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TNF stimulates expression of mouse MHC class I genes by inducing an NFκB-like enhancer binding activity which displaces constitutive factors

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We have dissected the mouse H-2K^b gene promoter in order to define the sequences responsible for induction by tumour necrosis factor (TNF-α). An enhancer element (−187 to −158) composed of two imperfect direct palindromic repeats has been shown to be necessary and sufficient for TNF-α induction of a heterologous promoter. A multimer of either repeat is also responsive, while a single copy is not: this is the situation in the β2-microglobulin (β2-m) promoter which contains a single palindrome and does not respond to TNF-α. We had previously found that the two repeats can bind a factor named KBF1. We show here that in the uninduced state the transcription factor AP2 binds to the inter-palindromic region, while in TNF-treated cells an NFκB-like activity is induced which displaces both KBF1 and AP2 and binds to the two palindromes. This strongly suggests that induction of an NFκB-like activity is responsible for TNF-α stimulation of mouse MHC class I genes.

Key words: MHC regulation/NFκB/TNF

Introduction

Class I and class II antigens of the major histocompatibility complex (MHC) play a key role in the recognition of foreign antigens by the receptor carried by cytotoxic and helper T cells (Unanue and Allen, 1987; Kourilsky and Claverie, 1989). Several substances modulate class I and class II gene expression and may, therefore, influence their function. These compounds include interferons (IFN) (reviewed in Guillemot *et al.*, 1988) and tumour necrosis factor (TNF) (Collins *et al.*, 1986; Pfizenmaier *et al.*, 1987) whose antitumoral and antiviral effects could, to a certain extent, be related to their action on MHC genes. It has been shown recently that TNF-induced stimulation of class I gene expression in human endothelial cells and fibroblasts takes place at least in part at the transcriptional level, and is blocked by protein synthesis inhibitors (Collins *et al.*, 1986). In this report, we document the mode of action of TNF-α on MHC class I genes.

TNF-α is a pleiotropic factor which participates in a regulatory network (Old, 1987) and has been implicated in a variety of actions important in host defence and inflammation. It stimulates a number of genes including those coding for c-*fos*, c-*myc* and interleukin-6 (IL-6) (Content *et al.*, 1985; Kohase *et al.*, 1986). Recent data (Zhang *et al.*, 1988) demonstrate that IL-6 induction by TNF-α involves an increase in intracellular cAMP and corresponding protein kinase activity, while another report (Sehgal *et al.*, 1987) suggests an involvement of the protein kinase C pathway.

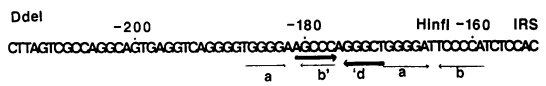
The promoter of mouse MHC class I genes has already been dissected in detail and various elements responsible for basal or induced activity have been characterized, together with the proteins that bind to these regions (Baldwin and Sharp, 1987; Israël *et al.*, 1987; Sugita *et al.*, 1987; Korber *et al.*, 1988; Shirayoshi *et al.*, 1988). An enhancer sequence located ~160 bp upstream of the transcription start point appears to be critical for the expression of the K^b gene. A short sequence called the interferon response sequence (IRS), which overlaps this enhancer sequence, has been shown to mediate response to the three types of IFNs (Friedman and Stark, 1985; Baldwin and Sharp, 1987; Israël *et al.*, 1986; Sugita *et al.*, 1987; Korber *et al.*, 1988). Short-term transfections with constructs containing various sub-fragments from the promoter have allowed us to localize the TNF-α responsive element in the same enhancer sequence but distinct from IRS. This region includes two imperfect palindromic repeats which appear to be necessary for TNF induction. In TNF-α treated cells, we observed a new enhancer-binding activity which we found similar if not identical to NFκB. NFκB is normally active in B lymphocytes, but an inactive form of it is present in all other cell types, where it can be induced by TPA (Sen and Baltimore, 1986b). Our results suggest that an NFκB-like activity displaces protein factors that bind the TNF-α responsive element in the non-induced state. The relationship between TNF-α activation and the action of cAMP and TPA are discussed in relation to the characterization of three cAMP-responsive elements (CREs) in the K^b promoter.

Results

Localization of the TNF-responsive element

Transcription of the mouse class I H-2K^b gene, as that of human class I genes (Collins *et al.*, 1986; Pfizenmaier *et al.*, 1987) is stimulated by TNF-α (data not shown). Promoter deletion mutants connected to a reporter gene (the bacterial chloramphenicol acetyltransferase or CAT gene), were assayed by short-term transfection in HeLa or mouse 3T3 cells. These experiments (not shown) indicated that the responsive element was localized in the −399 to −160 region of the K^b promoter. Stimulation by TNF-α was found to be higher in HeLa cells which were subsequently routinely used, but all the critical constructs were also tested in mouse 3T3 cells, with qualitatively similar results.

