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In vivo functional analysis of *in vitro* protein binding sites in the immunoglobulin heavy chain enhancer

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ABSTRACT

We have systematically investigated the functional role of protein binding sites within the mouse immunoglobulin heavy chain enhancer which we previously identified by in vitro binding studies (1,2). Each binding site was deleted, mutant enhancers were cloned 3' of the chloramphenicol acetyl transferase gene in the vector pA10CAT2 and transfected into plasmacytoma cells. We demonstrate that the newly identified site E, located at 324-338 bp, is important for enhancer function; previously identified sites B(uE1), C1(uE2), C2(uE3) and C3 were also shown to be important for enhancer activity. Sites A and D are not required for IgH enhancer function, as assayed by our methods. Thus, including the octamer site, six protein binding sites which bind at least six different proteins are important for enhancer function in vivo.

INTRODUCTION

The immunoglobulin heavy chain (IgH) transcriptional enhancer was the first eukaryotic cellular enhancer to be identified (3-6) and has subsequently served as a paradigm for studies on how enhancers activate transcription in a tissue specific manner (reviewed in 7,8). In vivo competition studies have demonstrated that cellular proteins bind specifically to IgH enhancer DNA and are required for enhancer function (9). In addition, in vivo dimethyl sulfate protection studies have identified several sites, designated "uE1-uE4," where B-cell specific protein contacts occur in vivo (10-11). Furthermore, specific protein binding sites on the IgH enhancer have been identified in vitro using a variety of methods (1,2,12-14).

It is important to correlate protein binding sites, identified either in vivo or in vitro, with enhancer function. In vitro transcription systems have been described (14-17) but the

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effect of the IgH enhancer in vitro is usually much smaller than that observed in vivo and sometimes difficult to reproduce. By contrast, transfection of enhancer- containing constructs allows the in vivo activity of these elements to be assessed. Thus, previous functional analyses of putative protein binding sites within the IgH enhancer have been carried out by mutation and transfection (18-21).

We have carried out an extensive in vitro analysis of protein binding sites on the IgH enhancer and have identified eight protein binding sites within the 1 kb Xba fragment which contains the IgH enhancer (Fig.1 and Fig.3; 1,2). We have identified three protein binding sites not reported by others: the E site (2) just 5' of uE1 at 324-338 bp (numbering of Ephrussi et al [10]) , site A having its 5' boundary at nucleotide 193 bp, and site D having its 3' boundary at nucleotide 727 bp (1). Five other binding sites, B, C1, C2, C3 and octamer, have been defined by our group (1,2) and by others as well (10,11,12,22). The octamer sequence at 541-548 bp is also found in the Simian Virus-40 (SV40) enhancer, immunoglobulin heavy and light chain promoters, and some non-immunoglobulin promotors (23-28). Sites B, C1 and C2 correspond to the B-cell specific motifs uE1, uE2 and uE3 identified by in vivo dimethyl sulfate protection assays (10). (Binding to site uE4, where protection was observed in vivo, has not been observed in vitro.) Binding in vitro also occurs just 3' of site C2 at site C3 which contains homology to a common enhancer GT core motif (3,4). Protein binding at sites E, B, and C2 has been precisely mapped by methylation interfence, DNase I footprinting, and orthophenanthroline/ copper (OP/Cu) chemical nuclease footprinting (2) whereas the remaining binding sites have been partially mapped by exonuclease III studies.

We report here results of functional analyses to determine which of these protein binding sites may be important for IgH enhancer function. Individual protein binding sites defined by in vitro methods were deleted and the mutated enhancers were cloned 3' of the chloramphenicol acetyl transferase (CAT) gene in the vector pA10CAT2 and transfected into plasmacytoma cells. The results show that the newly identified protein binding site, site E, located just 5' to site B, is important for enhancer function;

we also confirm the results of others that sites B, C1, and C2 are required for efficient enhancer activity. In addition, protein binding to site C3, which contains an SV40 GT box homology, was shown to contribute to enhancer function. Finally, sites A and D do not appear to be required for IgH enhancer activity in these transient transfection assays.

