An AP1 binding site upstream of the kappa immunoglobulin intron enhancer binds inducible factors and contributes to expression

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ABSTRACT

Expression of the kappa immunoglobulin light chain gene requires developmental- and tissue-specific regulation by *trans*-acting factors which interact with two distinct enhancer elements. A new protein–DNA interaction has been identified upstream of the intron enhancer, within the matrix-associated region of the J-C intron. The binding activity is greatly inducible in pre-B cells by bacterial lipopolysaccharide and interleukin-1 but specific complexes are found at all stages of B cell development tested. The footprinted binding site is homologous to the consensus AP1 motif. The protein components of this complex are specifically competed by an AP1 consensus motif and were shown by supershift to include c-Jun and c-Fos, suggesting that this binding site is an AP1 motif and that the Jun and Fos families of transcription factors play a role in the regulation of the kappa light chain gene. Mutation of the AP1 motif in the context of the intron enhancer was shown to decrease enhancer-mediated activation of the promoter in both pre-B cells induced with LPS and constitutive expression in mature B cells.

INTRODUCTION

The kappa immunoglobulin light chain gene intron enhancer is one of the most thoroughly characterized mammalian regulatory regions. The intron enhancer is comprised of a number of *cis*-acting elements spanning 250 bases of the J-C intron and is bound by numerous *trans*-acting transcription factors that are either ubiquitously expressed or interact with the enhancer at specific stages of B cell development. A number of inducible protein–DNA interactions at the intron enhancer have been previously described, including NF- κ B which is primarily responsible for intron enhancer activity (for review see 1) and κ B-FA, an inducible interaction found in pre-B cells (2). The region upstream of the intron enhancer has been identified as a matrix-associated region (MAR) (3). While this region is bound

by protein components of the nuclear matrix or scaffolding, it has also been proposed that these MAR proteins may include transcription factors (4,5).

Very little is known about protein–DNA interactions upstream of the kappa intron enhancer that includes the MAR. In this study a novel site of protein–DNA interactions was identified, upstream of the core intron enhancer, within the MAR of the kappa light chain gene. We found a number of different protein–DNA complexes are formed at this site, at distinct stages of B cell development. One of the DNA–protein complexes formed is greatly increased in a pre-B cell line when induced with LPS or IL-1. Transient transfection assays of enhancer-containing reporter gene constructs suggest that this interaction results in the positive regulation of enhancer activity. Competition binding studies as well as supershifts with antibodies to the c-Fos and c-Jun proteins indicate that this motif is an AP1 site.

MATERIALS AND METHODS

κ AP1 DNA probes and competitors

The complementary oligonucleotides used as probes or competitors in electrophoretic mobility shift assays are listed below. For DNA probes, single-stranded oligonucleotides were end-labeled with T4 polynucleotide kinase and [γ -³²P]ATP, annealed to the complementary strand, and purified with Biospin 6 columns (Bio-Rad). The κ AP1 oligonucleotides used were: 5' ATGCAAAAATATGACTAATAATAATTTAGCACA 3' and 5' TGTGCTAAATTATTATTAGTCATATTTTGCAT 3'. The AP1 consensus oligonucleotides used were: 5' CTAGATCCTCTAGAACTGACTCATCGGATCTAC 3' and 5' GTAGATCCGATGAGTCAGTTCTAGAGGATCTAG 3'. The non-specific oligonucleotides used as competitor DNA were: 5' CAGAGGGGACTTTCCGGCCGGCATCTGGCAG 3' and 5' CTGCCAGATGCCGGCCGGAAAGTCCCCCTCTG 3', which contain the κ B and κ E1 binding sites. The human κ AP1 oligonucleotides used were 5' GCTTTCCTTGACTCAGCCGTGCC 3' and 5' GGCAGCGGCTGAGTCAAGGAAAGC 3', derived from the sequence 320 bp 3' of the NF- κ B site. The

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mutated mouse κ API oligonucleotides (designated m κ API) used were 5' ATGCAAAAATACTGTTAACAATAATTTAGCAC-A 3' and 5' TGTGCTAAATTATTGTTAACAGTATTTT-TGCAT 3'.

