Analysis of the imperfect octamer-containing human immunoglobulin V_H6 gene promoter

Zijie Sun and Geoffrey R. Kitchingman*

Department of Virology and Molecular Biology, St Jude Children's Research Hospital, 332 N
Lauderdale Street, Memphis, TN 38101-0318, USA

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ABSTRACT

The octamer sequence ATGCAAAT is highly conserved in the promoter of immunoglobulin heavy and light chain genes and is one of the sequence motifs involved in the control of transcription of these genes. The promoter region of an human immunoglobulin heavy chain variable gene, the sole member of the V_H6 gene family, was found to differ from other V_H gene promoters: it contains neither the conserved octamer motif nor a heptamer sequence, and generally bears little resemblance to other V_H gene transcriptional control regions. An imperfect octamer sequence with a single nucleotide substitution (AgGCAAAT) is located 108 bp upstream of the ATG translation start site, and 81 bp upstream of the transcription initiation site. We sought to determine which sequence elements within the V_H6 promoter were responsible for transcription initiation by creating progressive deletions of a 1 kb fragment from this region and testing their ability to function as promoter elements in B and non-B cells (HeLa). The minimum fragment required for full promoter function was 110 bp, but a fragment with only 65 bp retained 30–50% activity in B cells. Similar levels of transcription were seen when the –146 bp promoter containing two point mutations in the imperfect octamer was tested. Mutation of a possible pyrimidine box sequence located downstream of the TATA box was shown to have only a minor effect (10–30%) on transcription when three nucleotides were changed. Surprisingly, CAT activity was not B cell-specific, as all constructs had virtually the same activity in several B cell lines and in HeLa cells. Removal of the TATA box led to a 50% reduction in CAT activity, and the region upstream of the TATA box functioned as a promoter in both orientations. The transcriptional activity of the V_H6 promoter was virtually enhancer independent: only a minor increase was observed when the immunoglobulin or SV40 enhancer was added to the promoter construct. Electrophoretic mobility shift assays of transcription factor binding to the region around the imperfect octamer indicated that binding was weak when nuclear extracts from either B cells or

HeLa cells were used. The amount of complex shifted was increased by mutating the imperfect octamer to a perfect one. Chimeras produced between the V_H6 promoter and a B cell-specific promoter from a member of the human V_H2 gene family demonstrated that the lack of tissue specificity was due to the absence of a repressor of non-B cell transcription in the V_H6 promoter. These results indicate that the V_H6 promoter is relatively simple, requiring little more than the TATA element and the imperfect octamer, and transcription from this promoter lacks B cell specificity and is not dependent on the enhancer element.

INTRODUCTION

Immunoglobulin (Ig) genes are assembled from a series of discontinuous germline gene segments that are juxtaposed during B lymphocyte differentiation (1–3). For the human Ig heavy chain genes, several hundred variable (V_H) region gene segments (4), at least twenty diversity (D) region gene segments (5), and six functional joining (J_H) region gene segments contribute to the generation of a diverse repertoire of antigen combining sites. Additional diversity can be added through the addition or deletion of nucleotides at the D–J_H and V_H–D junctions.

V_H gene sequences can be grouped into different families based on amino acid or nucleotide similarity (4, 6, 7), and seven human V_H gene families have been identified recently (4, 6). Members of each family are at least 80% or more identical at the nucleotide level. The V_H6 family contains a single member and is the V_H gene segment most proximal to the heavy chain D gene region. It is also thought to be first V_H gene used in ontogeny and it contributes significantly to the fetal heavy chain repertoire (8).

Ig heavy-chain gene transcription is controlled by a cell type-specific regulation mediated through the interaction of sequence-specific DNA binding proteins with *cis*-acting elements (9–13). The Ig heavy chain genes contain two cell type-specific regulatory elements that function independently to promote transcription: the enhancer and promoter regions (14–19). The human heavy chain enhancer is a relatively large region of about 400 bp located

*To whom correspondence should be addressed

between J_H and the mu switch region, and includes binding sites for specific proteins that act as activators or, perhaps in some cases, as repressors of transcription (20, 21). Human Ig heavy chain gene promoters are relatively short stretches of DNA that extend about 150–200 bp upstream of the transcription initiation site and contain several cell type-specific transcriptional elements (16, 22–27). One of these elements is the octanucleotide sequence, 5'-ATGCAAAT-3', and its precise inverse. This sequence is found in virtually all sequenced V_H and Ig light chain (V_L) gene promoters (25, 28). *In vivo* and *in vitro* analyses have shown that this element is required for tissue-specific promoter function (23, 24, 29). A second conserved sequence motif in Ig heavy chain gene promoters, 5'-C-TCATGC-3' (referred to as the heptamer element), is located from between 2 to 22 bp upstream of the octamer element and is required for the full lymphoid cell-specific activity of several immunoglobulin V_H promoters (26, 30). The octamer element is also a functional component of the Ig heavy chain gene enhancer and several non-immunoglobulin gene promoters (13, 31–33). Studies using gel mobility shift assays and DNase I or methylation interference footprint assays have shown that there is a family of transcription factors capable of binding to the octamer motif. Two of these factors, Oct-1 and Oct-2, have been studied in considerable detail (30, 34). Oct-1 is a ubiquitous octamer-binding factor that has been found in all mammalian cells analyzed thus far (35–39), while Oct-2 has been found only in B cells and is considered the octamer-binding protein responsible for cell type-specific transcription of Ig genes (39–43). Recently, it has been shown that the specificity of transcriptional activation by Oct-1 and Oct-2 is determined by the combination of multiple functional domains (39, 44–46).

