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Pentadecamer-Binding Proteins: Definition of Two Independent Protein-Binding Sites Needed for Functional Activity

MIKAEL SIGVARDSSON,* MATS BEMARK, AND TOMAS LEANDERSON

Immunology Unit, Lund University, S-223 62 Lund, Sweden

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The SP6 κ-promoter pentadecamer (pd) element was found to be unable to stimulate transcription when present in one copy as the only promoter element in a minimal promoter but showed weak stimulatory activity when present as a multimer (four copies). One copy of the pd element acted synergistically with an octamer element, but not with a SP1 site, to stimulate transcription. The effect was orientation dependent with regard to the pd element. Gel shift analysis showed that pd-binding proteins were expressed in transformed as well as nontransformed B lymphocytes, irregardless of their differentiation stage, and in HeLa cells. Two major complexes, binding to different sites within the pd element, were observed in gel shifts. A low-molecular-weight form dominated in resting cells, while a higher-molecular-weight form appeared after mitogenic stimulation. Southwestern analysis showed that the low-molecular-weight pd-binding protein had a molecular mass of 35 kDa, which was confirmed by fractionation by denaturating polyacrylamide gel electrophoresis and molecular sieving. The higher-molecular-weight complex was sensitive to detergent treatment, while the low-molecular-weight complex was not. Mutation analysis showed that the two pd-binding complexes interacted with distinct sites within the element and that dual occupancy was required for functional activity. The functional synergy between the pd element and the octamer was more pronounced in plasmacytomas than in B-cell lymphomas.

Transcriptional stimulation from immunoglobulin (Ig) promoters has been studied in great detail. The most prominent conserved sequence element in the Ig promoters is the octamer element (5) which interacts with the Oct family of transcription factors. In the Ig heavy-chain promoters, another conserved element called the heptamer element (11) is also present. Although this element has only limited sequence homology with the octamer motif, it also interacts with the Oct proteins, but this interaction is dependent on the presence of a proximal octamer motif (11). While an octamer-proximal heptamer element is absent in Ig light-chain promoters, these promoters contain another conserved sequence motif called the pentadecamer (pd) element with the consensus sequence 5′-TGCA GCTGTGNCNAN-3′ (5). The pd element has so far not been extensively analyzed with regard to protein interactions but often includes an E box (5′-CANNTG-3′) (4, 6). It has been analyzed functionally and shown to act synergistically with the octamer element to stimulate transcription of a reporter gene in transient transfections (7). The notion can hence be raised that the pd element is the functional homolog to the heptamer element in the Ig light-chain promoters and that these two elements have been conserved to ensure a high rate of transcription from rearranged Ig genes together with the octamer elements. Since it has been shown that a single octamer element in a promoter position suffices to create a B-cell-specific promoter (16), the effect of the heptamer and pd element should be quantitative rather than qualitative, which indeed has been confirmed (7, 11).

In this article, we characterize the SP6 κ-promoter pd element-binding proteins functionally as well as biochemically and find that their expression is not restricted to B lymphocytes. Hence, while the Ig heavy-chain promoters rely on binding multiple copies of the Oct proteins to achieve high transcriptional stimulation, the Ig light-chain locus has evolved a different mechanism using a ubiquitous transcription factor in synergy with the Oct proteins to fulfill the same function.

* Corresponding author. Mailing address: Immunology Unit, Lund University, Sölvegatan 21, S-223 62 Lund, Sweden.

MATERIALS AND METHODS

Purification of B cells and transient transfections. The spleens were aseptically removed from the animals, and single-cell suspensions were prepared and washed in balanced salt solution. Erythrocytes and T cells were removed from the suspensions, and then the cells were cultured in RPMI medium (Gibco, Paisley, United Kingdom) containing 7.5% fetal calf serum and preactivated by the addition of 25 μg of lipopolysaccharide (LPS) (*Escherichia coli* O55:B5; Sigma) per ml for 48 h. To obtain resting B lymphocytes, cell suspensions were separated on discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradients, while activated cells were recovered by Ficoll (Pharmacia) separation prior to transfection. The B-cell lines were grown in RPMI supplemented with 2.5% fetal calf serum, and log-phase cells were used directly in transfections after washing.

The expression vectors were constructed in the pGem3Z plasmid (Promega, Madison, Wis.) using the chloramphenicol acetyltransferase (CAT) gene as a reporter gene and an Ig heavy-chain intron enhancer 3′ of the reporter gene (7). The sequences of the promoter variants analyzed are given in the figures. DEAE-mediated transient transfections of LPS-stimulated mouse splenic B cells and B-cell lines were performed essentially as described previously (7), and all experiments were repeated at least three times. The cells were transfected with 10 μg of plasmid DNA for 45 min in 20°C and recultured for 48 h before analysis. After the cells were harvested, protein extracts were prepared from the cells. The protein extracts were tested for CAT activity by incubation with 1 μCi of ^{14}C-labelled chloramphenicol (Amersham, Amersham, United Kingdom), the acetylated products were separated by thin-layer chromatography, and then the silica plates were exposed to X-ray film. For comparison, CAT conversion is expressed as a factor of the activity seen with a promoter containing an octamer with random flanking sequence and a TATA box for comparison throughout.

Gel shift assay. Protein extracts were made by the method of Schreiber et al. (13). Five micrograms of protein extract was then mixed with 2 μg of poly(dI-dC) (Boehringer, Mannheim, Germany) and binding buffer. The mixture was incubated for 5 min at room temperature. If a competitor was used, the competitor was mixed with the other components and incubated at 37°C for 15 min. ^{32}P-labelled probe (20,000 cpm) was then added, and the sample was incubated at 37°C for 25 min. The samples were separated on a 5% polyacrylamide–Tris-borate-EDTA gel. The gel was fixed, dried, and autoradiographed. Detergent sensitivity was tested by adding the indicated detergents Nonidet P-40 (Fluka, Buchs, Switzerland) and/or sodium dideoxycholate (Gibco) to the gel shift incubation mixture just prior to loading.

