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Multiple DNA sequence elements are necessary for the function of an immunoglobulin heavy chain promoter

(mutagenesis/sequence conservation/transfection/transcription/B lymphocyte)

SUZANNE EATON* AND KATHRYN CALAME†‡

Departments of *Microbiology and †Biological Chemistry and the ‡Molecular Biology Institute, University of California, Los Angeles, CA 90024

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ABSTRACT **Sequences required for the function of the mouse V1 immunoglobulin heavy chain variable-region (V_H) promoter were identified by transient transfection of the normal and mutated promoters into plasmacytoma cells. Our results identify four regions required for normal promoter function: (*i*) the octamer ATGCAAAT, previously identified by others; (*ii*) a heptamer, CTAATGA; (*iii*) a pyrimidine-rich region; and (*iv*) a region between positions −125 and −251 relative to the transcription start site. Sequence analysis of 19 mouse and human V_H 5′ flanking regions shows that the heptamer and pyrimidine stretch are strongly conserved. We have also demonstrated that the octamer functions in an orientation independent manner in the V_H promoter.**

Transcription of immunoglobulin heavy chain (IgH) variable-region genes is controlled in a complex fashion that is both B-lymphocyte- and developmental-stage-specific. Heavy chain variable-region promoters are preferentially active in cells of the B-lymphoid lineage (1, 3, 4). In early pre-B cells, unrearranged IgH variable gene segments (V_H) are transcribed in an apparently enhancer-independent manner (5). It has been suggested that this early transcriptional activity is critical for permitting subsequent rearrangement of the V_H gene segments to form a functional heavy chain gene (5). Later in B-cell ontogeny, after V_HDJ_H rearrangement (where D and J_H are diversity and joining gene segments, respectively), V_H promoters become enhancer-dependent so that promoters that are within functional proximity of the IgH enhancer are transcriptionally active, whereas those further removed are transcriptionally silent (5–10). This dependence on the tissue-specific heavy chain enhancer only partially accounts for the tissue specificity of transcription; both the enhancer (1, 7, 8, 11) and promoter (1, 3, 4) elements restrict expression of the heavy chain gene to B cells. At later stages of B-cell development, the steady-state level of heavy chain mRNA increases dramatically. Both transcriptional (12, 13) and posttranscriptional (14) mechanisms are involved in this event, suggesting that V_H promoters may be subject to further regulation late in B-cell development.

Given the complexity of this regulation, V_H promoters might be expected to comprise several controlling elements, one or more of which might function in a B-cell-specific way. At present, however, only one DNA sequence has been shown to be important for V_H promoter function (1, 2). This element is a conserved octanucleotide present 5′ of all V_H genes and, in the inverted orientation, 5′ of all light chain variable genes, where it is also required for promoter function (15–17). The octanucleotide also occurs 5′ of the gene encoding the J chain (18). Interestingly, the sequence forms a functional part of several nonimmunoglobulin gene promoters; it is required for expression of the genes for U2 and U1 small nuclear RNAs, histone H2B, and herpes simplex virus thymidine kinase (19–22). Although the ubiquitous expression of these genes initially seemed to rule out a B-cell-specific role for the octamer, recent data suggest that B-cell extracts contain a unique octamer-binding protein in addition to a general octamer-binding protein that is found in all cells (23, 24). Thus, the octamer sequence appears to be responsible for at least part of the B-cell-specific activity of V_H promoters. One might expect additional sequences outside the octamer to be involved in the binding of the lymphoid-specific factor or perhaps in preventing the binding of the general factor, since there is no consistent variation in the sequence of the octamer between variable genes and more generally expressed genes. Additionally, nonoctamer sequence elements might act independently to modulate V_H promoter activity.

We have examined the 5′ flanking regions of a series of V_H genes and identified two sequences 5′ of the octamer that are strongly conserved. A functional analysis of a series of V_H promoter mutations demonstrated that the octamer is necessary but not sufficient for transcriptional competence and that three regions 5′ of the octamer are also required for full V_H promoter activity in plasmacytoma cells. Two of these functionally important regions correspond to heptamer and pyrimidine regions conserved in all V_H genes.