Mutagenesis experiments have shown that the octamer sequence is an essential component of the immunoglobulin promoter (23, 27, 29). Promoter constructs containing a single octamer in front of a TATA box are preferentially transcribed in B cells (22, 27). Binding of Oct-2 to its recognition sequence is highly specific, as oligonucleotides containing single base pair changes within this sequence compete very poorly or not at all for binding (47).

Because the V_H6 promoter is the first heavy chain variable region promoter activated during ontogeny, and differs so markedly from other V_H promoters, we speculated that unique elements might be involved in controlling transcription of this gene. Through a series of deletion mutants, we determined the minimal requirements for promoter activity, and found that the V_H6 promoter is virtually enhancer-independent and lacks cell type specificity.

MATERIALS AND METHODS

Cell lines

Three human B cell lines were used. Nalm-1, a human acute lymphoblastic leukemia (ALL) pre-B-cell line that expresses CD10, CD20, CD24 and cytoplasmic Ig (cIg) and contains a functionally rearranged V_H4 gene family member (48). ML-1 is an Epstein–Barr virus (EBV)-transformed human early B cell line that expresses surface Ig (sIg) with the heavy chain being from the V_H6 gene family (49). REH cells are a pre-B cell line with only heavy chain gene rearrangement (50). As a non-B cell line control we used a human cervical carcinoma cell line, HeLa. Nalm-1 and ML-1 were propagated in RPMI 1640 containing 20% fetal calf serum, 50 μ M β -mercaptoethanol, 0.03%

glutamine and antibiotics. REH cells were grown in RPMI 1640 with 10% fetal calf serum; HeLa cells were grown in Joklik modified S-MEM (GIBCO) with 10% fetal calf serum.

Construction of plasmids

The genomic clone, M2, containing a rearranged V_H6 gene and 1 kb of sequence upstream of the transcription start site, was isolated from a lambda library from a patient with ALL (51). A 1 kb fragment starting nine nucleotides upstream of the V_H6 gene transcription initiation site was cloned into pBluescript II KS(+) (Stratagene) as an *Eco*RI to *Hind*III fragment, and sequenced with Sequenase 2.0 using protocols supplied by the manufacturer (US Biochemical). This fragment was then transferred into the pCAT-Basic plasmid (Promega). A 515 bp fragment of the human Ig heavy chain enhancer was synthesized with oligonucleotide primers containing *Bam*HI sites by the polymerase chain reaction, using the M2 clone as a template. Following digestion with *Bam*HI, the fragment was ligated into the *Bam*HI site downstream of the CAT reporter gene in the same plasmid (Fig. 1).

Deletion mutants were constructed using the exonuclease III/mung bean nuclease procedure (52). *Bst*XI and *Nhe*I sites located at the 5' end of the V_H6 promoter fragment were used to prepare the plasmid for *Exo*III treatment. Deletion mutants containing 980, 810, 682, 562, 505, 390, 299 and 146 bp of upstream sequence were initially isolated. Large scale growth of these plasmids and CsCl purification were as described (53). The 308 bp deletion of the promoter was constructed by digesting with *Sal*I, which cuts in the polylinker adjacent to the *Hind*III site at the 5' end of the upstream sequences and again at nucleotide –316, followed by recloning into the *Sal*I site of the polylinker of the CAT plasmid with the Ig heavy chain enhancer. Constructs containing 110, 84, 70, 65 and 39 bp of upstream sequence were produced by PCR amplification of these regions with oligonucleotide primers, cloning into a pBluescript-derived T-vector (54), and then moved into the pCAT plasmid. The 78 bp promoter fragment from –66 to –144 was prepared in a similar fashion. Many of the same promoter constructs were also inserted into a CAT expression plasmid containing the SV40 enhancer (Promega).

Point mutations were produced using a PCR-based mutagenesis scheme (17). The imperfect octamer was mutagenized to one with a 5 of 8 match to the perfect octamer by using sets of primers containing two nucleotide changes in conjunction with upstream and downstream primers. A similar strategy was used to mutate the pyrimidine box sequence from a 10/12 match to one with a 7 of 12 match. The following primers were used:

5/8 octamer	5'-AAGTGACGCACACgGCcAATGCCAGGGTGTGGTTT-3'
7/12 pyr box	5'-TGTCAGCaTCggCCGCTGCAGAACTGCA-3'
5' upstream	5'-ATGATTACGCCAAGCTT-3'
3' downstream	5'-GCTCAGATCCTCTAGA-3'

These primers were used to synthesize two PCR fragments separately, which were then mixed, denatured, and filled in with the Klenow fragment of DNA polymerase I. The resulting fragment was then amplified using the outside primers. The mutated DNA fragments were then digested with *Hind*III, and cloned into the pCAT-Basic plasmid.

The V_H2 promoter fragment was originally isolated from a genomic clone termed M8 (51). A 226 bp fragment starting at the translation start site was cleaved at *Nco*I sites, converted to

blunt ends with mung bean nuclease, and ligated to *Eco*RI linkers. Following digestion with *Eco*RI, the promoter was cloned into pBluescript II KS(+). The fragment was then transferred into the pCAT-Basic plasmid which had been modified to contain a 515 bp fragment of the human IgH enhancer. Chimeric promoters were generated using PCR. All constructs were sequenced to ensure that no misincorporation occurred during amplification.