Southwestern blotting. Southwestern blotting was performed essentially as described by Miskimins et al. (10). Protein extract (50 μg), made by the method of Schöler et al. (12), was mixed in sample buffer, separated on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel, and electroblotted onto nylon membranes. The membrane was prehybridized with 10 mM HEPES (*N*-2-hydroxy-

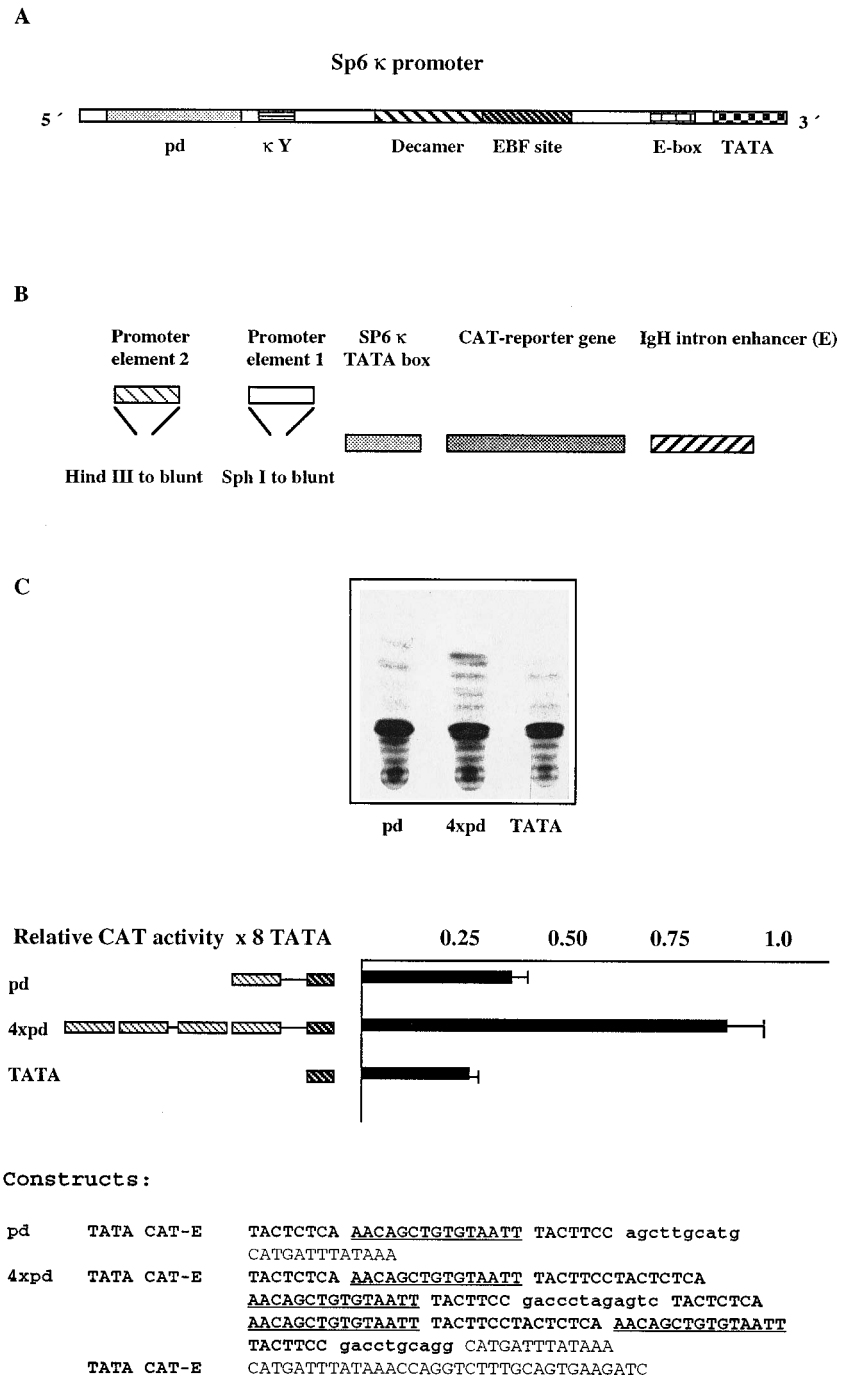
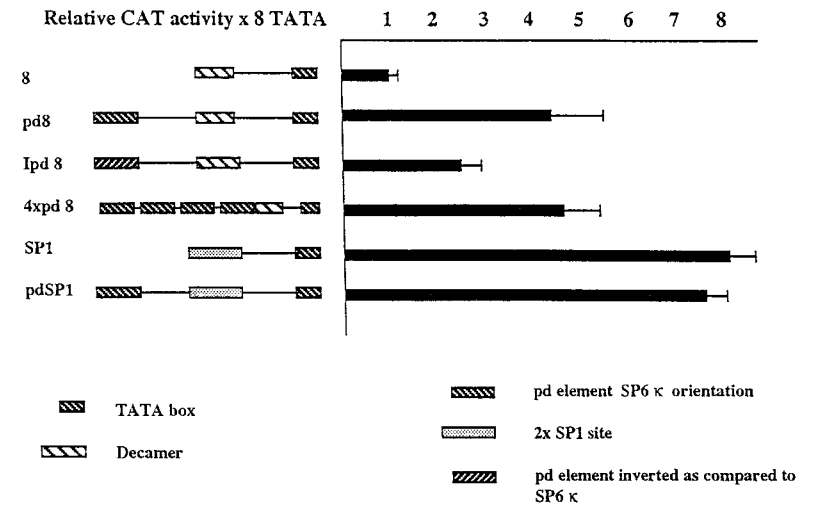


FIG. 1. Schematic figure of the SP6 κ promoter (A) and the plasmid constructs used for transfections (B). (C) Results of a CAT assay in which the indicated constructs had been transfected into nontransformed, LPS-stimulated mouse splenic B cells. (Top) Data from a representative experiment. (Middle) Data assembled from four independent experiments. (Bottom) Promoter sequence of the constructs in which all constructs contained the same TATA box sequence. The complete sequence is shown only for the TATA CAT-E construct. Capital letters refer to wild-type SP6 κ-promoter sequence, while lowercase letters indicate random sequence. Boldface type is used for sequence elements upstream of the TATA box. Underlining indicates the pd element. EBF, early B-cell factor.

ethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 8) with 5% nonfat dry milk, and after 1 h, oligo-pd probe (500,000 cpm/ml) was added. The probe was a tetramer of the pd element. Labelling was performed via a PCR utilizing SP6 and T7 promoter primers and ^{32}P-labelled dCTP. The probe was gel purified prior to use.

Preparative SDS-polyacrylamide gel electrophoresis (PAGE). The fractionation and gel shift assays of the fractions were performed as described by Baeuerle and Baltimore (1). Protein extract was precipitated at −20°C by the addition of 4 volumes of acetone, and then the pellet was dissolved in SDS loading buffer and boiled for 5 min. After electrophoresis, bands were cut out from different molecular weight regions on the basis of the position of a Rainbow molecular weight marker (Amersham) in a parallel lane. Proteins were eluted overnight at 4°C in 500 μl of elution buffer (10 mM Tris-HCl [pH 7.9], 0.1% SDS, 0.1 mg of bovine serum albumin per ml, 1 mM dithiothreitol, 0.2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 2.5% glycerol). The sample was cleared by centrifugation and concentrated by precipitation with acetone. After the pellet was washed with methanol, the dry pellet was dissolved in 2.5 μl of saturated urea solution and diluted with 125 μl of buffer (20 mM Tris-HCl [pH 7.6], 10 mM KCl,