EXPERIMENTAL PROCEDURES

Mutagenesis. The V1 DNA we subjected to mutagenesis was derived from ChM2-423 (25), a bacteriophage λ Charon 4A clone containing the rearranged IgH gene from the hybridoma HPCM2 (26). Mutants were constructed either by progressive 5′ deletion with exonuclease BAL-31 or by specific alteration using a synthetic oligonucleotide and phage vector M13. In the former case, a 2.8-kilobase-pair (kb) *Bgl* II fragment containing the V_HDJ_H exon and 2 kb of 5′ flanking sequence was cloned into $pA_{10}cat_2$ (27). This construct was cleaved with restriction endonuclease *Hin*dIII and treated with BAL-31. Following religation in the presence of *Bgl* II linkers, several clones were isolated that contained variable amounts of the 5′ flanking region. Deletion endpoints were determined by nucleotide sequencing (28). To construct site-directed mutants, the same *Bgl* II fragment was cloned into an M13 derivative, M13ΔE-lac (29). Oligonucleotides were designed to produce the appropriate alteration, and mutagenesis was performed by standard procedures (30). Where confirmation was not possible using a newly generated restriction site, the mutants were confirmed by sequencing.

Transfections. P3X63-Ag8 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in 7% CO_2 and transfected by calcium phosphate coprecipitation essentially as described (11, 31).

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Abbreviations: IgH, immunoglobulin heavy chain; V_H, heavy chain variable-region gene segment; J_H, heavy chain joining-region gene segment; D, diversity-region gene segment.

RNA Preparation. RNA was prepared ≈44 hr after transfection (32). After phenol extraction, RNA was resuspended in 400 μl of TE (10 mM Tris, pH 7.5/1 mM EDTA). To this was added 3 μl of 1 M $MgCl_2$, 40 μl of 100 mM dithiothreitol, 50 units of RNasin (Promega Biotec, Madison, WI), and 12 μg of RNase-free DNase. After incubation at 37°C for 10 min, RNA was extracted with phenol and ethanol-precipitated, and poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography according to standard protocols (59).

RNase Protection. Poly(A)$^+$ RNA (15–30 μg) was combined with 2–5 × 10^5 cpm of uniformly labeled probe synthesized *in vitro* from the bacteriophage SP6 promoter (33). This probe was complementary to the 2.8-kb *Bgl* II fragment comprising the V_HDJ_H and leader exons along with 2 kb of 5′ flanking sequence. RNA and probe were denatured at 85°C for 10 min, hybridized overnight at 49°C in 80% (vol/vol) formamide, and treated with RNase according to the protocol of Melton *et al.* (33). Protected RNA was denatured and electrophoresed in an 8% acrylamide/7 M urea gel. As a control for poly(A)$^+$-selection efficiency, β_2-microglobulin RNA was also measured. Poly(A)$^+$ RNA (0.5–1.0 μg) was hybridized at 53°C with an SP6-synthesized probe complementary to 325 base pairs (bp) of a β_2-microglobulin cDNA (34) and treated as described previously. Band intensities were quantitated by the method of Suiza (35).

Chloramphenicol Acetyltransferase Assays. These were performed as described (36).

Sequence Analysis. We used a data base and programs provided by T. Hunkapiller (California Institute of Technology, Pasadena, CA) to search for homologies between V_H gene segments. Additional homology studies were performed using GenBank§ and the NAQ program from the Protein Identification Resource.