DNA transfection

Transfection of plasmid DNA was carried out by electroporation. All transfections were carried out with 20 μ g of test plasmids and 10 μ g of a reference plasmid expressing β -galactosidase (Promega) as an internal control, unless otherwise indicated. The electroporation procedure was modified from a previous report (55). Briefly, both B cells and HeLa cells for electroporation were spun down and resuspended in fresh medium 24 h prior to electroporation. Immediately prior to electroporation, cells were collected by centrifugation, and resuspended at a concentration of 1×10^7 cells per 250 μ l per cuvette. Electroporation was performed using the Cell-porator (BRL), with the voltage and capacitance set at 250 V and 800 μ F. Twenty-four hours after electroporation, cells were collected by centrifugation and washed twice with phosphate-buffered saline, and lysates prepared by freezing and thawing cells three times following resuspension in lysate buffer [250 mM Tris-HCl (pH 7.8), 0.1% Triton X-100]. Cell lysates were centrifuged for 10 min at 14 000 rpm at 4°C, and the supernatant used for the CAT assay.

CAT assays

CAT activity assays were carried out as described by Gorman (53). We added 5 μ l of 35 mM acetyl-coenzyme A and 1 μ l of 25 μ Ci/ml 14 C-chloramphenicol to 80 μ l of cell extract, and incubated samples for 5 h at 37°C. After ethyl acetate extraction and evaporation to dryness, samples were resuspended in 20 μ l of ethyl acetate and spotted onto a TLC plate. After chromatography, the dried plates were autoradiographed using Kodak XAR film and intensifying screens (DuPont). For quantification, bands were cut out and counted. Results represent the mean activity of 2–4 independent experiments. β -galactosidase levels were measured using ONPG as a substrate.

Gel mobility shift assay

Oligonucleotides used for the mobility shift assay were 44 bp fragments, located between –110 and –67 in the V_H6 promoter (6). The wild-type probe contained an imperfect octamer with the sequence AGGCAAAT, and the mutant oligonucleotide contained a perfect octamer (ATGCAAAT). The fragments were generated by PCR amplification. Prior to protein binding, the primer was end-labeled using [γ - 32 P]-ATP and polynucleotide kinase. The gel shift assay was performed essentially as described by Singh *et al.* (37). Each assay contained 12 μ g of crude nuclear extract with 2 μ g of poly(dI-dC)poly(dI-dC) and 1×10^5 cpm of [32 P]-labeled DNA fragment in 20 μ l of a buffer containing 20 mM HEPES (pH 7.9), 50 mM KCl, 5 mM EDTA, 1 mM DTT, 3 mM MgCl₂, and 5% glycerol. After a 20 min incubation at room temperature the binding reaction was loaded onto a 4% polyacrylamide gel (20:1 acrylamide to bisacrylamide) in 0.25 $\times$ TBE buffer, and electrophoresed at 5 V/cm for 4 h at room temperature. The gels were then dried and autoradiographed using Kodak XAR film in the presence of Dupont Lightning Plus intensifying screens.

RESULTS

We previously cloned the V_H –D– J_H region of a rearranged Ig heavy chain gene containing the V_H6 gene segment from a patient with ALL (51). To identify elements required for transcription of this gene, we sequenced a fragment containing the region from –1024 to +51 relative to the transcription initiation site. The first 148 bp upstream of the transcription initiation site had previously been sequenced by Perlmutter and colleagues (6), and our sequence through this region is identical to theirs. A 1 kb fragment of the promoter region, from –8 to –1024, was then subcloned into the pCAT expression vector which also contained the human Ig heavy chain gene enhancer located downstream of the CAT coding region (Fig. 1). A series of 5' deletion mutants was constructed using this plasmid as a starting point, with the smallest promoter fragment in this series containing 146 bp of upstream sequences (Fig. 2A).

The CAT activities of the various constructs following electroporation into three B cell and HeLa cells are shown in Fig. 2B. The wild-type and 7 deletion mutants all promoted similar levels of CAT activity in HeLa, Nalm-1 and REH cells, and nearly comparable levels in ML-1 cells. The V_H6 promoter is a relatively strong one, as the levels of CAT expression were often greater in some cell lines than that promoted by the SV40 promoter/enhancer CAT construct that we used as a positive control for these experiments. Promoter activity was ablated when sequences from –8 to –316 were deleted (ΔV_H6 -700/Imm). The high level of CAT expression under the control of the V_H6 promoter in combination with the Ig heavy chain enhancer in HeLa cells indicates that this combination did not confer B cell specificity. One of the rationales for using a variety of B cell lines was that the V_H6 promoter is different in architecture from other human Ig heavy chain promoters, and this might require a unique set of transcription factors for maximal transcription, and that only B cells expressing this gene would contain this set. The results shown in Fig. 2B would argue against this possibility.

Figure 1. Structure of the CAT plasmid containing the immunoglobulin V_H6 heavy chain gene promoter and heavy chain enhancer. A 1011 bp promoter fragment of the human Ig V_H6 gene was inserted upstream of the CAT gene in the pCAT-Basic plasmid (Promega). A 515 bp fragment of the human Ig heavy chain enhancer was inserted downstream of the CAT gene. This plasmid was used to derive promoter deletion mutants.

