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A novel upstream element compensates for an ineffectual octamer motif in an immunoglobulin V_χ promoter

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The octamer (or dc/cd) motif is considered to be a critical component of all immunoglobulin (Ig) promoters. Although the sequence of this motif is highly conserved among most Ig promoters, there are some notable examples in which efficiently expressed Ig genes contain divergent octamers with base substitutions that are demonstrably deleterious when tested with heterologous proximal promoter elements. To elucidate the mechanisms that enable these naturally occurring Ig genes to cope with divergent octamers, we analyzed two such promoters with regard to their ability to interact with relevant transcription factors. We found that the divergent octamer in the χ^0 germline promoter strongly binds both Oct-1 and Oct-2 factors, presumably because of compensatory contributions by flanking DNA sequences. A more surprising result was obtained with the $V_\chi 19$ promoter. In this case, the divergent octamer is a very weak Oct factor binding site and, without help from another upstream element, is inadequate for efficient promoter function. This additional element, termed χY because of its high pyrimidine content (CTTCCTTA), serves as a binding site for a novel lymphoid-specific factor. When the divergent $V_\chi 19$ octamer was converted to a strong Oct factor binding site by a single point mutation, the need for χY was obviated. Interestingly, V_H promoters that contain the same divergent octamer also contain an upstream element that is very similar to χY.

Key words: immunoglobulin genes/octamer factors/promoters

Introduction

The transcription of immunoglobulin (Ig) genes is regulated by promoter and enhancer elements, both of which function preferentially in cells of the B-lymphoid lineage (see Calame and Eaton, 1988 for review). The most prominent sequence feature of Ig promoters is the so-called octamer (Parslow *et al.*, 1984) or decanucleotide motif (Falkner and Zachau, 1984), located ~50–70 bp upstream of the transcriptional start site. This motif, which has the consensus sequence TNATTTGCAT (dc) in Ig light chain promoters and its inverted complement (cd) in Ig heavy chain promoters, is essential for efficient Ig promoter activity both *in vivo* and *in vitro* (Bergman *et al.*, 1984; Falkner and Zachau, 1984; Mason *et al.*, 1985; Atchison and Perry, 1986; Ballard and Bothwell, 1986; Scheidereit *et al.*, 1987; LeBowitz *et al.*, 1988; Poellinger *et al.*, 1989). The octamer serves as a binding site for the lymphoid-specific transcription factor Oct-2 (a.k.a. OTF-2 and NF-A2) and its ubiquitous counterpart Oct-1 (a.k.a. OTF-1, NF-A1 and OBP100) (Singh *et al.*, 1986; Landolfi *et al.*, 1986, 1987; Staudt *et al.*, 1986). Both Oct factors can activate transcription from Ig promoters *in vitro* (LeBowitz *et al.*, 1988), although they may differ in their ability to interact with other transcription factors, possibly accounting for their tissue specific usage *in vivo* (Müller *et al.*, 1988; Tanaka *et al.*, 1988).

Octamer mutations that prevent Oct 1/Oct 2 binding are generally detrimental to promoter function. Experiments in which synthetic dc/cd motifs were placed upstream of a β-globin TATA box indicated that a single transversion at any position is sufficient to abolish or reduce markedly both Oct factor binding and promoter activity (Staudt *et al.*, 1986; Wirth *et al.*, 1987). However, such stringent sequence conformity is evidently not required when octamers occur in their natural context within Ig promoters since there are several examples of actively expressed mouse and human Ig genes with octamers that diverge from the normal consensus sequence (Table I). Such divergent octamers are present in the mouse χ^0 promoter, which drives transcription from the unrearranged C_χ locus, and in most or all members of certain V_χ and V_H gene families. Presumably, mutations in the V gene octamers occurred during the evolutionary expansion of these multigene families and were tolerated because of compensatory features of neighboring sequences.

Two types of compensatory features may be envisioned (Mocikat *et al.*, 1986; Rosales *et al.*, 1987; Baumruker *et al.*, 1988; Currie and Roeder, 1989). First, the sequences adjacent to the divergent octamer might modify the DNA structure so that Oct factors could bind with high affinity despite the nucleotide substitutions. Alternatively, the neighboring sequences might constitute binding sites for auxiliary factors that either reinforce weak Oct factor binding or that interact directly with proximal promoter elements to diminish the dependence on octamer function. An example of a sequence that could play such a role is the heptamer motif, CTCATGA, which is located upstream of the octamer in many V_H genes (Eaton and Calame, 1987) and which, in cooperation with the octamer, can also bind Oct factors (LeBowitz *et al.*, 1989; Poellinger and Roeder, 1989).

In principle, it is clear that sequence context can influence Oct factor binding or the dependence on octamer function. However, such contextual effects have not heretofore been related to the activity of naturally occurring divergent octamers in extant Ig promoters. Therefore, to elucidate the mechanisms that are actually used to cope with divergent octamers, we have analyzed the properties of two promoters that contain such motifs, χ^0 and $V_\chi 19$ (Table I). We

Table I. Variant octanucleotide (dc/cd) motifs in expressed[a] immunoglobulin genes

Motif	Gene family	Examples	References
TNATTTGCAT	most mouse and human V_κ genes		
TCCTTTGCAT	V_κ19 (mouse)	κ^+, MPC11	Kelley *et al.* (1982)
TACTTTGCAT		κ_{TNP}(ig κ-20 hybridoma)	Hawley *et al.* (1984)
TGCTTTGCAT	V_κ28 (mouse)	V_κSer(C.C48)	Boyd *et al.* (1986)
TGCTTTGCAT	V_κIII (human)	κ^+,IARC/BL41	Klobeck *et al.* (1985a)
		V_g, V_h	Pech and Zachau (1984)
		kv305	Chen *et al.* (1986)
		V_κRF	Radoux *et al.* (1986)
GNTTTTGCAT	V_κIV (human)	κ^+,J1	Klobeck *et al.* (1985b)
CNATTTGCTT	V_κI (human)	V_d	Pech *et al.* (1984)
ATGCAAATNA	most mouse and human V_H genes		
ATGCAAAGCA	V_HQ52 (mouse)	MOPC 141;	Sakano *et al.* (1980)
		PCG1-1	Stenzel-Poore *et al.* (1987)
		MC101	Kataoka *et al.* (1982)
ATGCAAAGCG		A 8.1	Gerondakis *et al.* (1984)
AGGCAAATGC	V_H6 (human)	fetal monocyte cDNA	Schroeder *et al.* (1988)
ATGTAAATNT	C_κ locus (mouse)	κ^0 promoter	Van Ness *et al.* (1981)

[a]The expressed status of rearranged genes in tumor cell lines and hybridomas was directly demonstrated. The expressed status of germline genes was inferred by sequence comparison with corresponding cDNAs or, in a few cases (V_g, V_h and V_d), by the lack of any apparent defect. MOPC 141 and PCG1-1 may be somatically mutated variants of the same V_H gene; all of the other listed V genes are distinct in the germline.

observed that the divergent octamer in κ^0 binds strongly to its cognate factors, and may therefore be sufficient for normal promoter function. In contrast, Oct factors bind very weakly to the V_κ19 promoter. In this case, promoter activity is strongly dependent on the presence of an upstream element, CTTCCTTA, that serves as a binding site for a distinct lymphoid-specific factor. Conversion of the divergent V_κ19 octamer to the canonical sequence restores strong Oct factor binding and obviates the need for the upstream element. Thus, this element and its cognate factor play a critical role in enabling the V_κ19 family to cope with a divergent octamer sequence. Interestingly, promoters of the V_HQ52 family, which contain the same divergent octamer, also contain a very similar upstream motif.