Nuclear extracts, electrophoretic mobility shift assays and footprinting

Nuclear extracts were prepared by the method of Dignam and Roeder (6) with previously described modifications (2). Protein concentration was determined by the method of Bradford (7). Mobility shift assays were performed essentially as described (4). Binding reaction mixtures contained 10 μ g nuclear extract, 0.5–1 μ g poly(dI·dC), and 20,000 cpm (0.2–1.0 ng) end-labeled double-stranded oligonucleotide probe, in a final reaction volume of 20–25 μ l, and were incubated at room temperature for 15 min. Competition binding reactions were preincubated for 10 min at room temperature with the indicated molar excess of unlabeled probe before the addition of radiolabeled probe. DNase I footprinting was performed essentially as previously described (2). Briefly, a 95 bp *Mbo*II–*Alu*I restriction fragment was labeled at one end and incubated with LPS-induced 70Z nuclear extract. 2 units/ml DNase I were added and incubated for 90 s, followed by addition of EDTA to 10 mM. The reaction was loaded onto a polyacrylamide gel as described for the mobility shift, and the B4 complex (see Fig. 1) was excised from the gel, the DNA eluted, then denatured and run on an 8% sequencing gel along with an A/G sequencing ladder of the same fragment. After drying the gel it was exposed to X-ray film.

Binding reactions for antibody complex supershifts included the preincubation of 1.5 μ g rabbit polyclonal c-Jun antibody (Ab-2, Oncogene Science), rabbit polyclonal c-Fos antibody (Ab-2, Oncogene Science), or NF- κ B p65 antibody (Pharmingen) for 15 min at room temperature.

Plasmid constructs

A wild-type 595 bp kappa enhancer fragment was amplified using the κ API oligonucleotide (see above) and an oligonucleotide of the sequence downstream of the enhancer at the closest *Alu*I site (5' TAGCCAAGCTTAACCTACTG 3') and was subcloned into the TA cloning vector, pCRTMII (Invitrogen). A similar amplification and subsequent TA cloning was performed with the same downstream primer and an upstream primer containing four base changes within the κ API site (m κ API) (sequences given above). The *Sac*I–*Xho*I fragments containing these enhancers were then isolated and ligated into the compatible site of KpLUC (28) to form Kp 595 and Kp (m κ API) 595. All plasmids were prepared independently twice and two independent isolates of each construct were typically assayed. Mutations and the integrity of the enhancer were confirmed by sequencing all constructs.

Transient transfection

Murine B cell lines were transiently transfected by the DEAE–dextran method as described by Grosschedl and Baltimore (8) with some modifications as described by Nelms *et al.* (2). For pre-B cell transfections 12.5 μ g/ml lipopolysaccharide (LPS) were added to half of the cells transfected. The cells were then cultured for 24 h at 37°C with 7% CO₂. Cells were lysed with 60 μ l of 1× Cell Culture Lysis Reagent (Promega). Supernatants were collected and assayed for protein according to the method of Bradford (9).

Luciferase assay

100 μ l Luciferase Assay Reagent (Promega) was added to 20 μ l of the protein extract and the reaction was counted for 15 s in a Lumat 9501 luminometer (EG & G Berthold) for each transfection. The amount of luminescence from RSV β gal was used to normalize the activity levels for transfection efficiency. β -Galactosidase assays were performed using the Galacto-light kit from Tropix (Bedford, MA). 5 μ l of cell extracts were incubated with 200 μ l of AMPGD reagent for 60 min at room temperature in the dark. 100 μ l of Emerald Accelerator reagent were then injected and the activity was counted in the Lumat 9501 luminometer for 15 s. In order to normalize for transfection efficiency, the luciferase activity levels were divided by the amount of β -gal luminescence and corrected for the differences in protein concentration in each extract. The results are represented as relative activities. In pre-B cell experiments the relative activity from the LPS-induced cells was divided by the level of activity from the uninduced cells transfected by the same plasmid and is expressed as fold induction. Relative luciferase activities from at least three independent experiments, done in duplicate or triplicate, were determined using two plasmid preparations of two different isolates of each plasmid construct.