RESULTS

Human and Murine V_H Promoters Contain Three Regions of DNA Sequence Conservation. We examined the 5′ flanking sequence of 2 human and 17 murine V_H gene segments derived from the T15, J606, Q52, and J558 families (37). Fig. 1*A* depicts three regions that we found to be highly conserved. The 3′ sequence, ATGCAAAT, is the previously identified octamer (15, 16). A heptamer, CTCATGA, is also strongly conserved at a variable distance (2–22 bp) upstream of the octamer; 11 of the 19 V_H regions show perfect conservation, and 8 of the 19 have a single base change from the consensus. Other genes that contain at least 6 of the 7 bp of the heptamer in their promoter regions are κ (V_κ; 4 out of 6 examined) and λ (V_λ; 1 out of 2 examined) light chain variable gene segments, the murine genes encoding the β chains of the class II histocompatibility antigens I-A and I-E, the murine gene encoding α_2(I) collagen, and two genes encoding α-amylase (*Amy-2a* I and II) (46–57). Vh81X, a member of a murine IgH gene family not included in the figure, also has a heptamer sequence, but it is 3′ to the octamer (58). Several of the V_H genes depicted in Fig. 1*A* contain multiple copies of the heptamer (not shown). In V_H genes of the J558 family, the heptamer lies only 2 bp upstream of the octamer and is part of an extended region of sequence conservation observed by Siu *et al.* (38) and by Ballard and Bothwell (2) for this V_H family. Upstream of the heptamer, there is a pyrimidine-rich region spanning 8–14 bp. This sequence is present in all the genes analyzed, not more than 41 bp 5′ of the heptamer. Regions in which purines and pyrimidines are asymmetrically distributed between strands have also been observed in a promoter fragment derived from

§National Institutes of Health (1986) Genetic Sequence Databank: GenBank (Research Systems Div., Bolt, Beranek, and Newman, Cambridge, MA), Tape Release 46.0.

A

	PYRIMIDINES	CTCATGA	ATGCAAAT
V1	CTTCACTCTCT....(18)....	CTAATGA....(14)...	ATGCAAAT
V11	CTCCTCCT.....(41)....	CCCATGA....(22)...	ATGCAAAT
V13	CCCATTTTC.....(41)....	CCCATGA....(22)...	ATGCAAAT
V14A	CTTCCACCCCT....(3).....	CCCATGA....(14)...	ATGCAAAT
V14B	TTTCTACCCCT....(3).....	CTCATGA....(14)...	ATGCAAAT
101	CTTCCTTTTATTCC.(0).....	CTCAGGA....(10)...	ATGCAAAG
141	CTTCCTTTTATTCC.(0).....	CTCAGGA....(10)...	ATGCAAAG
A8.1	CTTCCTTTTATTCT.(0).....	CTCAGGA....(10)...	ATGCAAAG
PJ14	CTTCCTTTTATCCT.(0).....	CTCAGGA....(10)...	ATGCAAAT
108A	TCTTCTTTCTCC...(33)....	CTCATGA....(2)....	ATGCAAAT
108B	TCCTTCCTCTCC...(46)....	CTCATGA....(2)....	ATGCAAAT
105	TCCTTCCTCTCC...(46)....	CTCATGA....(2)....	ATGCAAAT
111	TCCTTCCTGCCC...(46)....	CTCATGA....(2)....	ATGCAAAT
104	TCCTTTCTGTTC...(46)....	CTCATGA....(2)....	ATGAAAAT
BCL1	N.S.....	CTCATGA....(2)....	ATGCAAAT
H10	TTTCTTCTTCTCC..(33)....	CTCATGA....(2)....	ATGCAAAT
H4A	TTCCTTCTTCTCC..(33)....	CTCATGA....(2)....	ATGCAAAC
*HG3	TCTTCTTTCTCC...(36)....	CTCATGA....(2)....	ATGCAAAT
*HA2	TCTTTCTC.....(29)....	CTCATGA....(2)....	ATGCAAAT

B

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-280  ATACCAGTGA AGACATGAAG AATAGGTAGT CTTAGATATT GTTAGTTGGA

-230  AGGTGAACTT GTTAAATCAC AATAAAATAT TGAAGTGTTA TCACATACAC

-180  ATACTAAACA ATTTCTAACA TTGTTACTGA TAGCTGATTC ATTCACAATA
                                  PY
-130  TCCCTGCATT TTGTAATAAT AACTTCACTC TCTACAACTT CAATCCTAGA
        7                     8
-80   GCTAATGATA TAGCAGAAAG ACATGCAAAT TAGGCCACCC TCATCACATG

