Selective synergy of immunoglobulin enhancer elements in B-cell development: a characteristic of kappa light chain enhancers, but not heavy chain enhancers

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ABSTRACT

We have examined the interactions of the enhancers of the kappa immunoglobulin light chain gene as well as the interactions of the intron, μ , and 3' α enhancers of the heavy chain locus in mouse. We have observed that each of the kappa enhancers is very weak in comparison with the heavy chain intron enhancer. The mouse heavy chain 3' α enhancer is relatively weak as well. However, two kappa enhancers together synergistically activate transcription of a luciferase reporter gene to a level that is roughly equivalent to the heavy chain μ enhancer. Additionally, dimerization of either kappa enhancer results in synergistic increases in transcription. This property of synergism appears to be confined to the enhancers of the kappa locus, as addition of the 3' α E to μ E containing constructs increases transcription only modestly, and neither heavy chain enhancer synergizes when dimerized. We have gone on to characterize some of the minimal requirements for synergism between the kappa enhancers and find that the KB and E2 sites are required, but not the E3 site. The implications of these results for the coordinate regulation of the heavy and light chain transcription are discussed.

INTRODUCTION

Many genes of the immunoglobulin superfamily (1, 2), as well as a number of other genes (3–5), have been found to contain multiple enhancers. The interaction of the two enhancers of kappa immunoglobulin gene has been reported to be developmentally regulated, with synergy in later cell stages (6, 7). There are no reports of enhancer interactions in other members of the immunoglobulin supergene family.

The mouse immunoglobulin heavy chain is known to contain an intron enhancer (μ E), and at least one enhancer in the region downstream of the alpha constant region, 3' α E (8–10). The μ enhancer has been reported to be 10–20 times stronger than the kappa intron enhancer (11). The strength of the mouse 3' α E appears to be much less than that of the μ enhancer (8), but the

potential interaction of these enhancers has not been addressed. One aspect of these differences in enhancer strengths that has remained puzzling is that the rate of transcription of the endogenous heavy and light chains has been observed to be roughly equal (12).

Only recently has it been appreciated that the kappa intron enhancer may synergize with the kappa 3' enhancer to effect high level transcription. This synergism has been observed in both transient and stable transfection assays, using artificial reporter gene constructs as well as rearranged genomic transgenes (6, 7). The kappa intron enhancer has been extensively characterized with regard to its protein binding sites (13), tissue specificity, and developmental activity (1, 14). What role the individual binding sites may play in the synergistic activation of the kappa locus has not been delineated.

In the studies presented here, we sought to determine whether the heavy chain μ and 3' α enhancers are capable of synergistic activation similar to that observed in the kappa locus. We also sought to characterize the synergistic interaction of the kappa enhancers further. Specifically, we examined the requirement for previously identified intron enhancer binding factors within the larger context of a two enhancer system. Deletions and mutations which disrupted particular factor binding sites were evaluated in the presence of the κ 3'E. Transient transfection assays were performed using luciferase reporter genes driven by either a kappa V-region promoter or an HSV-TK promoter. A variety of mouse B-cell lines representing different stages of development were utilized for the analysis to examine the factors that contribute to coordinate control of heavy and light genes during B cell differentiation.

METHODS

Cell lines

The pre-B cell lines 1-8 and 3-1, the non-secreting B cell line A20, and the plasmacytoma cell line S194 have been characterized and referenced previously (6). The cell lines A20 and S194 were obtained from the American Type Culture Collection. The plasma cell line, S107, was kindly provided by

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Michael Atchison (University of Pennsylvania). Cells were maintained in RPMI 1640, 10% FCS, 25 U/ml penicillin, 25 μ g/ml streptomycin, 2 mM L-Glutamine. 50 μ M β -mercaptoethanol was added to pre-B cell cultures.

Plasmid construction

Luciferase expression vectors were made by cloning the indicated sequences into pGL-2, Promega (Madison, WI). The V κ -21E promoter, κ 3'E and the kappa intron enhancer used have been previously described (6). The HSV-TK promoter was provided by H. Towle (University of Minnesota). The mouse heavy chain μ enhancer was provided by T. Kadesch (University of Pennsylvania). The mouse 3' α enhancer was the gift of M. Neuberger (MRC, England). Subclones generated as indicated in the text and figures using standard techniques. In all cases enhancer segments were placed in a 5' to 3' orientation relative to the promoter sequence.

Transfection

Mouse B-cell lines were transfected by a modification of the DEAE-Dextran method (15). 1×10^6 cells per transfection were washed $1 \times$ in sterile TS (137 mM NaCl, 5 mM KCl, 0.6 mM Na₂HPO₄·7H₂O, 25 mM Tris, 0.5 mM MgCl₂, 0.7 mM CaCl₂, pH 7.4) and resuspended in 50 ml TS at 37°C. The cells were dispensed into sterile 24-well plates. 1 μ g test plasmid, and 250 ng of the control plasmid, pCH110, a β -galactosidase expressing vector, were resuspended in 75 ml TS containing 800 mg/ml DEAE-Dextran and warmed to 37°C. The plasmid/TS/DEAE-Dextran solution was added to the cells and mixed by pipetting. The mixture was allowed to sit at RT for 20 min. The cells were then overlaid with 1.12 ml RPMI 1640, 10% FCS, 25 U/ml penicillin, 25 μ g/ml streptomycin, 2 mM L-glutamine, 50 μ M β -mercaptoethanol, (+/- 10 μ g/ml lipopolysaccharide, Difco), and placed in a 37°C incubator with 7% CO₂. Transfections were harvested after 18–24 h. Transfection efficiency was normalized by co-transfecting with the β -galactosidase expression vector, pCH110, and dividing luciferase activity by β -galactosidase activity. The luminescence ratio was plotted in arbitrary units. Typically, a minimum of two preparations of each construct were transfected in duplicate, and average values were plotted. Estimation of the error was calculated as follows. The standard deviation for each preparation, N=2, was determined. Error values for the independent preparations were combined by calculating one half the square root of the sum of the squares of the standard deviations.