A



B

CONSTRUCT	TPA	cAMP	TNF
DdeI-HincII	3.2	3.5	3.3
DdeI-HinfI	4.0	3.7	7.8
IRS	1.1	1.2	0.9
(-210.-181)	1.3	3.6	1.2
(ab''dab)	4.1	1.9	3.7
$(ab''dab)_3$	3.6	1.8	19
(ab')	0.8	0.9	1.1
(ab)	1.0	1.3	1.2
$(ab)_4$	6.5	1.3	43
beta-2m Dde	1.3	1.2	1.3
$(beta\text{-}2m)_4$	5.6	0.9	28.2
AP1	4.3	1.4	0.9
AP2	3.5	3.3	1.1
(ab''d)	1.5	2.1	1.2
$(ab''d)_3$	3.9	2	8.5
(-108.-84)	1.2	4.3	0.9

Fig. 1. (**A**) Sequence of the −215 to −152 region of the H-2K^b promoter with some relevant regions indicated. The exact sequence of the oligonucleotides quoted under the region is given in Materials and methods. (**B**) Response to TPA, cAMP inducers and TNF-α of various sub-fragments from the H-2K^b promoter. The sequence of the cloned oligonucleotides is shown in Table II. *Dde*I−*Hin*cII corresponds to the −213 to −99 sequence, *Dde*I−*Hin*fI to −213 to −160. AP1 and AP2 are derived from the human metallothionein IIA gene. HeLa cells were transfected with 5 μg of the indicated construct, and stimulation was for 16 h with either TPA (100 ng/ml), 25 μM forskolin + 0.5 mM IBMX for cAMP induction or TNF-α (500 U/ml). Measurement of CAT activity is as described in Materials and methods. The values indicated represent the average stimulation observed in at least four experiments, as compared to untreated transfected cells. The β2-m-Dde construct is described in Kimura *et al.* (1986).

Sub-fragments of the −399 to −160 region were then cloned just upstream of the chicken conalbumin promoter, itself connected to the CAT gene (Kimura *et al.*, 1986). The −213 to −99 construct (*Dde*I−*Hin*cII) was stimulated by TNF-α (Figure 1B). The −213 to −160 construct (*Dde*I−*Hin*fI), which lacks the IRS (required for induction by the three types of IFNs) was even better stimulated while the IRS region alone (−163 to −137) displayed no stimulation. Thus, the TNF-α response element is separate from the IRS, and located in the −213 to −160 region of the K^b promoter.

Functional analysis of the −213 to −160 region

The −213 to −160 region includes a series of overlapping palindromes which constitute conspicuous sites for DNA binding factors (see below and Figure 1A). A perfect palindrome, denoted (ab), is the core region of the previously described enhancer element (enhA; Kimura *et al.*, 1986; Baldwin and Sharp, 1987; Shirayoshi *et al.*, 1987). A few nucleotides upstream lies an imperfect palindrome, denoted (ab') which is an imperfect copy of (ab) with regard to the b sequence. Factor KBF1 binds both palindromes (ab) and (ab') (Israël *et al.*, 1987; Yano *et al.*, 1987) (see Figure 2A) although (ab) with a higher affinity. Finally, these two palindromes are spaced by a short sequence denoted 'd, because it is symmetrical to b' and therefore somewhat analogous to a. Accordingly, a third, perfect palindrome, shown as (b' 'd) overlaps (ab'). The −188 to −160 region can thus be described as (ab' 'dab) (Figure 1A) including two KBF1 binding sites.

We asked which elements were important for TNF-α induction and whether induction proceeds through one of the classical second messenger pathways. Therefore, we treated the transfected HeLa cells in parallel with TNF-α, TPA and cAMP.

The −213 to −160 construct (*Dde*I−*Hin*fI) was stimulated by all three inducing agents (Figure 1B). The −188 to −160 region (which includes the three palindromes) was stimulated by TNF-α and TPA, but more weakly by cAMP. When polymerized, it displayed increased TNF-α induction but not cAMP induction. The (ab) palindrome was then tested separately. It displayed no stimulation by either TNF-α, cAMP or TPA. Neither did the (ab') palindrome. However, a multimer of (ab') or (ab) was strongly stimulated by TNF-α and TPA, not by cAMP. On the other hand a construct (ab' 'd) was stimulated by cAMP only, while polymerization restored TNF-α inducibility. It thus appeared that two adjacent palindromes were required for TNF-α induction. The promoter of the β2-microglobulin (β2-m) gene contains only one copy of a palindrome similar to ab' and able to bind KBF1 (Israël *et al.*, 1987). As expected, we found that this promoter is not stimulated by TNF (Figure 1B).

In summary, the TNF-α-responsive element must contain a pair of palindromes, and the response to TNF-α correlates with induction by TPA, not by cAMP. The relative degrees of stimulation by TNF-α and TPA are not, however, identical with all constructs. For example, constructs containing four or more sites [like $(ab)_4$ or $(\beta2\text{-}m)_4$] show a much stronger stimulation by TNF than by TPA. This may reflect a difference in the mode of action of these two inducers. The influence of the spacing and number of sites has not been systematically studied (see also Discussion).