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Constructs:
 8      TATA CAT E          TGATTTGCAT cgcatgagacgctaacatcgtacgttc CATGATTTATAAA
pd8     TATA CAT E   TACTCTCA AACAGCTGTGTAAT TACTTCC aagcttggatc
                           TGATTTGCAT cgcatgagacgctaacatcgtacgttc  CATGATTTATAAA
Ipd8   TATA CAT E    GGAAGTA AATTACACAGCTGTT TGAGAGTA aagcttgg
                           TGATTTGCAT cgcatgagacgctaacatcgtacgttc  CATGATTTATAAA
4xpd8   TATA CAT E   TACTCTCA AACAGCTGTGTAATT TACTTCCTACTCTCA
                     AACAGCTGTGTAATT TACTTCC gaccctagagtc TACTCTCA
                     AACAGCTGTGTAATT TACTTCCTACTCTCA
                     AACAGCTGTGTAATT TACTTCC gacctgcagg
                     TGATTTGCAT cgcatgagacgctaacatcgtacgttc          CATGATTTATAAA
SP1     TATA CAT E                CCGGCCCGCCCATCCCCGGCCCCGCCCATCC  CATGATTTATAAA
pdSP1   TATA CAT-E    TACTCTCA AACAGCTGTGTAATT TACTTCC agcttg
                          CCGGCCCGCCCATCCCCGGCCCCGCCCATCC  CATGATTTATAAA
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FIG. 2. Functional activity of the pd element in cooperation with other promoter elements after transfection into LPS-stimulated splenic B cells. The data were assembled from at least three independent experiments. Schematic diagrams of the promoters used are shown, and the sequences are shown in the lower part of the figure.

2 mM dithiothreitol, 10 μM phenylmethylsulfonyl fluoride). Renaturation was allowed to proceed for 18 h, and the protein was then stored at 4°C.

Ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography. Fifty microliters of J558 protein extract was precipitated with 40% ammonium sulfate for 15 min on ice and then spun in a tabletop centrifuge at maximum speed (12,000 × *g*) for 15 min at 4°C. The pellet was resuspended in 50 μl of a solution containing 20 mM Tris (pH 8.0), 100 mM NaCl, and 1 mM EDTA. The supernatant was moved to a new tube, and the ammonium sulfate concentration was increased to 50%. The proteins were precipitated for 15 min on ice and spun for 15 min as described above. The higher percentage precipitations were made as described above. Four-microliter samples of the fractions were used for electrophoretic mobility shift assay (EMSA) with the pd probe (1-μl samples with the decamer probe).

J558 protein extract (250 μl) was dialyzed against gel filtration running buffer (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA) for 3 h at 4°C with one buffer change and thereafter cleared by centrifugation before loading onto a Superose 12 column (Pharmacia). The flow rate was 0.4 ml/min, and 1-ml fractions were collected. Five microliters of each fraction was checked for binding activity by EMSA. Proteins used as size standards were bovine serum albumin (Sigma) (66 kDa), ovalbumin (Sigma) (46 kDa), and aprotinin (Sigma) (6.5 kDa) separated under the same conditions as described above.

For separation with ion-exchange chromatography, 250 μl of J558 protein extract was dialyzed into loading buffer (20 mM Tris [pH 8.0]) for 3 h at 4°C with one buffer exchange and then cleared by spinning before loading onto a prepacked Mono Q HR 5/5 (Pharmacia). Proteins were eluted by a linear gradient of elution buffer (20 mM Tris [pH 8.0], 500 mM NaCl) for 20 min at 0.5 ml/min, and 0.5-ml fractions were collected. Five microliters from each fraction was diluted to adjust the NaCl concentration and checked for pd-binding activity by EMSA.

Methylation interference. Radiolabelled oligonucleotides were methylated by the addition of 1 pmol of oligonucleotide to 200 μl of 50 mM Na cacodylate (pH 8), 1 mM EDTA, 1 μg of carrier DNA (pGem3Z), 20 μg of tRNA, and 0.5 μl dimethyl sulfate during 2 min. The reaction was terminated by the addition of 50 μl of 1.5 M Na acetate, 1 M 2-mercaptoethanol, 100 μg of tRNA per ml, followed by the addition of 750 μl of ethanol. The mixture was kept in liquid nitrogen for 2 min and then pelleted in a microcentrifuge. The pellet was dissolved in 250 μl of a solution containing 0.3 M Na acetate, 0.1 mM EDTA, and 25 μg of tRNA per ml and reprecipitated with 750 μl of ethanol, redissolved in 50 μl of 0.4 M NaCl, and precipitated with 1 ml of ethanol as described above. The precipitate was washed three times with 95% ethanol and left to dry at room temperature, and then the methylated DNA was used in a 10-times-scaled-up band shift reaction. The wet gel was maintained at 4°C for 2 h, and the bands representing bound and free DNA were excised, extracted in Tris-EDTA overnight, cleaved by boiling in 1 M piperidine for 25 min, and separated on a 10% polyacrylamide sequencing gel.

RESULTS

Functional analysis of the SP6 κ-promoter pd element. The SP6 κ-promoter pd element has previously been shown to stimulate transcription by acting synergistically with an octamer element (7). To investigate whether proteins interacting with the pd elements could activate transcription independently, we constructed transfection vectors containing the SP6 κ-promoter TATA box as the only promoter element and one or four copies of the SP6 κ-promoter pd element in a 5′ position. All the constructs used the CAT gene as a reporter gene and contained an Ig heavy-chain intron enhancer (2) in a 3′ position (Fig. 1B). These constructs were transfected into LPS-stimulated mouse splenic B cells and analyzed for transcriptional stimulation. The rationale for using untransformed cell populations for the functional analysis was to avoid any bias caused by aberrant expression of transcription factors or *onc* gene products in a chosen tumor cell line (8, 14). As shown in Fig. 1C, while one copy of the pd element did not stimulate transcription over background levels seen with a construct containing a TATA box only (1.5 times higher), the tetrameric