Results

The divergent κ^0 octamer is capable of binding the Oct-1 and Oct-2 factors

The κ^0 promoter possesses a divergent cd motif with C → T and A → T substitutions in the 4th and 10th positions, respectively (Table I). To determine whether this divergent motif can bind the Oct factors, we carried out a mobility shift analysis (Fried and Crothers, 1981) with a 170 bp DNA fragment from the κ^0 promoter region and nuclear extracts from the 3-1 line of pre-B cells (Figure 1). Extracts were prepared from untreated 3-1 cells, which contain mainly the Oct-1 factor, and from lipopolysaccharide (LPS) treated cells, which have an increased content of Oct-2 factor (Staudt *et al.*, 1986). This analysis revealed a set of retarded bands which is very similar to that observed with canonical octamer motifs (Landolfi *et al.*, 1986; Staudt *et al.*, 1986; Rosales *et al.*, 1987). Complexes characteristic of Oct-1 (O-1), Oct-2 (O-2) and an Oct multimer (O′) were observed (Figure 1A). The specificity of these interactions was demonstrated by competition experiments with unlabeled DNA fragments either containing or lacking a canonical octamer motif (Figure 1B). Inclusion of an octamer-containing DNA fragment derived from the V_κ21E promoter abolished nuclear factor−DNA interaction (lanes

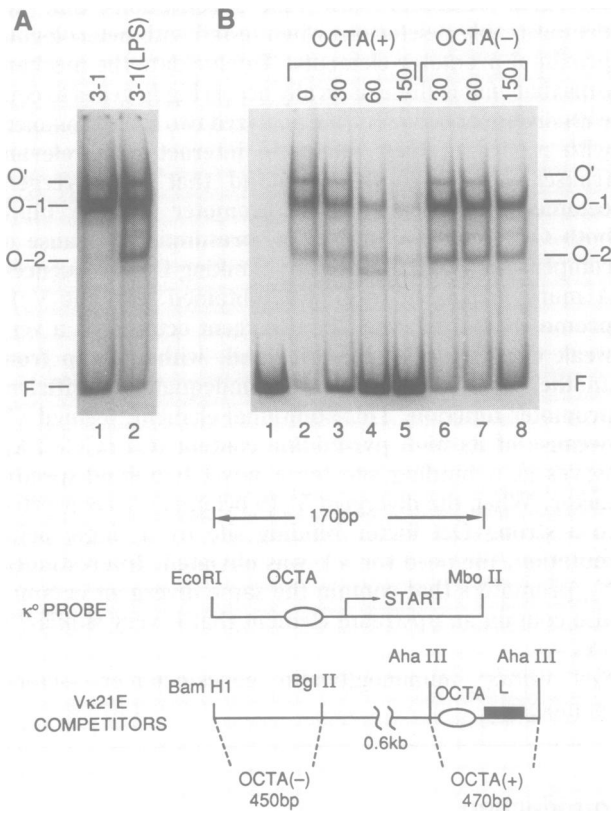

Fig. 1. Gel mobility shift assays of the κ^0 germline promoter region. (**A**) A labeled 170 bp fragment spanning the κ^0 promoter (diagrammed below) was incubated with extracts from untreated and LPS-treated 3-1 cells and analyzed by electrophoresis through a 4% polyacrylamide gel. (**B**) The incubation mixtures contained LPS-treated extract, labeled κ^0 fragment and 0−150 ng of unlabeled fragments from the octamer-containing [OCTA(+)] or an octamer-lacking [OCTA(−)] region of the V_κ21E promoter (diagrammed below). The sample in lane 1 lacked extract and competitor. Complexes presumed to contain Oct-1, Oct-2 and an octamer factor multimer are designated O-1, O-2 and O′ respectively. F designates free fragment. In the diagrams, the ovals indicate the positions of the octamer elements and the solid bar indicates the leader exon (L).

3–5), whereas inclusion of a DNA fragment lacking the octamer sequence had no effect on factor binding (lanes 6–8).

To verify that the DNA–nuclear factor interactions observed with the κ^0 DNA fragment were due to the divergent octamer motif, DNase I footprinting and methylation interference assays were performed. As expected, DNase I footprint assays showed a protected region of ~15 bp centered on the divergent octamer motif (Figure 2, bracket). In addition, methylation interference assays showed contacts at the same octamer nucleotides (Figure 2, arrows) that are observed with a canonical octamer motif (Staudt *et al.*, 1986). Therefore, the divergent κ^0 octamer appears capable of interacting with both the ubiquitous and tissue-specific octamer factors. Interestingly, DNase I hypersensitive sites and enhanced binding to methylated G residues (asterisks in Figure 2) occurred in the region of the transcriptional start site (CAP) of the κ^0 promoter. The significance of these additional interactions is presently unclear.

The V_κ 19A divergent octamer binds poorly to Oct factors. Identification of a novel DNA–nuclear factor interaction

The V_κ19A promoter, which efficiently drives transcription from the productive (κ^+) allele in MPC11 cells, contains an A → C transversion at the third position of the dc motif (Table I). To assess the ability of this divergent octamer motif to interact with nuclear factors, mobility shift assays were performed with an end-labeled 78 bp *Bst*NI–*Pvu*II DNA fragment containing the V_κ19A divergent octamer. Incubation of this DNA fragment with a nuclear extract prepared from LPS treated 3-1 cells yielded one prominent and several faint retarded bands (Figure 3A, lane 1). All of these complexes were efficiently outcompeted by an unlabeled DNA fragment derived from the V_κ19A promoter (Figure 3A, lanes 2–4). However, only complexes corresponding to three of the faint bands were outcompeted by an unlabeled V_κ21E promoter fragment containing the canonical octamer motif; no significant competition for the prominent complex was observed (lanes 5–7). This unexpected result indicated that the V_κ19A promoter is capable of only very weak Oct factor binding, and that it contains an additional binding site for an unrelated nuclear factor. The poor Oct factor binding capability of V_κ19A promoter was confirmed by a reciprocal competition experiment (Figure 3B). In this experiment, binding to the canonical dc motif of the V_κ21E promoter was successfully outcompeted by homologous unlabeled V_κ21E fragment (lanes 2–4) but not by excess V_κ19A fragment (lanes 5–7). The nuclear factor that specifically recognizes the V_κ19A promoter was later termed κY in view of its pyrimidine-rich binding site (see below).

