
Functional analysis of the murine IgH enhancer: evidence for negative control of cell-type specificity

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ABSTRACT

We have carried out a mutational analysis of the mouse IgH enhancer. Consistent with previous reports, deletions extending from either the 5' side or the 3' side of the enhancer fail to reveal distinct boundaries which define enhancer function in lymphoid cells. Interestingly, internal point mutations and deletions within the "enhancer core" regions fail to identify any necessary functional role for these conserved elements. When tested in CV1 cells, which do not normally respond to the IgH enhancer, certain deletions exhibit significant enhancer activity. We take these findings to indicate that the functional domains of the IgH enhancer are complex and that cell type specificity is defined in part by negative factors present in non-lymphoid cells.

INTRODUCTION

Enhancers are regions of DNA that act in cis to increase the rate of transcription of nearby genes. The unique property that defines these elements is their ability to function from distances of up to several kilobases when located either 3' or 5' to a gene (1). The mechanism by which enhancers function is unknown. It has been postulated that they may act as bidirectional "entry sites" for RNA polymerase or as DNA topoisomerase binding sites. Recent experiments indicate that they bind and can compete for cellular factors (2,3,4,5). Although many enhancers are active in a large variety of cell types, relatively little homology at the level of DNA sequence is observed between enhancers: a finding that suggests that the cellular components that interact with enhancers are complex.

The enhancers located in the J-C intron of the kappa and heavy chain immunoglobulin genes display the features of classical enhancer elements. Unlike most of their viral counterparts which show only slight variations in "host range", the immunoglobulin enhancers are active only in lymphoid cells (6,7,8,9). This cell type specificity, taken with the particular location of these enhancer elements within the genes led to an elegant model of

immunoglobulin transcriptional activation in B lymphocytes. It was reasoned that unrearranged V genes would be transcriptionally inert simply because they are not in the vicinity of the enhancer element. Upon rearrangement, they would come under the influence of the enhancer and thus be transcribed, leading to a mature immunoglobulin mRNA. Recent evidence indicating 1) that certain V genes are transcriptionally active before rearrangement (10) and 2) that the enhancer may be dispensable in late stages of B cell development (11,12) suggests that regulation of immunoglobulin genes is far more intricate.

The initial studies that defined the murine heavy chain enhancer mapped the functional elements qualitatively to a region of DNA comprising approximately 700 nucleotides with DNA fragments as small as 140 nucleotides possessing some activity (6,7). Surveys of the nucleotide sequences within the enhancer element reveal the presence of three regions that bear homology to enhancer "core" sequences and one octanucleotide that is highly conserved within the promoters of both heavy and light chain immunoglobulin genes (13,14). The importance of these conserved sequences was not established in those initial studies. In addition to these four, Ephrussi, *et al.* (4) identified four additional elements defined by regions that exhibit perturbed patterns of methylation when nuclei of lymphoid cells are treated with DMS. The early deletion studies of the enhancer clearly indicate that all four of these latter elements are not simultaneously required for enhancer function.

We have carried out a mutational analysis of the murine IgH enhancer using a transient assay system consisting of the gene encoding chloramphenicol acetyltransferase linked to the human beta globin promoter. Deletion mutants and site-directed point mutants have been linked to this transcription unit and tested for enhancer function upon transfection of both lymphoid and non-lymphoid cells. Our results indicate that the conserved "core" sequence elements may play only a minor role in enhancer function and that cell type specificity is, in part, negatively controlled.

MATERIALS AND METHODS

Plasmid DNAs

All plasmids were constructed using standard techniques (33). The plasmid pSVABGcat(x) (see Figure 1) represents a modification of pSV2cat (15) in which the SV40 early promoter has been replaced with the human beta globin promoter. The human beta globin promoter DNA fragment was deleted from the 3' side to a position one nucleotide past the initiating ATG and modified by the addition of a Hind III linker (T.K., in preparation).

Deletion mutations of the IgH enhancer employ the natural restriction sites indicated in Figure 2 and, with the exception mentioned below, these sites were modified with either Xba I linkers or Eco RI linkers for subcloning into pSVABGcat(x). All deletion mutants were inserted into pSVABGcat(x) in the same orientation relative to the CAT gene as found in the heavy chain intervening sequence. Deletions involving the Pst I site of the enhancer were not modified with Xba I linkers, but cloned directly into pSVABGcat(x) using the Pst I site in the plasmid.

