by cycloheximide but not by TPA treatment (Figure 9B). This inability of TPA to induce the TC-IIA binding activity suggests that F9(ND) cells lack the appropriate protein kinase C activity (Nishizuka, 1988) or another essential downstream component in the signal transduction pathway which is present in HeLa and pre-B cells (see also Shirakawa and Mizel, 1989). However, deoxycholate (DOC) treatment of whole cell (data not shown) or cytoplasmic extracts of untreated F9(ND) cells induced the formation of complex A (Figure 9B), indicating that, in these cells, the NF-κB-like protein TC-IIA is fully sequestered in the cytoplasm by an inhibitor protein probably similar to IκB (see above), which is partially inactivated following cycloheximide treatment or retinoic acid induced differentiation.

Other proteins with DNA binding specificities analogous to TC-IIA (NF-κB-like) have been identified in non-lymphoid cells (Clark *et al.*, 1988; Wu *et al.*, 1988). Notably EBP-1, a 57 kd HeLa cell protein, binds specifically to the TC-II and the κB enhansons (Clark *et al.*, 1988, 1989; Clark and Hay, 1989). This protein may correspond to the TC-IIA protein described here, but further studies will be necessary to clarify this point. It is therefore possible that a family of closely related NF-κB-like proteins exists, some of which (i.e. NF-κB) exhibit cell type specific regulation, while others would be present in many cell types. However, we do not understand why we (as well as others, see above) have found a high NF-κB-like binding activity in untreated HeLa cell nuclear extracts that was only weakly induced by TPA and cycloheximide, whereas in other studies this activity was low and strongly induced by these agents (Baeuerle and Baltimore, 1988a,b). It is unlikely that in our case this discrepancy could be accounted for by differences in nuclear extract preparation protocols, since the same protocol was used for the preparation of F9(ND) nuclear extracts where complex A could not be detected unless the cells were treated with cycloheximide (see above). Moreover, the TC-II enhanson is active in untreated HeLa cells *in vivo* (see Kanno *et al.*, 1989), suggesting that the TC-IIA protein is 'normally' active in these cells. Cloning and comparison of the cDNAs encoding each of these NF-κB-like proteins will be necessary to understand their relationship.

The TC-IIB protein is a KBF1/H2TF1-like protein

Proteins TC-IIA and TC-IIB can be differentiated in several ways. Each protein forms a complex of distinct electrophoretic mobility and, while the formation of the TC-IIB complex appears to be ubiquitous, the TC-IIA complex is not formed with nuclear extracts of untreated F9(ND) cells. Limited proteolytic digestion of the TC-IIA and TC-IIB complexes results in the appearance of a faster migrating complex A′ and a concomitant decrease in complex A, but complex B remain unchanged, indicating that protein A contains a pepsin sensitive site absent in protein B. Moreover, the formation of the TC-IIA and TC-IIB complexes shows a differential response to TPA and cycloheximide treatment. In 70Z/3 and HeLa cell nuclear extracts, the TC-IIA activity is induced by each of these treatments, where it is induced only by cycloheximide treatment in F9(ND) nuclear extracts. In contrast, the formation of the protein TC-IIB complex remains unaffected in all cases.

Competition gel retardation results show that, while the forming complex A binds with similar affinity to the TC-II, κB and H-2K^b sequences, the protein forming complex B binds with a higher affinity to the H-2K^b sequence. Furthermore, TC-II enhanson methylation interference experiments (our unpublished results) indicate that methylation of G235 partially interferes with formation of complex A, while complete interference is seen for complex B formation (see Figure 7B). These latter two observations are consistent with those described for the protein KBF1/H2TF1 identified in MEL, HeLa and 3T6 cell extracts (Israël *et al.*, 1987; Yano *et al.*, 1987; Baldwin and Sharp, 1987, 1988), which binds to the palindromic sequence 5′-TGGGGATTCCCCA-3′ (see KB-1 in Figures 2 and 7B) in the upstream regulatory region of the H-2K^b gene. This protein, which has been implicated in the control of expression of the H-2K^b gene (Kimura *et*

al., 1986; Baldwin and Sharp, 1987), was shown to bind to the TC-II/κB sequence at least five times less efficiently than to the H-2K^b sequence, and methylation interference experiments indicate that methylation of all of the G residues on each strand of the palindrome interfere with protein binding (Baldwin and Sharp, 1987; Israël *et al.*, 1987; see Figure 7B). Thus, the properties of the TC-IIB protein closely resemble those of the KBF1/H2TF1 protein.