The V_κ19-specific nuclear factor binds to an upstream motif distinct from the divergent octamer

To identify the sequence motif that is recognized by the V_κ19-specific nuclear factor, we carried out DNase I footprint and methylation interference studies on the prominent band of Figure 3A. A DNase I footprint of the fragment's plus strand indicated that the DNA–nuclear factor interaction is centered over the sequence CTTCCTTA, which is located 11 bp upstream of the divergent octamer motif (Figure 4A). The 5′ end of this sequence is protected

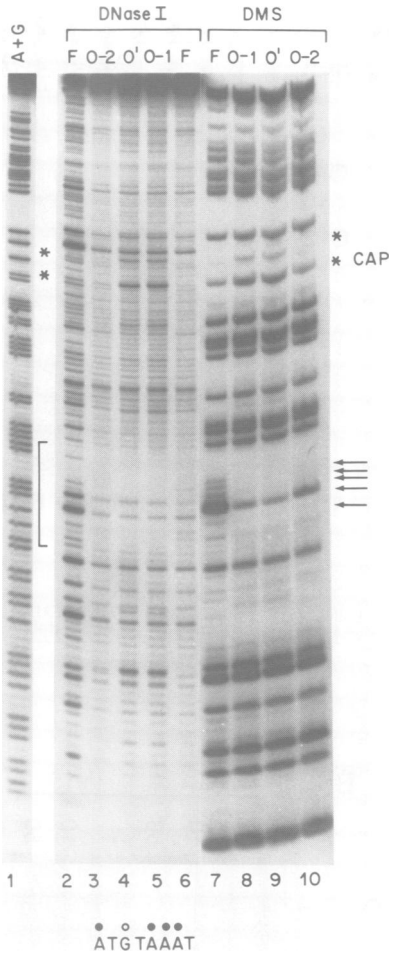

Fig. 2. DNase I footprint and methylation interference (DMS) analysis of the κ^0 promoter region. The octamer factor complexes O-1, O-2 and O′ and free fragment were isolated from a gel similar to that of Figure 1A, lane 2, and the 'plus' strands analyzed as described in Materials and methods. An A+G sequencing reaction of the labeled DNA fragment served as a position marker. The region protected from DNase digestion is delineated by a bracket; the methylated residues that inhibit factor binding are marked with arrows. These contact residues are shown below (closed circles represent complete inhibition, open circles represent partial inhibition).

from DNase I digestion while the 3′ end is hypersensitive. Methylation interference analysis of the minus strand indicated that methylation of two adjacent G residues (arrows) in the middle of this upstream element interferes with nuclear factor binding (Figure 4B). Thus, this pyrimidine-rich sequence constitutes a binding site for a nuclear factor (κY) that is clearly distinct from the Oct factors.

The κY factor is lymphoid specific

To establish whether the κY factor is present in cells representing different stages of B lymphocyte development and in cells of non-lymphoid origin, we carried out mobility shift assays with nuclear extracts from plasmacytomas (Ag8.653, S107, S194), an immature B cell lymphoma (38C-13), unstimulated pre-B cells (3-1), a human cervical carcinoma (HeLa), SV40 transformed monkey kidney cells (COS) and a mouse fibroblast (L cells). When assayed with the V_κ19A probe, all of the lymphoid cell extracts exhibited prominent κY and relatively faint Oct-1 bands similar to those observed with the 3-1 pre-B cell extract (Figure 5A and data not shown). In contrast, the κY factor was at least

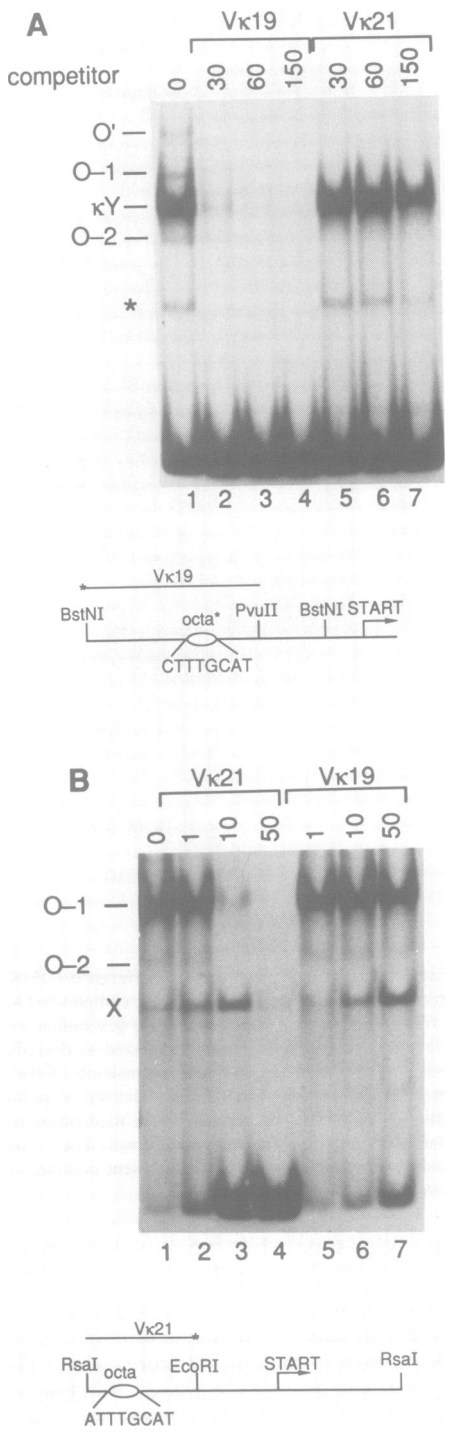

Fig. 3. Gel mobility shift assays of the V_x19A and V_x21E promoter regions. (**A**) A labeled 78 bp *Bst*NI–*Pvu*II fragment spanning the V_x19A promoter region (diagrammed below) was incubated with nuclear extract from LPS-treated 3-1 cells and the indicated amounts (in ng) of unlabeled competitor DNA corresponding to either a 98 bp *Bst*NI fragment encompassing the V_x19 promoter region or a 139 bp *Rsa*I fragment encompassing the V_x21E promoter region. Based on relative mobility, the complexes outcompeted by the V_x21 fragment are designated O-1, O-2 and O'. The major complex that is not outcompeted by the V_x21 fragment is denoted as κY. (**B**) A labeled 59 bp *Rsa*I–*Eco*RI fragment spanning the V_x21E promoter region (diagrammed below) was incubated with nuclear extract from S194 plasmacytoma cells and the same pair of unlabeled DNA competitors as in panel (A). The faint V_x19-specific band in panel (A) (*) may represent a complex with partially degraded κY factor; the minor band in panel (B) (X) probably represents non-specific binding.