-30   AAAACCACCC CAGAGTGACT CTACCAGTGG
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FIG. 1. Sequences conserved among V_H promoters and within the V1 promoter. (*A*) The pyrimidine-rich, heptamer, and octamer elements in IgH promoter regions (refs. 25 and 38–45; Steve Crews and Leroy Hood, personal communication) are written in columns under the consensus sequence. V1, V11, and V13 are members of the T15 family; V14A and V14B are from the J606 family; 101, 141, A8.1, and PJ14 are from the Q52 family. The rest of the murine V_H sequences and the two human V_H sequences are related to J558 (V_H subtype II). Numbers in parentheses indicate the number of bases intervening between elements. N.S. indicates that the region has not been sequenced. Asterisks denote human V_H genes; the other genes are of murine origin. (*B*) The sequence of the V1 heavy chain promoter is shown. Transcription initiates after the G at −1. The conserved elements are overlined (8, octamer; 7, heptamer; PY, pyrimidines).

the mouse I-E β-chain gene that demonstrates tissue-specific enhancing activity (55). Strong conservation of both the heptamer and pyrimidine elements in every V_H sequence examined suggests that they may have functional importance.

Previous work (25) mapped the *in vivo* transcription initiation (cap) site and determined the sequence of the V1 heavy chain gene that encodes the dominant idiotype in the BALB/c response to phosphocholine (Fig. 1*B*). Our approach in this study was to make mutations in the V1 promoter and to analyze their function by transient transfection into plasmacytoma cells. Fig. 2*A* summarizes the mutations of V1 used in these studies: (*i*) four progressive 5′ deletions with endpoints at −251, −125, −57, and −56, relative to the cap site; (*ii*) internal alterations or deletions that obliterated each of the conserved elements we identified; and (*iii*) an inversion (mutant i8) that places the heavy chain octamer in the light chain orientation.

Analysis of Mutant V_H Promoters Reveals a Functional Requirement for Multiple DNA Sequence Elements. To test the function of mutated V1 promoters, we used them to reconstruct complete μ heavy chain genes in the vector pIg (Fig. 3*A*). These constructs were transfected into plasmacytoma P3X63-Ag8 cells along with a constant amount of pSV2cat DNA (27). An aliquot of transfected cells was assayed for chloramphenicol acetyltransferase activity to normalize for transfection efficiency. Poly(A)$^+$ RNA was prepared from the remaining cells and hybridized with a probe for V1 RNA to quantitate V1 promoter activity. Inclusion of a probe for endogenous β_2-microglobulin mRNA provided a control for