Mutagenesis was carried out using M13 phage and synthetic oligonucleotides essentially as described by Kunkel (34).

Transfections and Assays of Chloramphenicol Acetyltransferase (CATase)

Transfections were carried out as follows. P3-X63Ag8 cells (17) were grown to a density of $3-6 \times 10^5$ cells/ml and washed with serum-free DMEM. Approximately $6-12 \times 10^6$ cells were resuspended in 2 ml of a CaPO₄ precipitate containing 50 ug plasmid (32) and incubated 15 min 37°C. At that time 8 ml DMEM containing 10% FCS was added and the cells were returned to 37°C. Cells were fed with an additional 10 ml medium after 24 hours and harvested and assayed for CATase after 48 hours as described (3,15). Assays were performed in the linear range of activity with regards to both the time of incubation and the amount of extract in the reaction. Transfections of CV1 cells were carried out essentially as described by Graham and van der Eb (32).

In experiments where CATase levels were normalized, taking into account the efficiency of the transfections, a plasmid expressing the human growth hormone gene from the HSV TK promoter and SV40 enhancer was included in each transfection (pSVTKGH). Growth hormone was assayed in the media of each flask as described (35) and this level was used to adjust the corresponding levels of CATase obtained.

RESULTS

Deletion analysis of the IgH enhancer.

The assay system used for our studies of enhancer activity is illustrated in Figure 1. The test plasmid contains pBR322 sequences linked to a hybrid eukaryotic transcription unit consisting of the bacterial CAT gene (encoding chloramphenicol acetyltransferase; CATase (15)) linked to the human beta globin promoter. We chose the CAT gene because of the sensitivity with which its expression can be detected and assayed and the beta globin promoter because of its profound transcriptional sensitivity to enhancer elements. The beta globin promoter has also been shown to correctly

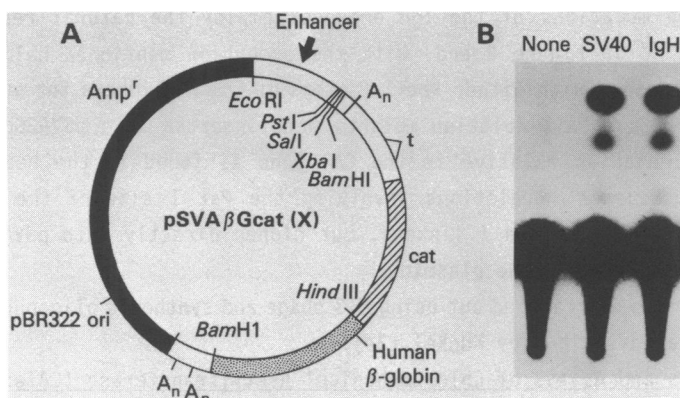


Figure 1. Assay of enhancer activity in P3-X63Ag8 cells. A. Structure of plasmid pSVABGcat(x) used for transfections denoting the position of enhancer insertions. "A_n" and "t" denote the SV40 early polyadenylation signal and small t-antigen intron, respectively. B. Effect of the SV40 enhancer and the Heavy chain immunoglobulin (IgH) enhancer on expression of CATase after transfection of plasmids into P3-X63Ag8 cells. Transfections and assays of CATase were carried out as described in Materials and Methods.

initiate transcription in response to both the SV40 (16) and the IgH enhancers (7). In addition, the plasmid contains a number of convenient restriction enzyme sites 3' to the CAT gene for the insertion of DNA fragments. When transfected into P3-X63Ag8 cells, a mouse myeloma cell line (17), this plasmid gives rise to only very low levels of CATase activity (Lane 1). However, when a segment of DNA corresponding to either the SV40 enhancer (SV40, lane 2) or the mouse heavy chain enhancer (IGH, lane 3) is inserted 3' to the CAT gene, the plasmid produces readily detectable levels of CATase activity as expected. This activity is entirely dependent on the presence of the globin promoter in the plasmid (data not shown).