MATERIALS AND METHODS

Mutant Enhancer Constructions

Binding sites B, C1, C2, C3 and D were deleted using synthetic oligonucleotide site-directed mutagenesis of a M13 phage vector containing the immunoglobulin heavy chain enhancer region. Oligonucleotides were designed to produce the appropriate deletion and mutagenesis was performed by standard procedures (29). Deletion end points were confirmed by DNA sequencing (30). Site E was removed by a cloning strategy which fused the DraI site at position 320 to the HinfI site at position 345. The mutant with sites B and C2 deleted was generated by cloning the restriction fragment XbaI-PstI isolated from the site B deleted mutant and the PstI-XbaI fragment from the site C2 deleted mutant into the XbaI site of vector the pA10CAT.2. The double mutant with sites C1 and C3 deleted was generated fortuitously with the oligonucleotide designed for C1 deletion. The 1 kb XbaI fragments containing the mutated enhancers, with individual binding sites deleted, were cloned into the XbaI site located 3' of the CAT gene in the vector pA10CAT.2 (5,31). Truncated enhancers including the restriction fragments PvuII-XbaI, XbaI-DdeI and DdeI-DdeI were end-filled with the Klenow fragment of *Escherichia coli* polymerase I and ligated into the end-filled XbaI site of pA10CAT.2.

Transfections

P3X63-Ag8 and L cells which were grown in Dulbecco's modified Eagles's medium (Gibco) with 10% fetal bovine serum (Irvine Scientific) and 20 micrograms per ml gentamycin in 7% CO_2, were transiently transfected by calcium phosphate coprecipitation essentially as described (9,32,33). Fifty micrograms of wild type enhancer plasmid or the molar equivalent of mutant constructs were cotransfected with 50 micrograms of plasmid

pCH110 (34) containing the beta-galactosidase gene into P3X63-Ag8 cells. After 48 hours half of the cells were assayed for CAT assays (9), while the other half was assayed for beta-galactosidase activity as described (35) except the assay was performed at pH 8.5 to reduce the endogenous beta-galactosidase activity. L cell transfections were performed using 10 micrograms of wild type enhancer or the molar equivalent of other constructs.

Gel Retardation Assays

Gel retardation assays and nuclear extract preparations were performed as previously described (2), except that the binding buffer contained final concentrations of 10% glycerol and 200 micrograms per ml of bovine serum albumin. The purification of proteins binding to sites E and C2 (termed uEBP-E and uEBP-C2) by oligonucleotide affinity chromatography will be described elsewhere (Peterson, C., Eaton, S. and Calame, K., manuscript submitted and Peterson, C. and Calame K., manuscript in preparation). Reactions with crude nuclear extract utilized 6ug of protein from the plasmacytoma cell line P3X-Ag8 and 5 ug of poly (dI-dC)-(dI-dC) (Pharmacia), reactions with affinity purified uEBP-E used 0.2 ng of uEBP-E and no nonspecific competitor, and reactions with uEBP-C2 used 0.5 ng of affinity purified uEBP-C2 and 50 ng of poly (dI-dC)-(dI-dC).

RESULTS

Functional analyses demonstrate that multiple sites are required for IgH enhancer activity.

Individual binding sites were deleted as described in Materials and Methods. Deletion end points and the region defined by orthophenanthroline/copper footprinting of each binding site are shown in Fig. 1. Sites E, C1, C3 and D deleted the known sites. The C2 deletion extended 3' of the C2 site into the C3 site and the B deletion removed only the 3' portion of the B site. We were not able to delete site A using oligonucleotide directed mutagenesis, probably because of the high AT content in this region. In order to assess the functional consequence of the removal of both the A and D sites, we transfected PvuII-XbaI truncated enhancer fragments. We did not delete the octamer site