A factor analogous to AP2 binds to the −188 to −160 region in the absence of TNF

To characterize the protein factors that bind to the TNF-α responsive element, we first performed retardation experiments using the (ab) or (ab') palindromic sequences as probes (Figure 2A). As expected, nuclear extracts from uninduced HeLa cells displayed a pattern (lanes 1 and 6) similar to that observed with KBF1 (lane 4). From previous work (Yano *et al.*, 1987) the latter is known to bind both (ab) and (ab'), with a higher affinity for (ab) [which explains the faint band observed with (ab') as compared to (ab)]. However, when we used the (ab' 'd) oligonucleotide, we found a band migrating more slowly than with KBF1 (Figure 2B, lane 1), suggesting that inclusion of the 'd sequence allowed binding of an additional factor. To confirm this, we performed a methylation protection assay of the entire (ab' 'dab) region in the presence of an excess of cold (ab) oligonucleotide in order to eliminate protections due to KBF1 binding (Figure 3). It then appeared clearly that extracts of

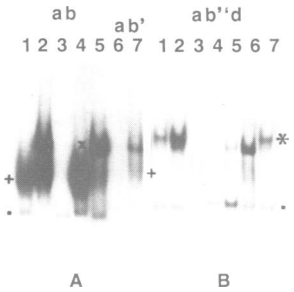


Fig. 2. Bandshift assay of DNA binding proteins from TNF-stimulated or control HeLa cells nuclear extracts that specifically interact with various sub-fragments of the TNF-responsive region of the H-2K^b promoter. Labelled oligonucleotide (0.25 ng) was incubated in 10 μl with 3–5 μg of nuclear extract and 0.2 μg of poly(dI·dC) in the buffer described in Materials and methods. (**A**) Experiments in lanes 1–5 have been done with the (ab) probe and in lanes 6 and 7 with the (ab′) probe. **Lanes 1** and **6**, extracts from uninduced HeLa cells; **lanes 2**, **3** and **7**, extracts from HeLa cells treated for 3 h with 500 U/ml of TNF-α; lane 3 includes a 40-fold excess of cold (ab) oligonucleotide (which exhibits equivalent affinity for both KBF1 end NF$\varkappa$B: Baldwin and Sharp, 1988). **Lane 4**, purified KBF1; **lane 5**, purified NF$\varkappa$B. (**B**) All lanes with the (ab′ ′d) probe except lane 2 where the hMTIIa (AP2) probe has been used. **Lanes 1–4**, extracts from uninduced HeLa cells; **lanes 5–7**, from TNF-treated HeLa cells; **lane 3** includes a 40-fold excess of cold (AP2) oligonucleotide, **lane 4** a 40-fold excess of both (AP2) and (ab) oligonucleotides and **lane 7** a 40-fold excess of (ab) oligonucleotide. **Lane 6** includes 3 mM GTP in the binding reaction. +, KBF1; ×, NF$\varkappa$B-like; *, AP2-like; ■, non-specific band.

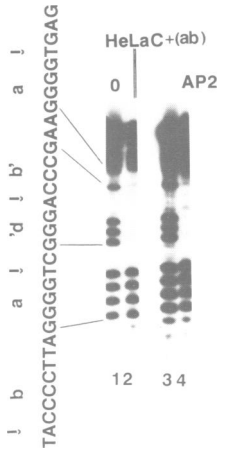


Fig. 3. DMS protection experiments using the labelled (ab′ ′d ab) oligonucleotide. (ab′ ′dab) was labelled at the 3′ end of the coding strand with the Klenow enzyme, incubated with nuclear extract in a scaled-up binding reaction, briefly treated with DMS before phenol extraction as described in Materials and methods. After ethanol precipitation, the samples were analysed on a 20% acrylamide–urea sequencing gel. **Lanes 1** and **3** show the result when no extract was added to the reaction. **Lane 2** shows the result obtained with the extract from control HeLa cells and a 40-fold excess of cold (ab) oligonucleotide. **Lane 4** shows the pattern obtained with purified AP2.

non-induced HeLa cells contain an activity (which had escaped our earlier investigations) which binds to the b′ ′d element (lanes 1 and 2).

Factor AP2 was a logical candidate, since it has recently been found that purified AP2 binds in the enhancer region of K^b (Imagawa *et al.*, 1987; Mitchell *et al.*, 1987). We performed a DMS protection experiment using purified AP2 (kindly provided by Drs W.Klump and M.Karin) and obtained a pattern (Figure 3, lane 4) identical to that seen in lane 2. We also used a typical AP2 site (derived from the hMTIIa gene) in retardation experiments. Incubation of this AP2 oligonucleotide with extracts from uninduced HeLa cells yields a band displaying the same mobility as the factor binding to ab′ ′d (Figure 2B, lane 2). Competition with an excess of the AP2 oligonucleotide displaces the AP2-like activity from the (ab′ ′d) oligonucleotides (lane 3) and allowed binding of KBF1 similarly to what can be seen with the (ab′) oligonucleotide (Figure 2A, lane 6).

From these data, we conclude that the protein factor, distinct from KBF1, which binds to the (ab′ ′dab) region, is analogous, if not identical, to AP2, and can bind to (b′ ′d) while probably excluding KBF1 from (ab′)—but not from (ab). We suspect that this AP2-like factor also binds this region *in vivo*. This can be inferred from the cAMP responsiveness of the −188 to −160 region, because AP2 sites have been shown to mediate response to cAMP (Imagawa *et al.*, 1987 and Figure 1B) but KBF1 sites do not respond to cAMP (Figure 1B).

In summary, in extracts from non-induced HeLa cells, the (ab′ ′dab) region binds one AP2-like and one KBF1 molecule (or a dimer of each, as suggested by the palindromic binding sites), with contact points shown in Figure 6 (A).

A protein factor analogous to NF$\varkappa$B binds the TNF-α responsive element upon TNF-α induction

We next observed, in retardation assays, that extracts of cells treated with TNF-α for 3 h displayed a new binding activity in the (ab) region (Figure 2A, lanes 1 and 2). A protein with a similar mobility binds to the (ab′) and (ab′ ′d) regions (panel A, lane 7 and panel B, lane 5).