Figure 2. Structure of the mutants used in deletion analysis of the V_H6 promoter and their promoter activities. (A) Scale at the top indicates distance from the transcriptional start site. Deletion mutants were constructed using the *exoIII*/mung bean nuclease procedure (52) from the parent CAT plasmid described in Fig. 1. Eight deletion mutants containing different amounts of V_H6 promoter region were isolated and used for transfection. The 308 bp deletion of the promoter (V_H6-700/Imm) was constructed by digesting with *Sall*, which cuts in the polylinker adjacent to nucleotide -1011, and again at nucleotide -316, followed by re-cloning into the *Sall* of the polylinker of the CAT plasmid with the Ig heavy chain enhancer. (B) Relative CAT activity was measured after transfection of the deletion mutants into both B (ML-1, Nalm-1 and REH) and non-B (HeLa) cell lines by electroporation, and assay of CAT activity as described in Materials and Methods. For each cell line, two to four independent experiments were performed. A plasmid containing the CAT gene under the control of the SV40 promoter and enhancer (Promega) was used as a positive control.

The 146 bp sequence element located upstream of the transcription initiation site in the V_H6 gene was as strong a promoter as the 1 kb fragment. Upstream of the TATA box there are several potential binding sites for transcription factors, and a potential pyrimidine box (56, 57) is located immediately downstream of the TATA element (Fig. 3A). To address the importance of these sites for transcription of the V_H6 gene, a second series of deletion mutants was constructed using a PCR-based technique. In the V_H6-110/Imm construct, a potential transcription factor binding site that resembles one found in Herpes simplex virus and Vaccinia thymidine kinase gene promoters was deleted (58). The V_H6-84/Imm construct has had

the USF-2 site (59) removed; in the V_H6-70/Imm plasmid the imperfect octamer (29) has also been. The V_H6-65/Imm promoter has a deletion of the potential binding site for the G-C box binding factor (60). The V_H6-39/Imm mutant had the binding site for the remaining transcription factors including the TATA box factors deleted. As shown in Fig. 3B, CAT activity for the V_H6-110/Imm construct was nearly identical with that of the 146 bp-containing promoter in all four cell lines tested, although activity in REH cells was about 20% lower than the other cell lines. There was a progressive drop in promoter activity as the region between -110 and -39 was deleted. Removal of the USF-2 site led to a promoter with activities in the various cell lines ranging from 35% in HeLa cells to about 70% of the -110 construct in BJA-B and ML-1 cells. Promoter activity was diminished another 10–20% by removal of the imperfect octamer and slightly reduced by removal of the G-C box region. Removal of the TATA and pyrimidine boxes in the V_H6-39/Imm construct reduced promoter activity to levels just above background. These results indicate that the TATA and pyrimidine boxes, in cooperation with multiple upstream elements, were responsible for promoter activity. To determine if these upstream elements could function in the absence of the TATA box, a 3' deletion was constructed that contained sequences from -74 to -146. This construct retained about 30–60% of the activity of the 146 bp fragment in the four cell lines (Fig. 3B). When the orientation of this fragment was reversed, its function actually increased. Inspection of the upstream sequence indicated the presence of a potential TATA box element on the opposite strand between nucleotides -101 and -105 (6). It is possible that when the -74 to -146 fragment is put in front of the CAT gene in the reverse orientation, this potential TATA element functions to promote transcription. Of interest is that one of the reading frames upstream of this element is open for about 300 bp (Z.Sun and G.R.Kitchingman, accompanying paper).

The results with the deletion mutants indicate that the imperfect octamer only contributes about 10–20% of the promoter activity of the 110 bp fragment. To test this more directly, we constructed a point mutant with changes in the octamer, changing the imperfect octamer to one with a 5/8 match (V_H6-146T/Imm; Fig. 4A). This promoter with the 5/8 octamer match showed a 40–70% reduction in CAT activity in the three B cell lines, but only 15% in HeLa cells (Fig. 4B). The reduction seen in the B cell lines was more drastic than would have been predicted from the deletion mutants, whereas the slight decrease seen with HeLa cells is consistent with the deletion mutant studies.

The pyrimidine-rich region immediately downstream of the TATA box in the V_H6 promoter resembles a sequence in several mouse immunoglobulin gene promoters (56, 57) that is necessary for an imperfect octamer to function as an efficient promoter. While this region is found upstream of the imperfect octamer in the two mouse promoters, but still might be required for transcriptional activity of the human V_H6 promoter. Three nucleotides in the pyrimidine box were changed in the V_H6-146pMu/Imm-CAT construct relative to the wild-type promoter (Fig. 4A). These changes produced a 10% reduction in promoter activity in the HeLa cells and a 10–30% reduction in the B cell lines (Fig. 4C). These data indicate that the pyrimidine-rich element in the V_H6 promoter plays a small role in the activity of this promoter.

Most Ig promoters require the presence of enhancer elements to confer maximal activity (24). To determine if this is true for

To determine the nature of the complexes formed on a minimal V_H6 promoter element, we performed gel mobility shift assays using an oligonucleotide centered around the imperfect octamer (Fig. 6A). The 44-mer from the wild-type promoter (V_H6-44WT) bound very poorly to crude nuclear extracts from both B cells and HeLa cells (Fig. 6B). A similar set of bands was found with each of the four extracts, although the intensity of the bands differed significantly. For example, complex

formation was minimal with the extract from ML-1 cells, but readily detectable when extracts from Nalm-1 cells were used. However, in all cases binding was much lower than when control immunoglobulin promoters containing perfect octamers were used (data not shown). To determine if this relatively low level of binding was due to the lack of tight binding sites for transcription factors, we changed the imperfect octamer to a perfect one (Fig. 6A) and repeated the analysis. Use of the V_H6-44Mut oligonucleotide resulted in the formation of significantly larger quantities of shifted complexes with each of the four nuclear extracts, indicating that the wild-type oligonucleotide with the imperfect octamer binds factors only weakly (Fig. 6B). The major complex formed with each of the four extracts was of similar size, indicating that the same factors were probably bound in each case. This complex contained Oct-1, as indicated by the ability of an anti-Oct1 antibody to shift all of this complex (Z.Sun, unpublished data).