Analysis of the binding of TC-IIA and TC-IIB to a series of mutated templates also reveals differences between these two proteins. Mutations at positions 244 and 245 have a more detrimental effect on the binding of TC-IIA, than on that of TC-IIB (TC-6 and 7 in Figure 7a). In addition, mutation at position 242 also results in a weaker binding of TC-IIA compared with TC-IIB. As expected, mutations of A243 or 242 to G, bringing the TC-II sequence closer to the H-2K^b sequence (Figure 7B) had no effect on the formation of either the TC-IIA or B complexes (our unpublished results).

Binding of the TC-IIA and B proteins to sequences related to the TC-II enhanson

Several TC-II-related sequence motifs (see Figure 7C) located in the HIV LTR (Nabel and Baltimore, 1987; Franza *et al.*, 1987), the IL-2 promoter (Hoyos *et al.*, 1989; and references therein), the IL-2 receptor α (IL-2Rα) promoter (Leung and Nabel, 1988; Cross *et al.*, 1989; and references therein), and the β-interferon promoter (β-IFN PRDII site) (Fujita *et al.*, 1989; Hiscott *et al.*, 1989; Lenardo *et al.*, 1989; Visvanathan and Goodbourn, 1989) have been shown to bind NF-κB-like proteins. Each of these sites, with the exception of the HIV LTR, diverges from the consensus sequence exemplified by the TC-II/κB enhanson (in each case the differences from the TC-II/κB enhanson have been underlined—see Figure 7C).

In competition gel retardation experiments, the IL-2 sequence competed more efficiently for the formation of complexes between protein B and either the TC-II or the H-2K^b motifs than for the formation of complexes between protein A and the same motifs (our unpublished data), suggesting that *in vivo* the KBF1/H2TF1 factor or a related protein, and not a NF-κB-like protein, may be involved in the activation of the IL-2 promoter. The higher affinity of the IL-2 sequence for the TC-IIB protein is consistent with our observations (Figure 7) that the G → T mutation at position 244 of the TC-II enhanson has a more detrimental effect on the binding of TC-IIA than on that of TC-IIB, and that the A → G mutation at position 243 has no deleterious effect on the binding of proteins TC-IIA and TC-IIB (our unpublished results). The β-IFN PRDII motif (Figure 7C) competes for formation of the TC-IIA and TC-IIB complexes as efficiently as the TC-II/κB enhanson (our unpublished results), suggesting that some base changes, (A → T) at position 241 and (G → T) at position 240, have no effect on the binding of the proteins TC-IIA and B to the TC-II/κB enhanson. In agreement with this assumption, we have found here that the G → T transversion at position 240 has little effect on protein binding to the TC-II/κB enhanson (TC-II in Figure 7A). However, if a A → T change is tolerated at position 241, it is clear that the A → C transversion in the TC-II/κB enhanson at the same position (TC-10 in Figure 7A) is deleterious to the binding of both proteins TC-IIA and B. Our observations (Figure 7A and unpublished data) that the base changes, A → G at position 242 and G → T at position 240 of TC-II, have no effect on the binding of TC-IIA are in agreement with the observation that the IL-2Rα sequence which contains these changes (see Figure 7C) is a high affinity NF-κB binding site (Cross *et al.*, 1989).

It appears therefore that there are a number of positions within the TC-II enhanson where nucleotide changes do not affect the binding of proteins TC-IIA and B, but that different nucleotide substitutions at each of these positions may differentially affect the binding of the NF-κB and KBF1/H2TF1-like proteins, e.g. in the case of the IL-2 motif (see above). Thus, conclusions concerning the possible role of TC-IIA/NF-κB-like or TC-IIB/KBF1/H2TF1-like factors in the activation of transcription from TC-II/κB or H-2K^b-like motifs cannot be readily deduced by sequence comparisons (see also the Discussion of Kanno *et al.*, 1989), as exemplified by the observation that the IL-2Rα and κB sequences are not functionally interchangeable (Cross *et al.*, 1989).