Figure 2 summarizes the results obtained after transfecting P3-X63Ag8 cells with plasmids carrying a series of deletions extending from either the 5' side or the 3' side of the enhancer. The deletion endpoints are defined by the restriction sites indicated, and each deletion coming from the 5' side is paired with each deletion coming from the 3' side. These combinations were constructed in order to detect any possible redundancies in functional elements, such as those observed the cytomegalovirus enhancer (18) or those observed with enhancers defined by large repeats of DNA (e.g. SV40 (19)). Transfections were carried out either in the absence or presence of an additional plasmid that expresses human growth hormone. In

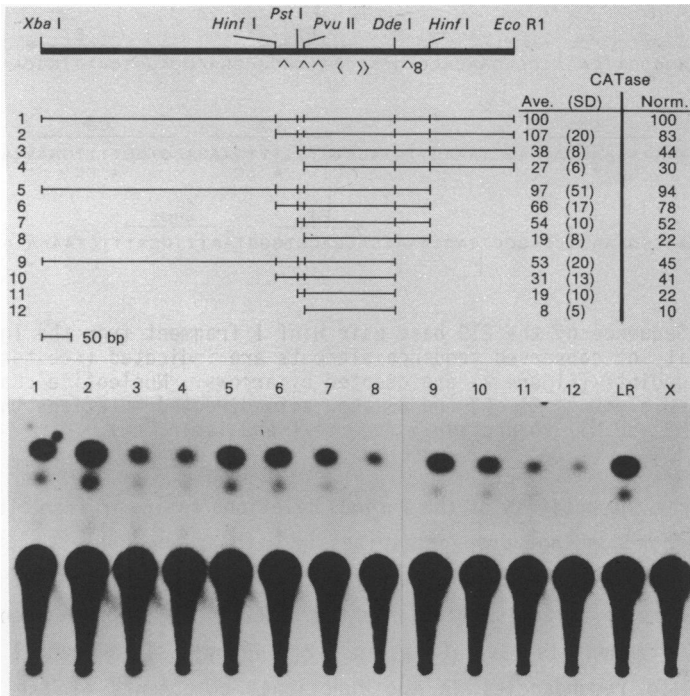


Figure 2. Deletion analysis of the IgH enhancer using transient assays in P3-X63Ag8 cells. CATase levels in the left-hand column (Ave.) represent the number averages (with standard deviations (SD)) of five separate transfections expressed relative to that value obtained with DNA fragment #1 (Eco RI to Xba I, 690 bp). Those values presented in the right-hand column (Norm.) represent values normalized for transfection efficiencies employing a plasmid expressing human growth hormone (average of two transfections). A representative autoradiogram of primary data from such an experiment is shown. "LR" indicates the SV40 enhancer and "X" indicates no enhancer. Symbols , > and "8" represent the positions of the conserved sequence elements diagrammed in Figure 3.

the first case, data represent the average values obtained from five separate transfections. In the second case, the expression of human growth hormone was used to normalize transfection efficiencies. The data obtained from each type of transfection gave essentially the same results, verifying that the plasmid expressing human growth hormone was not competing for transcription factors (or influencing the growth of the cells) and thus could be used to determine relative transfection efficiencies. Enhancer activities of each enhancer fragment are expressed relative to that observed with the largest element tested (Xba I to Eco RI; 690 bp).

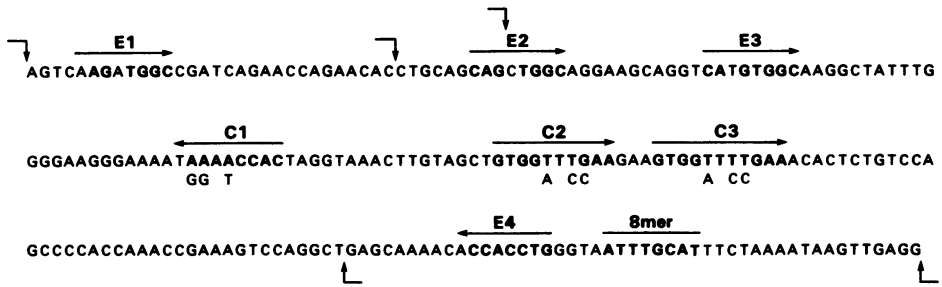


Figure 3. Sequence of the 219 base pair Hinf I fragment from the IgH enhancer. Identity of conserved sequence elements are indicated (see text) and deletion endpoints (Figure 2) are denoted by arrows. Nucleotide changes written below elements C1, C2 and C3 show site-directed mutations that create M1, M2 and M3, respectively (see text and Table One).