The V_H6 promoter displayed no tissue specificity, either with the imperfect octamer and the heavy chain enhancer or with a perfect octamer. The perfect octamer and the heavy chain enhancer have been found to limit expression of Ig promoters to B cells (23, 24, 29), and their inability to do so in the context of the V_H6 promoter was unexpected. To determine if some element of other Ig gene promoters absent in the V_H6 promoter was also required, we compared the promoter activity of the V_H6 gene to that of a V_H2 gene family member. The DNA sequence of the V_H2 promoter fragment is shown in Fig. 7. This promoter contains a perfect octamer centered around -80 bp from the translational start site, and a TATA box-like element is found between -44 and -50 . In preliminary experiments, we determined that this 226 bp fragment was as efficient as a 1 kb fragment when placed in front of a CAT reporter gene (Z.Sun, unpublished results). When the promoter activities of the 226 bp V_H2 gene fragment and the 146 bp fragment from the V_H6 gene were compared using CAT reporter constructs,

the results in Fig. 8 were obtained. The DNA fragment from the V_H2 promoter produced lower CAT activities in all four cell lines tested, ranging from 55% to 85% that of the V_H6 promoter in the three B cell lines, to less than 5% that of the V_H6 promoter in HeLa cells. This is the pattern of expression seen for many other Ig heavy chain gene promoters highly active in B cells and essentially inactive in non-lymphoid cells. To determine if there were specific DNA sequences in the V_H2 promoter that were required to inhibit expression of the promoter in HeLa cells, chimeric promoters were produced that were part V_H2 and part V_H6 (Fig. 9A). The $V_H2/V_H6/Imm$ construct contained V_H6 promoter sequences from -8 to the octamer, with the remainder of the promoter sequences coming from V_H2 (up through -226). The $V_H6/V_H2/Imm$ construct contained V_H2 sequences from the translation initiation site to the octamer, followed by V_H6 promoter sequences up to -146 . All constructs contained the immunoglobulin heavy chain enhancer. The wild-type and chimeric constructs were transfected into ML-1

Figure 4. V_H6 promoter point mutants. (A) DNA sequence of the V_H6 promoter from the transcriptional start site to -146 . The oval, open box and dashed box denote the locations of the imperfect octamer, the TATA box and the pyrimidine rich region, respectively. The sequences of the point mutants are shown below that of the wild-type sequence. (B) CAT activities of the V_H6 imperfect octamer (VH6-146/Imm-CAT) and double point mutant octamer (VH6-146T/Imm-CAT) containing expression plasmids. The relative CAT activity of the wild-type construct was set at 100% in each of the four cell lines. (C) CAT activity of the V_H6 promoter construct containing three nucleotide changes in the pyrimidine box region. The CAT activity of the mutant is expressed relative to that of the wild-type for each of the four cell lines tested.

and HeLa cells, and CAT activity measured. The results demonstrate that all constructs were active in the B cell line ML-1, but only two of the four constructs showed significant activity in HeLa cells (Fig. 9B). These were the wild-type V_H6 promoter, and the chimera in which V_H6 sequences replaced the sequences in the V_H2 promoter upstream of the octamer. The

chimera in which the V_H2 sequences upstream of the octamer were attached to the V_H6 promoter effectively silenced the V_H6 promoter in HeLa cells, implying that a non-B cell extinguisher is located somewhere between -80 and -226 in the V_H2 promoter.

DISCUSSION

The results presented in this paper demonstrate that the human immunoglobulin V_H6 promoter requires 110 bp of upstream sequence for full activity under the transient expression conditions we used. The promoter is very active in B cells of various differentiation stages, and is also very active in the epithelial HeLa cell line. The V_H6 promoter is not significantly stimulated by the Ig heavy chain gene enhancer element, nor does this element repress transcription in non-B cells. The activity of the V_H6 promoter in non-B cells is probably due to the lack of an element such as is found in the V_H2 promoter, that extinguishes expression in non-B cells. The lack of a positive or negative effect of the enhancer, combined with the lack of tissue specificity, make the V_H6 promoter unique among the Ig promoters studied thus far.

Architecture of the V_H6 promoter

The minimal promoter element for the V_H6 gene contains five potential factor binding sites in addition to the TATA box. Our analysis, using the CAT expression vectors containing the Ig heavy chain enhancer, has demonstrated that no one element in the promoter appears to be the major factor responsible for

Figure 5. CAT activity of the V_H6 -146 construct containing the immunoglobulin enhancer (V_H6 -146/Imm), the SV40 enhancer (V_H6 -146/SV40) or no enhancer (V_H6 -146/O). CAT activity was measured following transfection into B (ML-1 or Nalm-1) or non-B (HeLa) cells. Activities are expressed relative to those seen using the V_H6 -146/Imm construct. Each transfection was repeated four times, and standard deviations are indicated by the error bars.

