Transcription factor AP-4 is a ligand for immunoglobulin-κ promoter E-box elements

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Immunoglobulin (Ig)-κ promoters from humans and mice share conserved sequences. The octamer element is common to all Ig promoters and pivotal for their function. However, other conserved sequence motifs, that differ between Ig variable gene families, are required for normal promoter function. These conserved motifs do not stimulate transcription in the absence of an octamer. One example is an E-box of the E47/E12 type (5′-CAGCTG-3′), which is found in all promoters of the human and murine Ig-κ gene subgroups/families, with the exception of subgroups II and VI and their related murine families. In the present study we show that the ubiquitously expressed transcription factor AP-4, and not E47, interacts specifically with the κ promoter E-boxes when tested in electrophoretic mobility-shift assays using nuclear extracts derived from human and murine B-cell lines. Furthermore, AP-4, unlike E47, did not act as a transactivator, which is in agreement with previous studies on intact κ promoters, showing that transcription is absent when the octamer element has been mutated. Based on these data, and the conservation of the 5′-CAGCTG-3′ motif among human and murine κ promoters, we propose that AP-4 is the major ligand for Ig-κ promoter E-boxes.

Key words: bHLH-Zip protein, conserved sequences, immunoglobulin genes.

INTRODUCTION

Immunoglobulin (Ig)-κ V gene sequences from humans and mice have been classified into a number of subgroups and families, respectively, according to sequence similarities in their coding regions [1,2]. However, *V*κ gene conservation also extends into the promoter regions. Although the octamer motif and the TATA box are the only elements that are conserved in all Vκ promoters, other elements are conserved within different subgroups and families [3,4]. The octamer interacts with the Oct family of transcription factors [5]. It is essential for the normal function of Ig promoters, since mutations in the octamer that abrogate Oct binding result in levels of transcription similar to those observed with a TATA box only [6–8]. However, a minimal Ig promoter containing an octamer as the sole upstream element can only stimulate 10–20 % of the transcriptional activity seen with an intact κ promoter [7,9]. Hence, κ promoters are dependent on additional functional DNA elements for full activity [9,10]. These co-stimulatory elements, however, are not capable of stimulating transcription by themselves but are strictly dependent on the presence of an octamer element for activity. Thus κ promoter function is based on functional modularity [10].

Some of the co-stimulatory DNA elements are conserved within Vκ subgroups/families and between species, and some are not [4]. Examples of the former are the E-box motifs that are conserved within the human Vκ subgroups I, III, IV, V and VII and the related murine families, and the CCCT motif that is conserved within the human Vκ II subgroup and its related murine families [4]. Interestingly, the majority of the E-boxes found in Vκ promoters are of the E2A type (5′-CAGNTG-3′/5′-CANCTG-3′) [4], indicating that they are potential recognition sites for the *E2A* gene products E47 and E12 [11]. However, this represents a paradox, since the *E2A* gene products are intrinsically potent activators of transcription [12–14]. Previous studies have indicated that basic helix–loop–helix (bHLH) proteins interact *in vitro* with κ promoter E-boxes [15,16]. However, E47, although reported to bind optimally to the E-box sequence 5′-CAGCTG-3′ [17], interacted poorly with κ-promoter E-boxes [18], indicating that some other bHLH protein might be the E-box binding factor.

AP-4 is a ubiquitously expressed transcription factor of the bHLH-Zip subgroup of bHLH proteins, that binds the 5′-CAGCTG-3′ motif [19]. It was first identified as a factor binding to the simian virus (SV)40 enhancer, and was shown to interact synergistically with AP-1 to increase transcription *in vitro* from the SV40 late promoter [20]. Dimerization of bHLH proteins through the helix–loop–helix domain is a prerequisite for specific DNA binding via a short region of basic residues. In addition, bHLH-Zip proteins possess a second dimerization domain, the leucine zipper [21]. AP-4 is unique in that it contains an additional leucine zipper. Hence, AP-4 presents a specific tripartite dimerization structure, suggesting that AP-4 may interact with a wide variety of transcription factors [19]. However, there are no known heterodimerization partners of AP-4. Recently, the *Drosophila* homologue of human AP-4 has been cloned, and shows a high degree of similarity to the human and *Caenorhabditis elegans* counterparts within the bHLH-Zip domain, the second leucine zipper and a third conserved region of unknown function [22]. In the present work, we show that AP-4 is a ligand for κ-promoter E-boxes, indicating an important and novel role for this ubiquitously expressed regulator in the expression of lineage-restricted genes.

EXPERIMENTAL

Plasmids

A DNA fragment encoding full-length AP-4 [17] (a gift from Dr R. B. Gaynor, Department of Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75235-8594, U.S.A.) was subcloned into the pGEM®-3Z vector (Promega, Madison, WI, U.S.A.). To create an expression plasmid containing a Myc-

Abbreviations used: bHLH, basic helix–loop–helix; bHLH-Zip, bHLH zipper; EMSA, electrophoretic mobility-shift assay; HIV-1, human immunodeficiency virus type-1; Ig, immunoglobulin; LPS, lipopolysaccharide; pd, pentadecamer; SV, simian virus.

