An enhancer at the 3' end of the mouse immunoglobulin heavy chain locus

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ABSTRACT

A tissue-specific enhancer (E_{μ}) lies between the joining (J_H) and μ constant region (C_{μ}) gene segments of the immunoglobulin heavy chain (IgH) locus. Since mouse endogenous IgH genes are efficiently transcribed in its absence, the normal function of this enhancer remains ill-defined. Recently, another lymphoid-specific enhancer of equal strength has been identified 3' of the rat IgH locus. We have isolated an analogous sequence from mouse and have mapped it 12.5kb 3' of the 3'-most constant region gene (C_{α} -membrane) of the BALB/c mouse locus. The mouse and rat sequences are 82% homologous and share with other enhancers several DNA sequence motifs capable of binding protein. However, in transient transfection assays, the mouse sequence behaves as a weaker enhancer. The role of this distant element in the expression of endogenous IgH genes, both in E_{μ} -deficient, Ig-producing cell lines and during normal B cell development, is discussed.

INTRODUCTION

The immunoglobulin heavy chain enhancer (E_{μ}), located in the intron separating coding sequences for the heavy chain variable and constant regions, was the first transcriptional enhancer shown to function in a tissue-specific manner. (1,2). This tissue-specificity, along with the fact that E_{μ} was required for the efficient expression of cloned IgH genes, suggested that it was an essential controlling element in the developmentally-regulated expression of IgH genes. However, shortly after discovery of this enhancer, we and others (3-7) showed that its presence was not required for continued expression of endogenous IgH genes.

For example, in a derivative of the IgG2b-producing mouse myeloma line MPC11 (cell line 9921, ref.8), a class-switch DNA rearrangement resulted not only in a switch in Ig heavy chain class ($\gamma 2b$ to $\gamma 2a$), but also in loss of E_{μ} (6). Despite this loss, 9921 continued to produce heavy chain at the high levels characteristic of myeloma cells (7,8). A μ to δ class-switch event occurring in another cell line, similarly resulted in loss of E_{μ} without affecting IgH expression levels (4). A survey of myeloma lines revealed that functional, but E_{μ} -deficient IgH genes were not uncommon (3).

We suggested that a second enhancer might lie within the IgH locus, functionally substituting for the lost enhancer (7). However, we were unable to locate another enhancer in the newly-formed 9921 IgH transcription unit or within a region extending ~32kb 3' of it (7,9). As an alternate explanation for endogenous IgH expression in the absence of E_{μ} , we proposed that transcriptional activation and the maintenance of an active transcriptional state might involve separate mechanisms (7,9). That is, E_{μ} might be required for activation, but not for the maintenance of IgH gene transcription. We tested this hypothesis in a model system involving transfected IgH genes. Our results (9) and those of another, similar study (10) demonstrated that transfected IgH genes, at least when introduced into pre-B cells, required E_{μ} both to activate and to maintain transcription. As we have discussed previously (9), this difference between transfected and endogenous IgH genes might reflect a difference in chromosomal position, developmental history, and/or the developmental stage of the cells in which the genes are expressed.

Another observation in mouse myeloma cells supported the notion that a second enhancer might lie within the IgH locus, specifically in the region 3' of C_{α} . Studies of a myeloma variant with a reduced rate of transcription at the α -heavy chain locus revealed that sequences downstream of the α gene's polyadenylation site (secreted form) had been deleted (11). The deletion included all of the sequences that had as yet been mapped 3' of C_{α} (~4kb). These workers speculated that this might signal the presence of positively-acting regulatory sequences that had been lost by the deletion. Alternatively, they suggested, the DNA rearrangement might have led to the introduction of transcriptionally inhibitory sequences near the α -gene. Subsequently, Kakkis et al. (12) tested six DNA fragments from the region 3' of C_{α} (in mouse) for enhancer function and found none. However, a B cell-specific enhancer has been identified recently at the 3' end of the rat IgH locus after assay of ~30kb of DNA for enhancer function (13). The strength of this rat 3' IgH enhancer was essentially equivalent to that of mouse E_{μ} .

Using synthetic oligonucleotides with sequences derived from this rat 3' IgH enhancer element, we amplified and cloned, by the polymerase chain reaction (PCR) (14), an analogous region from mouse genomic DNA. Sequencing of the mouse clone revealed 82% identity with the rat 3'IgH enhancer. Using the mouse clone, along with two other DNA probes that we had

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previously used to map the 3' region of the mouse IgH locus (15), we were able to place the putative 3' mouse IgH enhancer (3'αE) 12.5kb 3' of BALB/c mouse Cα-membrane sequences. This is 2.4kb beyond the region previously tested for enhancer function by Kakkis et al. (12). Transient expression assays confirmed that 3'αE could behave as an enhancer. However, it proved to be relatively weak, showing an enhancer activity approximately 1/4 that of Eμ.

