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Functional modularity in the SP6 κ promoter

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ABSTRACT

The requirements of the SP6 κ promoter for transcriptional activation were studied in non-transformed murine B lymphocytes stimulated with lipopolysaccharide. Three different DNA motifs, besides the TATA-box, were needed for restoration of transcriptional activation to the same magnitude as seen with the native SP6 κ promoter. The decamer motif (TNCTTTGCAT) was found to induce transcription alone and point-mutation of this element reduced transcription to negligible levels, although the other two required elements were present. The penta-decamer element (TGCA^{G}/$_{C}$CTGTGNCCAG) did not stimulate transcription alone, but activated transcription synergistically in conjunction with the decamer motif. This synergism required the presence of a third pyrimidine rich element (CCCT) in the decamer 3′ flanking sequence. The pyrimidine rich element could partly be substituted for by an E-box core motif (CANNTG) 3′ of, but not by the κY motif (C-TTCCTTA) 5′ of, the decamer. Proteins interacting specifically with the penta-decamer element were detected by band-shift assay. The decamer 3′ flanking sequence of the SP6 κ promoter was found to modify the binding of endogenous Oct2 isoforms to the decamer motif i B lymphocytes, but not in CHO cells transfected with various Oct2 isoforms. Thus, complex protein/DNA interactions can be observed in the SP6 κ promoter which correlate functionally with a synergism in transcriptional activation.

INTRODUCTION

The genes encoding the heavy and light chains of the immunoglobulin (Ig) molecule are expressed in B lymphocytes only. This restriction of expression is primarily obtained by recombination events within both the heavy and light chain loci in B lymphocytes only (1, 2), as well as tissue specific transcriptional control mediated by promoter (3, 4) and enhancer regions (5–8). Within these regions conserved DNA motifs have been described. The most well defined motif is the decamer, TNATTTGCAT (3, 5, 7). This motif is highly conserved in the promoters for Ig heavy and light chain genes and in the Ig heavy chain (IgH) intron enhancer. It has also been found in the promoter region of other ubiquitously expressed genes (9, 10). At least three different proteins in B lymphocytes bind to the decamer motif. The first, Oct1 (11), is ubiquitously expressed whereas the other two, Oct2a (12) and Oct2b (13), are expressed in B lymphocytes, brain, kidney and sperm of the adult mouse but also during mouse embryogenesis (14). Oct1 is encoded by a separate locus while the Oct2-proteins are derived through differential splicing of a single gene (15). The IgH intron enhancer contains besides a decamer motif so called Ephrussi-boxes (E-boxes), with the core sequence CANNTG, which were initially described as protein binding sites by *in vivo* dimethyl sulphate (DMS) assays (21, 22). Protein/DNA interactions have also been defined by *in vitro* technique (23–27). Functional analysis revealed that the decamer (26, 28, 29) and the E-boxes E (30), μE1 (28, 30), μE3 (29, 30) and μE4 (29) were important for enhancer activity. The κ intron enhancer (8, 31) contains three E-boxes; all contributing to the enhancer activity (28). In addition, the κ intron enhancer contains a functionally important motif recognised by the transcription factor NFκB (32, 33, 28).

In the IgH promoter three functionally active promoter elements have been described (60, 3, 20). These are the pyrimidine rich region, the heptamer (CTCATGA) and the decamer motif. In Ig light chain (IgL) promoters the penta-decamer (pd) motif was defined by sequence homology studies (3), while expression studies have not revealed any function for this element (16, 17). The pd motif is also present in the IgH intron enhancer (3) and in the promoter of the joining chain (J-chain) gene (18). Recently a third DNA motif, κY (19) CTTCCTTA, was shown to stimulate decamer induced transcription in a κ promoter where the decamer motif contained one base mismatch as compared to the consensus sequence. In this report we analyse the SP6 κ promoter in detail and find novel functional interactions between distinct promoter elements. Thus, Ig transcription seems to be regulated by interactions between several proteins binding to discrete DNA elements as well as protein/protein interactions.

MATERIALS AND METHODS

Preparation of B lymphocytes, cell culture and transient transfection

In all experiments mice of the CBA/H strain were used (Alab, Stockholm, Sweden). Purification of B lymphocytes from spleen cells was performed by lysis of erythrocytes with Gay's solution as described (34), followed by lysis of T lymphocytes by treatment with the anti-Thy-1 antibody J-1-j (a kind gift from Dr. H. von Boehmer, Basel) and guinea-pig complement (Behring, Germany). The remaining B lymphocytes were washed in Hank's balanced salt solution (Flow Laboratories) and cultured at 37°C in Iscove's modified Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, antibiotics and

50 μM β-mercaptoethanol in humidified air with 5% CO_2. Lipopolysaccharide (*Escherichia coli* 055:B5, Difco) was added to the cultures at 50 μg/ml. A cell density of 5×10^6 cells/ml was used for the initial 48 h of culture and 1×10^6 cells/ml were used for reculture after the transient transfection. Cells were purified over Ficoll (Pharmacia) before the transfection. The transient transfection of normal murine B lymphocytes was performed exactly as described (35).

Chloramphenicol acetyltransferase assay

The transfected ceils were suspended in 50 μl sonication buffer (10 mM Tris, 0.5 mM $MgCl_2$, pH 7.4) after being washed in Hank's balanced salt solution. The cells were then freezed/thawed on dry ice/ethanol and 37°C waterbath three times and sonicated at 50 kHz for 15 minutes. The cytosol fraction was recovered after centrifugation at maximum speed in a microfuge (Eppendorf 5415) for ten minutes. One fourth of the obtained extract was mixed with 0.2 μCi ^{14}C-chloramphenicol (50 μCi/mmol) and 0.25 M Tris, pH 7.8, to final volume 100 μl. After five minutes of preincubation at 37°C 20 μl 4 mM acetyl coenzyme A was added and the incubation continued for 85 minutes at this temperature. The reaction was stopped by extraction with 400 μl ethylacetate. The organic phase was lyophilised, dissolved in 15 μl ethylacetate and applied onto a thin layer plate (Silica gel 60, Merck). Acetylated and non-acetylated chloramphenicol was separated in 95:5 (v/v) chloroform/methanol on a thin layer plate, visualised by autoradiography and the amount of acetylation determined by scintillation counting. The CAT-activity was expressed as percent acetylated chloramphenicol.