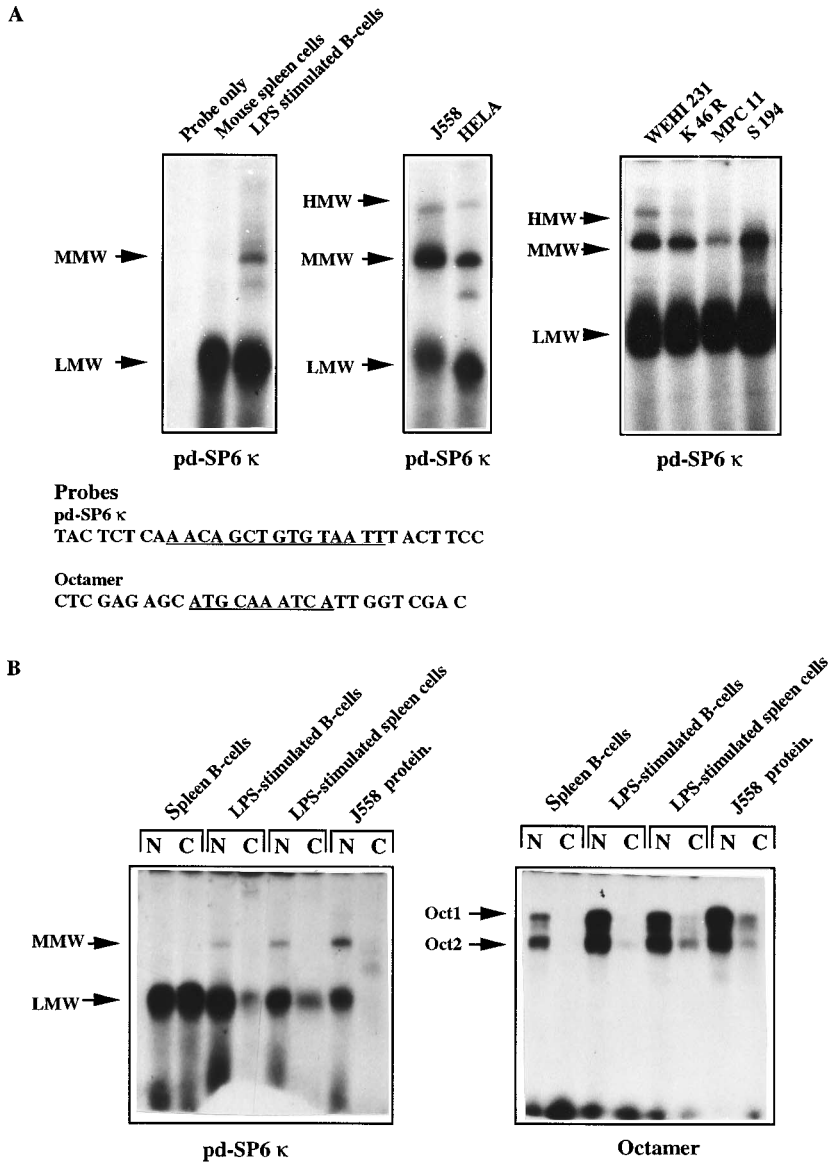

FIG. 3. (A) Protein-DNA complexes formed when the SP6 κ-promoter pd element was used as probe and mixed with protein extracts from the indicated cells in a gel shift assay. (B) Gel shift assays showing the differences in pd-binding activity between cytosolic (C) and nuclear (N) protein fractions from different cell populations (left gel). The right gel shows the same protein fractions tested for octamer-binding activity.

element showed a weak but significant stimulation of transcription (3.6 times the activity obtained with the TATA box only). We conclude that proteins interacting with the SP6 κ-promoter pd element contain weak activation domains for transcription.

Since the pd element is mostly present in only one copy in κ promoters, we further characterized its functional activity together with other transcription factor-binding sites (Fig. 2). First, DNA constructs were made in which a consensus octamer in the κ orientation with random flanking sequence was cloned 5′ of the SP6 κ-promoter TATA box in the presence or absence of a single pd element in the 5′ position (pd8 versus 8). A single copy of the SP6 κ-promoter pd element 5′ of the octamer had a synergistic effect on transcriptional stimulation, increasing the functional activity three times. Hence, a single-copy pd element exerts its transcriptional stimulatory effect via octamer-binding proteins. Inverting the pd element (Ipd8) decreased the synergistic effect between the two elements, indicating a need for properly arranged binding sites. Furthermore, the functional activity was not increased upon the addition of four copies of the pd element, showing that the binding of pd-binding factors was not a limiting parameter. To test whether the pd element could act synergistically with regard to transcriptional stimulation with any transcription factor, we constructed transfection vectors containing binding sites for SP1 in the presence and absence of the SP6 κ-promoter pd element in a 5′ position (pdSP1 versus SP1). As shown in Fig. 2, one copy of the SP6 κ-promoter pd element did not act synergistically with a SP1 site to increase transcriptional stimulation. We conclude that a single copy of the SP6 κ-promoter pd element acts synergistically with an octamer element but not with a SP1 motif to stimulate transcription, given that the elements are properly oriented relative to each other.

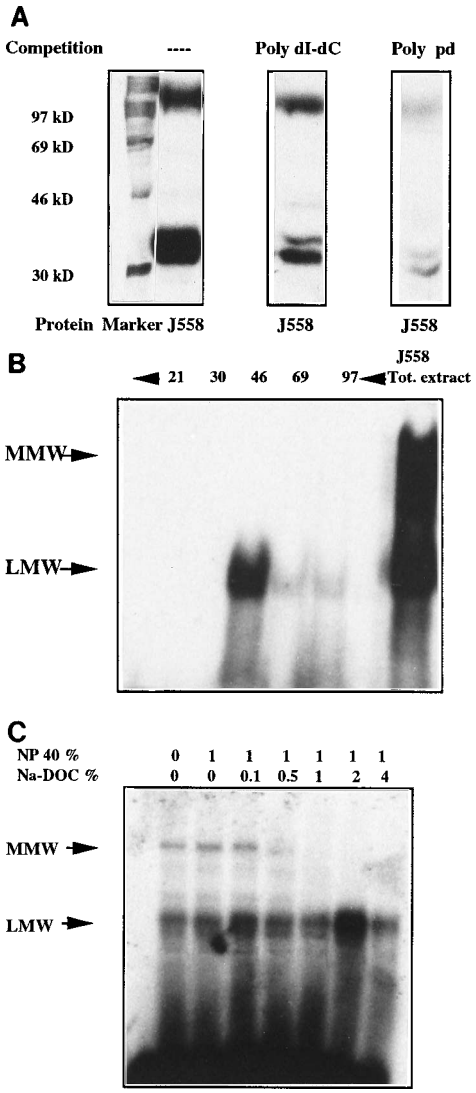

FIG. 4. (A) Southwestern blot of J558 protein extract, hybridized with a ^{32}P-labelled DNA probe consisting of four pd elements. The leftmost lane shows a molecular mass marker, and the next lane (----) shows the result obtained when no competitor DNA was added to the hybridization mixture. The other lanes shows the results of adding nonspecific [poly(dI:dC)] and specific competitor (unlabelled 4xpd probe). (B) Gel shift assays with J558 protein extract fractionated by SDS-PAGE are shown where the last lane shows the gel shift pattern of the unfractionated material. Tot. extract, Total extract. (C) Gel shift assay using protein extract from LPS-stimulated spleen cells where the indicated concentration of detergent was added to the gel shift incubation mixture. NP 40, Nonidet P-40; Na-DOC, sodium dideoxycholate.