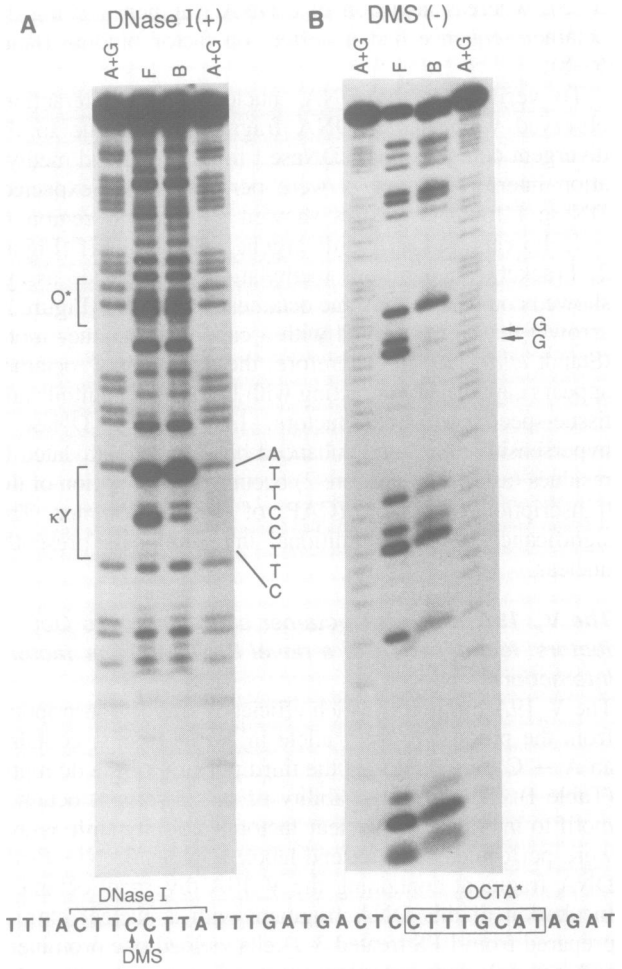

Fig. 4. DNase I footprint (**A**) and methylation interference (**B**) analysis of the V_x19A promoter region. The κY complex and free fragment (lanes B and F respectively) were isolated from a gel similar to that of Figure 3A, lane 1, and the 'plus' (DNase I) and 'minus' (DMS) strands analyzed as in Figure 2. The DNase I protected sequence and the minus strand contact residues for κY are marked by a bracket and arrows respectively. O* marks the location of the divergent octamer.

10–20 times less abundant in the non-lymphoid extracts (Figure 5B). As expected, all of the non-lymphoid cells contained the ubiquitous Oct-1 factor, which could be detected with either the weak binding V_x19 octamer (Figure 5B) or the strong binding V_x21 octamer (Figure 5C). The relative uniformity of the Oct-1 factor complex among the various extracts serves as a good internal control for the quality of the nuclear extracts, thus leading us to conclude that the κY factor is basically lymphoid specific.

In this series of experiments, the V_x19 probes did not form detectable Oct-2 complexes with any of the lymphoid cell extracts. The extracts derived from 3-1 cells (not LPS treated) and 38C-13 cells were deficient in Oct-2 factor, as indicated by the absence of a characteristic Oct-2 band in assays with the V_x21 probe (Figure 5C). Similar assays of the Ag8.653 and S107 extracts did reveal Oct-2 complexes, although their level was less than that of the Oct-1 complexes as judged by relative band intensities (data not shown). Such variability in Oct-2 factor content among extracts from different lymphoid cell lines is not unusual (Landolfi *et al.*, 1986; Staudt *et al.*, 1986; Wirth *et al.*, 1987; Nelms *et al.*, 1990). The lack of readily observable Oct-2 complexes with the V_x19 probe may be due to a combined effect of lower

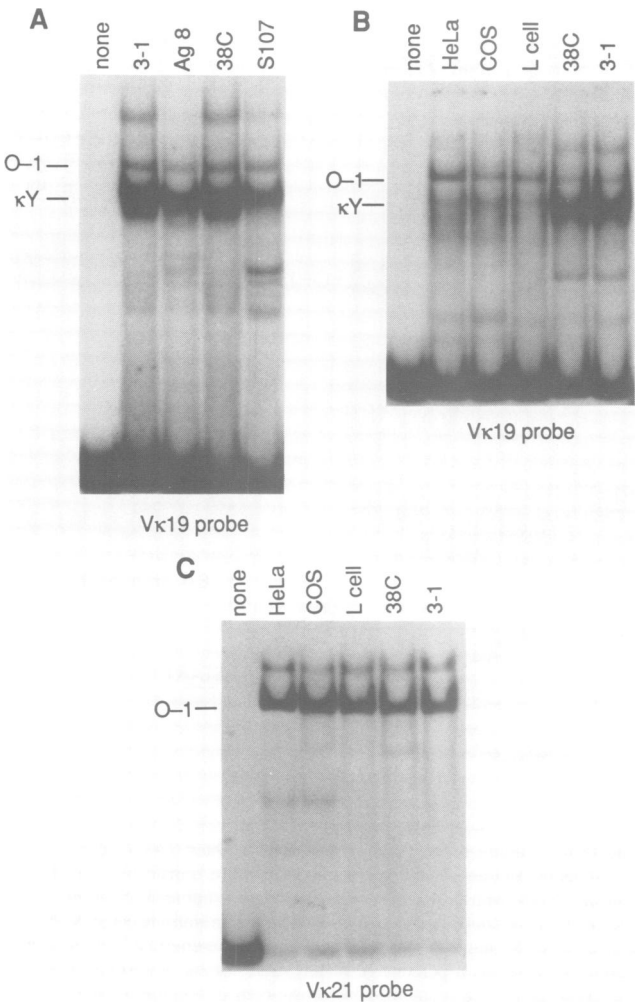

Fig. 5. Distribution of the κY factor among different cell types. Gel mobility shift analyses were carried out with nuclear extracts derived from various cell lines and fragment probes from the $V_κ19$ or $V_κ21$ promoters (see diagram, Figure 3. (**A**) Lymphoid cell extracts. (**B**) and (**C**) A comparison of non-lymphoid and lymphoid cell extracts.

factor concentration (relative to Oct-1) and reduced binding affinity of the divergent octamer.

The sequence embracing the κY binding site in the $V_κ19A$ promoter has some similarity to a motif (Pu box) in the enhancers of SV40 and a lymphotropic papovavirus that also binds to a lymphoid-specific nuclear factor (Pettersson and Schaffner, 1987). In the SV40 enhancer, the Pu box is located upstream of a divergent octamer, which although identical to that of $V_κ19$, strongly binds Oct factors because of a favorable sequence context (Rosales *et al.*, 1987; Baumruker *et al.*, 1988). When a 205 bp *Hpa*I–*Kpn*I fragment of SV40 DNA that contains the Pu box was used as a competitor in gel mobility shift assays, no competition for κY binding was observed, although, as expected, a 366 bp *Kpn*I–*Hin*dIII fragment of SV40 DNA which contained the divergent octamer readily outcompeted the weak Oct factor binding of the $V_κ19$ promoter (data not shown). These results indicate that κY and the Pu box binding factor are not the same.