Transfection vectors

All transfection vectors used originate from the plasmid construct L-CAT-E (35). The only differences between the constructs used in this study are located in the promoter regions 5′ of the TATA-box. In the κ′-CAT-E construct a 135 bp ScaI-BglII fragment of the SP6 κ promoter is inserted as a promoter (see Figure 1). All other constructs contain the oligonucleotide D which carry the TATA-box region of the SP6 κ promoter (see Figure 1). The different oligonucleotides used were ligated 5′ of this region in the promoter to obtain the different transfection vectors used.

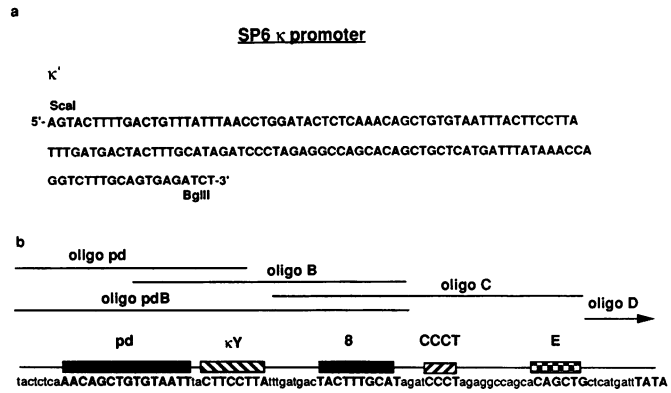

Figure 1. The SP6 κ promoter sequence. The sequence of the promoter fragment used for the analysis (κ′) is shown (panel a). The various sequence elements discussed are indicated as well as the position of the oligonucleotides used in the current analysis (panel b).

All the oligonucleotides were synthesised at the Department of Immunology, BMC, Uppsala, Sweden on a 380A DNA synthesiser, Applied Biosystems.

Oct expression vectors

Full-length mouse Oct2a and Oct2b cDNA clones were isolated by cross-hybridisation with a human Oct2 cDNA clone and subcloned into a RSV-neo expression vector. The pEV-oct-1 (58) vector was a kind gift from Dr Walter Schaffner, Zurich. The expression vectors were introduced into CHO cells by electroporation and transfected clones were selected by their ability to grow in G418 (BRL). In the case of the pEV-oct-1 vector, RSV-neo (1/20) was co-transfected to allow for selection in G418.

Electrophoretic mobility shift assay (EMSA)

The electrophoretic mobility shift assay (EMSA) was performed essentially as described (36) and the cell extracts used in the assays were made as described (14). Between 50 to 150 pg probe and 2 μg poly-$(dIdC)_2$ were used per reaction. Competition experiments were performed with 1000 fold excess of the unlabelled competitor DNA relative to the probe. Protein binding

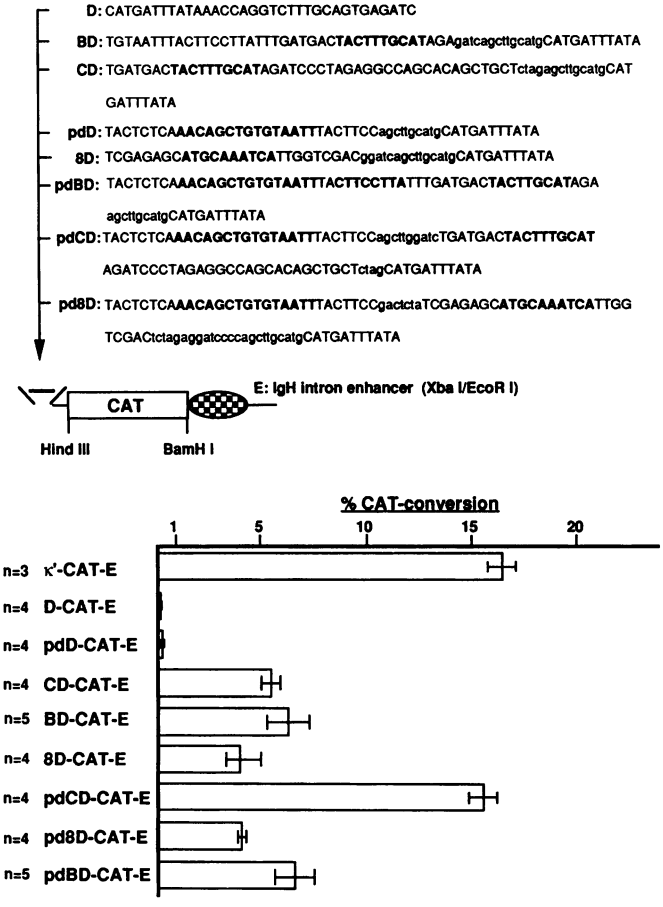

Figure 2. Synergism in transcriptional activation. Upper part: The sequence of the various promoter variants used for transfection experiments are shown in the upper part of the figure, for κ′ sequence see Figure 1 (small letters indicate plasmid sequence). Also a schematic build up of the transfection vector containing the CAT-gene as reporter gene with the IgH intron enhancer 3′, is shown. Lower part: Transcriptional activity of different promoter regions expressed as % CAT-conversion (calculation see Material and Methods). 'n' denotes the number of independent experiments.

was visualised by electrophoresis through a 6% polyacrylamide gel and subsequent autoradiography of the gel. All probes used in the EMSA were radioactively labelled with T4-polynucleotide kinase or the Klenow fragment as described (37).

RESULTS

Synergism between distinct DNA elements for transcriptional activation in the SP6 $\varkappa$ promoter

The sequence from −142 to −1 ($\varkappa'$) (ScaI to BglII) of the SP6 $\varkappa$ promoter (38) is shown in Figure 1a. In Figure 1b the promoter elements defined by others (3, 19) or in this study (see below) have been highlighted in the sequence for orientation. Also shown are the different oligonucleotides which were used for construction of promoter variants. The promoters were inserted into a plasmid vector, as indicated in Figure 2, containing the CAT gene as a reporter gene and the Ig heavy chain intron enhancer in a 3′ position. The actual sequences of these promoter variants are also shown in Figure 2, where the sequence in lower case letters represents polylinker sequence from the cloning vector.