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epitope tagged version of AP-4, AP-4 cDNA was PCR amplified using the 5′ primer: 5′-d(GCGGATCCCATATGGAGTATTTCATGGTGCCCACT) and the 3′ T7 primer: 5′d(TAATACGACTCACTATAGGG) with the AP-4–pGEM3Z vector as template. The amplified fragment was excised with *Bam*HI and *Eco*RI, and cloned into a *Bam*HI/*Eco*RI-excised MD3 vector resulting in plasmid AP-4MD3. The MD3 vector contains six copies of the myc-epitope inserted into pcDNA3, as described in [14]. The pcDNA3 vector encoding Syrian hamster E47 cDNA (E47-pcDNA3), and the expression plasmid encoding a Myc-epitope-tagged version of E47 (E47 cDNA cloned into MD3–E47MD3) are also described in [14].

The 4 × pd-luciferase reporter plasmid was made by inserting a PCR-amplified 4 × pd fragment into the basal *fos* reporter (pGL3*fos*) [14]. The pGL3*fos* contains sequences −56 to +109 from the c-*fos* promoter cloned into pGL3Basic (Promega). The 4 × pd-fragment was made using the 5′ SP6 primer: 5′d(TATTTAGGTGACACTATAG) and the 3′ primer: 5′d(ATAAGATCTAA TCATGCCTGCA), with the 4 × pdTATACAT-E construct as a template [15]. The PCR product was digested with *Bgl*II, and ligated into the *Sac*I/*Bgl*II-digested pGL3*fos*. The 4 × pdpGL3*fos* plasmid was sequenced over the 4 × pd insert.

The 2 × (μE2μE5)*fos* plasmid was made by introducing two copies of the μE2–μE5-containing region of the Ig heavy-chain intron enhancer into the pGL3*fos*, as described in [14]. Plasmids MD3, E47-pcDNA3, E47MD3, pGL3*fos* and 2 × (μE2μE5)*fos* were kindly provided by Dr. M. Sigvardsson.

Nuclear extracts, proteins translated *in vitro* and electrophoretic mobility-shift assay (EMSA)

Nuclear extracts from B-lymphoid (human: Raji, Namalwa, BJAB; murine: K46R and S194) and non-lymphoid (HeLa) cell lines, as well as from lipopolysaccharide (LPS)-stimulated (72 h) primary murine spleen cells were prepared according to Schreiber et al. [23]. *In vitro* translated AP-4 and E47 were made using the AP-4-pGEM3Z plasmid or the E47-pcDNA3 plasmid, respectively, and the TNT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. For EMSAs, 1 μl of *in vitro* translated proteins or 2 μl of nuclear extract were mixed with 1 μg of poly(dI-dC) in 15 μl of binding buffer [20 mM phosphate buffer (pH 6.0), 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, 0.01 % (v/v) Nonidet P-40, 0.1 mM NaCl, 100 μg/ml BSA and 4 % (w/v) Ficoll]. Samples were incubated for 5 min at room temperature before the addition of competitors or antibodies directed against E47 (1 μl, Santa Cruz sc-763 X; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or AP-4 (kindly provided by Dr R. B. Gaynor), and incubated for a further 15 min at room temperature. ^{32}P-Labelled probe samples (30000 c.p.m.) were added and the samples were kept at room temperature for 20 min before resolution by PAGE (5 % polyacrylamide gel) in TBE [0.09 M Tris/borate (pH 8.0)/2 mM EDTA].

Probes and competitors

^{32}P-Labelled or unlabelled oligonucleotides were annealed to unlabelled antisense oligonucleotides and used as probes or competitors respectively. The sense-strand sequence of each of the different oligonucleotides used in EMSA are shown in Figure 2(C).

Cells and transfections

Cells were cultured in RPMI with glutamine (Gibco BRL) supplemented with 7–8 % fetal-calf serum, 10 mM Hepes, 1 mM sodium pyruvate and 50 μg/ml gentamicin. HeLa cells were transfected with Lipofectin® (Gibco BRL) according to the manufacturer's protocol. Transfections were performed in 24-well plates and 2.5×10^5 cells/well were grown overnight to 40–60 % confluency. Subsequently, 0.6 μg of expression plasmid and 0.2 μg of reporter plasmid in 1 ml of serum-free medium (Optimem; Gibco BRL) containing 5 μl of Lipofectin® was added to each well. The transfection mixture was removed after 24 h and replaced with 1 ml of serum-containing medium. After incubation for a further 24 h, the cells were washed once in 1 ml of PBS (Gibco BRL) and harvested into 80 μl of reporter lysis buffer (Promega). The lysate was kept on ice for 10 min and then frozen at −70 °C for > 30 min. A sample (20 μl) of the lysate was assayed for luciferase activity using 100 μl of luciferase assay reagent (Promega) in a MicroLumat LB96D luminometer (EG & G; Berthold, Pforzheim, Germany). For each experiment, every combination of expression and reporter plasmids was co-transfected in triplicate, and experiments were independently repeated two or three times. Data shown are the mean values for each experiment normalized to the values obtained after co-transfection with reporter plasmid and empty expression plasmid.

SDS/PAGE and Western-blot analysis

SDS/PAGE and electroblotting were performed according to standard procedures [24]. Briefly, the reporter lysis buffer lysate from transfected cells (see above) was denatured in SDS loading buffer containing 100 mM dithiothreitol, and separated by SDS/PAGE (12 % gel). The proteins were transferred on to a Hybond-C nitrocellulose membrane (Amersham Pharmacia Biotech), which was blocked in 5 % (w/v) skimmed milk in PBST [0.05 % (v/v) Tween in PBS] for 30 min and washed three times for 5 min each time in PBST. Next, the membrane was incubated with a 1:2000 dilution of a mouse anti-myc antibody (Santa Cruz Biotechnology) in PBST and then washed as before. The secondary antibody, a rabbit anti-mouse horseradish peroxidase antibody (Santa Cruz Biotechnology), diluted 1:3750 in PBST, was added for 20 min and then removed by washing. Detection of the antibody–protein complexes by luminescence was performed using the ECL® kit (Amersham Pharmacia Biotech).