Note that the activity of the various deletions coming in from either side of the enhancer does not drop precipitously with any one deletion. Rather, the enhancer activity falls off gradually as DNA is removed from either side, leading finally to an activity of only ten percent of wild type when the Pvu II to Dde I fragment is used (Deletion #12; 140 bp). This gradual drop off is not due to redundancies in the functional components of the enhancer inasmuch as the relative effects of the 5' deletions are independent of which 3' end is present and the relative effects of the 3' deletions are independent of which 5' endpoints are used. However, when we tested plasmids that contain either one, two or three copies of fragment 12 (linked head to tail) or that contain either one, two or three copies of fragment 2, we observed a dose response of CATase expression to the number of enhancers present in the plasmid. This result would suggest that we could build a perfectly good enhancer just by employing the elements present on fragment 12 (to be presented elsewhere).

The nucleotide sequence of the central region of the enhancer element is shown in Figure 3 (Deletion #6, Fig.2), illustrating the conserved sequence elements postulated to be important for enhancer function. These include the three enhancer core elements (C1, C2 and C3), the octanucleotide ATTTGCAT (8mer) and the four elements defined by the perturbed DMS methylation patterns observed in isolated nuclei of B cells (E1, E2, E3 and E4). Also shown are the endpoints of our deletion mutants that map within this region. Note that simultaneous deletion of the octanucleotide and E4 (Fig.2, deletion #9) still retain roughly 50 percent of wild type enhancer activity. These

TABLE ONE
Effect of Core Mutations on Enhancer Activity

Enhancer	Exp.A	Exp.B	Exp.C
Fragment 1	100	100	100
M1	86	103	91
M2	98	106	86
M3	89	109	90
M12	94	106	86
M23	105	106	94
M13	107	99	96

Transfections into P3-X63.Ag8 cells were carried out as described in Materials and Methods and values were normalized to levels of human growth hormone.

conserved elements are therefore not vital for enhancer function. Furthermore, removal of E1 and E2 (Fig.2, deletions #3 and #4) also retain substantial enhancer activity, albeit lower than wild type levels. We conclude that these particular elements, while perhaps influencing the level of enhancer activity, are not absolutely essential.

Mutations within enhancer core elements.

In order to test the functional importance of the "core elements" (C1, C2 and C3) within the IgH enhancer, we point mutagenized each of these three elements and then tested the effect on enhancer activity in the context of the large Eco RI to Xba I DNA fragment (Fig. 2). Specifically, we created Bam HI restriction sites at each locus in order to unambiguously destroy each consensus sequence and to ease the identification and further manipulation of these mutant enhancers (see Fig. 3 for nucleotide substitutions). As shown in Table One, none of the three mutants, M1, M2 and M3 (corresponding to C1, C2 and C3, respectively) showed any significant diminution in enhancer activity.

In light of the degree to which these core elements are conserved between the SV40 enhancer, the polyoma enhancer and the IgH enhancer, this result was surprising to us. However, we reasoned that perhaps only two of the three or perhaps only one the three core elements is necessary for the enhancer to function. Instead of creating all the combinations of M1, M2

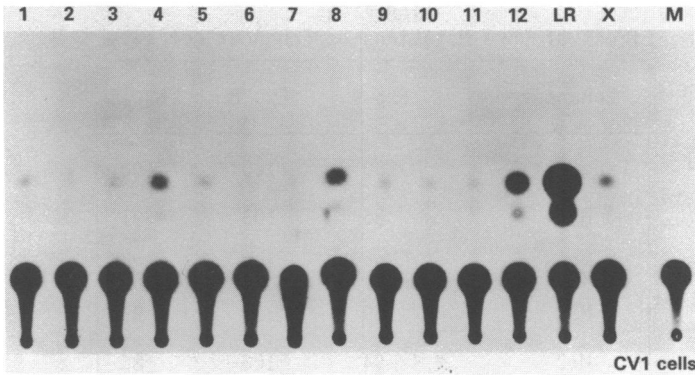


Figure 4. Deletion analysis of the IgH enhancer using transient assays in CV1 cells. CV1 cells were transfected with deletion mutants described in the legend to Figure 2 essentially as described in Materials and Methods.