The upstream binding motif is required for efficient $V_κ19A$ transcription

To determine whether the κY binding motif plays a functional role in the $V_κ19A$ promoter, a series of 5′ deletion constructs were prepared. In these extracts, $V_κ19A$ segments extending from various 5′ endpoints to position +123 were linked to an *Xba*I–*Bam*HI fragment which contains the κ enhancer and the κ constant region ($C_κ$) exon. These constructs were inserted at the *Eco*RI site of pSV2neo so that differences in transfection efficiency could be normalized by analysis of transcripts derived from the vector neomycin resistance gene. Constructs were transfected into S194 plasmacytoma cells and transcriptional activity of the $V_κ19A$ and the *neo* genes was measured by S1 nuclease protection analysis. Previously, we determined that a construct with 200 bp of 5′ flanking DNA sequence had the same transcriptional activity as a normal $V_κ19A$ rearranged gene with 1.8 kb of 5′ flanking sequence (data not shown). Progressive deletion of DNA sequences between −200 and −110 had no effect on expression (Figure 6A). Deletion to position −96 reduced activity to about half of the control level and deletion to position −84 (which totally removes the κY binding site) reduced expression to ~16%. Further deletion to the edge of or beyond the divergent octamer motif (−72 and −40 constructs) reduced expression to near background levels. To determine whether a canonical octamer motif could restore promoter activity, construct −84octa$^+$ was made. The activity of this construct, which is identical to the −84 construct except for a single base pair substitution that changes the divergent octamer to a perfect canonical octamer motif, was ~75% of the maximal level (Figure 6A). These results demonstrate that the κY binding motif is required for high level expression of the $V_κ19A$ promoter when the natural divergent octamer sequence is present, but not when this sequence is converted to a canonical octamer motif.

To relate the results of these functional studies to factor binding capability we carried out mobility shift assays of labeled DNA fragments with 3′ ends fixed at position −40 and with variable 5′ ends (Figure 6B). DNA fragments with 5′ ends at positions −110 or −96 exhibited typical strong κY and faint Oct-1 bands (more evident on longer exposures of this autoradiogram). Deletion of sequences upstream of −84 abolished the κY band, and deletion of sequences upstream of −72 eliminated all binding activity. The −84octa$^+$ fragment exhibited a very strong Oct-1 band, which was efficiently outcompeted by an unlabeled DNA fragment containing a canonical octamer motif but not by an unrelated DNA fragment (Figure 6C). Thus, the single base pair substitution results in a striking increase in Oct factor binding capability, which appears to obviate the need for the κY function.

The divergent octamer motif is also important for the efficient expression of the $V_κ19A$ promoter

To confirm the importance of the κY factor and to determine whether the divergent octamer also plays a role in $V_κ19A$ promoter activity, we tested the activity of mutants that contained deleterious mutations in the κY or Oct factor binding sites (Figure 7A). Compared with the wild-type $V_κ19A$ gene (κY$^+$O*), promoter activity was severely reduced in mutants that were incapable of binding either κY (κY$^-$O*) or Oct factors (κY$^+$O$^-$) (Figure 7B and C). Indeed, the Oct factor binding, albeit weak, may be even more critical for promoter function than κY binding, as judged by the relative activities of the κY$^+$O$^-$ and κY$^-$O* mutants (approximately 4 and 25% of the wild-type level respectively). A double mutant (κY$^-$O$^-$) which lacked