RESULTS

E-boxes in κ promoters

The regulation of Ig transcription using the SP6 κ promoter as a model (Figure 1A) has been studied previously [9,10,18]. This mouse κ promoter contains an octamer element [3,25] with a one-base mismatch from the consensus sequence, which is compensated for by octamer flanking nucleotides [26,27]. Furthermore, octamer-dependent, positive transcriptional-control elements are found 5′ and 3′ of the octamer. Functionally these can be divided into three regions: the pd-κY region, the CCCT motif and the 3′ E-box. The pd-κY region contains three protein binding motifs: an E-box of the E2A type, an A-T-rich region [3] and the κY element [28,29]. These three elements stimulate octamer-induced transcription in concert, since a mutation in any of the elements obliterates the positive effect of the whole region [16]. The CCCT motif and the 3′ E-box independently stimulate octamer-dependent transcription [10,18]. The activity of the various subregions of the SP6 κ promoter is summarized in Figure 1(B).

With the advent of sequence information from the human κ locus [2], it became evident that the SP6 κ promoter represents a composite of a typical human Vκ I and a Vκ II subgroup promoter [4]. Hence, the pd element is conserved in the human

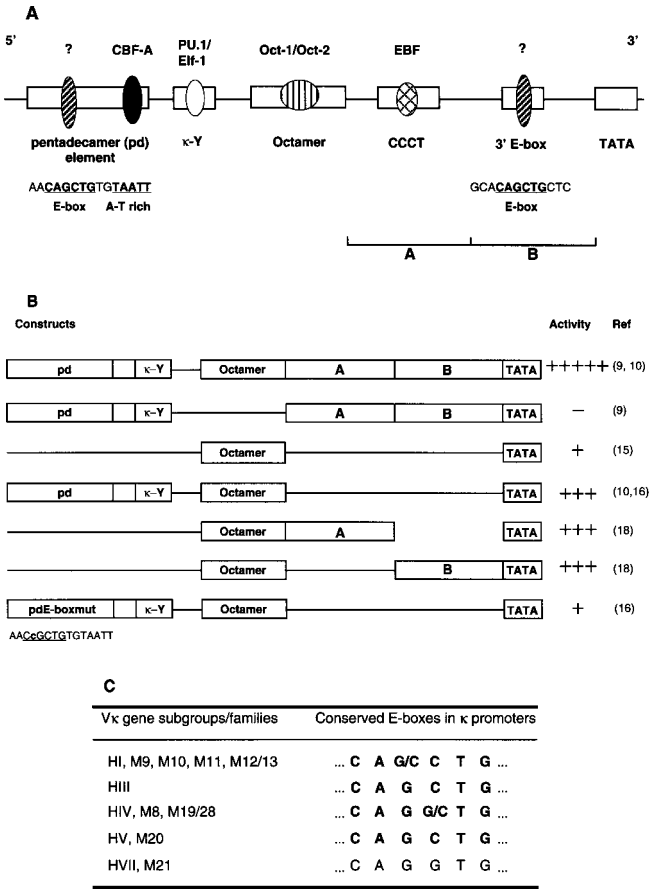


Figure 1 Schematic illustrations of the SP6 κ promoter and conservation of E-boxes in κ promoters

(**A**) The elements important for promoter function, and the proteins interacting with them are shown. Abbreviations: CBF-A, CarG-box binding factor-A; EBF, early B-cell factor. (**B**) Summary of the transcriptional activity of different promoter constructs. Ref, reference. (**C**) E-box sequences are conserved in human (H) and murine (M) κ promoters. Various human and murine Vκ subgroups and families are indicated on the left. The conserved E-box core consensus sequences are shown on the right. The sequences shown in bold typeface represent putative AP-4 binding sites.