and M3, we utilized the Bam HI sites we had built into these mutations and created deletions between each of the three core sequences. Hence, M12 is a deletion between C1 and C2, leaving only C3 intact. M23 is a deletion between C2 and C3, leaving C1 intact. M13 is a deletion between C1 and C3 with no core elements being left intact. As shown in Table One, none of these internal deletions had an appreciable effect on enhancer function. We therefore conclude that the enhancer core elements do not play a critical role in enhancer activity in lymphoid cells.

Activity of enhancer deletions in CV1 cells.

In addition to testing our various enhancer molecules in the B lymphoid cell line, P3-X63Ag8, we also tested them in CV1 cells, a monkey epithelial cell line (20). It has been reported that the IgH enhancer has no activity in a variety of non-B cells such as HeLa (7), and mouse L (6) cells. We have confirmed that this is also the case for CV1 cells using the large Xba I to Eco RI restriction fragment. However, when we examine the activity of the various deletions in CV1 cells we see the very striking result shown in Figure 4. Three of the twelve deletions, 4, 8 and 12, show substantially higher levels of enhancer activity in CV1 cells. Deletion 12, in fact, shows an activity that is as much as five percent that observed with the SV40 enhancer. Mutations affecting the core elements of the enhancer did not give rise to increased activity in CV1 cells (data not shown). Note that the three deletions that show increased activity in CV1 cells each possess the same 5' end, namely the Pvu II restriction site (see Fig. 2). We conclude

that some sequence elements within the IgH enhancer that lies 5' to that restriction site and 3' to the Dde restriction site repress the activity of this enhancer in CV1 cells.

DISCUSSION

We have carried out a functional analysis of the murine heavy chain enhancer. The assay system we have employed for this analysis relies on the ability of the enhancer to stimulate transcription of a hybrid transcription unit consisting of the human beta globin promoter linked to the CAT gene. It has previously been shown that the IgH enhancer stimulates proper initiation at the beta globin promoter (7) and that levels of CATase can be used to conveniently measure transcriptional activity (15). We have not re-confirmed those findings in our present study because 1) our various enhancer constructions do not physically disrupt the primary transcript and 2) the enhancers are placed 3' to the transcription unit and should therefore not interfere with utilization of the promoter elements per se. Because of the relatively large distance between the enhancers and the promoter, we have only determined the activity of the enhancers in the orientation that naturally occurs in the heavy chain gene. Although the assay system is artificial (i.e. it does not measure heavy chain gene transcription directly), and is thus limited in its ability to address more general questions concerning immunoglobulin gene regulation, we feel that is suited for the isolated examination of the IgH enhancer element.

Our results using transfections into lymphoid cells indicate that the DNA sequences important for activity of the heavy chain enhancer cannot be strictly defined. The essential elements are spread over a fairly large region of DNA (>220 bp) with no distinct boundary defining the enhancer. Our deletions therefore confirm the results of others which also indicate that enhancer activity is spread over a rather broad stretch of DNA (6,7). Our finding that the enhancer core elements are dispensable for activity is more surprising. These elements are highly homologous to those found in the SV40 and polyoma enhancers and are also conserved in the human heavy chain enhancer (although the core elements are not the only enhancer sequences conserved between mouse and human (21)). It is generally believed that conservation of DNA sequence is a hallmark of functional importance, yet we have been unable to confirm this for the IgH enhancer. It is possible, however, that these sequences possess a function that is not detected by our assay.

The role of the conserved octanucleotide, ATTGCAT, in the enhancer is particularly intriguing. This element is present not only in the mouse heavy chain enhancer (conserved at 7/8 nucleotides in human), but also in both the heavy chain and kappa promoters; in opposite orientations to one another (13,14). Recent evidence suggests that a common cellular protein binds to the octanucleotide present in each of these three elements (5). Our results indicate that this conserved octanucleotide is not critical for enhancer function, even though we have not yet demonstrated by point mutagenesis that it has no effect. The recent demonstration of promoter activity in the vicinity of the heavy chain enhancer suggests a more likely role for the conserved octanucleotide; namely as a promoter element (26). It has been shown that a region of the heavy chain promoter containing the octanucleotide is essential for promoter activity (23) and it is possible that our deletions would have a similar effect if they were tested for promoter activity per se.