It has been recently shown that the B cell specific transcription factor NF$\varkappa$B also binds to the (ab) palindrome (Baldwin and Sharp, 1988) and can be induced in HeLa cells after treatment with TPA (Sen and Baltimore, 1986b). Since we found that responsiveness to TNF-α correlated with responsiveness to TPA (Figure 1B), we examined whether NF$\varkappa$B was involved. Retardation experiments using (ab) or (ab′) labelled oligonucleotides show that the newly induced band observed in extracts from TFN-treated cells migrates similarly to the band obtained using purified NF$\varkappa$B (kindly provided by Drs K.Kawakami and R.G.Roeder; Kawakami *et al.*, 1988; Figure 2A, lane 5). This was confirmed by DMS-interference experiments which show that the protein binding to (ab) before induction (Figure 4, lane 2) and the induced one (lane 4) displays a profile identical to the one obtained with purified KBF1 and NF$\varkappa$B respectively (lanes 3 and 5).

The partial protection of the most external G of the palindrome observed in Figure 4 (lanes 4 and 5) is consistent with previous data obtained with NF$\varkappa$B (Baldwin and Sharp, 1988) while incomplete protection of the internal G has not been reported previously. Inclusion of 3 mM GTP in the binding reaction increased the intensity of the TNF-induced activity (Figure 2B, cf. lanes 5 and 6), consistent with the reported behaviour of *bona fide* NF$\varkappa$B (Lenardo *et al.*, 1988). Competition with an excess of cold (ab) oligonucleotide which binds KBF1 and NF$\varkappa$B with similar affinities (Baldwin and Sharp, 1988) prevents binding of the TNF-induced activity and allows detection of the AP2-like activity (Figure 2B, lane 7).

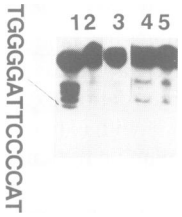


Fig. 4. DMS interference experiments using labelled and methylated (ab) oligonucleotide and either crude extracts or purified KBF1 or NFκB. (ab) oligonucleotide was labelled at the 3′ end of the coding strand, partially methylated with DMS and assayed in a scaled-up bandshift assay. The retarded bands were electroeluted and treated with piperidine as described in Materials and methods, before loading on a 20% acrylamide–urea sequencing gel. **Lane 1** represents the non-retarded band; **lane 2**, the band seen in Figure 2 with the extract from control HeLa cells; **lane 3**, the band obtained with purified KBF1; **lane 4**, the induced band seen with extract from TNF-treated HeLa cells; **lane 5**, the band obtained with purified NFκB.

Table I. Response to TNF by various sub-fragments of the H-2K^b promoter

Fragment	− TNF	+ TNF
conaCAT	1	1
(ab′′dab)	10	170
| Competition with ab	5.2	72
| Competition with NFKB	9.2	84
(m′d)1	3.1	150
(mab)1	4.0	6.5
(NFκB)2	1.5	110

The indicated oligonucleotides were cloned in the conaCAT vector. Transfection and measurement of CAT activities in control (−) or TNF-treated (+) HeLa cells were carried out as described in the legend to Figure 1B. Competition lanes include a 40-fold molar excess of ligated oligonucleotides in the calcium phosphate co-precipitate.

The NFκB-like factor replaces the AP2-like factor and KBF1 upon in vivo stimulation by TNF-α

The above results suggested a model in which, in TNF-treated cells, the (ab′ ′dab) region binds two molecules (probably dimers; Baueuerle and Baltimore, 1988a,b) of an NFκB-like factor, which apparently displaces the KBF1 and AP2-like molecules which are bound in the uninduced state (see Figure 6).

Does this actually happen *in vivo*? The results of *in vivo* footprinting experiments were obscured by the high background due to the numerous other MHC class I genes which display high homology with K^b (data not shown). We therefore used another more functional approach. We mutated individual binding sites in the (ab′ ′dab) composite element, cloned the corresponding oligonucleotides into the conaCAT vector and transfected the resulting constructs into HeLa cells, with or without TNF treatment. Results are shown in Table I. When the ′d element is mutated [construct (m′d)1] in a way that prevents binding of the AP2-like factor (not shown), the basal level of expression of the reporter gene is decreased, but the induced level is similar to that obtained with the non-mutated oligonucleotide. A mutation in the (ab) element which prevents binding of KBF1 (see Israël *et al.*, 1987) and of NFκB (not shown) also reduces the basal level but eliminates TNF induction [construct (mab)1]. These results indicate that both AP2-like and KBF1 bindings are important for the basal expression and confirm the non-involvement of the AP2-like factor in TNF