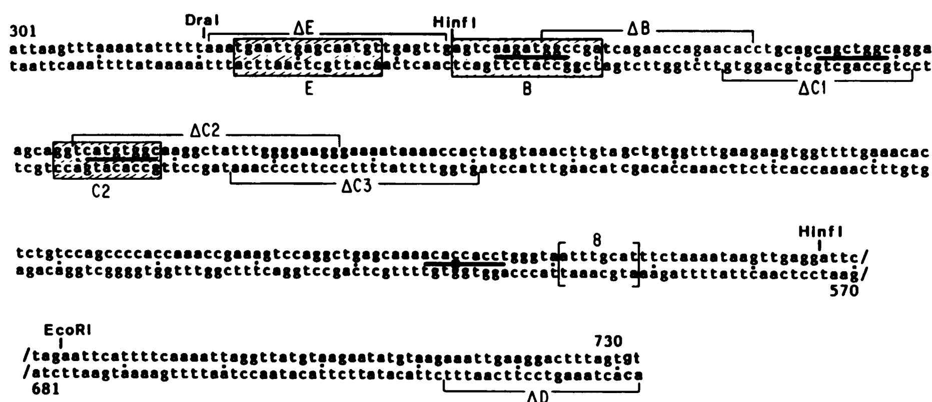

Figure 1. A map of protein-binding sites and end points of binding sites deletions studied in the functional analysis. Shaded boxes designate binding site boundaries by ortho-phenanthroline/copper chemical nuclease footprinting (2). Horizontal brackets designate deletion end points determined by sequencing (30). Horizontal lines indicate uE motifs (10,11). Vertical brackets denote the octamer sequence element. The 5' boundary for site C3 lies at nucleotide 429 and the 3' boundary for site D lies at nucleotide 727 as determined by exonuclease III protection experiments (1).

since the functional importance of this site has been demonstrated previously (19,21,36).

Mutated or wild type IgH enhancer fragments were cloned 3' of the CAT gene in the vector pA10CAT.2, such that the SV40 early promoter minus the SV40 enhancer was dependent upon activation by the IgH enhancers. Placement of the IgH enhancer fragments 3' to the promoter in these constructs mimics its in vivo placement 3' to VH promoters in rearranged heavy chain genes (5). We measured the activity of wild type and mutant enhancers after transient transfection of these constructs into P3X63-Ag8 plasmacytoma cells. In all experiments, variation due to transfection efficiency was corrected by co-transfecting a plasmid expressing beta-galactosidase activity (34,35).

Results of a typical transfection experiment utilizing wild type and mutant constructs are shown in Fig.2. The wild type enhancer stimulated transcription 10-15 fold relative to the enhancer-minus control pA10CAT2. Deletion of sites C1, C2 or E

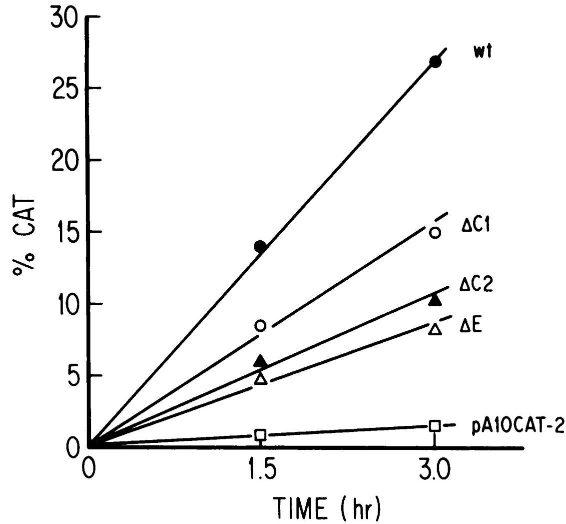

Figure 2. CAT activity from individual deletion mutants. Percent CAT activity was determined by quantitating the amount of labeled chloramphenicol converted into the acetylated forms by scintillation counting (5). A representative CAT assay with time points taken at 0, 1.5 and 3 hours is shown here.

resulted in 56%, 38%, and 31% of wild type activity, respectively. A summary of our results for transfections into P3XAg8 cells is presented in Figure 3. Each construct was transfected 3-10 separate times using different preparations of plasmid DNA and values were corrected for transfection efficiency based on beta-galactosidase assays. The results illustrate that deletion of sites E, B, C1 or C2 significantly reduces the activity of the 1kb IgH enhancer to values of 36+/-8%, 37+/-15%, 49+/-15% and 40+/-15% of the wild type activity, respectively. Deletion of site C3 showed a slight diminution of enhancer activity (78+/-22%); however deletion of sites C1 and C3 had a greater effect (14+/-8%) than deletion of site C1 alone (49+/-15%). Thus, the data suggest that site C3 is functionally important but to a lesser extent than sites E, B, C1 and C2.