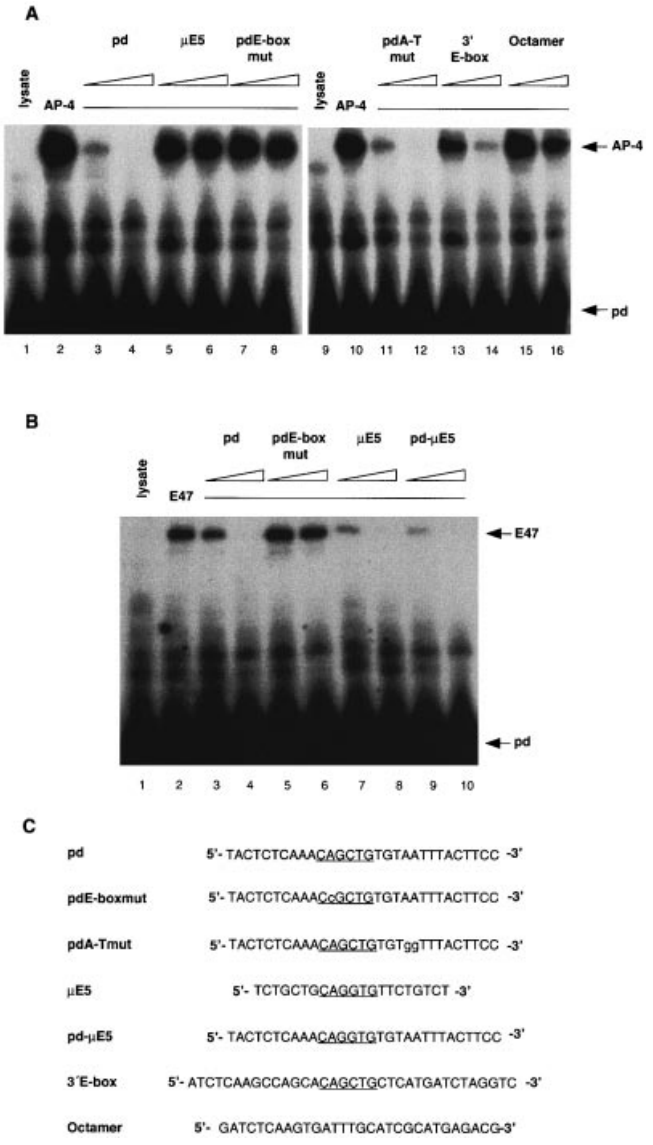


Figure 2 Both AP-4 and E47 bind the pd-element E-box in EMSA

(**A**) Binding of AP-4, translated *in vitro*, to a radiolabelled pd probe in the absence of competitor oligonucleotides (lanes 2 and 10), or in the presence of different unlabelled competitors as indicated (lanes 3–8 and 11–16). Competitors were added at 100- and 1000-fold molar excess. Lanes 1 and 9 contain reticulocyte lysate without template DNA. (**B**) Binding of *in vitro*-translated E47 to a radiolabelled pd probe, as in (**A**). (**C**) Sequences of the sense strands of the different oligonucleotides used in EMSAs. E-box core sequences are shown underlined. Mutated nucleotides are indicated by lowercase letters.

Vκ I promoters, whereas the CCCT element is conserved in Vκ II promoters. In addition, the E-boxes in κ promoters are well conserved, both with regard to sequence (5′-CAGCTG-3′; Figure 1C) and to their position relative to the octamer and the TATA box [4]. Given that the E-boxes in κ promoters show this high degree of conservation and functional activity, we favour the view that they are relevant transcriptional control elements for developmentally regulated Ig expression.

In vitro translated AP-4 and E47 bind to the SP6 κ promoter pd element

We have noted previously that, although the SP6 κ promoter E-boxes are candidate targets for *E2A* gene products, the results of an investigation of the relevant interaction using nuclear extracts were ambiguous [18]. Since the pd-E-box sequence 5′-CAGCTG-3′ is also a putative binding site for the bHLH-Zip protein AP-4 [20], the ability of E47 and/or AP-4 to bind the pd element *in vitro* was investigated. As shown in Figure 2(A), in an EMSA using AP-4 translated *in vitro* and the pd element probe, a complex was formed (lanes 2 and 10). The AP-4 complex was effectively removed by the addition of an excess of unlabelled pd element (lanes 3 and 4), and by a pd element with a mutated A-T-rich region (pdA-Tmut; lanes 11 and 12). In contrast, a pd element carrying a mutation in the E-box (pdE-box mut; lanes 7 and 8) failed to compete, as did the μE5 oligonucleotide (lanes 5 and 6) and the octamer oligonucleotide (lanes 15 and 16). These results indicate that *in vitro* translated AP-4 binds specifically to the pd-region E-box. We also investigated whether the E-box present in the SP6 κ promoter 3′-region interacted with *in vitro* translated AP-4. This was indeed the case, since an unlabelled 3′-region oligonucleotide also competed with the AP-4 complex (lanes 13 and 14).

The pd-element E-box was, as expected, also a target for *in vitro* translated E47 (Figure 2B). A complex formed with E47 and labelled pd probe (lane 2), was efficiently removed by an excess of unlabelled pd element (lanes 3 and 4) but not by pdE-box mut (lanes 5 and 6). The μE5 oligonucleotide (a known target for binding by E47 [30]) was at least as good a competitor as the pd element itself (lanes 7 and 8), and so was a composite oligonucleotide, pd-μE5, containing the μE5-motif and the flanking sequences from the pd element (lanes 9 and 10). Thus E47 translated *in vitro* has affinity for both the pd-element E-box as well as the μE5 motif (Figure 2C). Furthermore, the specificity of the interaction appeared to reside in the E-box core sequence and not in the flanking regions (compare μE5 with pd-μE5). AP-4, on the other hand, showed a strong preference for the pd-E-box, although it also bound the μE5 oligonucleotide when it was used as probe (results not shown).

AP-4, but not E47, binds to the pd element in nuclear extracts

Having established that *in vitro* translated AP-4 and E47 have affinity for SP6 κ promoter E-boxes we then investigated whether the pd element was bound by either AP-4 or E47 in nuclear extracts, and a series of gel retardation experiments, using nuclear extracts from human non-lymphoid (HeLa) and B-lymphoid cell lines (Raji, Namalwa and BJAB), were performed. Figure 3(A) shows the results obtained using the Raji cell line with the pd element as a probe; two major gel-retarded complexes were observed. The higher mobility band corresponds to CArG-box binding factor-A [31], whereas the lower mobility band showed a similar pattern of competition as *in vitro* translated AP-4 when using the same set of competitors. The addition of an excess of the unlabelled pd element (Figure 3A, lanes 2 and 3), the pdA-T mut (lanes 9 and 10) or the 3′ E-box (lanes 11 and 12) inhibited binding of the nuclear factor. Neither pdE-box mut (lanes 6 and 7) nor the octamer oligonucleotide (lanes 13 and 14) prevented formation of the specific complex, whereas μE5 competed slightly for complex formation in nuclear extracts (lanes 4 and 5). It should be noted that μE5 strongly inhibited complex formation between *in vitro* translated E47 and the pd element probe, whereas this was not the case when using *in vitro* translated AP-4 (compare Figure 2B, lanes 7 and 8, and Figure 2A, lanes 5 and 6). Similar results were obtained using nuclear extracts from the Namalwa, BJAB and HeLa cell lines (results not shown).