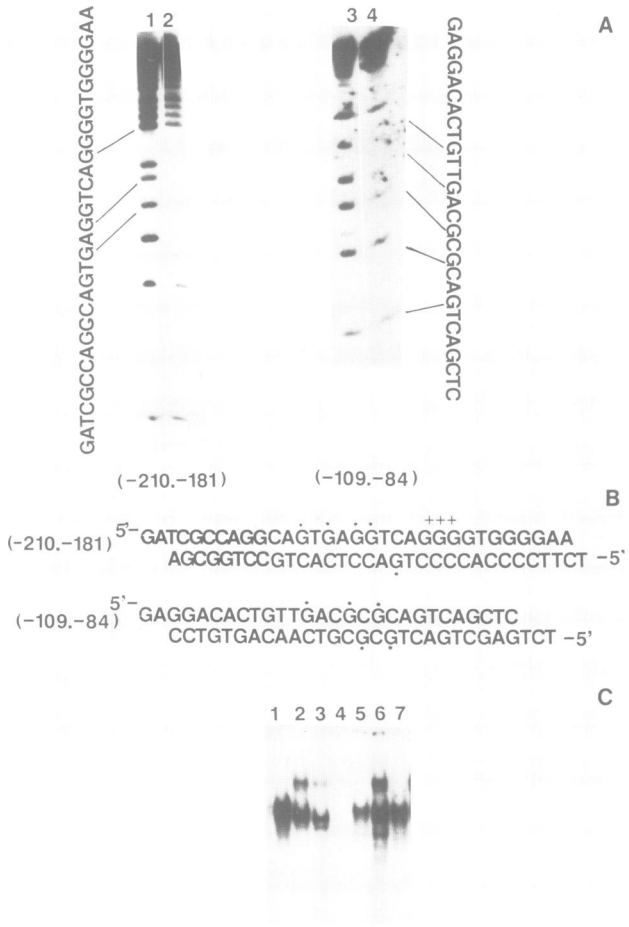


Fig. 5. (**A**) DMS protection experiments using the −210 to −181 and −109 to −84 oligonucleotides and extracts from control HeLa cells. The procedure is similar to that described in the legend to Figure 3. −210 to −181 was labelled with γ-ATP on the 5′ end of the coding strand, while −109 to −84 was labelled with the Klenow enzyme on the 3′ end of the coding strand. **Lanes 1** and **3** correspond to reactions with no extract added while **lanes 2** and **4** correspond to extracts of control HeLa cells. (**B**) The results obtained on both strands. A dot corresponds to a complete protected G while a + indicates incomplete protection. (**C**) Bandshift analysis of various CREs incubated with crude extract or purified AP1. The assay was performed as described in Materials and methods. **Lanes 1–4** correspond to CA oligonucleotide, **lane 5** to the human metallothionein IIA derived AP1 site, and **lanes 6** and **7** to the −210 to −181 oligonucleotide. **Lanes 1** and **7** show the result obtained with purified AP1 while **lanes 2–6** correspond to extract from control HeLa cells. In **lane 3** a 40-fold excess of AP1 oligonucleotide was added, and in **lane 4** a similar excess of the −210 to −181 region was used.

stimulation. This is further established by the lack of responsiveness of the hMTI1a AP2 site to TNF-α (Figure 1B, lane AP2) and by the positive response to TNF-α of the (ab′ ′dab) construct in the HepG2 hepatoma cell line (data not shown) where the AP2 activity has been shown to be absent (Chiu *et al.*, 1987).

In order to try to sort out the respective roles of KBF1 and NFκB in TNF induction, we took advantage of the fact that the NFκB site in the immunoglobulin Igκ enhancer binds KBF1 with a low affinity (Baldwin and Sharp, 1988; our unpublished results). We cloned two copies of the corresponding oligonucleotide into the conaCAT vector and assayed this construct in HeLa cells. We found (Table I) a low basal level consistent with the low affinity of KBF1 for the NFκB site, but a very high TNF-induced activity,

consistent with the hypothesis that NFκB induction is responsible for the observed stimulation. We also performed *in vivo* competition experiments with a 40-fold molar excess of ligated (ab) or (NFκB) oligonucleotides, as earlier (Israël *et al.*, 1987). Competition by the (ab) oligonucleotide reproducibly reduces both basal and induced CAT activities while competition by the (NFκB) oligonucleotide reduces only the TNF-induced CAT activity (Table I). Since the (ab) site binds KBF1 and NFκB with similar affinity while the (NFκB) site binds KBF1 poorly, these results support the above model.

Three cAMP-responsive elements are present in the H-2K^b promoter

If, as the above results indicate, the AP2-like factor is displaced from the (b′ ′d) palindrome by binding of NFκB to the ab′ site, co-stimulation by TNF-α and cAMP in the (ab′ ′dab) region of the promoter appears unlikely. However, our earlier unpublished work using the almost entire promoter had shown synergistic action of TNF and cAMP. If there were no other cAMP-responsive element(s) (CREs), the data would be contradictory. Therefore, we decided to map these elements in the K^b promoter.

The −210 to −181 sequence, cloned in the conaCAT vector, displays sensitivity to cAMP (Figure 1B). Similarly, the −122 promoter deletion mutant is responsive to cAMP while the −61 deletion is not (data not shown). An oligonucleotide spanning the −109 to −84 region, cloned in conaCAT, conferred responsiveness to cAMP (Figure 1B). Thus, the K^b promoter contains at least two other CREs (in the −210 to −181 and −109 to −84 regions) in addition to the one located within the (b′ ′d) region of the major enhancer.