We have shown that the cell type specificity displayed by the IgH enhancer is due in part to negative control. That is, we have identified mutations within the enhancer that lead to a substantial increase in enhancer activity in non-lymphoid cells. As mentioned above, the most pronounced effect is observed in those DNA fragments which have been deleted to the Pvu II site just 5' of the core elements. However, deletions which extend from the 3' side of the enhancer also have an effect since deletion #12 gives more activity than either Deletion #4 or Deletion #8 (Fig. 2). We have noted that this activity is still low compared to the SV40 enhancer (5%) and is also sensitive to the efficiencies of our transfections (the better the transfection, the more pronounced the effect). However, even though the activity is low, it should be kept in mind that these same mutations have an adverse affect on the activity of the enhancer in lymphoid cells. Hence, they likely have an adverse affect on the "potential" enhancer activity in CV1 cells as well. It is possible that both lymphoid and CV1 cells express the same factors capable of activating the IgH enhancer, but that CV1 cells also possess factors capable of inhibiting this activation.

Recently, Wasylyk and Wasylyk (36) have reported similar findings for the IgH enhancer. They have shown that, when placed immediately 5' to either a beta globin or conalbumin promoter, certain deletions of the enhancer are active in non-lymphoid cells. Consistent with our results, the enhancer fragment that they obtain the highest activity with in fibroblast cells extends from the Pvu II to Dde I restriction sites (our Fragment 12). They

also note that the relatively low activity of the IgH enhancer is influenced by the efficiency of transfection. However, one inconsistency between the two sets of experiments lies in our ability to measure the enhancement at a distance (1.6 kb). They state that they cannot observe enhancement in fibroblasts unless the enhancer is located adjacent to the promoter. The reason for this discrepancy is not clear, but may be related to the particular DNA sequences that lie between the promoter and enhancer in their plasmids.

Ephrussi, *et al.* (4) have shown that perturbations in the DMS methylation pattern of guanines within the IgH enhancer are seen only in lymphoid cells. One interpretation of this result is that the bound proteins that presumably cause those perturbations are present only in lymphoid cells. This is not necessarily the case if the immunoglobulin genes in non lymphoid cells are inaccessible to those factors. This type of phenomenon has been demonstrated with 293 cells which are able to transcribe transfected beta globin genes, yet do not express their endogenous beta globin genes (27). Presumably, the endogenous gene is inaccessible to those factors that activate the transfected gene. In fact, recent studies employing *in vitro* binding indicate that at least some of the proteins that specifically bind to the heavy chain enhancer and both the heavy chain and kappa promoters are present in both lymphoid cells and HeLa cells (5). For these reasons, the issue as to whether any of these binding activities account for the cell type specificity displayed by the IgH enhancer remains open.

Early studies of immunoglobulin gene expression using somatic cell hybrids showed that expression of the immunoglobulin genes in lymphoid cells could be extinguished by fusing those cells with fibroblasts (28). One explanation of this type of result is to postulate that the fibroblasts express a repressor that is capable of shutting off transcription of the immunoglobulin genes. In support of this model, it has been reported that a heavy chain gene transfected into mouse L cells can be transcriptionally induced by inhibitors of protein synthesis (29). It was postulated that this leads to the disappearance of short-lived repressors of immunoglobulin transcription. Considering that cell type specificity is displayed by both the IgH enhancer and immunoglobulin promoters (22,23,24,25), it is interesting to speculate that such a repressor may be acting on two control elements. A similar type of negative control may also apply to the light chain genes since the ability of LPS to induce transcription of a rearranged kappa gene can be mimicked with inhibitors of protein synthesis (30,31). This latter

finding is perhaps directly relevant to our results with the heavy chain enhancer since both LPS and protein synthesis inhibitors induce a DNase hypersensitive site in the vicinity of the light chain enhancer.

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