The transcriptional activity of these promoters was tested in transient transfections of LPS stimulated mouse B lymphocytes, as previously described (35), and the result of these experiments is shown in the lower part of Figure 2. The $\varkappa'$ promoter, as a positive control, induced 16 to 17% CAT-conversion while a TATA-box as the only promoter element, negative control, gave approximately 0.1% CAT-conversion. The 8D promoter, containing a consensus decamer element flanked by random sequence, stimulated transcription less efficient than the $\varkappa'$ promoter. This was also true for the promoters BD and CD, both containing the decamer motif and 5′ or 3′ flanking sequence derived from the SP6 $\varkappa$ promoter. These two latter promoters were, however, 1.5 to 2 times more efficient than the 8D promoter in stimulating transcription. The pdD promoter had no significant effect on transcription in our system. In contrast, when the pd element was fused to the SP6 $\varkappa$ decamer motif with intact 3′ flanking sequence (pdCD), the transcriptional stimulation equalled that obtained with the $\varkappa'$ promoter; the effect on transcriptional activation being synergistic. In contrast, when the pd element was linked to the SP6 $\varkappa$ decamer containing the 5′ flanking sequence (pdBD), or to the consensus decamer containing random flanking sequence (pd8D), no synergy on transcriptional activation was observed. We conlude from these experiments that an additional sequence motif present in the SP6 $\varkappa$ decamer 3′ flanking region was needed for the observed functional synergy between pd and decamer elements.

The SP6 $\varkappa$ decamer 3′ flanking sequence contains two DNA elements that stimulate transcription

The constructs made to analyse the 3′ flanking sequence are schematically described in Figure 3. In the pdC5D promoter the 13 bp sequence directly 3′ of the decamer motif is derived from the same position in the $\varkappa'$ promoter and the next 13 bp sequence

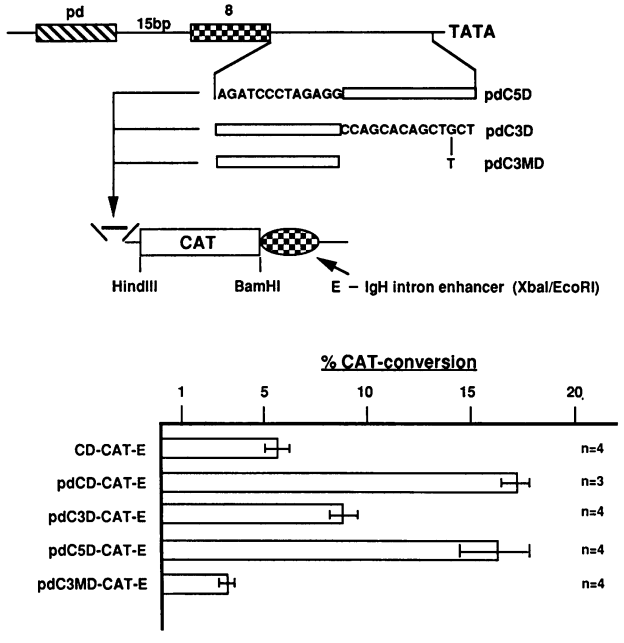

Figure 3. Decamer flanking sequence required for full promoter activity. Upper part: A schematic description of the three promoters used to analyse the sequence region between the decamer motif and the TATA-box. Open bars denote exchange of bases in the sequence of this region as compared to the $\varkappa'$ sequence of the same region. The sequences for conserved parts of this region in different promoters are written. Lower part: Transcriptional activity of different promoter regions expressed as% CAT-conversion (calculation see Material and Methods). 'n' denotes the number of independent experiments.

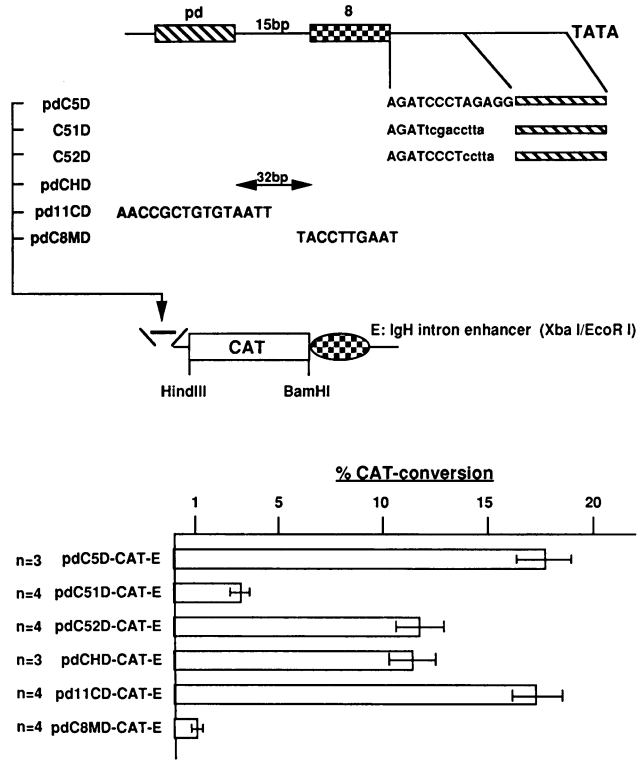

Figure 4. Mapping of the decamer 3′ flank sequence. Upper part: A schematic description of the promoters pdC5D, pdC51D and pdC52D used to analyse the 3′ flank sequence of the decamer motif (lower case letters in pdC51 and pdC52 indicate sequence diverging from pdC5). The striped bar denotes other sequences than that of the $\varkappa'$ promoter. Control promoters containing alterations in: a) the spacing between the pd element and the decamer (pdCHD); b) the pd element (pd11CD); c) the decamer (pdC8MD) are also shown. Lower part: transcriptional activity of different promoter regions expressed as% CAT-conversion. 'n' denotes the number of independent experiments.