The AP-2 factor binds to a sequence overlapping with, but distinct from the TC-II enhanson

A third protein was detected which binds to the SV40 enhancer region containing the TC-motifs and can be efficiently competed by the human metallothionein IIA enhancer sequence containing the AP-2 binding site (Figures 1, 2 and 10). This binding activity probably corresponds to the AP-2 protein (Imagawa *et al.*, 1987; Mitchell *et al.*, 1987), since its binding site, as deduced from competition with templates TC-0, TC-1, TC-2 and TC-20 to TC-26 (Figures 2 and 10, and unpublished data), corresponds closely to the AP-2 consensus site 5′-CC^C_GC^A_GGGC-3′ (Mitchell *et al.*, 1987). However in contrast to a previous report (Imagawa *et al.*, 1987), the SV40 sequences appear to be at least five times less efficient as a competitor when compared with the metallothionein sequence (MET-1, in Figure 2). This discrepancy may be due to the method of comparison, competition using the gel retardation assay in our case, and titration using DNase I footprinting in the other report. Our competition results also indicate that AP-2 cannot bind efficiently to the TC-I motif, which is in contrast to the DNase I footprinting data of a previous report which suggested that the TC-I motif within the whole SV40 enhancer sequence is also an AP-2 binding site (Mitchell *et al.*, 1987). At present no clear role for the AP-2 protein in the activation by the SV40 enhancer *in vivo* can be assigned. Although this protein activates moderately SV40 transcription *in vitro* (Mitchell *et al.*, 1987), the bases mutated in TC-22, which are specific to, and deletrious for AP-2 binding *in vitro*, are not detrimental to the SV40 enhancer activity *in vivo* (Zenke *et al.*, 1986). Moreover, there is no evidence indicating that the SV40 AP-2 site can act as an enhanson *in vivo* (Kanno *et al.*, 1989), although a moderate stimulation of transcription was seen using a reporter gene containing the metallothionein AP-2 binding site in *Drosophila* cells where the AP-2 protein was overexpressed (Williams *et al.*, 1988).

Materials and methods

Preparation of nuclear extracts

Nuclear extracts were prepared at 4°C from HeLa, F9(ND), MPC11, LMTK$^-$ and 70Z/3 cells according to the method of Dignam *et al.* (1983)

with the modifications of Wildeman *et al.* (1984) and dialysed for 3 h against buffer C [20 mM HEPES pH 7.9, 2 mM dithiothreitol (DTT), 1 mM $MgCl_2$, 20 mM KCl, 25% glycerol (w/v) and 0.2 mM phenylmethylsulphonyl-fluoride (PMSF)]. Induced 70Z/3 cell extracts were obtained by treating the cells with 10 μg/ml LPS (Difco) for 4 h prior to extract preparation, as previously described (Paige *et al.*, 1978; Staudt *et al.*, 1986). 70Z/3 extracts were also prepared from cells treated with cycloheximide (Aldrich-Chimie) added at a concentration of 10 μg/ml 20 min before the addition of LPS (Sen and Baltimore, 1986b). For HeLa and F9(ND) cell extracts, the phorbol ester tumor promoter TPA was added at a concentration of 100 ng/ml for 4 h, and cycloheximide at 35 μg/ml for 4 h (Wan *et al.*, 1987). Protein concentration was measured by the method of Bradford (1976).

Construction of recombinant plasmids and preparation of DNA templates

Synthetic oligonucleotides, TC-1F and TC-2F (Figure 2), were cloned between the *Kpn*I and *Bam*HI sites of a modified pUC18 vector (Rosales *et al.*, 1987) by 'shotgun' ligation (Grundström *et al.*, 1985). Plasmids were prepared by standard methods (Zenke *et al.*, 1986) and purified over two CsCl gradients. The nucleotide sequence of the inserts was verified by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). The plasmids were cleaved at the *Bam*HI site, dephosphorylated with calf alkaline phosphatase, and labelled with [γ^{32}P]ATP (5000 Ci/mmol; Amersham) using T4 polynucleotide kinase. The end-labelled templates were excised with *Kpn*I, purified on a 5% polyacrylamide gel and recovered by eletroelution to yield the 123 bp template fragments TC-1F and TC-2F. All other templates (Figure 2) were obtained as follows. Two chemically synthesized complementary oligonucleotides (5 pmol) were ^{32}P-5′-end labelled separately with T4 polynucleotide kinase, mixed, incubated for 10 min at 65°C, and then left at room temperature for 1 h. The resulting double stranded DNA templates were purified on a 10% polyacrylamide gel and recovered by eletroelution.