RESULTS

An inducible factor binds 360 bases upstream of the mouse kappa intron enhancer within the kappa MAR

In the last few years a number of transcription factor binding sites have been identified within the region upstream of the originally defined kappa intron enhancer (1). This includes the matrix-associated region (MAR) (3–5), which is thought to be the site of nuclear matrix protein interaction, although no specific protein interactions have been identified. In order to determine whether there were any regions of DNA–protein interaction that had not yet been described, a DNA–protein binding study was undertaken upstream of the mouse intron enhancer, within the MAR. By using electrophoretic mobility shift assays, an inducible binding activity within the MAR was identified (Fig. 1). A series of nuclear extracts prepared from both B and non-B cell lines revealed a number of DNA–protein complexes formed with an oligonucleotide corresponding to a 33 bp region 360 bases upstream of the NF- κ B site. Nuclear extracts prepared from the pre-B cell lines, 3-1 and 70Z/3, stimulated with bacterial LPS, showed the highest level of DNA–protein binding activity (band B4, Fig. 1A), although extracts from all of the cell lines tested, including pre-B, mature B, plasma cell, T lymphoma and HeLa cell lines, showed some binding activity. As described below, we subsequently identified this as a site homologous to an API motif and designate it κ API. One complex formed is common to all extracts tested (B2). One prominent complex formed with HeLa cell extracts only (B3). The T cell extract, EL4, also results in a unique lower complex (B5) which consistently runs at a different mobility than the inducible, B cell-specific complex (B4).

To demonstrate the specificity of these interactions, competitive binding assays were performed. Excess non-radioactively labeled double-stranded κ API oligonucleotides were included in the binding assay mixtures. A 50-fold excess of non-labeled oligonucleotide can effectively compete for all of the protein binding seen with the LPS-induced pre-B cell extract 70Z/3 (Fig. 1B). However, the same excess of an oligonucleotide containing the NF- κ B binding motif does not compete for any of the

Figure 1. Binding of nuclear factors to the κ AP1 oligonucleotide probe. (A) Mobility shift analysis of the κ AP1 probe with a variety of nuclear extracts. The cervical carcinoma cell line HeLa and the T lymphoma line EL4 were examined as well as the pre-B cell lines 3-1 and 70Z/3, the B lymphoma line WEHI 279, and the plasma cell lines S194 and MPC11. The pre-B cell lines were either unstimulated or induced with 15 μ g/ml LPS for 24 h before nuclear extract preparations. Five different bound complexes were identified, labeled B1–B5. (B) κ AP1 binding inducibility and specificity. 70Z/3 cells were grown in the presence or absence of 15 μ g/ml LPS for 24 h. Extracts from induced cells were incubated with the κ AP1 probe in the presence or absence of 50-fold excess unlabeled κ AP1 or non-specific (NS) competitor oligonucleotides. The bold arrow indicates the LPS-inducible (B4) complex.

complexes. This implies that all of the complexes formed with the 70Z/3 extract are specific to the sequence of the κ AP1 33 bp oligonucleotide. Similar competition assays analyzing the protein–DNA interactions formed with the other cell extracts confirmed the specificity of the complexes formed at all stages of B cell development and in non-B cells (data not shown).

We focused our attention on the B cell-specific and major inducible protein–DNA interaction (complex B4, Fig. 1A). To test whether the binding activity is affected by other inducers of kappa expression, 70Z/3 cells were grown in the presence of LPS for 8 h, phorbol 12-myristate 13-acetate (PMA) for 4 or 8 h or IL-1 for 24 h. As seen in Figure 2, both LPS and IL-1 induce the B4 protein binding activity. PMA did not affect the LPS-inducible binding activity in pre-B cells. Figure 2 also demonstrates the results of binding assays performed with 70Z/3 pre-B cell extracts induced with 100 U/ml IL-4 and 50 or 100 U/ml interferon- γ (INF- γ). Neither IL-4 nor INF- γ induce protein binding by the 33 bp kappa oligonucleotide. Therefore, we identified a specific DNA–protein binding interaction present in all stages of B cell development, but which is highly inducible in pre-B cells by both LPS and IL-1.

Figure 2. Induction of κ AP1 binding. The 70Z/3 pre-B cell line was untreated or treated with 100 U/ml IL-1 for 24 h, 15 μ g/ml or 10 μ g/ml LPS, 100 U/ml IL-4, 50 or 100 U/ml of interferon- γ (INF- γ) or 100 ng/ml PMA. The plasma cell line S194 was also untreated or treated with 10 μ g/ml LPS. The inducible, B cell-specific (B4) complex is marked by a solid arrow.