Figure 6. Gel mobility shift assays using oligonucleotides containing the wild-type or mutated V_H6 promoter sequence. (A) Sequence of the V_H6 promoter from -12 to -148 with the potential TATA box and imperfect octamers underlined. The brackets adjacent to V_H6 -44WT and V_H6 -44Mut define the 44 bp oligonucleotide containing either the imperfect (V_H6 -44WT) or perfect (V_H6 -44Mut) octamers. (B) Gel mobility shift assays using the imperfect or perfect octamer-containing oligonucleotides and nuclear extracts prepared from the indicated cell lines.

CCATGGTGCTGAAATGAGATAGATTTCCTGATGGAGACACACGTGGACTTCCACACCTGA
 GGGCTCACTGCTCTCCACACAGATGCACTCCCTACTGAGTCTGAGACCTGAGTGACC
 CCATAGAGTAGGGCTCAGATGAGGGGATGCAAAATCTCCACGCTCCACCCTCCCTGGGT
TCAAAAGACGAGGACAGGGCCTCGCTCAATGAACTCTGCTCCACCATG

Figure 7. Nucleotide sequence of the V_H2 promoter. The nucleotide sequence of an NcoI fragment of the V_H2 promoter is shown. The downstream NcoI site includes the ATG initiation codon. The underlined sequence at positions -44 to -50 represents a TATA-box-like element, while the next underlined sequence is the octamer binding site.

Figure 8. CAT activity in cells transfected with V_H6 and V_H2 promoter constructs. CAT reporter gene constructs driven by a 226 bp fragment from the V_H2 promoter or a 146 bp fragment from the V_H6 promoter were electroporated into each of the four cell lines, extracts prepared, and CAT activity measured as described in Materials and Methods. Three different amounts of each plasmid were tested in each cell line. (A) Autoradiograph of a TLC showing the conversion of chloramphenicol to acetylated chloramphenicol. (B) Quantification of the autorad. The relative amount of chloramphenicol converted to the acetylated form is shown with the V_H6 promoter construct activity arbitrarily set at 100% for each cell type.

transcription. Starting with the V_H6 -110/Imm construct, which retains full activity compared to 1 kb of upstream sequence, there is a gradual diminution in promoter activity as potential factor binding sites are removed. For example, in BJA-B cells, the -84 construct, in which the potential USF-2 binding site has been removed, retains about 71% of the activity of the -110 construct. The -70 construct, where the imperfect octamer has been deleted, still has about 60% of the promoter activity of the -110 construct, while deletion of the potential E2a and G-C boxes reduces activity to about 43%. It is not until the promoter is deleted down past the TATA box in the -39 construct that activity falls below 10%. The promoter activity of the various constructs is similar in the three B cell lines used, which represent two sIg-expressing cell lines and one pre-B cell line. We chose to use the ML-1 cell line (49) because this EBV-transformed lymphoblastoid cell line expresses immunoglobulin containing

Figure 9. Structure and activity of the V_H6/V_H2 chimeric promoters. (A) The structures of the wild-type V_H2 and V_H6 promoter constructs are shown at the top and bottom. Chimeric promoters were constructed by PCR using overlap in the octamer binding site. The light lines represent V_H2 derived sequences, while the heavy lines represent sequences derived from the V_H6 promoter. The TATA-box elements are indicated by the boxes, while the oval denotes the location of the octamer sequence. The actual sequences of the octamer in the various constructs are shown above the oval. (B) Autoradiograph showing the relative CAT activity of ML-1 or HeLa cells electroporated with the four constructs.

the V_H6 variable region, and we wanted to determine if unique factors were involved in its transcription. The results indicate that the promoter is equally active in V_H6 - and non- V_H6 -expressing B cell lines. While there was no real difference in the importance of the potential factor binding sites when the various constructs were analyzed in B cell lines, the region between -110 and -84 seems to be more important for transcription of the V_H6 promoter in HeLa cells than in B cells. Deletion of this region in the -84 construct results in a drop in activity to about 28% compared to the 112% activity of the -110 construct (relative to the V_H6 -146/Imm construct). Removal of the octamer binding site drops the activity of the V_H6 promoter to almost background levels in HeLa cells. Thus, while the V_H6 promoter is equally active in B and non-B cells, the relative contribution of the various potential factor binding sites to promoter activity may differ between cell types. The results with the deletion mutants were strengthened by the results obtained with point mutants. Changes in the first and fifth position within the imperfect octamer reduced promoter activity by 40–70% in the three B cell lines, but only by about 14% in HeLa cells. While an intact imperfect octamer is clearly required for full activity in all cells, it appears that sequences between -110 and -84 are relatively more important in HeLa cells. This region contains the potential USF-2 binding site, but at the present time we do not know whether or not it is this site within the 27 bp that is the important element for transcription in non-B cells. Using crude nuclear extracts from B cells and non-B cells, we do not find protection of this region in methylation interference experiments (Z.Sun, unpublished data). Experiments with promoters containing mutations in the potential USF-2 binding

site and adjacent regions will be required to determine the element(s) in this region important for promoter activity in HeLa cells.

The V_H6 promoter does not require the immunoglobulin enhancer in the construct that we have used, and the SV40 enhancer has no effect on the level of transcription. We have used both a minimal enhancer element, which has been shown by others to be functional, and a much larger region surrounding the enhancer, to determine if transcription of the V_H6 promoter in HeLa cells could be due to the absence of the non-lymphoid cell silencers found in the mouse heavy chain enhancer. Our results demonstrate that if such sequences are present in the human heavy chain enhancer, they are not active under the conditions we used (61). Although we have only analyzed the requirement for the enhancer with this element located downstream of the CAT reporter gene, this enhancer has been shown to be relatively position- and orientation-independent and should function at this location. The lack of tissue specificity seems to be due to the lack of another element, found in the promoter of the Ig heavy chain gene from the V_H2 family, but absent from the V_H6 promoter. It should be noted that while the constructs we used, with the exception of the wild-type V_H2 promoter, contained imperfect octamers, similar results were obtained with promoter constructs containing perfect octamers (Z.Sun, unpublished results). We are currently constructing chimeric promoters with smaller portions of the V_H2 upstream region to further localize the element(s) involved in tissue specificity.