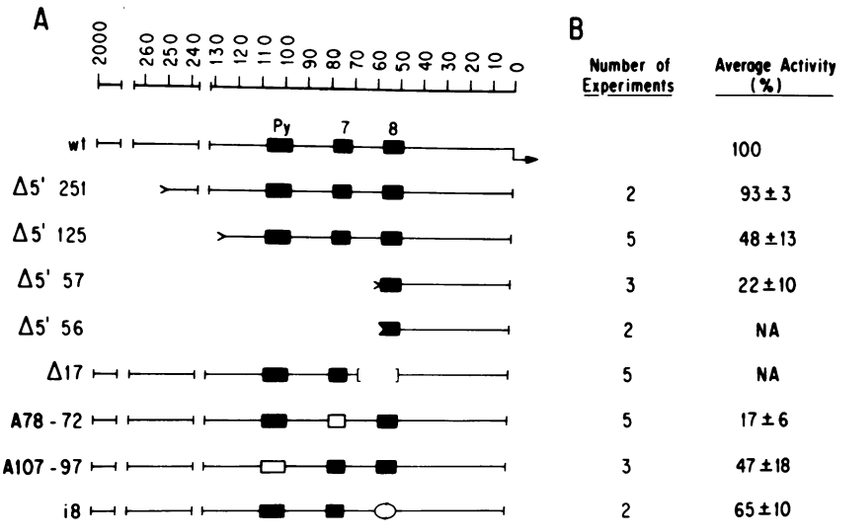

FIG. 2. Mutants used in deletional analysis of the V1 promoter and their activities. (*A*) Scale at the top indicates distance from the cap site. Conserved sequence elements are represented by black rectangles. The wild-type (wt) promoter contains ≈2000 bp of 5′ flanking sequence, and the progressive 5′ deletion mutants Δ5′ 251, Δ5′ 125, Δ5′ 57, and Δ5′ 56 contain the indicated amounts of sequence. Δ5′ 57 has an intact octamer, whereas Δ5′ 56 is missing the first base pair of the octamer. Δ17 is an internal deletion of the octamer and 9 bp 5′ to it. A78-72 is a mutant in which bases −78 to −72 (the heptamer) have been changed from CTAATGA to TGATCAC. A107-97 is a mutant in which bases 107–97 (the pyrimidine element) have been changed from CTTCACTCTCT to GTACACACACA. The mutant i8 contains the sequence ATTTGCAT in place of the heavy chain octamer, ATGCAAAT. (*B*) Results from multiple experiments are corrected for transfection efficiency (chloramphenicol acetyltransferase activity) and RNA level (β_2-microglobulin mRNA). The number of experiments is indicated and the averages are expressed as a percentage of the wild-type level of activity. If a mutant was tested more than twice, the average is expressed ± the standard deviation. If a mutant was tested twice, the average is expressed ± the range of the two experiments. NA indicates *no apparent* activity, even when autoradiograms were exposed for 7 days.

the amount of poly(A)$^+$ RNA loaded in each lane. RNase-protection data from three such experiments are shown in Fig. 3*B*, along with the correction factor derived from the two controls; results from multiple experiments are normalized for transfection efficiency and RNA level and summarized in Fig. 2*B*.

Our data indicate that all promoter activity resides within 251 bp of the cap site, since the 251-bp promoter is fully active (Fig. 2 and Fig. 3*B*, lane c vs. d). Δ17, a mutant in which the octamer has been deleted internally, has undetectable activity even after autoradiograms are exposed for 7 days (Fig. 2 and Fig. 3*B*, lane c vs. g). We estimate our limit of detection