To more specifically define the DNA–protein contact sites, DNase I footprint experiments were performed using the induced 70Z/3 pre-B cell nuclear extract and a 95 bp *Mbo*II–*Alu*I restriction fragment encompassing the oligonucleotide sequence. Footprinting of the B4 complex resulted in detectable protection on both the coding and non-coding strands (Fig. 3), along with a number of DNase I hypersensitive sites both within and flanking the protected region. The protected region contains sequences homologous to a site previously described as a nuclear factor binding site. The identified region is a 7 of 8 base match to a consensus AP1 site, known to bind the Fos and Jun families of transcription factors (10).

The kappa intron in both mouse and human contains an AP1 site that binds c-Jun and c-Fos

If the κ AP1 site contributes to regulation of mouse kappa expression, it might be expected to be a conserved motif within the human kappa intron. Indeed, an AP1 consensus motif, TG-ACTCA, was identified in the human intron almost equidistant from the NF- κ B binding site, however, it is located 320 bp 3' rather than 5' of the NF- κ B site. In order to determine whether the protein complex formed with the κ AP1 probe bound the same factors as the consensus AP1 (cAP1) motif (10) and the human

κ AP1 (hAP1), these sites were used as competitors in mobility shift assays. As shown in Fig. 4, the κ AP1, cAP1 and hAP1 sequences all effectively competed for complex B4 binding by the radiolabeled κ AP1 sequence. In contrast, a non-specific competitor sequence and a five base mutation of the κ AP1 sequence (see Fig. 6) did not compete. When the cAP1 or hAP1 probes were radiolabeled and used in mobility shift assays, identical complexes to those formed with the mouse κ AP1 probe were observed (not shown). The sequence similarity among the probes is confined to the AP1 motif and demonstrates that flanking sequences do not contribute to the specificity of the protein–DNA interaction. No complex was observed with radiolabeled mutant κ AP1 probe (not shown). These results serve to define the specificity of the sequence recognition and strongly suggest it is an AP1 motif.

AP1 sites have been shown to be bound by protein dimers of the Fos and Jun families (11). Jun forms homodimers and Fos and Jun family members can heterodimerize and bind to AP1 sites (reviewed in 11). The Fos and Jun proteins have been demonstrated to bind AP1-containing regulatory regions and can activate transcription (12), or in some cases down-regulate transcription (13,14). To determine whether Fos and Jun interact

Figure 3. DNase I footprint of the B4 complex and identification of AP1 homology. A 95 bp *Mbo*II–*Alu*I restriction fragment of the kappa J-C intron, encompassing the κ AP1 oligonucleotide sequence, was used in a preparative binding reaction with LPS-induced 70Z/3 pre-B cell nuclear extract. After DNase I treatment and electrophoresis, the B4 complex was excised from the gel, the DNA was isolated and denatured before loading on a sequencing gel. The region of protection is indicated. Sequence numbering is as in reference 26. The homology to the consensus AP1 site is depicted.

Figure 4. Competition analysis of κ AP1 binding activity. Radiolabeled κ AP1 oligonucleotide probe was incubated with LPS-induced 70Z/3 nuclear extract in the absence of competitor (NC) or in the presence of unlabeled κ AP1, an oligonucleotide containing a 4 bp mutation of the κ AP1 sequence (mut κ AP1), the human κ AP1 motif (hAP1), a consensus AP1 motif (cAP1), or a non-specific competitor (NS). See Materials and Methods for sequence details of probes used. Arrow designates the B cell-specific (B4) complex.

Figure 5. κ AP1 supershifts with c-Fos and c-Jun antibodies. (A) Antibodies specific for c-Fos and c-Jun were preincubated in binding assays with nuclear extracts from different cell lines before the addition of κ AP1 oligonucleotide probe. Two non-B cell line extracts, HeLa (a cervical carcinoma line) and EL4 (a T lymphoma cell line), two LPS-induced pre-B cell line extracts, 3-1 and 70Z/3, and the mature B cell extract WEHI 279 were preincubated with no antibody, a polyclonal c-Jun antibody (AB-2, Oncogene Science), a polyclonal c-Fos antibody (AB-2, Oncogene Science) or an antibody specific for the p65 component of NF- κ B (Pharmingen). Presence of antibody is indicated by + above the corresponding lane. Supershifted complexes are indicated with arrows. (B) A second supershift analysis of LPS-induced 70Z nuclear extracts. Antibodies to c-Jun, c-Fos, and p65 component of NF- κ B were added as indicated.