Imperfect octamer-containing promoters

There are at least 16 human and mouse Ig heavy and light chain gene promoters that have been found to contain variant octamers, usually with one nucleotide change in the canonical sequence ATGCAAT (for a complete list, see ref. 56). The V_H6 promoter element studied here is the only human Ig heavy chain promoter discovered thus far that contains a variant octamer. Several variant octamer-containing Ig gene promoters from mice have recently been studied (56, 57), and results from those studies have indicated that other transcription factors that bind adjacent to the imperfect octamer are necessary to compensate for inefficient promotion of transcription by the variant octamer. In one case, binding of a lymphoid-specific factor called κ Y could activate a mouse kappa gene promoter (56), while a ubiquitous factor termed NTF activated the transcriptional capability of a variant octamer-containing mouse heavy chain promoter (57). Both of these factors recognize relatively pyrimidine-rich sequences located immediately upstream of the variant octamer. The V_H6 promoter contains a region that resembles the NTF binding domain, with a 10/12 nucleotide match. These sequences are located between -37 and -48 (6) just downstream of the putative TATA box. The transient transfection assay has shown that this element is required for maximal V_H6 promoter activity, although the double point mutant that we studied retained more activity than similar mutants in the κ Y or NTF binding sites. Removal of the κ Y site from the mouse kappa promoter reduced transcriptional activity by 84% (56), and mutation of the NTF site reduced *in vitro* transcription by 3-fold (57). These results are in contrast to the minimal reductions (10–30%) seen with the V_H6 promoter when the 10/12 pyrimidine box was changed to one having only a 7/12 match. We are cautious in interpreting these results, as there are a number of differences in the constructs and the methods used to analyze expression that make

comparisons difficult. For example, in the studies with the mouse kappa chain promoter, the plasmid constructs contained an authentic gene including enhancer, but promoter activity was not measured in non-B cells (56). The studies with the mouse heavy chain promoter used a construct without an enhancer, but utilized both B cell and non-B cell extracts for *in vitro* transcription studies. This promoter was active in HeLa cell extracts when the NTF binding site was present, regardless of whether the variant octamer was intact, but the promoter was dependent on both the octamer and NTF binding sites for full activity in B cells (57). Further work is clearly needed to determine the exact role that the variant octamer, alternative factor binding sites, and enhancers play in controlling the level and tissue specificity of transcription of Ig promoters. We have used gel shift assays to attempt to determine whether a factor similar to κ Y or NTF binds to the pyrimidine-rich region in the V_H6 promoter, but no complexes have been observed (Z.Sun, unpublished observations).

Analysis within the natural context

Promoters generally rely on more than one transcription factor, or factor binding site, to interact with the generalized transcriptional machinery to effect mRNA synthesis. In many Ig promoters, the binding of two octamer binding proteins to the octamer and heptamer elements promotes a sufficiently strong interaction to allow efficient transcription (23). In other Ig promoters that do not contain heptamers, pyrimidine-rich elements such as κ Y and NTF synergize with the octamer element to promote strong binding of the transcription factors to allow for high levels of transcription (56,57). A third combination of factor binding sites known to promote efficient transcription of Ig promoters is one where the octamer and a USF site are paired. Such a combination has been shown to stimulate the basal level of transcription of the mouse λ 2 gene (62). The V_H6 promoter does not contain a heptamer and mutation of a pyrimidine-rich sequence in the promoter region does not have a significant effect on the transcriptional activity of the promoter in plasmid constructs. The V_H6 promoter does contain a USF-2-like sequence immediately upstream of the imperfect octamer, and deletion of this region reduces transcriptional activity in B cells (by about 30%) and non-B cells (by about 70%). The USF-2-like site is the only transcription factor recognition motif found by computer search of the 27 bp region deleted in the V_H6-84/Imm construct, but we have not directly tested the role of this potential binding site in V_H6 promoter activity. As indicated above, this site is not protected in DNA methylation protection experiments, so all we can conclude at the present time is that some site within the 27 bp region is important for V_H6 promoter activity, and that this site is more crucial for activity in non-B cells than in B cells.

Several groups have reported that mutation of any single nucleotide in the octamer sequence would cause either, (1) loss of binding activity in gel mobility shift assays with both the B cell-specific transcription factor Oct-2 and the ubiquitous transcription factor Oct-1, or (2) abolition of promoter activity in *in vitro* transcription assays (29,47). However, subsequent work by Herr and co-workers demonstrated that the binding of both Oct-1 and Oct-2 to octamer-containing promoters is very dependent on the context within which the octamer lies, and variant octamers with only a 3 of 8 match can bind both Oct-1 and Oct-2 tightly in the proper context (63). The results from the electrophoretic mobility shift assays performed with the wild-

type and mutant V_H6 promoters (Fig. 7B) demonstrate that the affinity of factors, including the octamer binding factors, is not high for the imperfect octamer, but that this does not affect the ability of this promoter to function. The lack of tight binding of factors to the oligonucleotide containing the imperfect octamer contrasts with the strong promoter activity for V_H6 *in vivo*, which is in contrast to several other promoters that have been studied previously (29). Further, increasing the factor binding strength by changing the imperfect octamer to a perfect one in V_H6 only slightly increases the *in vivo* promoter activity (Z.Sun, unpublished data). Elements outside of the 44-mer used for the mobility shift assays may compensate for the poor binding of the octamer binding factors in the context of the natural promoter, although gel mobility shift assays with DNA fragments scanning the promoter region have yet to identify such an element (Z.Sun and G.R.Kitchingman, unpublished data).