is replaced by a random sequence. The pdC3D promoter, on the other hand, has the first 13 bp region replaced by a random sequence while the next 13 bp is derived from the same position in the $\varkappa'$ promoter. In a variant of the pdC3D promoter, pdC3MD, a point mutation is introduced into a E-box core motif present in the 3′ 13 bp region. The CAT-conversions obtained with the different promoters after transfection of LPS activated B lymphocytes are shown in the lower panel of Figure 3. The pdCD and the CD promoter were included as controls. The pdC5D promoter stimulated transcription as efficient as the pdCD promoter, while the pdC3D promoter stimulated expression less efficient. The pdC3D expression, however, was slightly better than that obtained with the CD promoter. The pdC3MD promoter, on the other hand, stimulated transcription five times less efficient than the pdCD promoter. This activity was lower than that observed with the CD promoter and in parity with what was observed with the consensus decamer with random flanking sequences (8D, Figure 2). Thus, the E-box core motif 3′ of the decamer in the SP6 $\varkappa$ promoter is active as a stimulatory element for transcription, but an additional stimulatory element is present within the immediate 3′ flanking sequence of the decamer.

A motif with a CCCT core stimulates transcription in the SP6 $\varkappa$ promoter

In order to map the element in the 3′ flanking sequence a third generation of CAT-vectors was prepared and transfected into normal B lymphocytes (Figure 4). In the pdC51D promoter the first 4 bp directly 3′ of the decamer are kept intact, as compared to the $\varkappa'$ promoter, while the rest of the sequence to the TATA-box is altered. This promoter induced a transcriptional activity

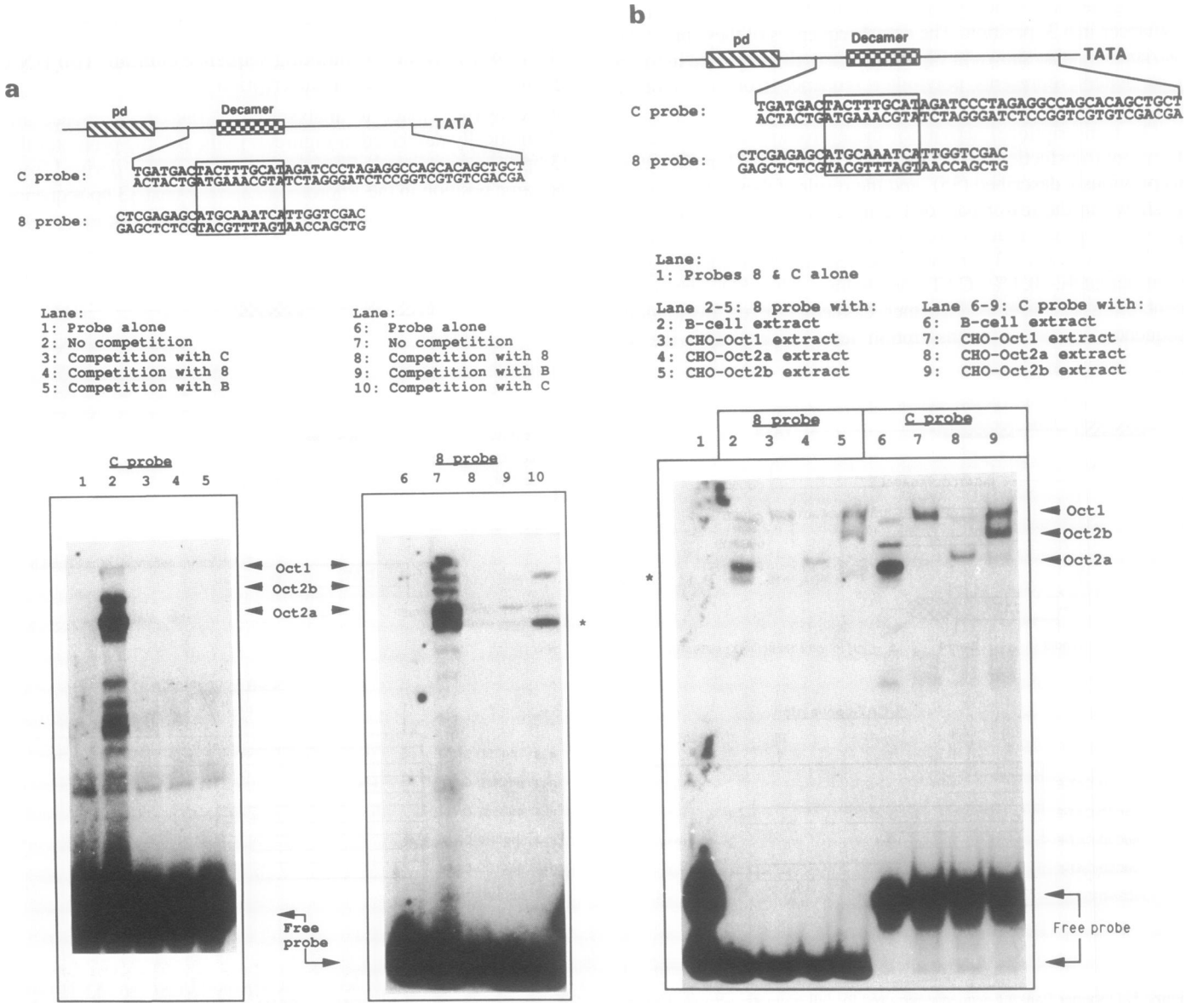

Figure 5. **(a)** Importance of the decamer flanking sequence in protein binding. The probes used in the EMSAs are indicated in the top part of the figure. The decamer sequence is boxed in both probes. Extract from B cells stimulated with LPS was used in all lanes except 1 and 6, where no extract was added. Shifts corresponding to Oct1, Oct2a and Oct2b are indicated. The star indicates unspecific binding of the probe. **(b)** Preferential Oct2 binding is B cell specific. The probes used in the EMSAs are indicated in the top part of the figure. The decamer sequence is boxed in both probes. Extracts used in this experiment were either from B cells stimulated with LPS or CHO cells stably transfected with Oct1, Oct2a or Oct2b. Shifts corresponding to Oct1, Oct2a and Oct2b are indicated. The star indicates unspecific binding of the probe.

comparable to that obtained with the pdC3MD and the 8D promoters. The first 8 bp 3′ of the decamer are kept intact while the rest of the sequence is altered in the pdC52D promoter which induced 70−80% transcriptional activity as compared to the pdCD promoter. Hence, the DNA element needed for synergistic stimulation of transcription by interactions between the pd and decamer elements centers around a CCCT core sequence.