To unequivocally identify the pd-element binding factor, the specific complex was further analysed by the addition of antibodies. As shown in Figure 3(B), a polyclonal antibody against AP-4 [17] prevented formation of the complex (lanes 2–4), whereas an antibody directed against E47 did not (lane 5). Since both *in vitro* translated AP-4 and E47 could bind to the same site, but only AP-4 appeared to be involved in complex formation in nuclear extracts, we next addressed the question whether E47 was present in the nuclear extract preparations. The μE5 probe with Raji nuclear extracts as a source of protein was used in an EMSA. As shown in Figure 3(C), three gel-retarded complexes were obtained. The addition of antibodies against E47 (lane 3) and AP-4 (lane 2) showed that the slower migrating complex contained E47, whereas the faster migrating complex contained AP-4. We conclude from these data that AP-4 is the preferred pd-element ligand in nuclear extracts, even in the presence of E47, which has an intrinsic affinity for the pd-element E-box (Figure 2B).

AP-4 also binds to the 3′ E-box in the SP6 κ promoter

Figures 2(A) and 3(A) show that the addition of an excess of the 3′ E-box oligonucleotide inhibited binding of AP-4 to the pd probe, indicating that the 3′ E-box might also be a binding site for AP-4. In order to confirm this, we performed EMSAs with human B-cell lymphoma nuclear extracts and a labelled 3′ E-box probe (Figure 4A). Two complexes were formed, a specific low-mobility band and an non-specific band with higher mobility. Formation of the specific complex was inhibited by the addition of an excess of unlabelled 3′ E-box (lanes 2 and 3) and by the pd element (lanes 4 and 5), whereas addition of μE5 (lanes 6 and 7), pdE-boxmut (lane 8) or the octamer oligonucleotide (lane 9) did not compete for binding. The low-mobility band was shown to contain AP-4, since its formation was prevented by the addition of an anti-AP-4 antibody (Figure 4B, lanes 2–4) but not by an anti-E47 antibody (lane 5). In conclusion, these results demonstrate that AP-4 also is the preferred ligand for the SP6 κ 3′ E-box.

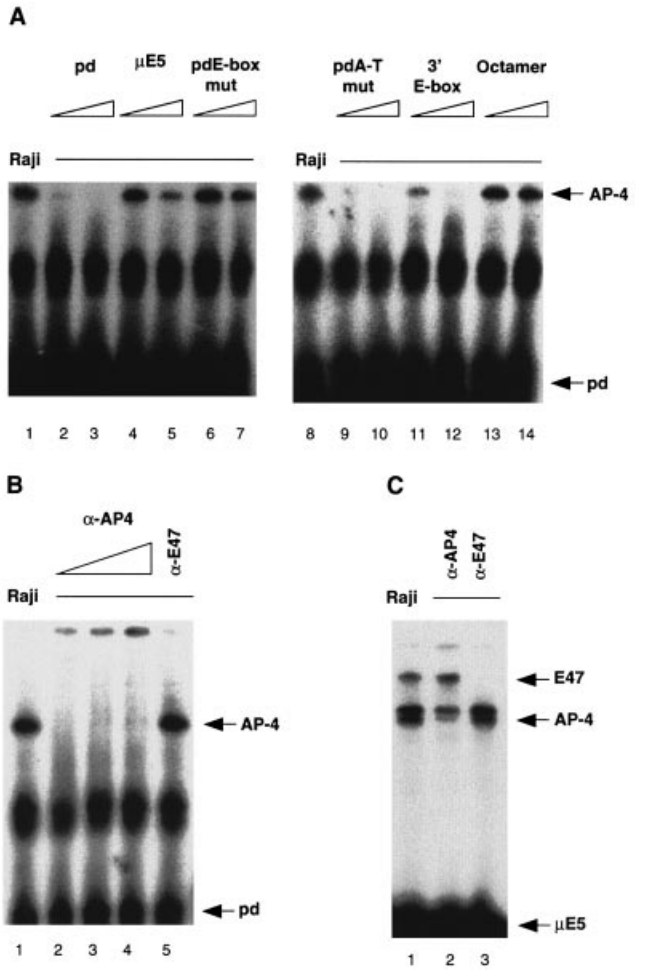


Figure 3 AP-4, but not E47, binds the pd-element E-box in nuclear extracts derived from the Raji cell line

(**A**) EMSA with nuclear extracts from the Raji (human B-cell lymphoma) cell line as a source of protein and a radiolabelled pd-element probe. Various unlabelled competitors, as indicated, were added in 100- and 1000-fold molar excess. (**B**) EMSA performed with Raji nuclear extract and a pd-element probe (lane 1), and with the addition of polyclonal antibodies against AP-4 (α-AP4; lanes 2–4, antibody diluted 1:10, 1:5, 1:2 respectively) or E47 (α-E47; lane 5). (**C**) EMSA with nuclear extracts from the Raji cell line as a source of protein and a radiolabelled μE5 probe, showing that the nuclear extract contains E47. Lane 1 shows the three gel-retarded complexes formed with the μE5 probe; the lower mobility band was absent after the addition of anti-E47 antibody (α-E47; lane 3), whereas the highest mobility band was removed by the addition of AP-4 antibody (α-AP4; lane 2).