Analysis of proteins binding to the SP6 κ-promoter pd element. To study the pd-binding proteins, gel shift assays were performed with a pd probe. Purified, resting mouse splenic B lymphocytes generated a low-molecular-weight gel shift, LMW (Fig. 3A). When the same cells had been stimulated for 48 h with the mitogen LPS, a medium-molecular-weight (MMW) gel shift appeared in addition to the LMW shift present in the resting cells. Protein extracts from the B-cell lymphoma cell line WEHI 231, the plasmacytoma cell line J558 and HeLa cells all contained LMW and MMW complexes and an additional high-molecular-weight complex, HMW. Extracts from the B-cell lymphoma cell line K46R and from the plasmacytoma cell lines MPC11 and S194 contained the MMW and

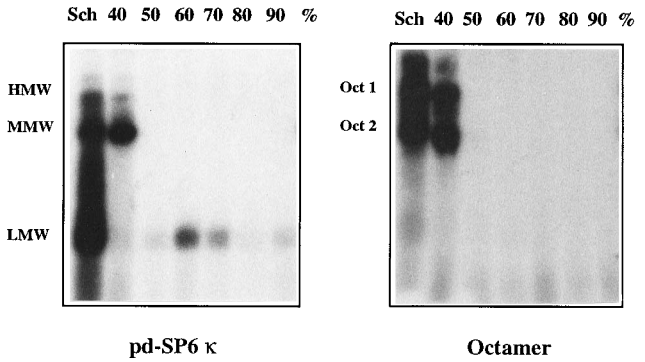

FIG. 5. Separation of MMW and LMW complexes by sequential ammonium sulfate precipitation. The percent saturation of ammonium sulfate is indicated above the gels. Sch, protein extract made by the method of Schreiber et al. (13) used as starting material.

LMW complexes. Thus, the pd-binding proteins are ubiquitously expressed and do not vary with the differentiation stage of the B cell line analyzed but vary with cellular activation.

We next analyzed the subcellular distribution of the pd-

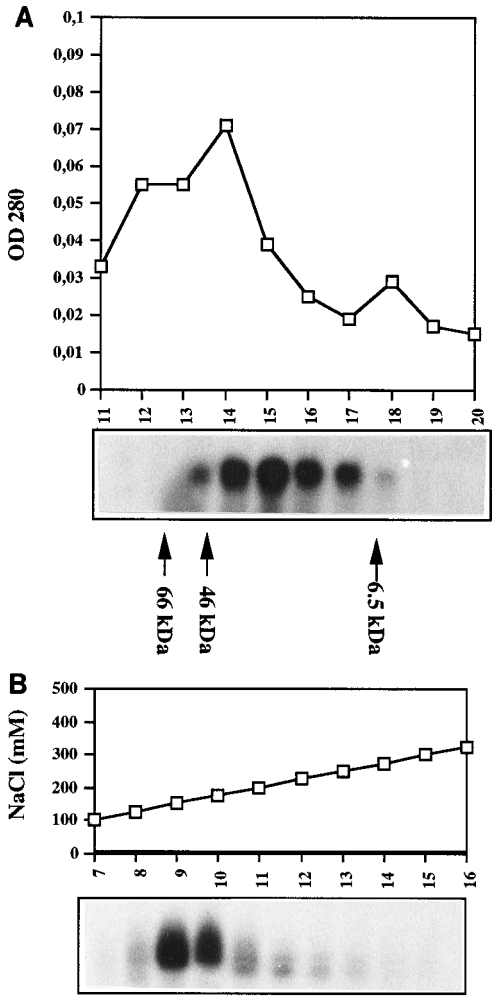

FIG. 6. Separation of protein extracts by size on Superose 12 column (A) and by ion-exchange chromatography Mono Q columns (B), as described in Materials and Methods. OD 280, optical density at 280 nm.

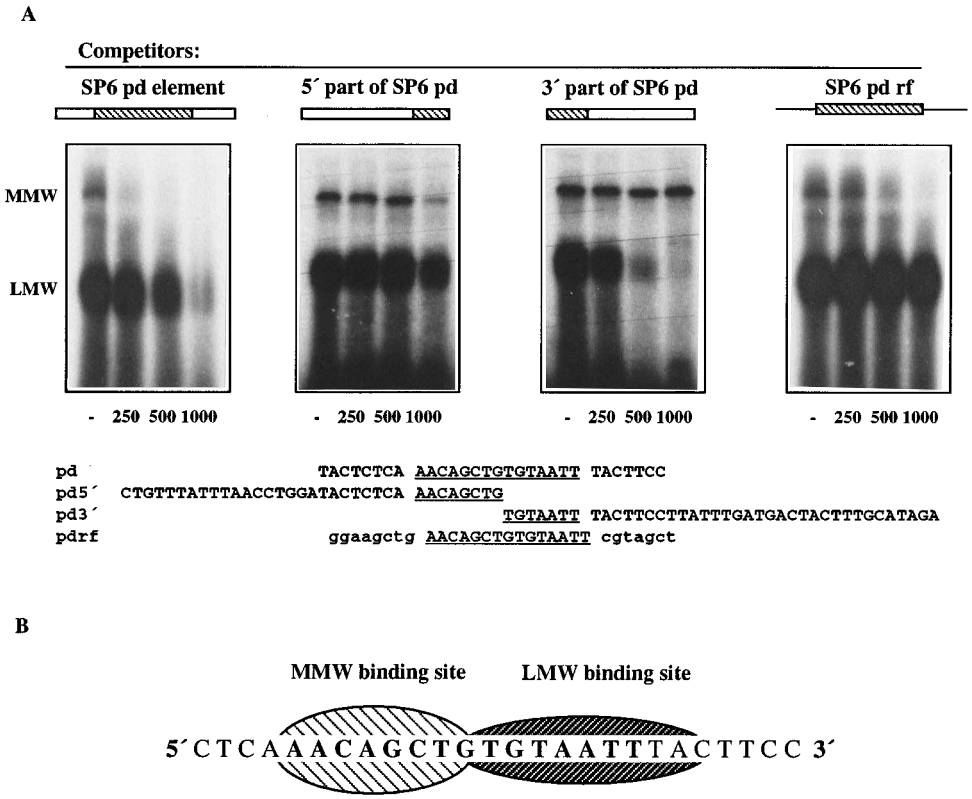

FIG. 7. (A) Characterization of the binding specificities of the LMW and MMW complexes by gel shift analysis using extracts from LPS-stimulated spleen cells. The SP6 κ-promoter pd element was used as a probe, and the competitors are indicated in the figure. (B) Schematic diagram of the binding sites for the two complexes.

binding proteins with cytoplasmic protein extracts and compared them in gel shift assays with the same amount of nuclear protein extracted from the same cell populations (Fig. 3B). Extracts from all cell types tested, except the normal resting B cells, had MMW and LMW shifts in the nuclear fraction (HMW could be detected in the J558 extract upon longer exposure), while only extracts from the cytosol generated LMW complexes. The resting B cells had only LMW protein both in the nucleus and the cytosol, but upon activation with LPS, the LMW activity in the cytosol became less abundant while there was additional formation of MMW complexes in the nucleus. When the experiment was repeated with a consensus octamer probe, the nuclear fraction contained virtually all the octamer-binding activity, giving an indication of the purity of the fractions as well as being a control for extract quality.