Included in Figure 4 are also experiments performed with some control constructs. In the pdCHD promoter the spacing between the pd element and the decamer motif has been changed from 15 bp to 32 bp. In the pd11CD promoter the pd element from the SP6 $\varkappa$ promoter, that contains an E-box core motif (C-ANNTG), was exchanged for the pd element of the MPC11 $\varkappa$ promoter (39) that does not contain such a motif. The last control promoter, the pdC8MD, has a mutated decamer motif. Transcriptional activity from the pd11CD promoter was as high as that of the pdCD promoter, indicating that the E-box core motif in the pd element of the SP6 $\varkappa$ promoter is not critical for transcriptional activity. The change in spacing between the pd element and the decamer in the pdCHD promoter decreased the activity by 20% as compared to the pdC5D promoter. Mutation of the decamer motif (pdC8MD) in the presence of the pd and CCCT element decreased the transcriptional activity to just over background levels, indicating a pivotal role for the decamer motif in the regulation of $\varkappa$ transcription.

Decamer flanking sequence influences Oct2 binding in B cell extracts

We next analysed protein/DNA with electrophoretic mobility shift assays (EMSAs) using the C (SP6 $\varkappa$ decamer and 3′ flanking sequence) and 8 (consensus decamer with random flanking sequence) oligonucleotides. In the first experiment the probes were incubated with an extract from LPS activated B lymphocytes (Figure 5a). The results show that while the 8 probe gave the expected EMSA pattern described for B lymphoid extracts, the C probe did not. While both probes gave bands corresponding to Oct1 and Oct2a (11, 12), the C probe was bound inefficiently to complexes with a mobility intermediate to these and corresponding to Oct2b (13). This result is also evident in competition studies where the unlabelled C oligonucleotide was unable to compete with the Oct2b complex when 8 was used as a probe, while the B oligonucleotide (containing the same decamer but a different flanking sequence) competed with all the specific complexes generated by the 8 probe. In the reverse experiment, where C was used as a probe, both the B and the 8 oligonucleotide competed with all the observed complexes with a similar efficacy. To exclude that this observation had a trivial explanation we decided to investigate whether or not the preferential Oct2b binding was B cell specific. To this end, cDNA

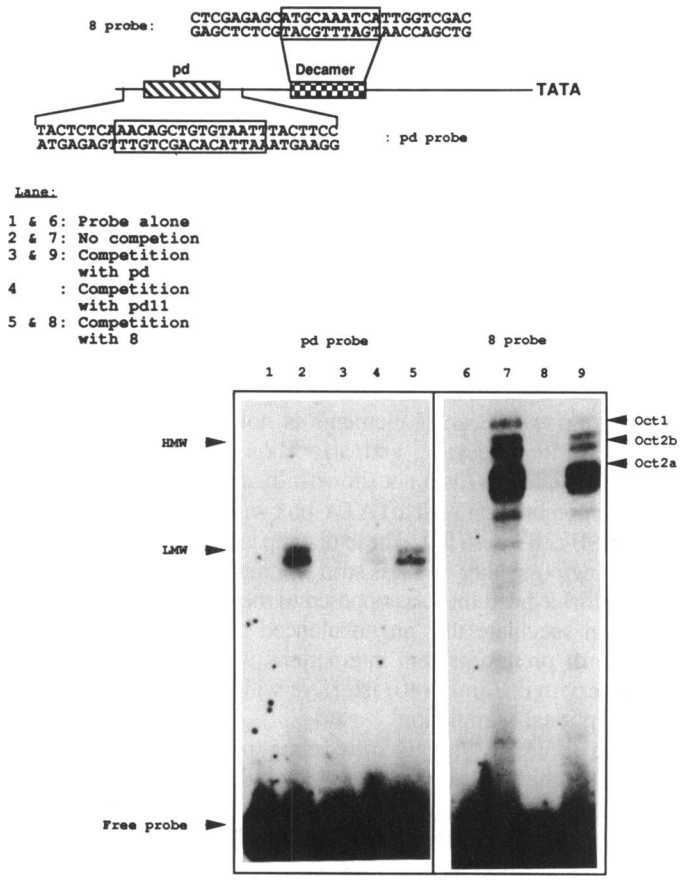

Figure 6. The pd element interacts with non-decamer binding proteins. The probes used in the EMSAs are indicated in the top part of the figure. Extract from B cells stimulated with LPS was used in all lanes except 1 and 6, where no extract was added. Oct1, Oct2a and Oct2b are indicated for shifts obtained with the 8 probe. High molecular weight (HMW) and low molecular weight (LMW) shifts obtained with the pd probe are indicated. Note that the apparently same position of the HMW and the Oct2b shift is not correct since the two panels are derived from two separate gels. The HMW shift has higher mobility than the Oct2a shift when both are run on the same gel.