with the κ AP1 site, antibodies specific for c-Fos and c-Jun were preincubated in binding assays with nuclear extracts from a number of different cell lines (Fig. 5). Two non-B cell line extracts, HeLa (a cervical carcinoma line) and EL4 (a T lymphoma cell line), both of which are known to express Fos and Jun (15), were tested along with three B cell lines. Two LPS-induced pre-B cell line extracts, 3-1 and 70Z/3, the mature B

Figure 6. κ AP1 reporter gene constructs and transfection results. (A) Plasmid constructs used in transient expression assays. KpLUC 595 and KpLUC m595 contain a 595 bp intron enhancer fragment with the endpoints indicated. The m595 enhancer contains 5 base substitutions within the region of sequence indicated. The sites of base changes are indicated. The enhancer fragments were subcloned into the KpLUC reporter gene vector which contains the V κ 21E promoter and the luciferase reporter gene (30). Numbering is as in reference 16. (B) Transient expression assays in 3-1 pre-B cells uninduced (open bars), or induced with LPS (lightly stippled bars), PMA (medium stippled bars) or both LPS and PMA (darkly stippled bars). (C) Transient transfection results of the myeloma cell line S194. The activity of the mutated enhancer is given relative to the activity of the wild-type enhancer construct. Values are averages of 2-4 independent experiments as described in Materials and Methods. Normalized LUC activity is the units of luciferase activity relative to the units of β -galactosidase activity resulting from the co-transfection of the RSV β -gal plasmid. Errors are represented as standard error of the mean. In both cases differences between wild-type activity and mutant were significant to $P < 0.05$.

cell extract WEHI 279, and the two non-B cell line extracts were preincubated with no antibody, a polyclonal c-Jun antibody (AB-2, Oncogene Science), a polyclonal c-Fos antibody (AB-2, Oncogene Science) or a control antibody specific for the p65 component of NF- κ B (Pharmingen). The mobility shift analysis in Figure 5A shows a strong new complex of larger mobility forms when the c-Fos antibody is added (lanes 4, 8, 12, 16 and 20), and two faint new complexes when the c-Jun antibody is added (lanes 3, 7, 11, 15 and 19). The mobility shifts and antibody supershifts were repeated in a second, independent analysis using 70Z nuclear extracts (Fig. 5B). In this second experiment the antibody supershifts are readily apparent only in induced extracts when either c-Fos or c-Jun, but not when the non-specific p65, antibodies are added. The two complexes seen in the anti-c-Jun supershift likely reflect Jun-Jun and Fos-Jun dimers. In all cases the inducible B4 complex decreases in intensity and smears upward whenever a specific supershift is seen. It is difficult to determine whether the minor upper complexes also decrease in intensity due to the smeared supershift of the lower, most intense complex. These experiments demonstrate the presence of Fos and Jun family members in the major complex formed with the newly identified kappa binding site in B and non-B cell nuclear extracts, thereby confirming its identity as an AP1 site.

The κ AP1 site alone or in multimers does not enhance transcription but can influence kappa expression in the context of the enhancer

The murine κ AP1 motif was hypothesized to bind factors that activate transcription in induced pre-B cells and mature cells in a manner similar to the κ B site, which has a similar factor binding profile. The κ B site, when subcloned in multiple copies upstream of a promoter-containing reporter gene significantly enhances reporter gene transcription (16). A more recently identified inducible binding activity at the κ A site (2) can also independently enhance transcription when the κ A site is multimerized (Nelms and Van Ness, unpublished results). An experiment was therefore designed to examine the ability of 1–4 copies of the κ AP1 motif to activate transcription of the reporter gene in pre-B or mature B cells. The oligonucleotide containing the κ AP1 motif was subcloned in 1–4 copies upstream of the kappa promoter and the CAT reporter gene. These plasmids were transfected into the pre-B cell lines 3-1 and 1-8 which were induced with LPS for 24 h. No significant activity was seen, even upon induction of any of the transfected cells (data not shown). Similar results were obtained with another pre-B cell line, 1-8, and the plasma cell line S194 (data not shown). Thus, the κ AP1 site is unable to act independently to regulate kappa expression in B cells.