It has been shown recently that a protein binds to the −203 to −185 region (Shirayoshi *et al.*, 1988) and that the purified factor AP1 binds to the −210 to −181 and −101 to −88 sequences *in vitro* (Korber *et al.*, 1988). Since AP1 is responsive to TPA but not cAMP (e.g. see Deutsch *et al.*, 1988 and references therein, and construct AP1 in Figure 1B), it is likely that another factor can bind these two regions *in vivo*. The binding sites located in these two regions were characterized by DMS protection, shown in Figure 5(A) and summarized in (B). Careful examination of the sequences involved show similarity to the CRE located in various cAMP-responsive genes which bind CREB, ATF and probably other factors (Montminy *et al.*, 1986; Montminy and Bilezikjian, 1987; Comb *et al.*, 1988; Lin and Green, 1988; Sassone-Corsi, 1988; Dean *et al.*, 1989) but can also bind AP1 (Deutsch *et al.*, 1988). Retardation experiments using the −200 to −181 region, and consensus sites for AP1 and CREB (the oligonucleotide CA was derived from the somatostatin promoter; Montminy *et al.*, 1986) showed that (Figure 5C) the −210 to −181 region can bind purified AP1 (kindly provided by Drs S.Hirai and M.Yaniv: lane 7) in a similar fashion as the CA oligonucleotide (lane 1) and to the band obtained with an AP1 site and crude extract (lane 5). However, using crude extract with either CA or the −210 to −181 region results in a series of bands (lanes 2 and 6), one of them corresponding to AP1 (cf. lanes 2 and 3). Besides, an excess of cold −210 to −181 oligonucleotide competes all bands formed with labelled CA (lane 4). Similar results have been obtained with the −109 to −84 oligonucleotide (not shown) and are summarized in Figure 5(B). These results strongly suggest that the proteins which bind *in vivo* to the two cAMP-responsive regions outside the enhancer belong to the CREB/ATF family.

Discussion

We have shown here that TNF-α stimulates the transcription of the mouse H-2K^b gene through the induction of an NFκB-like binding factor. The latter attaches to two sites in the enhancer and displaces two other factors present in uninduced HeLa cells, namely KBF1 and an AP2-like protein (Figure 6). The TNF-α activation pathway appears to be distinct from the pathways of induction by IFNs as well as by cAMP, although interactions between these pathways can be observed, as discussed below.

Involvement of an NFκB-like factor in TNF-α induction

NFκB, originally characterized as a transcription factor involved in the expression of the κ chain of immunoglobulins (Sen and Baltimore, 1986a), has been shown to be present in the cytoplasm of non-B cells under an inactive form, probably bound to a repressor. Activation of pre-B cells by LPS or of HeLa cells by TPA leads to dissociation from the repressor (a step probably involving protein kinase C), translocation to the nucleus, binding to the DNA and activation of target genes (Baueuerle and Baltimore, 1988a, b). Such activation of NFκB does not require protein synthesis.

We found that at least two adjacent NFκB sites are required for stimulation to occur. This is the normal situation in the H-2K^b promoter and in all mouse and human class I genes encoding major transplantation antigens characterized so far. This is also true for the HIV-1 enhancer whose stimulation by TNF has recently been shown to be mediated through induction of an NFκB-like activity (Osborn *et al.*, 1989). We have further shown that the presence of only one site in the β2-m promoter (Israël *et al.*, 1987) correlates with the absence of TNF-α stimulation. This has been confirmed on the endogenous gene in mouse 3T3 cells by Northern analysis (not shown). This result is interesting because the expression of class I heavy chains and β2-m appears, under most circumstances, to be co-regulated. Thus, IFNs induce both heavy chains and β2-m, but TNF-α behaves differently. The reason why two NFκB sites are necessary for activation is unknown. The distance between the two sites does not seem to be crucial: (ab′ ′dab)$_1$ or (ab)$_4$ are both stimulated, and even a multimer in which the (ab) palindromes are separated by 25 bp of the IRS sequence is stimulated by TNF-α (not shown).

It may be noted that our results suggest that KBF1 might be functionally replaced by NFκB or NFκB-like factors in situations where the latter are activated. In this regard, it is interesting that strong stimulation of class I gene expression has recently been observed in activated mouse B cells (Southern *et al.*, 1989). It is possible that transcription of class I genes under those circumstances is driven by NFκB rather than KBF1.

One may wonder whether NFκB could be a post-translationally modified form of KBF1 (Baldwin and Sharp, 1988). In this case, activation by TNF-α would be readily understandable as an increase of the enhancer activity. However, the simultaneous presence of an inactive form of NFκB in the cytoplasm and of a constitutive form of KBF1