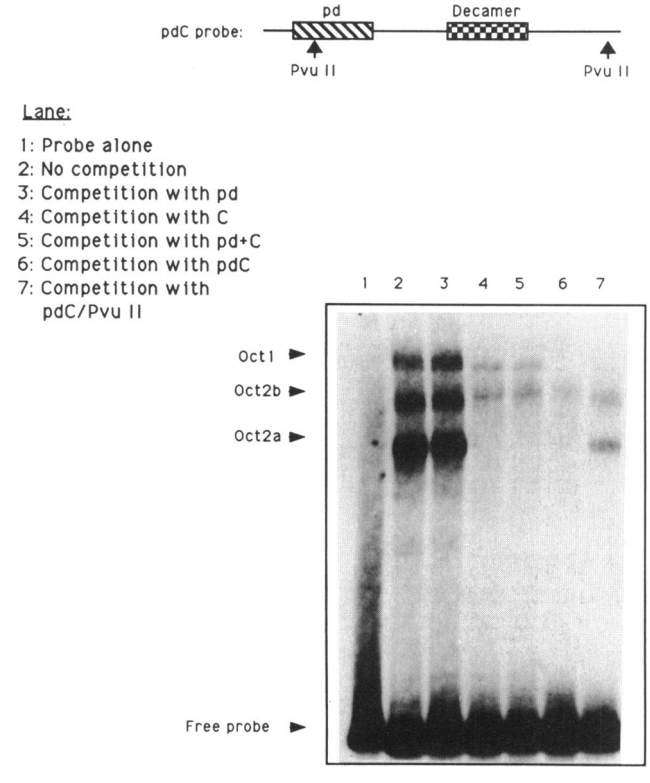

Figure 7. Complex protein/DNA interactions in the SP6 $\varkappa$ promoter. The probe used is indicated in the top part of the figure. The PvuII sites used to obtain the shorter promoter fragment (pdC/PvuII), used as competitor in lane 7, are indicated. Extract from B cells stimulated with LPS were used in all lanes except 1, where no extract was added. The positions of Oct1, Oct2a and Oct2b are indicated.

clones for Oct2a and Oct2b were cloned from a cDNA library generated from LPS activated mouse B lymphocytes, subcloned in expression vectors and transfected into CHO cells. As a control an expression vector containing Oct1 (58) was used. After selection in G418 extracts were made and analysed in EMSA with the C and the 8 probe. In such an experiment (Figure 5b), both Oct2a and Oct2b interacted equally well with both probes. We conclude from these experiments that B cell specific proteins interact with the SP6 κ promoter 3′ flanking sequence and modify the interaction of Oct2 proteins to their target sequence.

The pd element interacts with non-decamer binding proteins

The functional data indicate that the pd element interacts with a protein or proteins differing from the Oct-proteins. To analyse this, an EMSA was performed using the pd oligonucleotide as a probe. Two complexes were observed when cell extracts from B lymphocytes were incubated with the pd probe (Figure 6, left panel). These complexes could be competed out by cold pd or pd11 oligonucleotide but not with cold 8 probe. The extract was also incubated with the 8 probe as a control for degradation (Figure 6, right panel) and while the cold 8 probe was an efficient competitor in this experiment, no competition with the pd oligonucleotide was observed. Thus, the pd element is bound by DNA-binding proteins that have no detectable affinity for a consensus decamer motif.

Complex protein interactions in the SP6 κ promoter

In order to study the specific band pattern obtained in EMSA with a promoter fully active in stimulating transcription a promoter region lacking the TATA-box was used (pdC in Figure 7). Three major complexes were obtained after incubation of the probe with cell extracts from B lymphocytes, all which could be competed out with the cold probe. When the pd element alone was used as a competitor, no competition could be observed with any of the complexes ('pd-shifts' would not be visible on this exposure). The C oligonucleotide, containing the decamer motif present in the SP6 κ promoter, could not compete with the pdC probe for binding to the complex with intermediate mobility, even when it was used in competition experiments together with the pd probe. In addition, if the pdC fragment itself was digested with PvuII before being used in competition experiments it was no longer able to compete efficiently with all the observed complexes. We conclude that complex protein/DNA interactions involving several distinct DNA elements occur in the SP6 κ promoter.

κ chain promoters

	Decamer		
Sp603	TACTTTGCAT	AGATCCCTAGAGGCCAGCACAGCTGCTCATGATTT ATAAA	(39)
MPC11	TCCTTTGCAT	AGATCCCTAGAGGCCAGCACAGCTGCTCATGATTT ATAAA	(39)
MOPC41	TAATTTGCAT	ACCCTCACTGCATCGCCTTGGGGACTTCTTT ATATA	(16)
Vk-1B (NZB)	GCTTTTGCAT	ATTCGCCCCCACCCTTACTAGTCTGAG ATAAA	(47)
Vk-1C (BALB/c)	GCTTTTGCAT	ATTGCTCCCCAGGGAGGGCCCACCCTTACTAGTCTGAG ATAAA	(47)
Vk-1C (CE)	GCTTTTGCAT	ATTGCTCCCCAGAGAGGGCCCACCCTTACTAGTCTGAG ATAAA	(47)
Vk-1A (BALB/c)	GCTTTTGCAT	ATTGCTCCCCAGGGAGGGCCCACCCTTACTAGTCTGAG ATAAA	(47)
K2	TGATTTGCAT	GTACCCAAAGCATAGCTTACTGACATGAGGATTTCTTCATAGTCAGGTCACACCCTTTGCTGGAGTC AGAAT	(48)
Vk-Ser (BALB/c)	TGCTTTGCGT	AGATTCTTGCAGGTCAGCCCAGCTGCCCATGTTTT ATAAA	(49)
Vk-Ser (C.C58)	TGCTTTGCAT	AGATTCTTGCAGGTCAGCCCAGCTGCCCARGTGTT ATAAA	(49)
37A4	CTATTTGCAT	ATTTCATTTTCAGTAACTAGAAATATTTCACAGCTGTTTTTAAAGCAAAGTACTTATG ATAAT	(50)
Vk-21B	TTATTTGCAT	ATGAAATAATTTTATAACAGCCCAGGCTTCTTTAAGGGCAGCTGCCAGGAGCCTA AGAAA	(51)
Vk-21C	TGATTTGCAT	ATGAAATAATTTTATAACAGCCCAGGCTTCTTTAAGGGCAGCTGCCAGGAGCCTA ATAAA	(51)
Tumour T	TGATTTGCAT	GCTCTCACTTCACTGCCTTGGGGACTTCTTC ATATA	(52)