Previously identified DNA protein binding motifs have been shown to activate transcription in the presence of other binding motifs even when the motif alone has no detectable enhancer activity (17). In order to determine whether the functional activity of the κ AP1 site requires the presence of other factor binding sites within the enhancer, a 595 bp enhancer construct containing five base changes within the κ AP1 motif was compared for activity with a wild-type κ AP1-containing enhancer (Fig. 6). The base substitutions made within the κ AP1 motif are the same as used in the competitive binding assay described above. An oligonucleotide containing these changes has no protein binding activity detectable by mobility shift assay and cannot compete for protein binding to a wild-type AP1 oligonucleotide (Fig. 4).

The wild-type enhancer construct, KpLUC 595, and the mutated enhancer construct, KpLUC m595 (Fig. 6A), were transiently transfected into 3-1 pre-B cells in duplicate as well as S194 plasma cells in quadruplicate. Luciferase activity was normalized for transfection efficiency by co-transfection with a RSV β -galactosidase reporter gene as described in Materials and Methods. The endogenous kappa enhancer activity is inducible in the pre-B cell line with mitogens and is constitutive in the myeloma cell line S194. Thus, our functional analyses of the mutation of the κ AP1 site were examined for effects on both inducible and constitutive enhancer activity.

Pre-B cells were transfected with KpLUC 595 and KpLUC m595 and induced with LPS, PMA, or both for 20 h before cell harvest. Cell extracts were then assayed for luciferase activity. As shown in Fig. 6B, enhancer activity was evident in extracts derived from transfection of both constructs (compared to KpLUC control), however, mutation of the AP1 site resulted in an ~80% loss of induction in response to LPS ($P < 0.05$). In these experiments neither enhancer construct responded to the PMA induction. When co-stimulated with both LPS and PMA a loss in the mutated reporter gene induction was seen, similar to that seen with LPS alone. The pattern of inducible κ AP1 site binding activity, as determined by mobility shift assays, was the same as the pattern of transcriptional activation attributed to this site through the κ AP1 mutation analysis. The binding and activating potential of this site is increased by LPS induction but not by PMA induction. The transfection of these constructs into S194 plasma cells also demonstrated a loss of constitutive enhancer activity upon the mutation of the κ AP1 site (Fig. 6C). A 25% loss in activity ($P < 0.05$) was seen when comparing the mutated enhancer to the wild-type enhancer. Therefore, the factors binding to the AP1 site are responsible for a portion of the transcriptional activating capacity of the kappa intron enhancer both early and late in B cell development. However, as indicated by the inactivity of the site alone or in multimers, this activity requires the presence of other factor binding motifs within the kappa intron.

DISCUSSION

A number of transcription factor binding sites have been identified within the region upstream of the kappa intron enhancer over the last few years (reviewed in 18). This upstream segment includes the nuclear matrix-associated region where no specific factor interactions have yet been identified. In scanning the DNA sequence upstream of the well-characterized kappa intron enhancer for possible protein interactions, a DNA binding activity was detected 360 bp upstream of the NF- κ B binding site (mouse) or 320 bp downstream of the NF- κ B site (human). We have shown that binding activity is inducible in pre-B cells and that it is also present, albeit at apparently lower levels, in later stages of B cell development. Nuclear extracts prepared from pre-B cells, induced with either LPS or IL-1, show significantly more binding activity than uninduced extracts (Figs 1 and 2).

DNase I footprinting revealed a region of protection containing a sequence motif with a seven of eight base match to the consensus AP1 binding site. IL-1 and PMA have both been demonstrated to induce c-Fos and c-Jun expression (19,20), which bind as heterodimers to consensus AP1 motifs (11). Induction of κ AP1 protein binding activity with the phorbol ester PMA was not seen, which suggests that some differences exist between the induction of factors interacting at this AP1 site in B cells and the classic PMA-inducible heterodimeric Fos/Jun complex previously

described (20,21). The same sequence that is present in the κ AP1 motif has been used in studies examining variant AP1 motifs of other genes and their ability to bind Fos and Jun (22,23). These two studies confirm the ability of a sequence identical to that found in the κ AP1 motif to bind Fos and Jun or to compete for factor binding in mobility shift assays (22,23). Our results, which specifically identify a variant AP1 in the kappa intron, are consistent with these studies, and demonstrate the ability of a consensus AP1 sequence motif to compete for the binding of the B cell protein complex formed with the murine κ AP1 probe. The inability of a mutated AP1 motif to compete for protein binding to this motif confirms the specificity of the interaction (Fig. 4).