To prepare competitor DNA, an equal quantity (200 pmol) of the two complementary oligonucleotides was added to a hybridization buffer containing 10 mM Tris−HCl pH 7.5 and 10 mM $MgCl_2$ and the mixture was incubated for 1 h at room temperature.

Gel retardation and methylation interference assays

Gel retardation assays were performed essentially as described by Strauss and Varshavsky (1984) with the modifications of Singh *et al.* (1986). 5 μg of protein was mixed with 2−4 μg of poly(dI-dC) (Pharmacia) in 15 μl of 15 mM NaCl, 1 mM $MgCl_2$, 0.02 mM EDTA, 3 mM Tris−HCl pH 7.5, 0.1 mM DTT, 0.4 mM sodium phosphate pH 7.0 and 6% glycerol. The mixture was preincubated at 0°C for 15 min followed by the addition of 2−5 fmol (1−1.5 × 10^4 c.p.m.) of either the ^{32}P-5′-end-labelled DNA fragments or synthetic oligonucleotides. Incubation was continued at 20°C for 15 min and the DNA−protein complexes were loaded onto a low ionic strength 6% polyacrylamide gel (30:1 cross-linking ratio) containing 6.7 mM Tris−HCl pH 7.5, 3.3 mM sodium acetate and 1 mM EDTA. Treatment of cytoplasmic extracts with deoxycholate (DOC) and NP40 after the addition of the ^{32}P-5′-end-labelled probe was performed as described by Baeuerle and Baltimore (1988a). Electrophoresis was performed at 150 V until a suitable separation had been achieved. The gel was then dried and autoradiographed.

The methylation interference experiments were performed essentially as previously described (Hendrickson and Schleif, 1985). 1 × 10^6 c.p.m. of ^{32}P-5′-end-labelled DNA was methylated at 20°C for 4 min in 50 μl of 50 mM sodium cacodylate pH 8.0, 1 mM EDTA by the addition of 2 μl of DMS. The methylated DNA was ethanol-precipitated, washed with 70% ethanol and used in the retardation assay as described above. After electrophoresis the wet gel was autoradiographed at 4°C overnight. The complexed and unbound DNA were excised from the gel and electroeluted. After two precipitations with ethanol, the DNA fragments were cleaved at the methylated G residues as described by Maxam and Gilbert (1980), and electrophoresed on an 8% polyacrylamide−7.5 M urea gel. The gel was then fixed, dried and autoradiographed.

Gel retardation competition assays

Binding reactions and gel electrophoresis were carried out as described above, the exception being the addition of cold competitor DNA (as indicated in legends to figure) at the same time as the labelled DNA following the preincubation period.

Proteolysis experiments

A pepsin stock of 1 mg/ml (Sigma) was diluted in 10 mM Tris−HCl pH 7.9 and 50% glycerol to 100, 10 nd 1 ng/μl; pepsin was added to the binding reaction as indicated in the legend to Figure 4, and after incubation at 25°C for 10 min, inactivated with 250 ng of the protease inhibitor pepstatin (Sigma) prior to loading the 'retardation' gel as described above.

Acknowledgements

We extend our gratitude to A.Staub and F.Ruffenach for the synthesis of the oligonucleotides. We thank also C.Werlé and B.Boulay for the illustrations, the secretarial staff for typing the manuscript and M.Gilbert for technical assistance with the cell culture. I.D. was supported by fellowships from the Royal Society of Great Britain and the Fondation pour la Recherche Médicale, M.M. by the American Cancer Society (PF2640), M.K. by the French Ministère des Affaires Etrangères and R.R. and J.-H.X. by the Université Louis Pasteur. This work was supported by grants from the CNRS, the INSERM, the Fondation pour la Recherche Médicale and the Association pour le Développement de la Recherche sur le Cancer.