H chain promoters

	Decamer		
pCh104	TTTTACCTAAGAAGTACCCTGCTCATGAAT	ATGAAAATTA CCCAAGTCTATGGTAGTAAATACATGGATGTCCACACCC TGAAA	(53)
pCh108B	TCTTATGTACAAAGTCCCCTGCTCATGAAT	ATGCAAATTA CCGTTCTCTATGTTGGTTAAAAACAGGGATGTCAACACCC TGAAA	(53)
pCh105	CCTTAACTAAGTAATGCACTGCTCATGAAT	ATGCAAATCA CCTGGGTCTATGGCAGTAAATACAGGAATGGATGTCCACACCC TGAAA	(53)
MC101	TTCCTTTTATTCTCTCAGGAACCTCCCCCA	ATGCAAAGCA GCCCTCAGGCAGAGG ATAAA	(54)
MOPC141	TTCCTTTTATCCTCTCAGGAACCTCCCCCA	ATGCAAATCA GCCCTCAGGCAGAGG ATAAA	(55)
V11 (T15)	ACCCATGAGCTGAAGTTATAGCAGGAAGAC	ATGCAAATTG GAACTCACCCCCCTTCTT ATAAA	(56)
V13 (T15)	ACCCATGAGCTGAAGTTATAGCAGGAAGAC	ATGCAAATTG GAACCCACCCCCTTCTC ATAAA	(56)
pCh108A	TATTATATACAAAGTCCCCTGCTCATGAAT	ATGCAAATTA CCTAAGTCTATGGTAGTTAAAAACAGGGATATCAACACGC TGAAA	(53)
V1 (T15)	ATCCTAGAGCTAATGATATAGCAGAAAGAC	ATGCAAATTA GGCCACCCTCATCAC ATGAA	(56)
pCh111	CCTTGCTGAGTAATGCACCTGCTCATGAAT	ATGCAAATCA CCGCAAGTCTTTGGCAGTAAATACAAGGATGTCCACATTC TGAAA	(53)
37A11	TTCTTATCCAAGAAGTTAATGCTCATGAAT	ATGCAAATTA CTTGAGTCTATGTCAGTAAATACAGAGGTGACCACATGC AGAAA	(50)
H10	CTTATGTAAGAAAAGCCTTGCCTCATGAGT	ATGCAAATCA TGTGCGACTGTGATGATTA ATATA	(50)

Figure 8. Conservation of the pyrimidine rich motif among V gene promoters. κ promoter sequences are shown in the upper part of the figure and IgH chain promoters are shown in the lower part. The sequences are named as in the corresponding references. The decamer motif and the TATA-box are indicated by introduced spacing from the rest of each sequence. The pyrimidine rich motif and the CANNTG-motif are highlighted.

Conservation of the CCCT motif in Ig promoters

A sequence comparison of different published Ig heavy and κ light chain promoters was performed in order to see whether the CCCT core motif present in the SP6 κ promoter is present in other Ig promoters. Parts of the analysed sequences are shown in Figure 7. The CCCT core motif and E-boxes core motifs (C-ANNTG) are highlighted. The CCCT motif was present in the region between the decamer motif and the TATA-box in eight out of the fourteen κ promoters analysed. In the promoters lacking the CCCT core motif one or two E-box core elements were found in the analysed region except in the tumour T promoter. The distance to the decamer motif varied for the two elements. Among the twelve studied Ig heavy chain promoters nine contained the CCCT core motif. In eight of them the motif was found between the decamer and the TATA-box. Three promoters had the motif upstream of the decamer and two of the three also contained the CCCT core motif downstream of the decamer motif. One promoter contained both a CCCT core motif and an E-box core motif, while two others contained only an E-box core motif. One promoter did not contain any of the two motifs. The distance between the decamer motif and the other sequence motifs varied among the analysed Ig heavy chain promoters.

DISCUSSION

In this report the functional characteristics of the SP6 κ promoter are analysed in detail by transfection of untransformed mouse B lymphocytes. In B cell lines the synergistic mechanism between the pd and the decamer elements is not as pronounced as in normal B lymphocytes, and also shows variability between distinct B cell lines (data not shown). In addition, the pd element alone in conjunction with a TATA-box will activate transcription in some B cell lines (57). These discrepancies could indicate the interesting possibility of a less stringent regulation of transcription in transformed cell lines, as opposed to their normal counterparts. One can speculate that an unbalanced expression of proteins capable of protein/protein interactions with the transcriptional machinery in cell lines (40) interfere with the fine regulation of transcriptional stimulation.

Three different, synergistically acting, promoter elements were defined in the SP6 κ promoter. When the decamer motif was present as the only promoter element a significant transcriptional activation was observed, as previously shown (41). When this motif was mutated at two positions, which interfered with the binding of Oct-proteins (Figure 4), very low levels of transcription were seen, although the rest of the promoter was intact. Hence, the synergistic effect in between the three DNA motifs described herein is dependent on the binding of Oct proteins to the κ promoter. This synergism was also reflected at the level of protein/DNA interactions since a complete competition of protein binding to the SP6 κ promoter could only be obtained when the pd and the decamer element in *cis* were used as competitor, while the same elements in *trans* competed

as the decamer alone. Furthermore, deleting the five 5′ bases in the pd element also changed the competition pattern as compared to the intact probe used in the EMSA, again arguing for *cis* interactions and excluding fortuitous bands generated by the spacer sequence present in the pdC probe but absent in the oligonucleotides containing the individual elements. From the experiments performed here it is not clear exactly what proteins are represented in the individual complexes resolved in the EMSA. Clarification of that issue needs further analysis where the various complexes are isolated and analysed biochemically.