We have also tried to establish a relationship between the pd-binding proteins and other DNA-binding proteins by supershift experiments using antibodies (anti-Rel, -Ets, -Jun, -Oct1, -Oct2, and -Myc) as well as competition for pd binding with probes containing binding sites for known transcription factors (Oct, NF-κB, AP-1, AP-2, AP-3, and NF-AT). No interference with the pd-binding proteins has been detected with either of these approaches (data not shown).

One pd-binding protein has a molecular mass of 35 kDa and MMW complexes are formed by noncovalent interactions. To further characterize the pd-binding proteins and address the question of whether the multiple proteins bind the pd element, we made Southwestern blots with J558 protein extracts and hybridized them with a multimerized pd element (Fig. 4A). Two bands were readily detectable: one high molecular weight (>100 kDa) and one band with an apparent molecular mass of 35 kDa. When poly(dI-dC) was included in the hybridization mixture, the 35-kDa band was resolved into a doublet. Adding unlabelled, multimerized pd element as a competitor together with poly(dI-dC) in the hybridization mixture reduced the hybridization intensity with all bands.

The Southwestern data opened the possibility that several pd-binding proteins were expressed in J558 cells. Since the denaturation conditions for the Southwestern experiment were mild, we performed a preparative SDS-PAGE separation of the protein extracts (Fig. 4B). The protein was eluted from the gel slices and renatured, and the fractions were screened for pd-binding activity by gel shift. Figure 4B shows that protein extracts from J558 cells contained LMW pd-binding activity in the fraction ranging from 30 to 46 kDa. No pd-binding proteins could be detected in the higher-molecular-weight fractions, although the parallel lane with total J558 protein extract contains a high level of the MMW complex.

The result from the SDS-PAGE separation could be interpreted as indicating that the MMW complex was made up of noncovalently linked proteins. Complementation experiments in which negative fractions from the SDS-PAGE separation were mixed together in an effort to regenerate the MMW complexes were unsuccessful (data not shown). To address directly the stability of the MMW complexes, we performed gel shift assays with the pd element and protein extract from LPS-stimulated spleen cells after the addition of increasing amounts of detergent, as previously described (1). This experiment showed (Fig. 4C) that the MMW complex is sensitive to detergent treatment, while the LMW complex is not. We conclude that the main SP6 κ-promoter pd element-binding protein(s) (LMW) has an apparent molecular mass of 35 kDa,

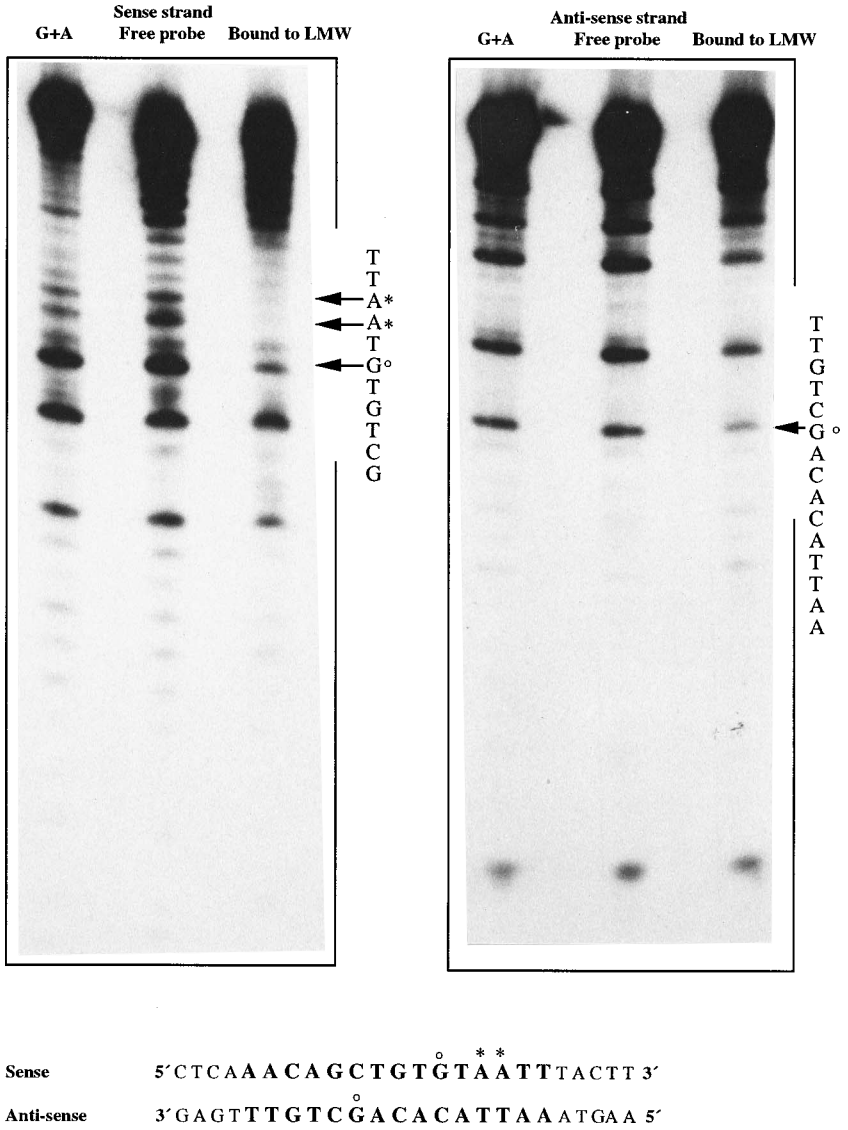

FIG. 8. Methylation interference assay of the LMW complexes from LPS-stimulated spleen cells. The asterisks indicate methylation-sensitive sites, and the circles indicate partially methylation-sensitive sites.

while the MMW complex is made up of noncovalently linked proteins.

To further characterize biochemically the pd-binding proteins, protein extracts were separated by either sequential ammonium sulfate precipitation, gel filtration, or ion-exchange chromatography. The Oct1 and Oct2 proteins precipitated at 40% ammonium sulfate saturation as did the MMW pd-binding protein(s) (Fig. 5). The LMW pd-binding protein(s) was retrieved after precipitation at 60 to 70% saturation. Hence, differential ammonium sulfate precipitation is a convenient procedure of separating the two pd-binding complexes. Upon separation on Superose 12 or Mono Q, only the LMW complex could be retrieved (Fig. 6A or B, respectively). The apparent molecular mass on the Superose column was in good agreement with that seen after preparative SDS-PAGE, while the LMW complex eluted from the ion-exchange column at 150 to 200 mM NaCl.