We note that none of these deletions completely abolish enhancer activity, suggesting that some of these sites may be functionally redundant. In addition, smaller restriction fragments containing subsets of protein binding sites, such as the

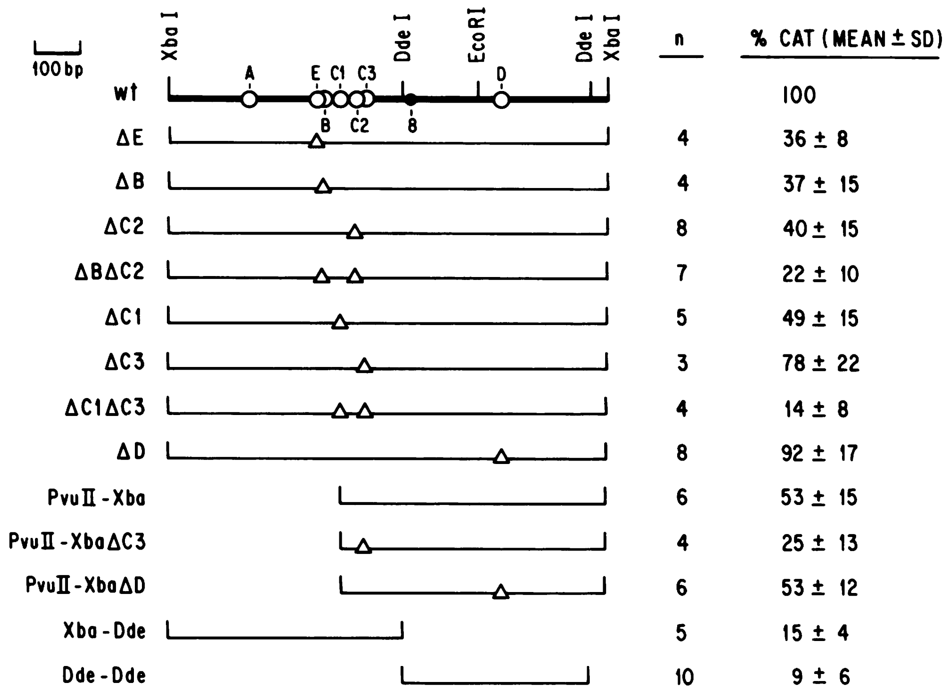

Figure 3. Summary of functional analyses of protein binding sites on the IgH enhancer. Restriction map of the 1.0-Kb XbaI fragment containing the entire IgH enhancer is shown with protein binding sites indicated by circles. The overlapping circles do not imply overlapping binding sites. The number of experiments performed is indicated and CAT activity of each mutant is expressed as a percentage of the wild-type level. Results from multiple experiments are corrected for transfection efficiency (beta-galactosidase) and presented as mean+/-standard deviation.

PvuII-XbaI fragment, the XbaI-DdeI fragment, or the DdeI-DdeI fragment showed activities which were not strictly additive, based on the activity of individual deletions (Fig. 3). These results suggest that in addition to functional redundancy, complicated interactions may occur between proteins bound at different sites with the net result that particular subsets of sites may show varying amounts of activity. It is particularly interesting that when we divided the enhancer at the DdeI site, so that sites A, E, B, C1, C2 and C3 were on the 518 bp XbaI-DdeI fragment and the uE4, octamer and D sites were on the 424 bp DdeI-DdeI fragment, neither fragment exhibited significant enhan-