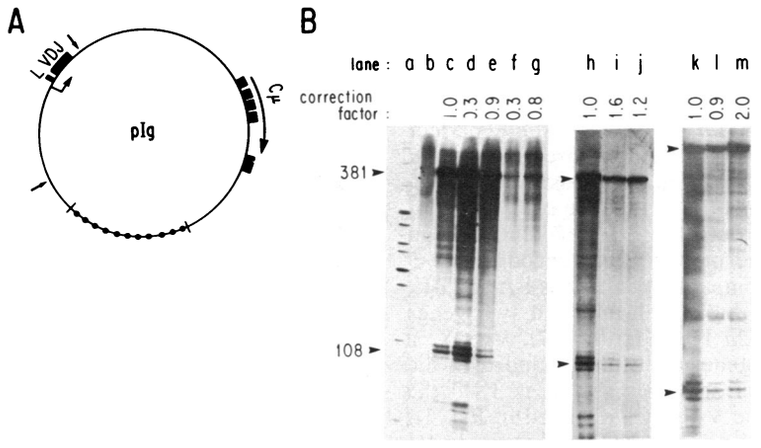

FIG. 3. Structure of pIg vectors and RNase-protection analysis of RNA from transfected cells. (*A*) pIg was constructed by insertion of two *Eco*RI fragments comprising the heavy chain gene of the hybridoma HPCM2 (25, 26) (represented by a solid line) into the *Eco*RI site of pSVODgpt (represented by the beaded line). pSVODgpt does not contain the simian virus 40 enhancer. Mutant promoters were inserted into this construct by replacing the 2.8-kb *Bgl* II fragment (ends indicated by short straight arrows) with a mutated *Bgl* II fragment (see *Experimental Procedures*). Black rectangles represent exons; in addition to the rearranged *VDJ* exon, these include the exon encoding the leader peptide (L) and those encoding the μ heavy chain constant region (C_μ). Bent arrow originates at transcription start site and shows direction of transcription. (*B*) Results from three separate RNase-protection experiments are shown; lanes a–g are from experiment 1, lanes h–j are from experiment 2, and lanes k–m are from experiment 3. Lane a: pBR322 cut with *Hpa* II. Lane b: 30 μg of RNA from untransfected cells. Other lanes: 10–30 μg of poly(A)$^+$ RNA from cells transfected with 100 μg of wild type (lane c), Δ5′ 251 (lane d), Δ5′ 125 (lane e), Δ5′ 56 (lane f), Δ17 (lane g), wild type (lane h), Δ5′ 57 (lane i), A78-72 (lane j), wild type (lane k), A107-97 (lane l), or i8 (lane m) was probed for V1 RNA. Properly initiated transcripts result in protection of a triplet centered at 108 bp. This corresponds to the leader exon and 5′ untranslated sequence. Protection of the V_HDJ_H exon results in a band at 381 bp. Correction factors for each lane indicate the amount by which band intensities must be adjusted to correct for RNA level and transfection efficiency (data not shown). Preliminary experiments indicated that the amount of DNA transfected was in a linear dose–response range and that a 50% decrease in the amount of transfected DNA resulted in a 50% decrease in V1 protected message.

to be ≈2% of wild type; thus, heavy chain promoter activity in the absence of the octamer is reduced by at least a factor of 50. The low activity of Δ5′ 57, which retains only the octamer and sequences 3′ of it, establishes that this element is not sufficient for V_H promoter function and suggests that regions 5′ of the octamer are required (Fig. 2 and Fig. 3*B*, lane h vs. i). Further deletion to −56 results in a promoter with undetectable activity (Fig. 2 and Fig. 3*B*, lane c vs. f), again demonstrating the importance of the octamer.

Progressive 5′ deletion of sequences to −125 resulted in a 52% drop in V1 mRNA (Fig. 2 and Fig. 3*B*, lane c vs. e). Thus, a region that increases activity 2-fold is located near −125 or between −125 and −251; more mutants will be required to delineate this element precisely. Alteration of the conserved stretch of pyrimidines reduced promoter activity by a factor of 2–3 (Fig. 2 and Fig. 3*B*, lane k vs. l), indicating that this region is necessary for normal V_H promoter function. Similarly, alteration of the heptamer lowered the level of expression by a factor of 5 (Fig. 2 and Fig. 3*B*, lane h vs. j), thus establishing its role in V_H promoter function.

The Octamer Can Function in Either Orientation in V_H Promoters. The conserved octanucleotide occurs in opposite orientations in heavy and light chain variable genes. This indicates that it may be able to act in an orientation-independent manner. To test this possibility, we examined a mutant promoter in which the octamer had been inverted. This promoter retained 65% of the original activity (Fig. 2 and Fig. 3*B*, lane k vs. m), suggesting that this element is functional in both orientations.

DISCUSSION

We examined a V_H promoter in order to determine which regions are necessary for expression in plasmacytoma cells. We found the conserved octanucleotide to be necessary but not sufficient for full V_H promoter activity. We identified at least three additional upstream elements that contribute to promoter function and showed that two of them correspond to regions of sequence conservation in all V_H promoters. Finally, we determined that the octamer is active in both orientations in the V_H promoter.

The mutation with the most significant effect on promoter activity, aside from deletion of the octamer, was alteration of the heptamer. The functional importance of this sequence, combined with its strong conservation, defines the heptamer as a controlling element for heavy chain promoters. Although the conserved heptamer has not been identified in previous studies of heavy chain promoters, there has been some evidence to suggest its importance. Recently, Ballard and Bothwell (2) reported that progressive 5′ deletion of a 12-bp region abutting the octamer in V_H 186.2 significantly reduced the activity of this J558-family V_H promoter. In genes of the J558 family, the heptamer is part of a 21-bp region of conservation that includes the octamer motif. The deleted 12 bp contain the conserved heptamer. The identification of the heptamer also clarifies recent experiments reported by Landolfi *et al.* (23). They observed that a 30-bp, octamer-containing fragment derived from the BCL1 V_H gene produced three retarded complexes in an electrophoretic-mobility-shift assay when incubated in lymphoid extracts. When a 20-bp fragment was used instead, one of the three complexes disappeared. The 30-bp fragment contains the heptamer, whereas the 20-bp fragment bisects it. The existence of the heptamer may also be relevant to the results of Mason *et al.* (1) who examined a series of progressive 3′ deletions of the V47 V_H gene, which contains two copies of the octamer. They observed that a deletion endpoint that altered two of the bases in the 3′ octamer had no effect on transcription. A deletion that extended further 5′ but retained the second octamer had greatly reduced activity. The more extensive deletion removed half of the heptamer in addition to the 3′ octamer.