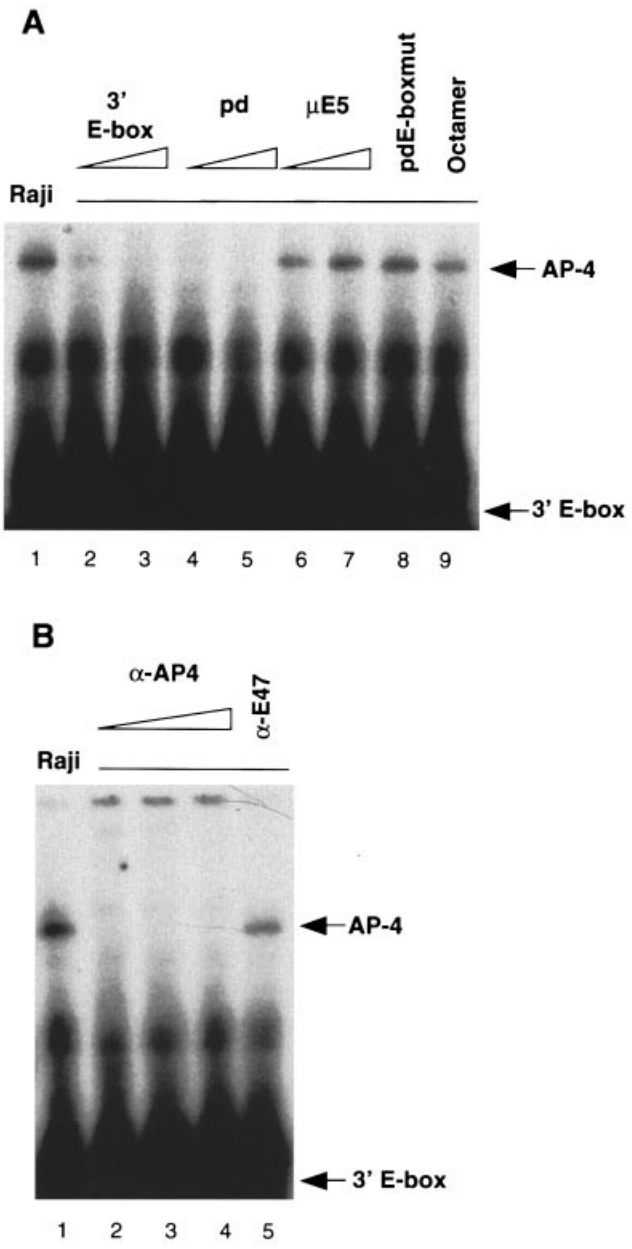


Figure 4 AP-4 binds the E-box present in the SP6 κ promoter 3′ region: EMSA with nuclear extracts from the Raji cell line and radiolabelled 3′ E-box probe

(**A**) The lower mobility band was removed by addition of a 200- or 1000-fold molar excess of unlabelled 3′ E-box (lanes 2 and 3) and pd element (lanes 4 and 5). The same concentration of the μE5 element did not affect the binding of the nuclear factor to the template (lanes 6 and 7). In a similar manner, a 1000-fold molar excess of the pd element with a disrupted E-box (pdE-boxmut; lane 8) and the octamer (lane 9) failed to compete. (**B**) EMSA with Raji nuclear extracts and the 3′ E-box probe (lane 1), and with the addition of an AP-4 antibody (α-AP4; lanes 2–4, antibody diluted 1:10, 1:5, 1:2 respectively), or an antibody against E47 (α-E47; lane 5).

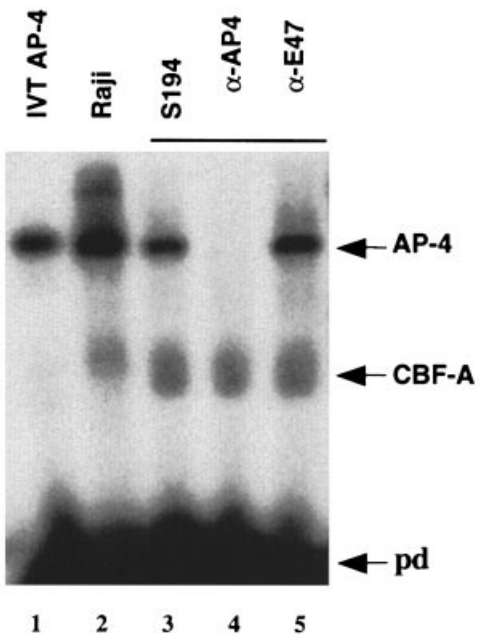


Figure 5 Complex between AP-4 translated *in vitro* and a pd element probe has similar mobility to the complex detected in nuclear extracts from the Raji and S194 cell lines