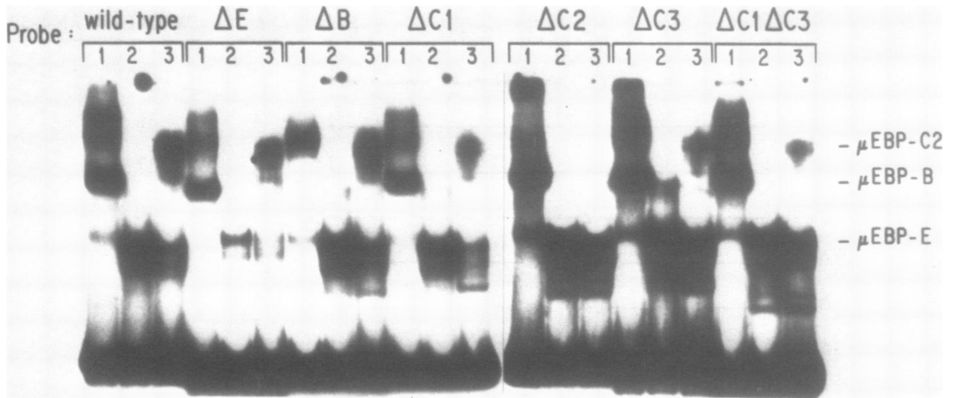

Figure 4. Gel retardation assays with mutant enhancer fragments. 200 bp DraI-DdeI gel retardation probes were prepared from the wild-type and mutant IgH enhancers indicated above the figure. Lanes labeled 1 utilized crude nuclear extract, lanes 2 used affinity purified uEBP-E, and lanes 3 utilized affinity purified uEBP-C2. Gel retardation complexes which correspond to protein binding at sites E, B, and C2 are indicated by arrows. The binding activity seen with the uEBP-C2 preparation with the C2 deletion probe is the result of contamination of this preparation with uEBP-E. The slowly migratory bands seen with crude nuclear extracts with either the C2 or the C3 deleted probe are unresolved complexes in the wells.

cer activity (Fig. 3). The simplest interpretation of these data is that one or more proteins binding within each fragment must interact for efficient enhancer activity.

Deletion of site D in the 1 kb-XbaI fragment resulted in wild type enhancer activity (92+/-17%). Previous exonuclease III competition studies suggested that the same protein binds to sites A and D. Thus, we wished to test the activity of an enhancer which lacked both binding sites. Since we were unable to delete site A directly, we tested the enhancing activity of a shorter restriction fragment lacking site A, the PvuII-XbaI fragment. This fragment, which also lacks sites E, B and C1, produces 53+/-15% of wild type activity compared to the 1 kb-XbaI fragment. Deletion of site D on the PvuII-XbaI fragment caused no further decrease in activity (53+/-12%). Deletion of site C3 in the PvuII-XbaI fragment did decrease enhancer activity (25+/-13%), consistent with site C3 deletion results with the 1 kb Xba I fragment. Thus, we conclude that protein binding at sites A

and D is not required for IgH enhancer function in transient transfection assays.

Several groups (18,20,21) have reported that deletion of certain regions of the IgH enhancer increases activity in fibroblasts, suggesting the presence of negative elements which inactivate the enhancer in non-lymphoid-cells. Each of our mutant IgH enhancer constructs was transfected into mouse L cells at least three times. Although mutants showed decreased activity relative to the wild type similar to that seen in the plasmacytomas, none of the constructs showed significantly increased enhancer activity (data not shown).

Binding site deletion does not affect protein binding at near-by sites.

Deletion of protein binding sites, rather than alteration by base pair substitutions, limits the formation of low affinity binding sites which might permit partial function. However, it is possible that deletions may artificially create novel binding sites or affect binding to the remaining sites by altering the spacing between binding sites. To test these possiblities we performed in vitro gel retardation assays and compared the binding of proteins to wild-type and mutant enhancer probes using crude nuclear extracts as well as purified preparations of uEBP-E and uEBP-C2. We did not assess the binding of proteins to sites C1, C3 and D because we have not worked out gel retardation assays or protein purifications for these proteins yet.