LMW and MMW complexes have distinct DNA-binding specificities. To determine the binding specificities of the LMW and MMW pd-binding complexes, competition experiments with various oligonucleotides in gel shifts were performed (Fig. 7A). The pd oligonucleotide competed with both protein-DNA complexes, while an oligonucleotide containing only the 3′ part of the pd element competed with only the LMW complex. An oligonucleotide containing only the 5′ half of the pd element competed poorly with both complexes. That no competition was observed for the MMW complex was surprising but is most likely explained by the fact that the competitor ends immediately after the last base in the E-box motif (see Fig. 9 for further analysis). To establish whether the protein-binding sites extended outside the classical pd element (5), an oligonucleotide containing a minimal pd element with random flanking sequence was used as a competitor. This oligonucleotide competed with the MMW complex but not with the LMW complex. Thus, the two protein complexes had different DNA-binding specificities; the MMW complex bound within the classical pd element in the E box containing the 5′ part of the element, while the LMW complex was formed with

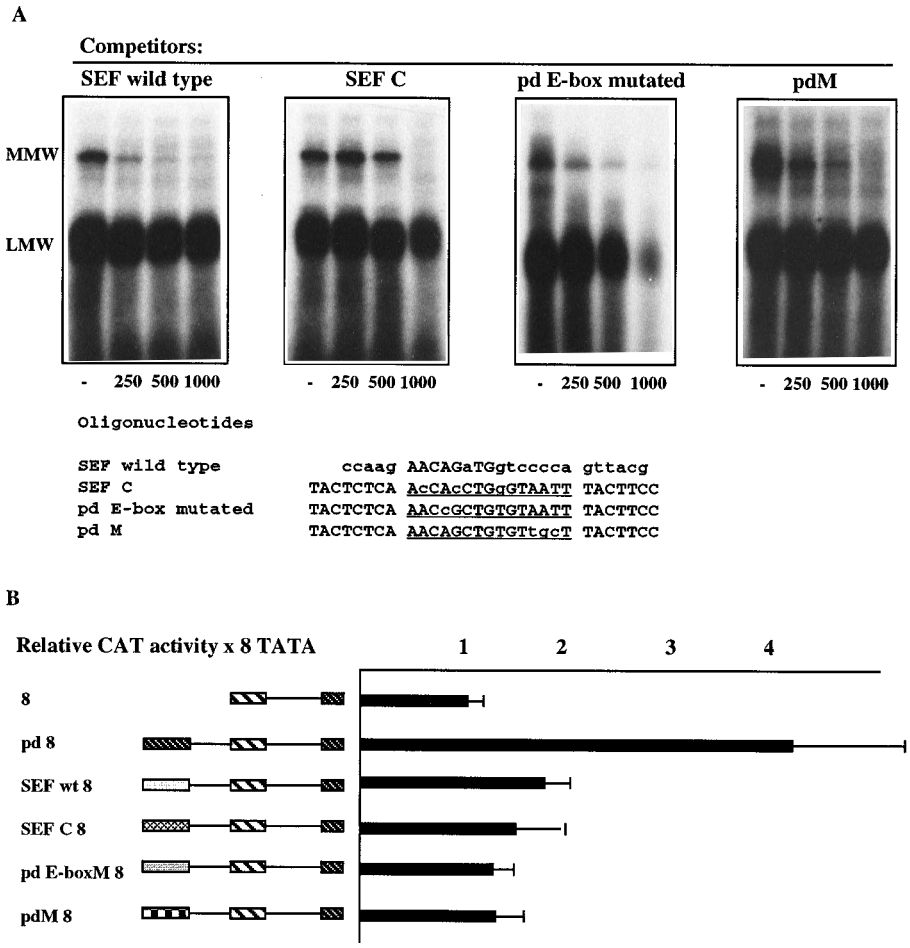

FIG. 9. (A) Binding activity of the indicated pd mutant by competition to binding of SP6 κ-pd element in protein extracts from LPS-stimulated spleen cells. (B) Functional activity obtained when the pd mutant oligonucleotides were coupled to a decamer element and transiently transfected into LPS-stimulated spleen cells.

the A/T-rich 3′ part of the element and its binding site extended outside the classical pd element (Fig. 7B).

To obtain a more detailed binding site for the LMW protein, a dimethyl sulfate methylation interference assay was performed with protein extract from LPS-stimulated spleen cells. Figure 8 shows the result of this analysis in which methylation of two A's on the sense strand in the 3′ end of the element interfered strongly with LMW binding. Partial interference was seen after methylation of a G on the sense strand proximal to the A nucleotides above and of a single G on the central part of the antisense strand as indicated in Fig. 8. These data confirmed that the 35-kDa protein contacts the DNA in the 3′ part of the element.

Double occupancy of the LMW and MMW pd-binding complexes is required for functional activity. To further clarify the positions essential for protein binding and functional activity of the pd element, a set of mutant SP6 κ-promoter pd elements were generated. These mutants were first used as competitors in gel shifts (Fig. 9). Since the MMW complex was hypersensitive to the addition of detergent and binds to the E box containing part of the pd element, the MMW complex might consist of a protein complex of the helix-loop-helix family. Indeed, the binding sequence shows a strong resemblance to that of a protein of the SL3-3 enhancer factor (SEF) family (3, 9), and SEF-binding sites were accordingly used as pd competitors. The wild-type SEF site has a low degree of homology to the pd element (Fig. 9A), with the homology restricted to the E-box motif, but competed efficiently for the formation of the MMW pd complex while not competing for the formation of the LMW complexes. The E box in the pd element matches perfectly with a SEF A site (3, 9), and to confirm the SEF-like features, competitions were made with a oligonucleotide in which the E-box region was turned into a SEF C site, thereby introducing three mutations in the pd element while conserving the E-box core element. Interestingly, this mutation created a perfect match to the μE4 site found in the Ig heavy-chain intron enhancer. This oligonucleotide competed less efficiently for the binding of the MMW complex than did the SEF wild-type oligonucleotide and only weakly for the binding of the LMW complex. Hence, the MMW complex has binding specificity resembling, but not identical to, a helix-loop-helix protein of the SEF family. A pd oligonucleotide containing a single point mutation disrupting the E box competed less efficiently with the MMW complex than did the SEF wild-type competitor while competing for the LMW complex (for comparison, see Fig. 7 for pd element competition). When the pd 3′ AAT motif, containing two methylation-sensitive A's, was changed to TGC, this oligonucleotide (pdM) did not compete for LMW pd binding but did compete for the formation of the MMW complex (Fig. 9A). The function of the pd element