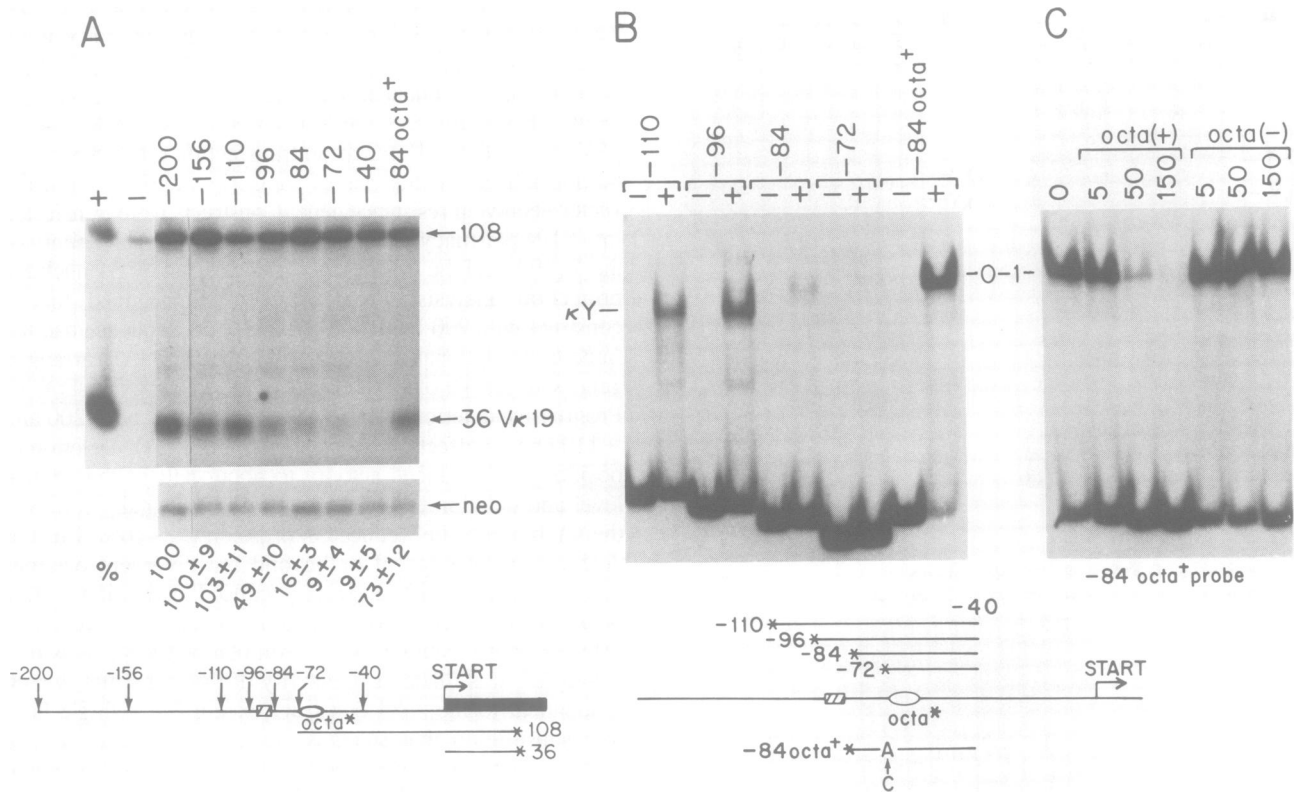

Fig. 6. The effect of 5′ deletions and an octamer up-mutation on the activity and factor binding properties of the V_x19 promoter. (**A**) κ gene constructs containing the indicated 5′ deletions of the V_x19 promoter region or a deletion and an octamer point mutation were transfected into S194 cells and their expression measured by S1 nuclease protection analysis of cytoplasmic RNA with a 108 bp probe. Transcripts initiated at the proper V_x19 cap site protect a 36 nucleotide fragment ($36V_x19$). Positive (+) and negative (−) S1 controls are assays of RNA from untransfected MPC11 cells (which contain an active V_x19A gene) and untransfected S194 cells respectively. An S1 nuclease assay of the SV40-*neo* gene that was present on each construct is also shown. Below each lane is the amount of V_x19 transcript expressed as a percentage of the value for the −200 construct. The values, which represent an average of two or three independent transfection experiments, were obtained by densitometric scanning of the autoradiograms and normalization to the *neo* signals. (**B**) Gel mobility shift analysis with a nuclear extract from unstimulated 3-1 cells of fragments derived from the constructs of panel (A). The positions of the κY and Oct-1 complexes are noted. (**C**) The complex formed with labeled $-84octa^+$ probe was competed for by the indicated molar excess of unlabeled DNA fragments containing [OCTA(+)] or lacking [OCTA(−)] a canonical octamer sequence (see Figure 1). In the schematic diagrams the hatched box and oval designate the positions of the κY and divergent octamer respectively.

ability to bind both factors was virtually inactive. These results indicate that the κY element and the divergent octamer are both required for optimal V_x19A promoter activity. Moreover, the mobility shift analysis with the κY^-O^* and κY^+O^- mutants indicates that κY and Oct factor binding occurs independently (Figure 7C).

Discussion

We have studied the ability of two naturally occurring divergent octamer motifs to bind *trans*-acting nuclear factors. One divergent octamer, ATGTAAAT, which occurs in the κ^0 promoter, strongly binds both the Oct-1 and Oct-2 factors. Previously, Staudt *et al.* (1986) demonstrated that nuclear factor binding to synthetic octamer oligonucleotides is abolished by a transversion at the very same position that is divergent in the κ^0 octamer. The ability of the κ^0 octamer sequence to bind strongly to its cognate factors could be due to the fact that it has a transition rather than a transversion at this position. However, Mocikat *et al.* (1986), using oligonucleotides with natural flanking sequences, demonstrated binding ability with another transversion (ATGTGCAT) that was also ineffective in the assay of Staudt. A more likely reason for the difference in results is the contribution of flanking Ig DNA sequences that are present in our κ^0 DNA segment and in the probe used by Mocikat. These flanking DNA sequences may compensate for weak nuclear factor binding to imperfect octamers by supplying additional factor−DNA interactions, as demonstrated by Baumruker *et al.* (1988).

A more surprising result was obtained with the V_x19A divergent octamer. Despite the fact that the V_x19A promoter is very active in MPC11 and other lymphoid cells (Schibler *et al.*, 1978; Atchison and Perry, 1986), its divergent octamer, CTTTGCAT, binds Oct factors very poorly and, without help from another upstream element, is inadequate for efficient promoter function. Apparently, the flanking DNA sequences in this promoter do not supply the additional contacts needed to produce a strong Oct factor binding site. Instead, the V_x19A promoter contains a pyrimidine-rich motif (κY) 11 bp upstream of the divergent octamer, which serves as a strong binding site for a novel lymphoid-specific nuclear factor. A deletion of the κY motif or a 4 bp mutation that abolishes its factor binding capability results in a severe reduction of promoter activity, thus demonstrating that the interaction of this motif and its cognate factor is essential for proper functioning of the V_x19 promoter.

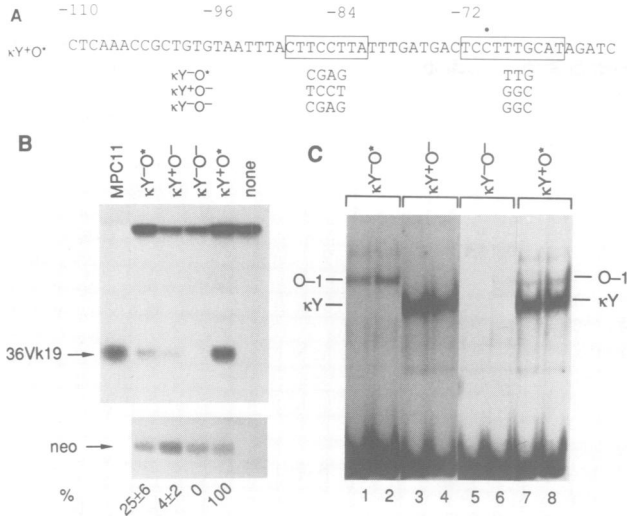

Fig. 7. The effect of κY and octamer mutations on the activity and factor binding properties of the $V_κ19$ promoter. (**A**) Sequences of the wild-type $V_κ19A$ promoter ($κY^+O^*$) and the substituted regions of the mutants. The κY and dc elements are boxed. (**B**) S1 nuclease protection assay of cytoplasmic RNA from S194 cells transfected with the wild-type and mutant genes. The values below each lane, expressed as a percentage of wild-type expression, represent an average of three independent transfection experiments. See Figure 6 for other details. (**C**) Gel mobility shift analyses of the wild-type and mutant genes. Odd- and even-numbered lanes correspond to assays made with 3-1 or 38C nuclear extracts respectively.

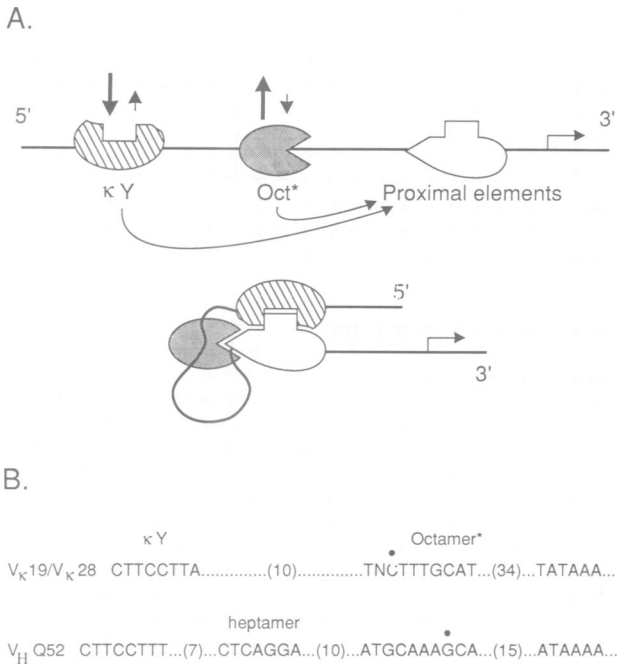

Fig. 8. Model for κY divergent octamer interaction in the $V_κ19$ promoter (**A**) and a sequence comparison of promoter elements in the $V_κ19/V_κ28$ and V_HQ52 gene families (**B**).