The results (Fig. 4) show that, as expected, deletion of sites E, B or C2 abrogates binding of the cognate protein. The residual binding observed with the site E deletion probe is due to a low affinity binding site for uEBP-E at 467-485 bp on the enhancer determined by chemical nuclease footprinting studies (Peterson, C., Eaton, S. and Calame, K. manuscript submitted). Furthermore, deletion of adjacent sites or other nearby sites does not completely abrogate binding of uEBP-E, uEBP-C2 or uEBP-B. This demonstrates that these proteins do not require interaction with other proteins in order to bind to their cognate DNA sequence, a result consistent with the fact that they can be purified by affinity chromatography using only the cognate binding site (Peterson, C., Eaton, S. and Calame, K. manuscript sub-

mitted and Peterson, C. and Calame, K. manuscript in preparation). The data also suggest that no new binding sites are created by these deletions since no new gel shifted complexes appeared when mutant enhancer probes were used to react with crude nuclear extracts (Fig.4).

Deletion of site C1 or C3 as well as sites C1 and C3 caused about a two-three fold decrease in the binding of purified uEBP-C2 (lane 3) which may decrease enhancer activity. However, these deletions may not affect uEBP-C2 binding in vivo since no decrease of uEBP-C2 was observed using crude nuclear extracts (lane 1). Thus we conclude that the primary effect of deleting sites C1 and C3 is due to lack of binding to proteins at these sites although a minor effect could be due to decreased uEBP-C2 binding.

DISCUSSION

Our purpose in undertaking these studies was to determine which of the IgH enhancer protein binding sites that we had identified by in vitro methods were important for enhancer function in vivo. We were particularly interested in testing the importance of sites A, D and E which had not been tested by others and of site B for which conflicting results have been reported. By systematically deleting each binding site, we have been able to establish their functional importance. The limitation of such an approach is that possible interaction among proteins and possible spatial constraints among sites cannot be assessed. However, having established which sites are necessary for function, we can purify the proteins which bind to them so that these more complex questions may be addressed in the future.

Proteins binding to many of the in vitro sites have been characterized and, in some cases, purified. The octamer is recognized by several proteins, at least one of which appears to be lymphoid specific (23,37,38); two octamer binding proteins have been purified recently (39,40). Proteins binding at all other identified sites have a ubiquitous tissue distribution (1,12,13,40). Three distinct proteins binding to sites E, B, and C2 (termed uEBP-E, uEBP-B and uEBP-C2 respectively) have been purified to near homogeneity (2; Peterson, C., Eaton, S. and

Calame, K. manuscript submitted). In vitro competition studies suggest that the same protein binds to sites A and D while two different proteins bind to sites C1 and C3 (1), but proteins binding to these sites have not yet been purified.

Our results clearly show that site E is important for enhancer function. Although protein binding at site E was not detected in early exonuclease III experiments (1), gel retardation assays (12), in vitro DNase 1 footprinting (13,14), or in vivo mapping studies (10), the probes used in most of these studies did not include the E binding site. The E site has recently identified and carefully mapped (2); uEBP-E, the protein binding to site E, has been purified to apparent homogeniety (Peterson, C., Eaton, S. and Calame, K. manuscript submitted). We have also noticed recently that purified uEBP-E binds with lower affinity to another site within the IgH enhancer which is 70 bp 5' of the octamer element and includes the third common enhancer GT box. A region containing the second E site was altered previously by mutation; it was found to have no function in lymphoid cells (18). It will be interesting to test the functionality of double deletions of both uEBP-E binding sites.

It is also clear from our data that the proteins which bind to sites A and D are not important for enhancer function, at least as assessed by transient transfection studies. Sites A and D are in the vicinity of nuclear matrix association regions and topoisomerase II consensus sequences (41,42), suggesting that these sites and the protein which binds to them may be important in organization of chromatin loop domains. Imler et al (20) defined large regions, which included sites A and D, on the 3' and 5' ends of the 1 kb XbaI IgH enhancer fragment which showed inhibitory activity in non-B cells. Our failure to observe increased enhancer activity in L cells transfected with constructs lacking the A and D sites argues that these specific sites are not involved in the inhibitory activity noted by Imler et al (20).