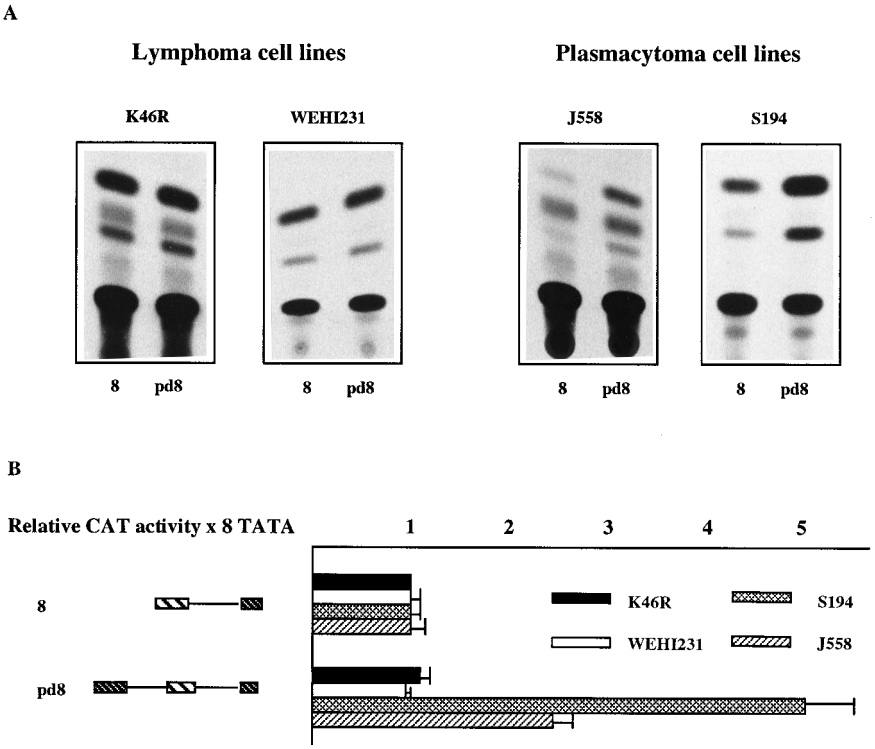

FIG. 10. The pd-decamer synergy obtained with the same 8 and pd8 constructs as used in Fig. 2 when transiently transfected into the indicated cell lines.

mutants was tested by transient transfections in LPS-stimulated spleen cells, as described above. None of the constructs could act synergistically with a decamer to the same degree as that of the wild-type pd element (Fig. 9B), showing that neither the MMW nor the 35-kDa protein alone suffices for the decamer costimulatory activity of the pd element.

The synergy between the SP6 κ-promoter pd element and an octamer motif may be differentiation specific. To address the question of whether cooperation between the pd and octamer elements to stimulate transcription were regulated during B-cell differentiation, we transfected two B-cell lymphoma cell lines (K46R and WEHI 231) and two plasmacytomas (S194 and J558) with the 8 and pd8 constructs, respectively. As can be seen in Fig. 10, a synergistic effect with regard to transcriptional stimulation between the SP6 κ-promoter pd element and the octamer was readily observed in the plasmacytomas while no synergy was detected in the lymphomas. Thus, the pd-octamer synergy may be regulated temporally during peripheral B-cell differentiation.

DISCUSSION

The pd elements are present in essentially all Ig light-chain promoters and often located within 20 bp from an octamer motif, although great variability exists. The pd element seems to be build up around a central conserved G, and the positions proximal to this nucleotide also show a low variability between the different pd elements, while the variability increases in the distal ends of the motif (5). The degeneracy of the pd element should be seen in the context of the octamer elements present in the same set of promoters. These promoters often contain more than one octamer element which frequently contain mismatches compared with the consensus octamer motif (15). Hence, both these promoter elements are frequently redundant as well as degenerated, arguing that compensatory mechanisms are active so that the final promoter configuration is determined by its ability to stimulate transcription, i.e., function, rather than a strict conservation of given sequence elements and the spacing between them.

In this article, we characterize the SP6 κ-promoter pd element with regard to function and protein binding. The SP6 κ-promoter pd element could activate transcription when present in four copies 5′ of a TATA box. This result would indicate that the pd-binding proteins contain activation domains capable of interacting with the transcriptional machinery. Since the pd elements are present in only one copy in Ig light-chain promoters, we would like to argue that its primary function is to act synergistically with the octamer element to stimulate transcription. The synergistic effect is restricted since the synergy was active together with an octamer element while not observed when the pd element was linked to a SP1 motif. In addition, the synergy was seen only when the pd element had the correct (Ig light chain) polarity with regard to the octamer. This result indicates that the communication between Oct proteins and pd-binding proteins involves distinct domains, and we envision that this communication involves even more complex protein interactions including transcriptional adapter molecules.

Utilization of the pd element as a probe in gel shift analysis of cell extracts showed that proteins binding specifically to the pd element were expressed in B cells as well as in HeLa cells. Furthermore, the similarity of the protein complexes seen in a B-cell lymphoma (WEHI 231) and those in a plasmacytoma (J558), which express significantly different levels of light-chain transcripts, argues that the expression of pd-binding proteins are not directly correlated either with the level of Ig light-chain expression or the differentiation stage of the B cell. An interesting point, however, is the observation that only the LMW

complex can be detected in gel shifts using cell extracts from resting B cells, while extracts from mitogen-stimulated B cells also contain the MMW complex. Hence, the pd-binding proteins are modified by cellular activation. Furthermore, the finding that the LMW complexes were present in the cytosol while MMW complexes were detected only in the nucleus suggests that the MMW is mainly a nuclear protein or a complex formed by a nuclear factor and the cytosolic protein. Upon cellular activation, the amount of cytosolic LMW complexes decrease, indicating that a translocation of the LMW complex components from the cytosol to the nucleus could be occurring. The characterization of the pd-binding proteins revealed that distinct proteins bind to the 5′ and 3′ part of the element, respectively. Mutation of one of these sites destroyed functional activity, showing that dual occupancy of the MMW and LMW complexes is critical for synergy between the pd and octamer elements. The MMW complex interacts with the 5′ part of the SP6 κ-promoter pd element, is precipitated by 40% saturation of ammonium sulfate, and is sensitive to detergent treatment. This complex is also related to helix-loop-helix proteins of the SEF type (3, 9), since SEF-binding sites can compete for MMW formation. The LMW complex consists of protein(s) with an apparent molecular mass of 30 to 46 kDa under reducing and nonreducing conditions. It is puzzling that we are unable to recover the MMW complex after gel filtration or ion-exchange chromatography. One possible explanation is that the MMW complex consists of two subunits that interact in a template-dependent fashion and that the subunits are separated by size and charge but are both precipitated at 40% ammonium sulfate saturation.

By methylation interference analysis, a contact region between the SP6 κ-promoter pd element and the LMW pd-binding protein(s) was mapped to the 3′ part of the element. Mutation of three nucleotides within this region severely impaired protein binding as well as functional activity, which was defined as the ability to cooperate with an octamer element to stimulate transcription. The last point that merits discussion was the finding that the increased transcriptional stimulation from an octamer motif induced by the presence of a pd element seemed to be active only in cell lines representing late stages of the peripheral B-lymphocyte differentiation pathway (plasmacytomas). The LPS-stimulated splenic B cells used for the transfection analysis also represents a late, secretory differentiation stage in the B-lymphoid pathway. Since no differences in the expression of pd-binding protein(s) could be detected in B lymphomas and plasmacytomas, the mechanism for the difference in functional activity cannot simply be at the level of accessibility of these proteins. Rather, we believe that transcriptional adapter molecules that interact with both the octamer-and pd-binding proteins may be differentially expressed during peripheral B-cell differentiation. The characterization and isolation of such adapter molecules is necessary in order to elucidate the mechanisms behind the regulation of κ transcription.

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