The heptamer sequence occurs in the promoter regions of various genes, some of which are expressed in B lymphocytes (V_H, V_κ, V_λ, *I-E*$_\beta$, I-A$_\beta$) and some of which are not [α_2(I) collagen, *Amy-2a* I and II]. This sequence has not yet been examined specifically for function in non-V_H promoters, although in the collagen promoter it occurs twice in a 473-bp fragment that affects transcriptional activity. It will be interesting to determine the role of the heptamer in these promoters.

Our experiments suggest that the region of purine/pyrimidine asymmetry and sequences between −251 and −125 are also involved in IgH gene expression. The effect of altering or deleting either of these elements is to decrease promoter activity by a factor of 2–3. This reduction is less than that observed for the heptamer or octamer element. It is possible that 2- to 3-fold changes in expression may represent artifactual differences. However, we believe this to be unlikely for two reasons: first, our assay was designed to allow quantitative interpretation by controlling for subtle variations in both transfection efficiency and RNA level; in addition, when one examines the standard deviations associated with the measurement of these mutant activities, they indicate a very significant difference from wild-type activity (Fig. 2). Neither the purine/pyrimidine asymmetry element nor the element between −251 and −125 was observed previously (1–3). Their identification here is allowed by the quantitative nature of our assay and by differences in the design of our mutations.

Since our data suggest a controlling element between −251 and −125 in the V1 promoter, we searched for homologies between this region and other immunoglobulin-associated sequences. We found four stretches of strong homology to the IgH enhancer, one of which was present in 6 of 11 V_H promoter regions (only 11 of the 19 V_H genes depicted in Fig. 1 were examined; the rest had not been sequenced in relevant regions). Further deletional analysis will be necessary to define this element more precisely, to determine whether other V_H promoters contain functionally similar elements, and to determine the significance, if any, of homologies with the IgH enhancer.

The fact that the octamer can be found in either orientation seems to suggest *a priori* that its function is orientation-independent. It is possible, however, that within a given promoter it may be constrained by a requirement for interaction with adjacent promoter elements. Our octamer-inversion mutant, i8, functions at a significant, though reduced, level (65% of wild type). This level is meaningful when compared to the complete lack of activity demonstrated by Δ17, the octamer-deletion mutant. Our results are consistent with those of Ballard and Bothwell (2) who examined the effect of octamer inversion in the 186.2 V_H gene promoter. The orientation-independence of the octamer has also been studied in two non-immunoglobulin promoters. Mattaj *et al.* (19) showed that the octamer located upstream of the *Xenopus* gene encoding U2 small nuclear RNA functions in either orientation. In contrast, Parslow *et al.* (22) observed that the octamer in the herpes thymidine kinase promoter is not active when inverted. Perhaps the orientation-independence of the octamer varies with its context of surrounding sequences. The partial reduction in activity we observe when the octamer is inverted may reflect this. Alternatively, the octamer-factor binding site may consist of a larger region than we have inverted. This would result in an imperfect inverted site of compromised affinity. The relation of orientation-independence to the octamer's mechanism of action awaits further study.

The three promoter elements identified in this study may be involved in conferring B-cell specificity on V_H promoters,

interacting with IgH enhancer factors, or responding to signals from lymphokines. It may also be that these elements are important early in B-cell development for the enhancer-independent expression of V_H genes.

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