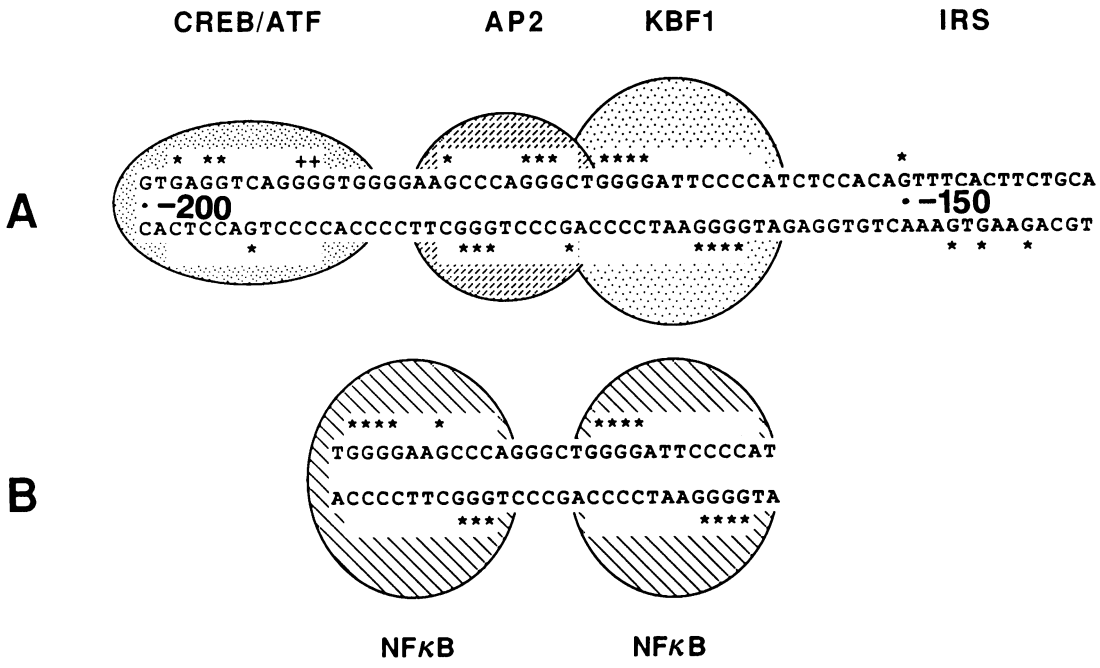


Fig. 6. Schematic illustration of the binding sites of proteins to the −200 to −137 region of the H-2K^b promoter, with the contact points on the Gs indicated. The data for the IRS are from Shirayoshi *et al.* (1988). (**A**) With an extract from control HeLa cells; (**B**) with an extract from TNF-stimulated HeLa cells. *, complete protection; +, incomplete protection.

in the nucleus, as well as differential responses of the binding activity of the two factors to the presence of nucleotides (Lenardo *et al.*, 1988) suggest that they actually are different factors.

Pathway(s) leading to the TNF-α mediated induction of MHC class I genes

We have found that, in the mouse K^b gene, induction by TNF-α does not utilize the IRS, and uses a pathway distinct from the cAMP second messenger pathway, but possibly similar to the TPA pathway. In contrast, in the induction of HIV-1 LTR by TNF, it was suggested that TNF uses a pathway different from the one used by TPA (Osborn *et al.*, 1989). Another report shows that stimulation of Igκ expression by cAMP in mouse pre-B cells is mediated by an induced NFκB-like activity (Shirakawa *et al.*, 1989). Activation of NFκB by TPA does not require protein synthesis, and we have observed (data not shown) that TNF induction of the NFκB-like activity in HeLa cells is not blocked by cycloheximide. However, it is known that TNF stimulation of class I gene transcription is blocked by inhibitors of protein synthesis (Collins *et al.*, 1986). It thus seems that, in addition to induction of an NFκB (or NFκB-like) binding activity, a cycloheximide-sensitive step is required to activate transcription. Thus, there may be several routes leading to NFκB or NFκB-like induction, with different requirements for protein synthesis.

We found (Figure 1B) that the constructs which respond to TNF-α do not all respond to cAMP inducers. This suggests that the TNF-α effect is not mediated through the cAMP-dependent protein kinase pathway in HeLa cells. Nevertheless, we have characterized three previously unknown CREs in the H-2K^b promoter. Two of these elements bind factors belonging to the CREB/ATF family (the −210 to −181 and −109 to −84 regions) while the third one is located in the (ab′ 'dab) region and binds an AP2-like factor. In spite of its location, the latter is not involved in induction by TNF-α, since a polymerized AP2 site is not responsive to TNF-α (Figure 1B) and a multimerized (ab′) site, which does not bind AP2 (not shown), is still responsive. We have also transfected the (ab′ 'dab) construct into HepG2 cells and found it to be still responsive to TNF-α (data not shown), although the AP2 activity has been shown to be missing in these cells (Chiu *et al.*, 1987).

The exact contribution of these elements to the overall stimulation by cAMP remains to be investigated by site-directed mutagenesis in the context of the entire promoter. An interesting point is the recent finding that early adenovirus promoters are induced by cAMP through sequences required for E1a *trans*-activation (Sassone-Corsi, 1988). It has been demonstrated that oncogenic adenoviruses strongly repress class I expression and that E1a gene is directly involved in this effect (Van der Eb *et al.*, 1984). We are currently testing the effect of E1a on the H-2K^b CREs.

As independent as they seem to be, the TNF-α, cAMP and IFN induction pathways may display a variety of interactions at the level of the K^b promoter itself. This is suggested by the fact that the enhancer−IRS region from −201 to −137 of the K^b promoter is covered with a dense array of proteins including at least the following (Figure 6): a CREB/ATF-like factor (−200 to −188), AP2 + KBF1 (or NFκB: −187 to −159) followed by two or three proteins binding to the IRS (−158 to −137: Shirayoshi *et al.*, 1988). The proximity of these binding sites suggest protein−protein interactions or exclusion by steric hindrance. A question one can ask, for example, is whether in B cells, where NFκB activity is constitutive, it is NFκB or AP2 + KBF1 that binds the (ab′ 'dab) sequence and regulates class I expression. Another intriguing point is the reproducibly lower in-