The interplay between the divergent octamer and κY is interesting. A single base pair substitution that converts the divergent octamer to a strong Oct factor binding site permits normal promoter activity to be achieved in the absence of the κY element. Conversely, a 3 bp mutation that eliminates the weak Oct factor binding capability of the divergent octamer drastically impairs promoter activity, even in the presence of the κY element. Thus, the κY element at its natural location in the $V_κ19A$ promoter can functionally complement a weakly binding Oct factor but cannot, by itself, interact effectively with proximal promoter elements. There is no evidence that κY acts by strengthening Oct factor binding to the divergent octamer. Indeed, Oct factor binding is usually easier to detect in the absence of κY factor binding than in its presence (compare, for example, lanes 1 and 2 versus 7 and 8, Figure 7C). Moreover, κY binding is not affected by the presence or absence of Oct factor interactions (compare lanes 3 and 4 versus 7 and 8, Figure 7C). Thus, as a plausible model, we envision a synergism between the κY and Oct factors at the level of their interaction with more proximal promoter elements (Figure 8A). This type of interaction contrasts with the proposed interplay between the heptamer and octamer motifs in V_H promoters, which appears to involve cooperative binding of the Oct factors (LeBowitz *et al.*, 1989; Poellinger and Roeder, 1989).

Whether the factor that normally participates in this interaction with κY is Oct-1 or Oct-2 is presently unclear. We consistently observe Oct-1 binding to $V_κ19$ promoter fragments, but had considerable difficulty detecting Oct-2 complexes, even with extracts which were known to contain Oct-2. This might reflect a preference for Oct-1 or it might simply be due to a technical problem of detecting complexes between the less abundant Oct-2 factor and a low affinity octamer binding site. Recently it was observed that extinction of $V_κ19$ gene expression in somatic cell hybrids of MPC11 cells and fibroblasts is accompanied by a loss of Oct-2 factor production (assayed by gel shift analysis with a synthetic consensus octamer probe) (Bergman *et al.*, 1990). If the lack of κ expression in these hybrids is indeed caused by the deficit of Oct-2 factor, this would imply that the $V_κ19$ promoter does utilize Oct-2.

The prevalence of κY factor in cells representative of various stages of the B lymphoid lineage and its scarcity in cells of non-lymphoid origin indicates that this factor is basically lymphoid specific. Although a factor with this particular binding site has not previously been reported, we suspect that κY may have a role in the expression of other lymphoid-specific genes. It is noteworthy that two other mouse $V_κ$ genes that contain the same divergent octamer, $κ_{TNP}$ and $V_κ$Ser (Table I), also have an identically located κY element. It would appear, therefore, that the relationship between κY and the divergent octamer was established before the evolutionary expansion of the $V_κ19/V_κ28$ multigene families and that it has been preserved because of its importance for promoter activity.

Interestingly, a 7/8 match to the κY binding site occurs in the promoters of various members of the V_HQ52 family of mouse heavy chain genes, all of which also contain the same divergent octamer that is found in the $V_κ19/28$ families (Table I). In these genes, the κY-like motif is located 24 bp upstream of the divergent octamer, 7 bp upstream from the heptamer motif, and 49 bp upstream of the TATA box (Figure 8B). A similar upstream pyrimidine-rich sequence has been shown to contribute modestly (~2-fold) to the activity of a V_H promoter that has a canonical octamer motif (Eaton and Calame, 1987). Conceivably, such sequences might have greater importance in the V_H promoters that contain divergent octamers.

It is not yet known how effective promoter activity is achieved in the various human V genes with divergent octamers. None of these genes contain readily discernible

κY motifs, so presumably they rely either on contextual effects to engender strong Oct factor binding, as is the case with the κ^0 promoter, or they contain binding sites for additional auxiliary factors. It is evident from the present study that Ig promoters have evolved more than one way to compensate for divergent octamer binding sites and that these compensatory mechanisms can be identified by an analysis of the factor binding capability and functional properties of the particular promoter of interest.

Materials and methods

Plasmid constructions

Plasmids −96, −84, −72 and −84octa$^+$ were prepared through the use of specific oligonucleotides (see below) with *Xba*I and *Xho*II compatible 5′ and 3′ ends respectively. Specific oligonucleotides were annealed to their complementary strands and subjected to a unidirectional three-step ligation protocol consisting of the annealed oligonucleotides, a 183 bp *Xho*II−*Hin*dIII DNA fragment (spanning sequences −60 to +123) and plasmid vector pUC18 cleaved with *Xba*I and *Hin*dIII. The DNA sequences of the resulting clones were confirmed by the method of Maxam and Gilbert (1980). Appropriate clones were cleaved at the polylinker *Hin*dIII site on the 3′ side of the promoter, filled in with Klenow polymerase, and blunt end ligated to a 3.9 kb filled-in *Xba*I−*Bam*HI DNA fragment that contains ~2.2 kb of the 3′ portion of the $J_\kappa-C_\kappa$ intron (which includes the κ enhancer), the C_κ region, and 1 kb of 3′ flanking sequence. These plasmids were then cleaved at the *Bam*HI site in the 5′ polylinker region and at the *Pvu*I site 116 bp 3′ of the polylinker region to release the constructs from vector DNA sequences. DNA fragments containing the κ constructs were fractionated by agarose gel electrophoresis, purified by electroelution, filled in with Klenow polymerase, and blunt end ligated to the filled-in *Eco*RI site of plasmid pSV2neo to yield the final constructs. In these and all other constructs, the κ and *neo* genes are in opposite transcriptional orientation. For constructs −156 and −110, a 320 bp *Bam*HI−*Pst*I V_κ19A DNA fragment containing 198 bp of 5′ flank, the leader exon, and 48 bp of intron DNA sequence was blunt end ligated to the *Bam*HI site of plasmid pGEM2. This plasmid was linearized at the *Eco*RI site 5′ of the V_κ19A sequences and digested with *Bal*31 exonuclease for various times. Samples were pooled, phenol extracted, precipitated with ethanol, filled in with Klenow polymerase and reprecipitated. The DNA fragments were cleaved at the polylinker *Xba*I site 3′ of the V_κ19A sequences, isolated by PAGE and ligated to *Hin*cII−*Xba*I cleaved pGEM4. After verification by dideoxy chain termination DNA sequencing (Promega Biotech), plasmids were cleaved at the *Xba*I−*Bam*HI polylinker sites 3′ of the V_κ19A sequences and ligated to the 3.9 kb C_κ-containing *Xba*I−*Bam*HI DNA fragment. The resultant clones were cleaved with *Pst*I−*Bam*HI to release the insert sequences from the vector, and after purification, DNA fragments were blunt end ligated to *Eco*RI cleaved pSV2neo. Constructs −200 and −40 were derived from plasmids L19 and L19.0$^-$ previously described (Atchison and Perry, 1986). The DNA inserts on L19 and L19.0$^-$ were released by *Sal*I−*Bam*HI digestion and inserted at the *Eco*RI site of pSV2neo as described above. The oligonucleotides used for preparing constructs −96, −84, −72 and −84octa$^+$ are: −96, CTAGATAATTTACTTCCTTATTTGATGACTCCTTTGCATA and GATCTATGCAAAGGAGTCATCAAATAAGGAAGTAAATTAT; −84, CTAGATTATTTGATGACTCCTTTGCATA and GATCTATGCAAAGGAGTCATCAAATAAT; −72, CTAGATCCTTTGCATA and GATCTATGCAAAGGAT; −84octa$^+$, CTAGATTATTTGATGACTCATTTGCATA and GATCTATGCAAATGAGTCATCAAATAAT.