References

Angel,P., Imagawa,M., Chiu,R., Stein,B., Imbra,R.J., Rahmsdorf,H.J., Jonat,C., Herrlich,P. and Karin,M. (1987) *Cell*, **49**, 729−739.
Atchison,M.L. (1988) *Annu. Rev. Cell Biol.*, **4**, 127−153.
Atchison,M.L. and Perry,R.P. (1987) *Cell*, **48**, 121−128.
Baeuerle,P.A. and Baltimore,D. (1988a) *Cell*, **53**, 211−217.
Baeuerle,P.A. and Baltimore,D. (1988b) *Science*, **242**, 540−546.
Baldwin,A.S. and Sharp,P.A. (1987) *Mol. Cell. Biol.*, **7**, 305−313.
Baldwin,A.S. and Sharp,P.A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 723−727.
Ballard,D.W., Böhnlein,E., Lowenthal,J.W., Wano,Y., Franza,B.R. and Greene,W.C. (1988) *Science*, **241**, 1652−1655.
Banerji,J., Rusconi,S. and Schaffner,W. (1981) *Cell*, **27**, 299−308.
Böhnlein,E., Lowenthal,J.W., Siekevitz,M., Ballard,D.W., Franza,B.R. and Greene,W.C. (1988) *Cell*, **53**, 827−836.
Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248−254.
Chambon,P., Dierich,A., Gaub,M.P., Jakovlev,S.B., Jongstra,J., Krust,A., Lepennec,J.P., Oudet,P. and Reudelhuber,T. (1984) *Rec. Prog. Horm. Res.*, **40**, 1−42.
Chiu,R., Imagawa,M., Imbra,R.J., Bockoven,J.R. and Karin,M. (1987) *Nature*, **329**, 648−651.
Clark,L. and Hay,R.T. (1989) *Nucleic Acids Res.*, **17**, 499−516.
Clark,L., Pollock,R.M. and Hay,R.T. (1988) *Genes Dev.*, **2**, 991−1002.
Clark,L., Nicholson,J. and Hay,R.T. (1989) *J. Mol. Biol.*, **206**, 615−626.
Cross,S.L., Halden,N.F., Lenardo,M.J. and Leonard,W.J. (1989) *Science*, **244**, 466−469.
Davidson,I., Fromental,C., Augereau,P., Wildeman,A., Zenke,M. and Chambon,P. (1986) *Nature*, **323**, 544−548.
Davidson,I., Xiao,J.H., Rosales,R., Staub,A. and Chambon,P. (1988) *Cell*, **54**, 931−942.
Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475−1489.
Fletcher,C., Heintz,N. and Roeder,R.G. (1987) *Cell*, **51**, 773−781.
Franza,B.R.,Jr, Josephs,S.F., Gilman,M.Z., Ryan,W. and Clarkson,B. (1987) *Nature*, **330**, 391−395.
Fried,M. and Crothers,D.M. (1981) *Nucleic Acids Res.*, **9**, 6505−6525.
Fromental,C., Kanno,M., Nomiyama,H. and Chambon,P. (1988) *Cell*, **54**, 943−953.
Fujita,T., Miyamoto,M., Kimura,Y., Hammer,J. and Taniguchi,T. (1989) *Nucleic Acids Res.*, **17**, 3335−3345.
Garner,M.M. and Revzin,A. (1981) *Nucleic Acids Res.*, **9**, 3047−3060.
Grundström,T., Zenke,M., Wintzerith,M., Matthes,H.W.D., Staub,A. and Chambon,P. (1985) *Nucleic Acids Res.*, **13**, 3305−3316.
Hatzopoulos,A.K., Schlokat,U. and Gruss,P. (1988) In Hames,B.D. and Glover,D.M. (eds), *Transcription and Splicing Frontiers in Molecular Biology Series*. IRL Press, Oxford, pp. 43−96.
Hendrickson,W. and Schleif,R. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 3129−3133.
Herr,W. and Clarke,J. (1986) *Cell*, **45**, 461−470.
Herr,W. and Gluzman,Y. (1985) *Nature*, **313**, 711−714.
Hiscott,J., Alper,D., Cohen,L., Leblanc,J.F., Sportza,L., Wong,A. and Xanthoudakis,S. (1989) *J. Virol.*, **63**, 2557−2566.
Hoyos,B., Ballard,D.W., Böhnlein,E., Siekevitz,M. and Greene,W.C. (1989) *Science*, **244**, 457−460.
Imagawa,M., Chiu,R. and Karin,M. (1987) *Cell*, **51**, 251−260.
Israël,A., Kimura,A., Kieran,M., Yano,O., Kanellopoulos,J., Le Bail,O. and Kourilsky,P. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 2653−2657.