MATERIALS AND METHODS

Oligonucleotides and polymerase chain reaction (PCR) conditions

Two oligonucleotides, corresponding to positions 1–25 and 712–735 (nucleotides numbered as in ref.13) were used as primers to amplify and clone the mouse-equivalent of the rat 3' IgH enhancer. A KpnI recognition sequence was appended to the 5' end of the 1–25 primer and a BamHI site to the 5' end of the 712–735 oligonucleotide. The sequences of these oligonucleotides were (coding strand) #1–25: 5' cagct agcgg tacc*c aagcc tgtct gtgga gggat tgc (the * precedes nucleotide #1 in the rat sequence) (non-coding strand) #712–735: 5'cagct agcgg atcc*t gaacag aggcg ttgct cacaa cat (* precedes the nucleotide complementary to #735 in the rat sequence).

Standard PCR conditions were used (10mM Tris-Cl, 50mM KCl, 1.5mM MgCl₂, 0.2mM of the four deoxynucleotide triphosphates), with 100pg of each of a pair of oligonucleotide primers added per reaction, 1–2μg mouse DNA as template, and 2.5U AmpliTaq (Perkin Elmer Cetus). The PCR was continued for 35 cycles in a thermal cycler (Perkin Elmer Cetus). The temperature cycle was 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 3 minutes. There was a final extension at 72°C for ten minutes.

Plasmid cloning and sequencing of the mouse 3' IgH locus element

Mouse PCR products obtained with the 1–25/712–735 primer pair were cut with KpnI and BamHI, size-fractionated by agarose gel (1.5%) electrophoresis, and cloned into KpnI/BamHI-digested pBS(+) (Stratagene). Bacteria containing the desired recombinant plasmids were infected with the K07 helper phage, and single-stranded M13 phage DNA isolated by standard methods (16).

A central EcoRI site within the 596bp mouse PCR product was used to subdivide it into 306bp and 290bp fragments, each of which was subcloned into the pBS(+/-) vectors (Stratagene). Both strands of each fragment were sequenced by the chain-termination method (17), using a Sequenase kit (United States Biochemical).

Genomic DNA blot analysis

Agarose gel electrophoresis, transfer to nitrocellulose or Nytran (Schleicher and Schuell), and DNA hybridizations were performed essentially as previously described (18). For all hybridizations, the mouse sequences were separated from vector fragments before labelling and use as radioactive probes. Ten to fifteen micrograms of restriction enzyme-digested DNA were run in each lane.

DNA probes included the mouse PCR product of these studies (3' α E), 3' α -1.3 (19) and 3' α -0.7 (15). 3' α -1.3 (a gift of Janet Stavnezer, U. Mass) is a 1.3kb fragment isolated from phage clone λ 57.1 which contains the α germline gene of the I strain mouse (19). It maps $\sim$ 3.7kb 3' of the EcoRI site adjacent to

the α -membrane (α_m) exon, but hybridizes with other regions 3' of $C\alpha$ as well (see map and 15). 3' α -0.7 was subcloned from a phage clone (Ch3' $C\alpha$ 12A, gift of Kathryn Calame, Columbia Univ.) containing the $C\alpha$ germline gene of BALB/c (19). It maps ~8.6kb 3' of the EcoRI site adjacent to α_m and additionally hybridizes to the regions detected with 3' α -1.3 (see map and ref. 15).

Transient transfections and CAT assays

J558L cells were transiently transfected by the DEAE-dextran method essentially as previously described (20). 2×10^7 cells, in logarithmic growth, were transfected with $10 \mu\text{g}$ plasmid and the cells harvested 44–48 hours later. After washing, the cells were resuspended in 0.15 ml of 0.25 M Tris-HCl, pH 7.5 and lysed by the freeze-thaw method. The resulting extracts were heated at 65°C for 10 minutes before use in the CAT assay in order to inactivate an endogenous acetylase activity. Protein

Figure 1. Sequence of 3'αE. The 596bp sequence of 3'αE is shown. Potential protein-binding motifs (black boxes above sequence) are indicated (AP-2, μE5, and octamer=octa). Sequences designated 1, 2, and 3 (open boxes above sequence) show some similarity to the AP-4 (CAGCTGTGG, ref.29), μE1 (TCAAGATGGC, ref.30), and AP-1 (T[T/G]AGTCA, ref.29) motifs, respectively. At positions where the rat 3'IgH enhancer and 3'αE differ, the nucleotide present in the rat sequence is indicated below the 3'αE sequence. [—] below the sequence indicates a nucleotide(s) missing from the rat sequence. The extra 184bp of sequence in the rat 3'IgH enhancer is indicated, as is one additional nucleotide (A) that is present in rat, but not in the mouse sequence.

concentrations of the cellular extracts were determined using the BioRad Protein Assay. CAT activity was measured by the two-phase fluor method (21) as modified by Han et al. (22).