Antibodies specific for c-Fos and c-Jun epitopes were capable of supershifting the protein complex bound to the κ AP1 site. This suggests the presence of c-Jun and c-Fos in the DNA-bound complexes. In all cases the supershift with c-Fos was much stronger than that with c-Jun, which may be due to different antibody affinities for their respective epitopes on the DNA-bound proteins. Differences were also seen in the intensity of c-Jun supershifts which are more apparent in HeLa and pre-B cell extracts than in mature T or B extracts. The differences could indicate the binding of Jun family members that do not cross-react with this c-Jun antibody in some extracts, and are therefore not detected in this analysis. The antibodies used are specific to c-Jun and c-Fos (Oncogene Science) and are not known to significantly cross-react with other family members. Therefore, other Fos and Jun family proteins may be present in the bound complexes and are not supershifted by the antibodies used in this study.

The presence of multiple specific κ AP1-protein complexes also supports the possibility that different Fos and Jun family member homodimers or heterodimers may bind the κ AP1 site in B cells. A number of Fos and Jun family members are expressed in B cells and are inducible by mitogens and cytokines (21,24). Further studies to examine the components of these B cell-specific complexes will be needed to identify all of the proteins involved and determine whether there is a B cell-specific Fos or Jun family member.

The human J-C intron also contains a perfect consensus AP1 site. This motif binds a protein complex which can also be supershifted by c-Fos and c-Jun antibodies (data not shown) and can compete for protein binding to the murine AP1 site (Fig. 4). The human AP1 site, however, is located 320 bases downstream of the κ B site, in the 3' region flanking the enhancer. Therefore, although in different relative positions, the presence of an AP1 site has been conserved between species. The conservation of an AP1 site within both intron enhancer regions supports the hypothesis that it plays a role in kappa gene regulation.

The inability of the AP1 oligonucleotide alone to activate transcription from a kappa promoter is consistent with a number of other factor binding motifs which demonstrate enhancer activity only in the presence of other motifs (5). The distal AP1 motif of the IL-2 promoter has been shown to activate transcription when placed in multiple copies upstream of a promoter and reporter gene, however, the fragment used contained other binding sites as well (7). Regardless of the site's inability to act as a transcriptional activator alone, the AP1 site in the context of the enhancer acts to increase its activity.

The contribution of the AP1 motif to intron enhancer activation was demonstrated by a decrease in enhancer activity upon mutation of the sequence motif. The loss in enhancer activity

was greatest in LPS-induced pre-B cells (Fig. 6). The significant loss of enhancer induction is somewhat surprising given the induction of other known activators, such as NF- κ B and κ B-FA. However, similar dramatic loss of enhancer activity has been noted upon deletion of other enhancer motifs. Notably, mutation of κ E1 and κ E2 resulted in a loss of 80 and 90% enhancer activity, respectively, despite the presence of NF- κ B (25). Thus, our results are consistent with the idea that within the context of the expanding array of enhancer motifs, individual binding sites can significantly influence transcriptional activation. Moreover, unlike previous transfection constructs that used smaller (200–300 bp) enhancer-containing fragments, our constructs contain additional flanking sequence, including newly described silencer regions (26). From these results it is becoming increasingly evident that additional portions of the J-C intron impact on enhancer function.

It has recently been demonstrated that the bZIP regions of c-Fos and c-Jun are capable of physically interacting with NF- κ B p65 and result in enhanced binding and activation (27). These authors suggest a combinatorial mechanism of gene regulation involving cross-coupling of different transcription factors. The presence of both motifs and the interaction of transcription factor families within the kappa intron may therefore ensure the highest possible level of transcriptional activation.

It is also noteworthy that this newly identified AP1 motif is present within the kappa matrix-associated region, upstream of the intron enhancer. AP1 binding activity has recently been described within the nuclear matrix fraction of HeLa cell extracts (29). The κ AP1 motif may therefore play a role in both the transcriptional regulation of kappa gene expression and in the association of the locus to the nuclear matrix.

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