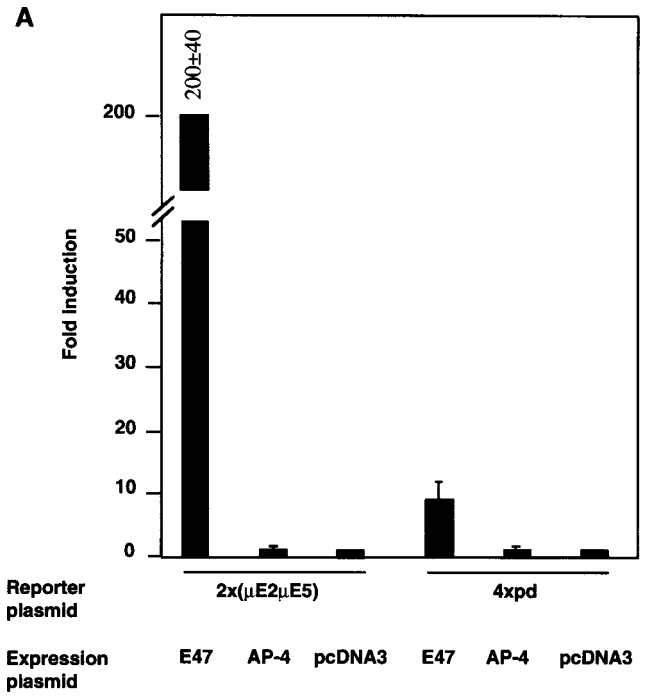
EMSA using the pd element as a probe with AP-4 translated *in vitro* (IVT AP-4; lane 1), nuclear extracts from the Raji (lane 2) or S194 cell lines (lanes 3–5). In lanes 4 and 5, antibodies against AP-4 (α-AP4) or E47 (α-E47) respectively were added.

AP-4 in nuclear extracts from murine B-cell lines binds to the pd element

The antibody against AP-4 was raised against the human AP-4 protein [17], and therefore all our initial experiments were made using nuclear extracts derived from human B-cell lines. However, since our model promoter is the murine SP6 κ promoter, we also tested for complex formation using the pd element and nuclear extracts derived from the murine lymphoid cell lines K46R (a B-cell lymphoma) and S194 (a plasmacytoma), as well as from primary LPS-stimulated murine spleen cells. Figure 5 shows the results of an EMSA using the S194 plasmacytoma cell line. A complex was detected (lane 3) with similar electrophoretic mobility to the complex formed using *in vitro* translated AP-4 (lane 1), and to that formed with nuclear extracts from the human Raji cell line (lane 2). Moreover, the S194 complex was removed by the addition of the anti-AP-4 antibody (lane 4) but not by the anti-E47 antibody (lane 5). Similar results were obtained with nuclear extracts from K46R and LPS-stimulated spleen cells (results not shown), indicating that AP-4, and not E47 or any other bHLH protein, was the preferred protein interacting with the pd element in murine B cells.

Ectopically expressed AP-4 does not transactivate a pd-element reporter construct

AP-4 was initially identified as an SV40 enhancer binding factor capable of stimulating SV40 transcription *in vitro*, and it has been reported to bind several viral and cellular enhancers. However, not much is known about the function of AP-4 in promoting transcription, in contrast to the well-known transactivation properties of E47. We wanted to compare the ability of AP-4 and E47 to transactivate a reporter construct containing the pd element as a regulatory element. We therefore made a reporter plasmid carrying four copies of the pd element linked to the c-*fos* minimal promoter and a luciferase reporter gene. This reporter was transiently co-transfected into HeLa cells, together



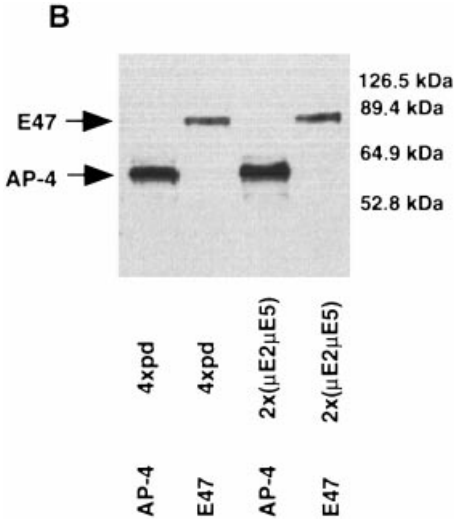


Figure 6 Comparison of the ability of AP-4 and E47 to transactivate reporter constructs carrying different E-box motifs as regulatory elements

(**A**) HeLa cells were co-transfected with plasmids expressing either AP-4 or E47 or empty expression vector (pcDNA3) and a luciferase reporter plasmid carrying either two copies of the μE2–μE5 sites [2 × (μE2μE5)], linked to a minimal *fos* promoter or a multimerized pd element (4 × pd). Fold induction was calculated relative to the level of luciferase expression obtained with pcDNA3. (**B**) Western-blot analysis of lysates used to measure luciferase activity as in (**A**), showing that Myc-tagged AP-4 is expressed equally well as the Myc-tagged E47. Lysates were from co-transfections using the indicated expression and reporter plasmids. The proteins were detected with a Myc antibody and the arrows indicate the positions of Myc-tagged AP-4 and Myc-tagged E47 respectively. Molecular-mass standards are indicated on the right.

with an expression vector encoding Myc-tagged versions of either AP-4 or E47. The ability of AP-4 and E47 to transactivate a luciferase reporter carrying two copies each of the μE2 and μE5 motifs as regulatory element was also analysed. As shown in Figure 6(A), E47 transactivated the pd-construct poorly (about 10-fold), whereas the μE2–μE5-construct was very efficiently activated (200-fold). In contrast, AP-4 did not transactivate any of the reporter constructs. The Myc-tagged versions of both AP-4 and E47 were efficiently expressed in the transfected cells, as seen in Figure 6(B), which shows a Western-blot of the same lysates as those used for assaying luciferase activity in a representative transfection experiment. In addition, the Myc-tagged version of AP-4 bound the pd probe efficiently in an EMSA (results not shown). In addition, co-transfection experiments using untagged AP-4 were performed and similar results to those where the Myc-tagged version was used were obtained (results not shown). Thus we conclude that, at least in the context of the pd element, AP-4 is not a transactivator but must act via alternate mechanisms (see below). The low level of activation induced by epitope-tagged E47 on the 4 × pd-reporter when compared with its effect on the μE2–μE5-construct is probably due to E47 being excluded from the pd-E-box site by endogenous AP-4, since no binding of E47 to the pd probe was detected in EMSAs when using nuclear exctracts as a source of protein (Figure 3B). In addition, we did not detect E47 binding to the pd probe in nuclear extracts prepared after transfection of HeLa cells with the E47 expression plasmid, whereas binding to the μE5 probe was readily detected (results not shown).