The CAT expression vectors used in these experiments were derived from the QM293 vector of M. Anderson and S. Morrison (University of California, Los Angeles). QM293 consists of the Ig λ_1 -light chain promoter (263bp XbaI/HaeIII fragment, refs. 23,24) ligated to the HindIII/BamHI fragment of RSVCAT, and inserted into the pAT153 plasmid (25). The HindIII/BamHI fragment of RSVCAT is a 1.6kb fragment containing CAT coding sequences, SV40 intron sequences, and an SV40 polyadenylation signal (26).

QM351 was also provided by M. Anderson and S. Morrison and differs from QM293 only by the insertion of the 678bp Xba-EcoRI $E\mu$ enhancer fragment at a site immediately adjacent to the λ_1 promoter fragment (position 1, Fig.4). We refer to this plasmid as $E\mu 1$ in Fig. 4. The same enhancer fragment (but with a functionally silent mutation in one of the enhancer 'core' sequences, ref.27,28) was also placed in two orientations at the BamHI site of QM293 (position 2, Fig.4). These are designated $E\mu 2+$ and $E\mu 2-$, respectively. The BamHI site is 1.6kb 3' of the λ_1 promoter fragment. In the two 3' α E CAT constructions,

the 596bp 3' α E fragment was inserted at this BamHI site (in both orientations: 3' α E2+ and 3' α E2-, respectively).

RESULTS

A pair of primers that corresponded to nucleotide positions 1–25 and 712–735 within the rat 3'IgH enhancer sequence were used to amplify and clone the analogous sequence in mouse. PCR reaction products from mouse DNA were size-fractionated by agarose gel electrophoresis, blotted to nitrocellulose, and hybridized to a similarly-amplified product from rat DNA (data not shown). The size of the rat PCR product was as predicted by the published sequence of the rat 3'IgH enhancer (13). The mouse fragment was 139bp smaller than the comparable (735bp) rat fragment. The mouse fragment, designated 3' α E, was sequenced and used to hybridize to blots of restriction-digested mouse DNA. The sequence is shown in Fig. 1 and the genomic DNA blots are shown in Fig.2.

The difference in size (139bp) between the mouse and rat fragments is due to a large gap in the mouse sequence as compared to the rat (184bp) and, reciprocally, a few small insertions in the mouse sequence. The 184bp gap in the mouse sequence corresponds to a stretch of alternating dinucleotides (primarily GT_n:GA_n) in the rat (13). When the 735bp rat sequence is aligned with the 596bp mouse sequence, there is an overall identity of 62%. However, when the 184bp gap is eliminated from the homology analysis, the mouse and rat sequences show 82% identity. By comparison, the IgH intron enhancers in mouse and rat show 91% identity.

As in the analogous rat sequence, there are several protein-binding motifs within 3' α E that have been shown to contribute to enhancer activity within the context of other enhancers. The μ E5 and octamer motifs of $E\mu$ are found in this element as is the AP-2 binding motif, first described in the SV40 enhancer (reviewed in 29). The octamer motif is one of two motifs within $E\mu$ that bind lymphoid-specific protein factors (reviewed in 30). The second motif that does so, μ B (31,32), was not present in 3' α E. Sequences that resemble μ E1, AP-1, and AP-4 sites (reviewed in 29) are found within 3' α E. In the 3'IgH enhancer of rat, the AP-1-like site matches the consensus for this motif (13). However, in mouse 3' α E, there is a base-pair change from this consensus. There are also one base-pair changes between mouse and rat μ E1-like and AP-4-like motifs (see legend, Fig. 1).

Because of its homology to the rat 3' IgH enhancer, we thought the mouse sequence might lie in an analogous region within the

Figure 2. Genomic Southern blots establishing placement of 3' α E within the IgH locus. BALB/c mouse genomic DNA was cut with EcoRI (E), BglII (Bg), BamHI (B), or SacI (S), or a combination of these enzymes as indicated at the top of each lane. Digested DNA was hybridized with either the 3' α E probe, 3' α 0.7, or 3' α 1.3 (indicated below lanes). Except for the EcoRI-digested DNA hybridized with 3' α E (first lane), all of the blots shown were sequentially hybridized with each of the three probes. Arrows in the 3' α 0.7 and 3' α 1.3 hybridizations indicate those fragments that are also detected with the 3' α E probe. DNA molecular weight markers are indicated to the left of the autoradiographs.