In general agreement with others (18-21), we find that sites B, C1, C2 and to a lesser extent C3 are important for IgH enhancer function. Our results showing the importance of site B are in agreement with Lenardo et al (19) who tested 3-5 bp al-

terations in this site and noted a significant decrease in enhancer activity. The inability of Kiledjian et al (21) to observe decreased activity after alteration of this site may be related to technical differences in the specific constructs used. Kiledjian et al (21) did observe decreased IgH enhancer activity when they altered uE2 (C1) and uE3 (C2), which agrees with our results. Wasylyk et al have shown that the SV40 core elements play an important role in IgH enhancer activity in fibroblasts (43), although Kadesch et al found little if any importance for the core elements in B-cells (18). Our finding of a slight effect upon deleting site C3 is consistent with the notion that other elements of the enhancer are more important for activity in B cells.

Although we did not test the functional importance of the octamer site, its importance has been demonstrated previously (19,21,36). Thus, it appears that six sites--E, B(uE1), C1(uE2), C2(uE3), C3 and the octamer are important for IgH enhancer function, although site C3 has minor importance in B cells. Site uE4 has also been shown by Kiledjian et al (21) to be important although no protein binding has been demonstrated at this site.

None of our deletions completely abolished enhancer activity. Similar results have been obtained by others for the IgH enhancer (18-21) and for the SV40 viral enhancer (44). This suggests that sites which bind different proteins may be functionally similar or redundant within the overall context of transcription activation. We also note a lack of additive effects for combined deletions or for fragments containing subsets of binding sites. It is difficult to interpret these results further until the exact functional interactions between different enhancer factors is understood more thoroughly.

Our finding that when the IgH enhancer was divided at the DdeI site neither fragment alone had significant activity is consistent with the results of Kiledjian et al (21) who also found, using slightly different constructs, that neither half of the enhancer had high activity. They also showed that amplification of either portion led to increased activity and argued that the enhancer was functionally divided into two domains. Our results are consistent with that notion and suggest that interaction be-

tween proteins bound in both domains is required for full activity.

There have been several reports of negative elements in the IgH enhancer which inhibit enhancer activity in non-B cells (18,20,21). Existence of such an element would be consistent with the observation that non-lymphoid cells contain trans-acting negative regulators which inhibit immunoglobulin gene transcription (46). The enhancer could be the target for such a trans-acting negative regulator. However, the reported locations of negative elements is confusing and inconsistent (18, 20, 21). None of the deletions we have made showed increased IgH enhancer activity, compared to wild type, when transfected into mouse L cells. The explanation for our finding is not clear but may be related to the particular constructions or cell lines which were used. In particular, based on the results of Kadesch et al (18) and Kiledjian et al (21), we expected that deletion of the B or C1 sites might lead to increased IgH enhancer activity in L cells. It may be that the negative effect can only be seen with particular promoters or that, as noted by Imler et al (20), spacing is critical for the negative effect. Alternatively, there may be no negative factors. Recent transgenic mice studies show that combinations of IgH and elastase enhancers led to expression of a reporter gene in tissues predicted by the sum of each enhancer activities, demonstrating that negative control of the IgH enhancer in non-lymphoid tissues either does not occur or is not dominant to the positive effect of the elastase enhancer (47).

In summary, we have demonstrated that protein binding sites E, B, C1, C2, and C3 are important for IgH enhancer function in vivo. Including the octamer element, six binding sites recognized by a minimum of six different proteins are required for efficient enhancer function. The results presented here and from previous studies show that there is general agreement with respect to positive elements required for the IgH enhancer. There is also agreement that interaction between different enhancer domains, probably at the protein level, is necessary for function. The number and location of inhibitory elements is not well-resolved at present. In order to understand the role of each functionally important IgH enhancer-binding protein and the

nature of their interactions with IgH enhancer DNA and each other, it will now be necessary to purify and study each protein.

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