Table II. Sequences of the various oligonucleotides used in this study

ab	5′-G A T G G G G A T T C C C C A T-3′ 3′- A C C C C T A A G G G G T A C T-5′
ab′	5′- G A G T G G G G A A G C C C A G G G C-3′ 3′- C A C C C C T T C G G G T C C C G C T-5′
IRS	5′- G A T T C C C C A T C T C C A C A G T T T C A C T T C T G C A-3′ 3′- A A G G G G T A G A G G T G T C A A A G T G A A G A C G T C T-5′
ab′ ′dab	5′- G A T G G G G A A G C C C A G G G C T G G G G A T T C C C C A T-3′ 3′- A C C C C T T C G G G T C C C G A C C C C T A A G G G G T A C T-5′
−210 to −181	5′- G A T C G C C A G G C A G T G A G G T C A G G G G T G G G G A A-3′ 3′- A G C G G T C C G T C A C T C C A G T C C C C A C C C C T T C T-5′
ab′ ′d	5′- G A T G G G G A A G C C C A G G G C T G G-3′ 3′- A C C C C T T C G G G T C C C G A C C C T-5′
−109 to −84	5′- G A G G A C A C T G T T G A C G C G C A G T C A G C T C-3′ 3′- C C T G T G A C A A C T G C G C G T C A G T C G A G C T-5′
β-2	5′- G A A A G G G A C T T T C C C A T-3′ 3′- T T C C C T G A A A G G G T A C T-5′
AP1	5′- G A T C G T G A C T C A G C G C G-3′ 3′- A G C A C T G A G T C G C G C C T-5′
AP2	5′ G A A C T G A C C G C C C G C G G C C C G T G-3′ 3′- T G A C T G G C G G G C G C C G G G C A C C T-5′
CA	5′- G T T G G C T G A C G T C A G A G A G A G A G-3′ 3′- C G A C T G C A G T C T C T C T C C A A C-5′
m′d	G A T G G G G A A G C C C A A T T A T G G G G A T T C C C C A T A C C C C T T C G G G T T A A T A C C C C T A A G G G G T A C T
mab	G A T G G G G A A G C C C A G G G C T G G G G A T T C C G G A T A C C C C T T C G G G T C C C G A C C C C T A A G G C C T A C T
NFκB	G A C A G A G G G G A C T T T C C G A G A G G G T C T C C C C T G A A A G G C T C T C C C T

ducibility by TFN of the *Dde*I−*Hin*cII construct as compared to *Dde*I−*Hin*fI (Figure 1B), which lacks the IRS. This difference is not due to a distance effect since it is still observed after reversing these two elements. We previously demonstrated that for IFN stimulation to take place, the enhancer (ab′ ′dab) region is required in conjunction with the IRS (Israël *et al.*, 1986). Our working hypothesis is that proteins binding to these two regions interact *in vivo* so that the IRS binding protein(s) which normally interact with KBF1 would interfere with NFκB activity induced upon TNF-α treatment.

Materials and methods

Cell culture

HeLa and NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. The same medium was used during the transfection experiments.

Human tumour necrosis factor

TNF-α was produced in CHO cells as described in Korn *et al.* (1988). It was purified chromatographically to close to homogeneity. It has a sp. act. of 6×10^3 U/μg protein.

Transient analysis of gene expression

Transfection of HeLa cells was performed as previously described (Kimura *et al.*, 1986) by the calcium phosphate co-precipitation procedure. TPA (100 ng/ml), forskolin (25 μM) + IBMX (0.5 mM), or TNF-α (500 U/ml) were left for 16 h after which a cytoplasmic extract was prepared, heated for 10 min at 65°C and CAT activity assayed with [^{14}C]dibutyryl coenzyme A as described in Neumann *et al.* (1987).

Competition was carried out by including a 40-fold molar excess of polymerized oligonucleotides in the calcium phosphate co-precipitate. This concentration was chosen from preliminary experiments similar to the one described in Israël *et al.* (1987).

Oligonucleotides

The different oligonucleotides used are listed in Table II. They have been synthesized on an Applied Biosystems 380A synthesizer and purified by gel electrophoresis.

These oligonucleotides were either labelled at both ends with γ-ATP for bandshift assays, or one strand was 5′-end-labelled and then annealed with the unlabelled complementary strand for DMS protection or DMS interference experiments.

For cloning in an expression vector, these oligonucleotides were filled in with the Klenow enzyme and cloned into the filled in *Bam*HI site located 100 bp upstream of the conalbumin cap site in the conaCAT vector (Kimura *et al.*, 1986). The protruding ends of these oligonucleotides are designed so as to allow multimerization in a head-to-tail fashion, e.g. in the constructs $(ab′\ ′dab)_3$ or $(ab)_4$.

DNA binding protein analysis

Nuclear extracts were prepared as described in Yano *et al.* (1987). Bandshift assay was performed as in Israël *et al.* (1987) except that 20 mM Tris−HCl, pH 7.8, was substituted for 10 mM phosphate buffer, pH 6.0.

Methylation interference and methylation protection experiments

DMS interference experiments were performed as described in Israël *et al.* (1987). Analysis was performed on a 20% acrylamide−8 M urea sequencing gel. For DMS protection experiments, 3 ng of oligonucleotide 5′-labelled at one end was incubated in 30 μl with 10−15 μg of nuclear extract and 0.5 μg of poly(dI·dC). After 15 min at room temperature, 0.5 μl of DMS was added for 1 min. The reaction was stopped by phenol extraction, followed by ethanol precipitation. After piperidine treatment and repetitive lyophilization, the samples were analysed on a 20% acrylamide−8 M urea sequencing gel. Control was performed with bovine serum albumin instead of the nuclear extract. When purified protein was used, poly(dI·dC) was reduced to 100 ng.

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