Figure 3. Localization of 3' α E within the BALB/c mouse IgH locus. Open boxes above the line designate $C\alpha$ coding sequences (α) and the α membrane exon (α_m). 3' α E is shown as a filled box above the line. Open and striped boxes below the map indicate regions that hybridize with one or both of the 3' α 1.3 and 3' α 0.7 probes. Specific restriction sites which flank 3' α E are indicated and include SacI (S), BglII (Bg), BamHI (B), and EcoRI (E). The map shown does not include all of the restriction sites for these enzymes. The map of the region 3' of $C\alpha$ in BALB/c was determined by genomic Southern and pulse field gel electrophoresis, predominantly using the 3' α 1.3 probe (15). 3' α 1.3 hybridizes to a single SfiI (~25kb) and a single KpnI (~33kb) fragment and two EcoRI fragments (27kb and 12kb). Double digestions with EcoRI and other enzymes, together with use of a second probe (3' α 0.7), indicated that an ~2kb EcoRI fragment lay between the 12kb and 27kb fragments (15).

mouse IgH locus. We have previously generated a map of the region 3' of α as part of an effort to identify and describe DNA rearrangements that quite frequently occur in this region in myeloma cells (15; also see map, Fig. 3). This map was determined by genomic DNA blot (Southern) analysis and pulse field gel electrophoresis, predominantly using the 3'-1.3 probe (15). Digestion of BALB/c mouse DNA with EcoRI generates two fragments (12kb and 27kb) that hybridize with both the 3'-1.3 and 3'-0.7 probes. As can be seen in the blots in Fig. 2, the cloned mouse PCR product of this study (3' α E) hybridized both to the smaller of these EcoRI fragments (12kb) and to an even smaller (2kb) fragment. The latter corresponds in size to an EcoRI fragment that separates the 12kb and 27kb fragments (see map, Fig. 3). The position and approximate size of this EcoRI fragment had been established in our previous mapping studies of this region, but it could not be directly visualized with either the 3'-1.3 or 3'-0.7 probe. As 3' α E has an EcoRI site within it, its hybridization pattern is consistent with a position at the junction between the 12kb and 2kb EcoRI fragments.

To confirm this mapping assignment, we analyzed mouse DNA digested with several additional restriction enzymes. For example, a 10.9kb BglII fragment straddles the 2kb EcoRI fragment. As expected, 3' α E hybridized to a BglII fragment of this size. BamHI and SstI fragments that span the 2kb EcoRI fragment have also been mapped previously with the 3'-1.3 and 3'-0.7 probes. Again, these fragments (8.8kb SacI, not shown; 6.9kb BamHI, Fig. 2) were detected with 3' α E. Double digestion with EcoRI and any of these three enzymes generated the expected two-fragment hybridization pattern with 3' α E (see Fig. 2). In this

way, we have been able to position 3' α E within the BALB/c mouse IgH locus, ~12.5kb 3' of the membrane exon of α .

To test 3' α E for enhancer function, we inserted it into an enhancerless reporter plasmid that contained chloramphenicol acetyltransferase (CAT) coding sequences under the control of the Ig λ_1 light-chain promoter. 3' α E was inserted, in both orientations, downstream of the CAT transcription unit ~1.6kb from the λ promoter. To compare the activity of 3' α E to E_μ , comparable modifications of the enhancerless plasmid were made in which E_μ , instead of 3' α E, was inserted either adjacent to the λ promoter or 1.6kb 3' of it. All plasmids were separately transfected into the mouse myeloma cell line J558L, and CAT activity was measured in cell extracts prepared 48 hours after transfection.

As summarized in Fig.4, when placed 3' of the λ promoter, 3' α E enhanced CAT expression (over the enhancerless CAT vector) an average of 4.3-fold in one orientation and 2.4-fold in the other. This was approximately 25% of the activity of E_μ when E_μ was placed in the same position ($E_{\mu 2+/-}$), and 10% of the activity of E_μ placed adjacent to the λ promoter ($E_{\mu 1}$) (Fig.4). It should be noted that E_μ is one of the stronger enhancers identified in mouse Ig loci; the κ light chain intron enhancer has approximately 5% of the activity of E_μ (33).