The upstream region also acts as a promoter in an orientation-independent manner, perhaps due to the presence of a TATA box-like element on the opposite strand. When the -74 to -146 fragment is reversed as in the V_H6 -74/Imm(R) construct, the TATA box is now positioned about 40 nucleotides away from the normal transcription initiation site for the V_H6 gene, and could potentially function to promote transcription. The presence of this TATA box in the inverse orientation opens up the possibility that bidirectional transcription is occurring in this region. Bidirectional transcription has been demonstrated as occurring from the mouse V_H441 promoter (64). In that case, the RNA produced from the upstream transcription unit is not polyadenylated, and apparently is non-coding. Examination of the upstream region of the V_H6 promoter demonstrates that one reading frame is open for over 300 bp, indicating the potential for a protein coding transcript from this region. Studies to identify potential transcripts and determine their coding potential are reported in the accompanying paper (Z.Sun and G.R.Kitchingman).

Tissue specificity of the V_H6 promoter

The immunoglobulin promoters studied to date require relatively little upstream sequence to promote basal level transcription, and studies from many laboratories have identified the octamer as the element most critical for conferring cell type-specific transcriptional regulation (4, 23-27, 30, 37). Transcription of the V_H6 gene is non-tissue-specific. Others have found that Ig promoters with high affinity octamer binding sites directed ubiquitous expression, while those constructs with weak octamer binding sites were more B cell specific (61). This is exactly the opposite of what we have found. Conversion of the V_H6 imperfect octamer to a perfect one by site-directed mutagenesis results in a more B cell specific promoter (Z.Sun and G.R.Kitchingman, unpublished results), and in a higher affinity binding site as judged by gel mobility shift assays (Fig. 6B). While these differences may reflect the natural differences between the human Ig heavy chain gene promoter we studied and the mouse Ig gene promoters studied by Schaffner's group, they also emphasize the need to analyze promoters within their natural contexts to determine if such differences really do exist. The mouse studies were performed using partial Ig gene promoters truncated immediately upstream of the perfect octamer elements, and were in a plasmid with an SV40 enhancer (61). The relative ratio of the Oct-1 and Oct-2 proteins in the cells used for transcription may have an effect on the relative strength

and tissue specificity of the Ig promoter (65). The cell lines that we chose to use have variable ratios of Oct-1 and Oct-2, ranging from high Oct-2 levels in BJA-B cells to no Oct-2 in HeLa cells, and this does not seem to affect the absolute level of transcription from the V_H6 promoter. It thus appears that the basal level of transcription from this promoter can be stimulated by either of the octamer binding factors. The tissue specificity seems to be due to the lack of a negative element in the promoter region that prevents expression in non-B cells (Fig. 9B). This factor(s) may bind to element(s) analogous to those found in the heavy chain gene enhancer in mice, which act as repressors of transcription in non-B cells (20, 21). From a series of V_H2 promoter deletions that we have made, the region containing this element has been narrowed to between -126 and -176, with there being some sequences between -176 and -226 that have a small effect on tissue specificity (Z.Sun, unpublished data). Whatever the identity of this element, our data demonstrate that the lack of tissue specificity of the V_H6 promoter is due to the absence of a repressor of transcription in non-B cells. We emphasize that the tissue specificity of promoter activity pertains only to the plasmid constructs, and not to the endogenous genes. In non-B cells the immunoglobulin genes are present in closed chromatin that lack the DNase I hypersensitive sites of transcribed regions (67), and for the heavy chain gene enhancer, do not have proteins bound to sites critical for activity (68). We expect that the V_H6 gene region will also be present in closed chromatin in HeLa cells and will not be transcribed, and in support of this we have found no evidence for transcription in the region surrounding this promoter (Z.Sun and G.R.Kitchingman, unpublished data). Thus, while there is the potential for V_H6 promoter activity in non-B cells, there is no evidence that this actually occurs.

We were interested in the V_H6 promoter for several reasons. The first is that it is the V_H region most proximal to the diversity elements (4, 8) and is probably the first variable region promoter activated in ontogeny. Consequently, the requirements for its optimal activity may differ from those of other variable region promoters. The second is that the human immunoglobulin heavy chain enhancer, which is the earliest Ig promoter used during B cell ontogeny and the first used during the rearrangement process, also contains two imperfect octamer sequences (20, 21). The presence of imperfect octamers is potentially functionally important because of several reports demonstrating the decreased octamer binding factor capability of such elements (47), and the *in vivo* decrease in promoter activity (29). Other elements must compensate for the imperfect octamer in this promoter and studies to identify such elements are currently in progress.

Our current studies have served to identify the minimal promoter region of the Ig V_H6 gene, and have determined that the promoter, even in the context of the Ig heavy chain gene enhancer, is not tissue specific. Studies to identify transcription factor binding sites in the V_H6 promoter that are necessary for optimal activity are in progress.

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