Jones,N.C., Rigby,P.W.T. and Ziff,E.B. (1988) *Genes Dev.*, **2**, 267–281.
Kanno,M., Fromental,C., Staub,A., Ruffenach,F., Davidson,I. and Chambon,P. (1989) *EMBO J.*, **8**, 4205–4214.
Kaufman,J.D., Valandra,G., Rodriguez,G., Bushar,G., Giri,C. and Norcross,M.A. (1987) *Mol. Cell. Biol.*, **7**, 3759–3766.
Kawakami,K., Scheidereit,C. and Roeder,R.G. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4700–4704.
Kimura,A., Israël,A., Le Bail,O. and Kourilsky,P. (1986) *Cell*, **44**, 261–272.
Laimins,L.A., Khoury,G., Gorman,C., Howard,B. and Gruss,P. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 6453–6457.
Landolfi,N.F., Capra,J.D. and Tucker,P.W. (1986) *Nature*, **323**, 548–551.
Lee,W., Mitchell,P. and Tjian,R. (1987) *Cell*, **49**, 741–752.
Lenardo,M., Pierce,J.W. and Baltimore,D. (1987) *Science*, **236**, 1573–1577.
Lenardo,M., Kuang,A., Gifford,A. and Baltimore,D. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 8825–8829.
Lenardo,M., Fan,C.M., Maniatis,T. and Baltimore,D. (1989) *Cell*, **57**, 287–294.
Leung,K. and Nabel,G.J. (1988) *Nature*, **333**, 776–778.
Maniatis,T., Goodbourn,S. and Fischer,J.A. (1987) *Science*, **236**, 1237–1245.
Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499–526.
Mercurio,F. and Karin,M. (1989) *EMBO J.*, **8**, 1455–1460.
Mermod,N., Williams,T.J. and Tjian,R. (1988) *Nature*, **332**, 557–561.
Mitchell,P.J., Wang,C. and Tjian,R. (1987) *Cell*, **50**, 847–861.
Moreau,P., Hen,R., Wasylyk,B., Everett,R., Gaub,M.P. and Chambon,P. (1981) *Nucleic Acids Res.*, **9**, 6047–6068.
Müller,M.M., Gerster,T. and Schaffner,W. (1988) *Eur. J. Biochem.*, **176**, 485–495.
Nabel,G. and Baltimore,D. (1987) *Nature*, **326**, 711–713.
Nelsen,B., Hellman,L. and Sen,R. (1988) *Mol. Cell. Biol.*, **8**, 3526–3531.
Nishizuka,Y. (1988) *Nature*, **334**, 661–665.
Nomiyama,H., Fromental,C. and Chambon,P. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 7881–7885.
Ondek,B., Shephard,A. and Herr,W. (1987) *EMBO J.*, **6**, 1017–1025.
Ondek,B., Gloss,L. and Herr,W. (1988) *Nature*, **333**, 40–45.
Osborn,L., Kunkel,S. and Nabel,G.J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 2336–2340.
Paige,C.J., Kincade,P.W. and Ralph,P. (1978) *J. Immunol.*, **121**, 641–647.
Picard,D. and Schaffner,W. (1984) *Nature*, **307**, 80–82.
Pierce,J.W., Lenardo,M. and Baltimore,D. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 1482–1486.
Rosales,R., Vigneron,M., Macchi,M., Davidson,I., Xiao,J.H. and Chambon,P. (1987) *EMBO J.*, **6**, 3015–3025.