An unexpected finding was the preferential Oct2a binding of the SP6 κ promoter decamer probe (TACTTTGCAT), as compared to the consensus decamer probe (TNATTTGCAT). There are two possibilities for this effect, namely that it is due to the flanking sequence of the decamer, or that the mismatch in the third position should influence the binding. Others have in extensive studies failed to detect any difference in sequence specificity between different Oct proteins (13, 42) and we would therefore favour the interpretation that the decamer flanking sequence influences the binding preference of Oct2 proteins (note that the SP6 κ promoter decamer competes well for Oct1 binding). This argument is strengthened by the fact that the B oligonucleotide, containing the 5′ flanking sequence of the SP6 κ promoter decamer, competes differently than the C oligonucleotide which contains the 3′ flanking sequence when the consensus decamer is used as a probe. An interesting finding is that the differential Oct2 binding of the SP6 κ promoter decamer probe seems to be B cell specific since it shows an identical EMSA pattern when used with extracts derived from CHO cells transfected with either Oct2a or Oct2b. This would indicate that the decamer 3′ flanking sequence dependent protein/DNA interaction is B cell specific. A caution should be made, however, since Wirth *et al.* (59) recently demonstrated the presence of multiple Oct2 forms and our definition of the Oct2b as the Oct2 form unable to interact with the SP6 κ promoter decamer probe is based on mobility. On the other hand, our cDNA clones were derived from the same cell population as used for making the cell extracts used in EMSA and Oct2a and Oct2b clones were in abundance. Therefore we still favour the interpretation that a B cell specific protein is involved in the flanking sequence specific modification of Oct2 interactions with the SP6 κ promoter decamer probe.

The second element to be defined by function was the pd element. This element could not stimulate transcription alone but activated transcription synergistically in conjunction with the decamer. These data are in contrast to earlier studies, where the pd element has been defined as unimportant for transcriptional activation (16, 17), a discrepancy most likely due to the fact that we use untransformed cells for our transfection studies. When the pd element was used in the EMSA it was found to bind proteins distinct from those binding to the decamer. The SP6 κ promoter pd element contains an E-box core motif (CANNTG) but this is apparently not vital for its functional activity since the MPC11 promoter pd element, which lacks a CANNTG motif, induced equivalent transcriptional activity in conjunction with the SP6 κ promoter decamer. It exists a variability in spacing, ranging between 7 to 46 bp, between the pd element and the decamer motif in native light chain promoters (3). When we increased the pd/decamer spacing from 15 to 32 nucleotides (pdC5D and pdCHD) a 20% reduction in transcriptional activity was observed. It would be interesting in future experiments to investigate if the same decrease in activity can be observed with elements from a promoter sequence with a different wild-type spacing.

The SP6 κ promoter also contains the recently described κY motif (19). When we compare the transcriptional efficacy of a promoter containing the SP6 κ promoter decamer and the 5′ sequence containing κY (BD) it is better than what is observed with a consensus decamer with random sequences (8D), and as active as the decamer with the 3′ flanking sequence containing the CCCT motif (CD). On the other hand, κY seems to be unable to synergise with the pd element to activate transcription (pdB). It should also be noted that in the functional studies of the κY element (19) the κ promoter used contains a CCCT element 3′ of the decamer. This would indicate that also κY can synergise with the CCCT element to stimulate transcription. In our constructs, however, the κY element can be destroyed without loss of function (e.g. pdC). This is not surprising since the κY element is not as conserved as the pd element (3, 19) but rather supports the concept of functional modularity, as discussed below.

The synergism between the pd element and the decamer motif was dependent on a third DNA element situated between the decamer and the TATA-box in the SP6 κ promoter. In addition this 3′ element also stimulated transcription together with the decamer element in the absence of pd (CD vs. 8D in Figure 2). The 3′ element centered around a pyrimidine rich motif (CCC-T). It should be pointed out that additional bases 3′ of the CCC-T core sequence must be important for transcriptional activation since the pdC52D construct, diverging from the wild-type sequence immediately after the T in the core sequence, gave only 80% of the activity seen with the positive control (pdC5D) containing an additional five bases of the wild-type sequence. The CCCT core motif was frequently found in the decamer flanking sequence in Ig promoters. In most promoters lacking a CCCT core motif an E-box core motif could be found in the decamer flanking sequence. In the SP6 κ promoter the E-box core motif between the decamer and the TATA-box is functional since a point-mutation of the motif reduces transcriptional activity. The presence of the E-box core motif, however, can not be critical for transcriptional activation since the pdC5D construct which contains only the CCCT core motif activates transcription as efficient as the wild-type κ promoter. Thus, we propose that Ig promoters have evolved based on functional modularity so that at least one of several possible DNA elements which are involved in stimulation of transcription is included. The common denominator for these DNA elements is that they bind transcription factors, which activate transcription only in the presence of Oct proteins. The Oct proteins being the central *regulatory* protein for transcription while the other transcription factors are needed to achieve a complete transcriptional activation from the promoter. Several other groups have reported the importance of pyrimidine rich motifs for the expression of immunoglobulin genes (19, 20, 43). All these motifs have been defined in assay systems based on transformed ceil lines and all have been located upstream of the decamer motif. No protein-DNA interaction studies of the CCCT core motif have been performed in this report but others (42, 44) have shown by DNaseI footprinting a protection of a region spanning both the decamer motif and the pyrimidine rich motif immediately downstream of it.

The detailed mechanism for the synergism between the pd element and the decamer motif remains to be elucidated. In one model the Oct proteins would bind accessory molecules, adaptors or co-stimulators, that would contain transcriptional activating

domains. One paradigm for such a mechanism is the interaction between VP16 and Oct1 (45). In another model, several distinct DNA binding proteins cooperate to bind transcriptional activators (46). Recently it was shown by *in vitro* transcription that additional κ transcriptional stimulating factors, besides Oct1 and Oct2, were present in B cell extracts (42). No protein/DNA interactions could be detected with Oct-depleted extracts to a κ promoter, suggesting the presence of an adaptor molecule specific for B cells. In our experiments we can show a clear sequence dependence for synergy of function and also complex protein interactions in the SP6 κ promoter. In addition, we show that flanking sequences influence the interactions of different Oct2 forms to the decamer motif. Hence, we favour a mixture of the two models depicted above where Oct proteins form protein/protein interactions with other transcriptional factors while these transcriptional factors in addition need to bind to a consensus DNA sequence. The generated protein complex can then either activate transcription directly or via adaptor molecules. Such a mechanism can explain how transcriptional factors recognising relatively short or degenerated DNA motifs still can aid in tissue-specific transcription. If the activation is dependent on the expression of specific Oct proteins, all the accessory transcription factors do not have to be expressed in a tissue-specific manner. In addition, in such a model, different Oct2 forms could vary in their efficacy of DNA binding and transcriptional stimulation dependent on flanking elements and in their strength of the protein/protein interactions with other transcription factors.

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