While our studies demonstrate that mouse 3' α E is a weaker enhancer than mouse E_μ , the rat 3'IgH enhancer appeared to be roughly equivalent in activity to E_μ in the studies of Pettersson et al. (13). Mouse 3' α E and the rat 3'IgH enhancer differ from one another by one nucleotide in three of the six protein-binding motifs identified in the sequences shown in Fig. 1. While these nucleotide differences may contribute to the rat and mouse elements' apparent functional differences, a more thorough functional analysis of each of these enhancers will be required to settle this issue.

DISCUSSION

We have identified a 596bp mouse sequence which lies 12.5kb 3' of the membrane exon of α in BALB/c. It shows 82% identity (excluding a 184bp gap) to a B cell-specific enhancer in the rat. 3' α E functions as an enhancer when assayed in transiently transfected mouse myeloma cells, albeit as a less powerful one than the previously-described IgH enhancer, E_μ .

A central issue is whether 3' α E normally regulates IgH genes. The IgH genes of several mouse cell lines lack E_μ and yet are expressed at high levels (3,5-7,34). This could signal an enhancer-independent stage in IgH gene expression (7,9). Alternatively, the expression of IgH genes may remain enhancer-dependent, with 3' α E functionally replacing E_μ in these cell lines. In the earlier report describing the rat 3'IgH enhancer, that enhancer and mouse E_μ were of equal strength. However, 3' α E is significantly less powerful than E_μ (approximately 1/4). Can 3' α E nevertheless function equivalently to E_μ within the endogenous IgH locus? Can it do so over the much greater distances involved?

Certainly, within this context, it will be of interest to determine whether 3' α E is the only element with enhancer activity 3' of the mouse IgH locus or, rather, is part of a larger controlling region. Preliminary findings suggest that sequences homologous to a part of 3' α E (bp 1 to 300) lie within 2.5kb upstream of 3' α E. While these sequences include only one known transcription factor-binding motif (AP-2), the duplication of this motif and/or of other as-yet-unidentified motifs, might contribute to overall enhancer activity.

Figure 4. Enhancement of CAT activity in 3' α E-containing constructs. A partial diagram of the CAT vectors is shown. Only the CAT transcription unit and the restriction enzyme sites into which enhancer fragments were inserted are included in the diagram. E = EcoRI; B = BamHI; P λ = λ -light chain promoter; CAT = CAT coding sequences. a. Enhancement factor was determined by comparing CAT assay values for each of the constructions relative to the enhancerless CAT vector (QM293). Values are averages from three to six experiments, using two different DNA preps in most cases. Representative experimental values (cpm/80μg extract): 278 (no enhancer); 13,656 ($E_{\mu 1}$); 1166 (3' α E2-); 789 (3' α E2+). b. The nomenclature for the CAT constructs is as follows. 1=position 1 (adjacent to promoter) in CAT construct. 2=position 2 (1.6kb 3' of promoter region) in CAT construct. $E_{\mu} +/-$ = 678bp XbaI-EcoRI fragment in natural (+) or inverted (-) orientation. 3' α E+/- = 596bp KpnI-BamHI fragment in same (+) or opposite (-) orientation to that of sequence shown in Figure 1.

If 3'αE normally enhances transcription of the IgH genes, it would necessarily be functioning at very great distances from the responding promoter. The distance between 3'αE and the IgH promoter of the Eμ-deficient myeloma 9921 can be estimated at 70kb (taking into account a tandem duplication of Cγ2a within this cell line, 35). The distance between 3'αE and the promoter for μ heavy chain genes could be as much as 200kb.

How might a control region several kilobases 3' of Cα 'communicate' with gene promoters at the opposite end of this 200kb locus? Cockerill has suggested that the IgH locus can be divided into at least four looped DNA domains on the basis of matrix attachment sites (MARS; 36). One of these sites maps in or near Eμ, another is in the Cδ-Cγ3 interval, a third lies between Cγ3 and Cγ1, and the fourth, between Cγ1 and Cγ2b. No attachment site was found at the 3' end of the locus, but the DNA studied did not extend as far as the enhancer-like element described here. MARS may provide the scaffolding necessary for bringing remote regions of the chromosome into proximity. Interestingly, another derivative of MPC11 provides some indication of such an interaction between the 3' and 5' ends of the IgH locus. In this cell line, a DNA recombination event juxtaposed V_H sequences with sequences 3' of Cα through an inversion (15). A potential intermediate in this inversion is a loop of DNA containing the expressed V_H gene and the entire C_H gene cluster, stabilized by an interaction between sequences 5' of V and 3' of Cα.

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