DISCUSSION

In this study our goal was to identify the ligand for the two E-boxes present in the SP6 κ promoter. Using synthetic mutant or wild-type pd element oligonucleotides, in addition to a wild-type 3′ E-box oligonucleotide in EMSA experiments, we have shown that the bHLH-Zip protein AP-4 interacts in a specific fashion with both E-boxes. Both *in vitro* translated AP-4 and nuclear-extract-derived AP-4 exhibited similar competition patterns and, in nuclear extracts, the major protein–DNA complex formed was shown to contain AP-4 when specific antibodies were used. Experiments were carried out with human B-cell lymphomas (Raji, Namalwa and BJAB) and with several murine cell lines and similar results were obtained. The majority of the E-boxes found in κ promoters are putative AP-4 binding sites (Figure 1C), and it has been speculated that the original κ gene promoter contained a TATA-box, an octamer element and possibly an E-box [32]. Since the latter element is well conserved in κ promoters [4], and our findings show that AP-4 binds to both E-boxes in the SP6 κ promoter, we would like to propose AP-4 as the major ligand for κ promoter E-boxes.

It should be noted that the pd element is a weak stimulatory element when present as four copies in a minimal promoter [15], indicating that none of the pd-element ligands should be strong transcriptional activators. Furthermore, the activity of each of the SP6 κ co-stimulatory elements is octamer-dependent, precluding the possibility of a strong activator binding to any of these sites and thereby overuling the function of the octamer-binding proteins. This functional profile of pd interacting proteins fits nicely with the transfection data shown in the present study, where no stimulation was seen with AP-4 but the potent activator function of E47 disqualified it as a pd-element ligand, given that all κ promoter transcription should be octamer-dependent [6–8,10]. *In vitro* translated E47 showed a robust interaction with the pd probe in EMSAs, whereas no interaction between E47 and the pd probe was detected when nuclear extracts were used. This might be due to AP-4 being more abundantly expressed than E47 and/or having a higher intrinsic affinity for the pd element.

AP-4 has been shown to interact with sites in both promoter and enhancer regions of viral and cellular enhancers. For

instance, a sequence conserved in several enhancers of pancreatic cell-specific genes is also conserved within the amylase 2A enhancer and contains an AP-4-recognition sequence. Mutation of this site inactivates enhancer activity and abolishes binding of AP-4 *in vitro* [33]. In addition, the enhancers of the human proenkephalin gene [34], the metallothionein IIA gene, the polyoma virus and the SV40 virus [20,35], as well as the bovine leukaemia virus long-terminal repeat [36], the distal regulatory region of the porcine insulin-like growth-factor binding protein-2 gene [37], the human angiotensinogen gene promoter region [38] and the human immunodeficiency virus type-1 (HIV-1) TATA element [17], have all been shown to interact with AP-4. The exact molecular mechanisms by which AP-4 influences transcription at these different sites is, however, not known and in several cases AP-4 has even been proposed to repress gene expression. For example, binding of AP-4 to the HIV-1 TATA element *in vitro* was found to compete for TATA-box binding protein binding to this element, and to inhibit *in vitro* transcription from the HIV-1 long terminal repeat [17]. Co-transfection of an expression vector containing AP-4 coding sequences down-regulated the expression of reporter constructs containing the human angiotensinogen gene promoter [38] and diminished Tax-activated expression from a construct containing the bovine leukaemia virus long-terminal repeat [36]. Within the late promoter region of SV40, AP-4 appears to be involved in conferring significant levels of nuclease sensitivity, thus implicating AP-4 in chromatin remodelling [35]. Based on its homology to transcription factors Myc and Max, it has been proposed that AP-4 might bind its target sequence even when incorporated into nucleosomes, thereby allowing other transcription factors to bind in a co-operative manner [35]. The results of the present study are in agreement with the function of AP-4 being that of a 'nucleating' protein in the context of the SP6 κ promoter. Here, its function would be to facilitate other protein interactions, rather than being an activator *per se*. This is also in agreement with earlier data on the orientation dependency of the pd element, and that the pd element does not synergize with an Sp1 element [16]. It is of interest that AP-4 also bound the pd-E-box in nuclear extracts from a murine plasmacytoma (S194), since this cell line has been used to show that the transcriptional synergy between the pd element and the octamer is specific for cell lines representing late stages of differentiation [15].

In conclusion, we have provided evidence that the ubiquitous transcription factor AP-4 is the major E-box ligand within κ promoters. This finding would indicate that there is a division of labour within the Ig loci, where the *E2A* gene products primarily interact with and regulate enhancer elements, whereas AP-4 would be the primary promoter E-box ligand. The further consequences of this finding with regard to developmental regulation of the Ig loci and communication between enhancer and promoter elements remain to be clarified.

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