Plasmids containing κY and octa mutations were created using an oligonucleotide directed mutagenesis protocol (DeChiara *et al.*, 1986). For the construction of κY$^-$O* and κY$^+$O$^-$, a 305 bp *Xba*I−*Aat*II fragment containing the V_κ19 promoter region was excised from the −110 V_κ19A plasmid and inserted into the pGEM4 blue vector. Plasmid κY$^-$O$^-$ was derived from a κY$^-$O* subclone. The oligonucleotides used for mutagenesis of κY$^-$O*, κY$^+$O$^-$ and κY$^-$O$^-$ are: (−104) CCGCTGTGTAATTTACTcgagTATTTGATGACTCCTTTGCAT (−63), (−80) TTCCTTATTTGATGACTCCTggcCATAGATCCCTAGAGGCC (−48) and (−88) TcgagTATTTGATGACTCCTggcCATA (−62), respectively. After verification by dideoxy chain termination DNA sequencing with a T7 primer (Sequenase USB kit), plasmids κY$^-$O*, κ$^+$O$^-$ and κY$^-$O$^-$ in pGEM4 blue vector were cleaved by *Xba*I−*Aat*II enzymes. The resulting 305 bp fragments were reintroduced in the −110 V_κ19A plasmid between the *Xba*I−*Aat*II sites.

DNA transfection assays

Cell culture of S194 plasmacytoma cells, transfection by the DEAE−dextran procedure, and isolation of total cytoplasmic RNA were performed as previously described (Atchison and Perry, 1986). Detection of correctly initiated transcripts from the V_κ19A promoter was accomplished by S1 nuclease analysis according to Atchison and Perry (1986) except that the hybridization was done at 37°C and 80 units of S1 nuclease (Pharmacia) was used. To analyze transcripts from the neomycin resistance gene, a 500 bp *Sph*I−*Bgl*II DNA fragment which spans the *neo* transcriptional start site was prepared. Plasmid pSV2neo was cleaved with *Bgl*II, treated with bacterial alkaline phosphatase (BAP; Worthington), and recleaved with *Sph*I. The 500 bp *Sph*I−*Bgl*II DNA fragment was isolated after PAGE, labeled with [^{32}P]γATP by polynucleotide kinase (Pharmacia), and hybridized with 100 μg of total cytoplasmic RNA for 6−12 h in 80% formamide, 10 mM PIPES, pH 6.5, 1 mM EDTA, 0.4 M NaCl at 50°C. After hybridization, S1 nuclease digestion and polyacrylamide−urea gel electrophoresis were performed as previously described (Atchison and Perry, 1986).

Nuclear factor binding assays

Nuclear factor binding assays and PAGE were performed essentially as described by Singh *et al.* (1986). Each assay contained 8 μg of nuclear extract protein (Dignam *et al.*, 1983), 0.1−0.5 ng of the ^{32}P-labeled DNA fragment to be analyzed, and 3.2 μg of poly(dIdC)·poly(dIdC) (Pharmacia). To prepare the DNA probe containing the κ^0 divergent octamer, a 900 bp *Eco*RI DNA fragment containing the divergent octamer was treated with BAP, then end-labeled with [^{32}P]γATP and polynucleotide kinase. The labeled fragment was cleaved with *Mbo*II and the 170 bp *Eco*RI−*Mbo*II κ^0 DNA fragment was isolated after PAGE. The V_κ19A divergent octamer probe was prepared from a 98 bp *Bst*NI DNA fragment. This DNA fragment was end-labeled as described above, cleaved with *Pvu*II, and the 78 bp *Bst*NI−*Pvu*II DNA fragment containing the V_κ19A divergent octamer was isolated after PAGE. The V_κ21E canonical octamer probe was prepared from a 59 bp *Rsa*I−*Eco*RI fragment of the PC6684 κ^+ gene (Atchison and Perry, 1986). The κY$^+$O*, κY$^-$O*, κY$^+$O$^-$ and κY$^-$O$^-$ probes were derived from the corresponding pGEM4blue subclones containing *Xba*I−*Aat*II 305 bp fragments. For each plasmid, a *Pvu*II (−43)−*Sau*96A (located in pGEM4blue upstream of −110) fragment was end-labeled with [^{32}P]γATP and isolated after PAGE. Various unlabeled DNA competitors were added to the binding assays as indicated in the figure captions.

Methylation interference and DNase I footprint studies

The V_κ19A plus strand DNA probe was identical to the probe used in the mobility shift assay (see above). The V_κ19A minus strand DNA probe was derived from a subclone (p320) containing the V_κ19A promoter region. This clone was digested with *Pvu*II, treated with BAP, end-labeled with [^{32}P]γATP and polynucleotide kinase, and recleaved with *Bst*NI. The labeled 78 bp *Bst*NI−*Pvu*II DNA fragment containing the divergent octamer motif was isolated after PAGE. The V_κ19A labeled DNA fragments and the 5′ end-labeled 170 bp *Eco*RI−*Mbo*II DNA fragment containing the κ^0 octamer motif (described above) were partially methylated by treatment with dimethyl sulfate (Aldrich) according to the method of Maxam and Gilbert as modified by Staudt *et al.* (1986). Partially methylated or untreated DNA probes were subjected to binding reactions that were scaled up 5-fold, and incubated at room temperature for 20 min. The binding reactions containing the unmodified DNA samples were incubated with 5 μg/ml DNase I (Worthington) and 5 mM $MgCl_2$ for 2 min. DNase I digestion was terminated by addition of EDTA to 5 mM. Samples were then electrophoresed on low ionic strength 4% polyacrylamide gels. Bands in the free and bound fractions were visualized by autoradiography, excised from the gel and isolated by electroelution. Each sample was extracted twice with phenol−chloroform, once with chloroform and precipitated twice with ethanol. Samples containing the partially methylated DNA probe were resuspended in 0.15 ml 1 M piperidine and incubated at 90°C for 30 min. Piperidine was removed by butanol precipitations and lyophilization. Equal amounts of radioactivity were subjected to electrophoresis on 8% polyacrylamide−urea sequencing gels and visualized by autoradiography.

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A recent study of V_HQ52 promoter [Yoza,B.K. and Roeder,R.G. (1990) *Mol. Cell Biol.*, **10**, 2145–2153] has revealed an element upstream of the divergent octamer that contributes to the overall efficiency of this promoter in a cell-free transcription system. This element, which partially overlaps the heptamer sequence and interacts with a ubiquitous nuclear factor, is clearly distinct from κY. Whether the κY-like element of this promoter is also required for optimal activity was not addressed in this study.