Sakaguchi,N., Kishimoto,T., Kikutami,H., Watanabe,T., Yoshida,N., Shimizu,A., Yamawaki-Kataoda,Y., Honjo,T. and Yamamura,Y. (1980) *J. Immunol.*, **125**, 2654–2659.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Sassone-Corsi,P. and Borrelli,E. (1986) *Trends Genet.*, **2**, 215–219.
Scheidereit,C., Heguy,A. and Roeder,R.G. (1987) *Cell*, **51**, 783–793.
Schirm,S., Jiricny,J. and Schaffner,W. (1987) *Genes Dev.*, **1**, 65–74.
Sen,R. and Baltimore,D. (1986a) *Cell*, **46**, 705–716.
Sen,R. and Baltimore,D. (1986b) *Cell*, **47**, 921–928.
Serfling,E., Jasin,M. and Schaffner,W. (1985) *Trends Genet.*, **1**, 224–230.
Shirakawa,F. and Mizel,S.B. (1989) *Mol. Cell. Biol.*, **9**, 2424–2430.
Shirakawa,F., Chedid,M., Suttles,J., Pollok,A. and Mizel,S.B. (1989) *Mol. Cell. Biol.*, **9**, 959–964.
Singh,H., Sen,R., Baltimore,D. and Sharp,P.A. (1986) *Nature*, **319**, 154–158.
Singh,H., LeBowitz,J.H., Baldwin,A.S. and Sharp,P.A. (1988) *Cell*, **52**, 415–423.
Staudt,L.M., Singh,H., Sen,R., Wirth,T., Sharp,P.A. and Baltimore,D. (1986) *Nature*, **323**, 640–643.
Strauss,F. and Varshavsky,A. (1984) *Cell*, **37**, 889–901.
Sturm,R., Baumruker,R., Franza,B.R.,Jr and Herr,W. (1987) *Genes Dev.*, **1**, 1147–1160.
Tooze,J. (1982) *Molecular Biology of Tumor Viruses*, 2nd edn, Part 2, revised. *DNA Tumor Viruses*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Visvanathan,K.V. and Goodbourn,S. (1989) *EMBO J.*, **8**, 1129–1138.
Wan,Y.-J.Y., Orrison,B.M., Lieberman,R., Lazarovici,P. and Ozato,K. (1987) *J. Cell. Physiol.*, **130**, 276–283.
Wasylyk,B. (1988) *CRC Crit. Rev. Biochem.*, **23**, 77–120.
Weiher,H., König,M. and Gruss,P. (1983) *Science*, **219**, 626–631.
Wildeman,A., Sassone-Corsi,P., Grundström,T., Zenke,M. and Chambon,P. (1984) *EMBO J.*, **3**, 3129–3133.
Wildeman,A.G., Zenke,M., Schatz,C., Wintzerith,M., Grundström,T., Matthes,H., Takahashi,K. and Chambon,P. (1986) *Mol. Cell. Biol.*, **6**, 2098–2105.
Williams,T., Admon,A., Lüscher,B. and Tjian,R. (1988) *Genes Dev.*, **2**, 1557–1569.
Wu,F.K., Garcia,J.A., Harrich,D. and Gaynor,R.B. (1988) *EMBO J.*, **7**, 2117–2129.
Xiao,J.H., Davidson,I., Rosales,R., Ferrandon,D., Vigneron,M., Macchi,M., Ruffenach,F. and Chambon,P. (1987a) *EMBO J.*, **6**, 3005–3013.
Xiao,J.H., Davidson,I., Macchi,M., Rosales,R., Vigneron,M., Staub,A. and Chambon,P. (1987b) *Genes Dev.*, **1**, 794–807.
Yano,O., Kanellopoulos,J., Kieran,M., Le Bail,O., Israël,A. and Kourilsky,P. (1987) *EMBO J.*, **6**, 3317–3324.
Zenke,M., Grundström,T., Matthes,H., Wintzerith,M., Schatz,C., Wildeman,A. and Chambon,P. (1986) *EMBO J.*, **5**, 387–397.

Received on August 1, 1989; revised on September 19, 1989