Luciferase assay

At harvest, all cells were transferred to 1.5 ml microcentrifuge tubes, washed $1 \times$ with phosphate buffered saline, and pelleted by centrifuging 5 min at 1500 rpm. 60 μ l of $1 \times$ Cell Culture Lysis Reagent from Promega, (Madison, WI) were added to the pellet and mixed by pipetting. 100 μ l Luciferase Assay Reagent (Promega) were added to 20 μ l of the lysate and was counted for 15 seconds in a Lumat 9501 luminometer (EG & G Berthold).

β -Galactosidase assay

β -galactosidase assays were performed using the Galacto-Light kit from Tropix (Bedford, MA). In micro-titer plates, 5 μ l of the lysate were incubated with 200 μ l of the AMPGD reagent (Tropix) for 60 min at room temperature in the dark. 100 μ l of the Emerald Accelerator reagent (Tropix) were injected immediately prior to measurement in the luminometer.

RESULTS

One of the striking features of immunoglobulin production is the coordinated regulation of heavy and light chain genes, resulting in approximately equal expression of each. Given the complex array of regulatory regions identified in each locus, we have been analyzing enhancer activities at various stages of B cell development. Vectors containing individual or paired enhancers were constructed to determine their role in the developmental regulation of each locus. In addition, specific mutations in individual factor binding sites were created in the light chain locus to determine critical elements involved in enhancer synergy. In every case a minimum of two independent vector isolates were transfected in duplicate. Transfection efficiency was normalized by co-transfection with a β -galactosidase expression vector. Values reported include standard error of the mean.

The combination of heavy chain enhancers is not synergistic

The organization of the immunoglobulin heavy chain locus is similar to the kappa light chain locus in that it contains an enhancer in the J-C intron (referred to here as μ enhancer), and at least one (9, 10) additional enhancer 3' (3' α E) of the most distal constant region. We were interested to see whether the synergistic interactions seen with enhancers of the kappa light chain gene (6, 7) represented a general pattern of immunoglobulin transcriptional regulation. We chose to investigate the possible interactions of the immunoglobulin heavy chain enhancers. Accordingly, we constructed a set of luciferase reporter vectors driven by a HSV-TK promoter which included either the 700 bp *Xba*I–*Eco*RI fragment of the μ enhancer (16), or a 900 bp *Sma*I fragment of the 3' α enhancer (9), or both together. Because immunoglobulin enhancers may display a promoter preference (17), comparisons of the enhancers of the heavy and light chain loci were made using a common heterologous TK promoter. Previous studies showed that the kappa light chain enhancer synergy was observed in the context of a natural kappa promoter as well as the TK promoter (6). Transfection of these plasmids into B-cell lines representing different stages of development was performed, and the activities of the dual enhancer constructs were compared with constructs containing single enhancers.

Transfections of the myeloma cell line S194 show that the 3' α E is a very weak enhancer relative to the μ enhancer, as are the individual kappa intron and 3' enhancers (Fig. 1A). Notably, the combination of the two different heavy chain enhancers results in no additional increase in transcription above the μ enhancer alone. Thus, the synergism of the kappa enhancers that is observed in this cell line (6, 7) appears to be specific to the kappa locus. Interestingly, the combined effect of the kappa enhancers, yields a transcriptional unit which is approximately equal in strength to the paired heavy chain enhancers (Fig. 1A).

Kappa enhancer activity was detected in the B cell lymphoma A20, with a significant increase in activity when the intron and 3' enhancers were paired (Fig. 1B). We also detected activity of the 3' α E in A20. The activity of the 3' α E in this line was approximately equivalent to the kappa 3' enhancer. Previously, the 3' α E was reported to be inactive in A20 cells (9). The contrast in these observations may result from the use of different reporter genes, or differences in the sensitivity of each assay system. As seen with the S194 cell line, the combination of the two heavy chain enhancers showed no evidence of synergy.

Transfections were also performed in the pre-B cell line 3-1. The activity of the 3' α E driven construct was no greater than

Figure 1. Developmentally regulated expression by the intron and 3' enhancers of the immunoglobulin heavy and light chain genes. A) Transfection of the mouse myeloma cell line, S194. B) Transfection of the B-lymphoma line, A20. C) Transfection of the pre-B cell line, 3-1. Solid bars represent un-induced activity, and stippled bars are activity after induction with 10 μ g/ml of LPS. Luminescence was corrected for transfection efficiency and plotted in arbitrary units.

that of the TK promoter alone. Thus, the 3'aE does not appear to be active at this stage of development (Fig. 1C). This developmentally regulated activation of the 3'aE appears to parallel the activity of the 3' kappa enhancer (6). We conclude that no cooperation appears to exist between the enhancers of the heavy chain locus, at any stage of development that we

Figure 2. Enhancer activity of immunoglobulin heavy chain gene enhancer combinations, transfected into the myeloma cell line, S194. Luminescence was corrected for transfection efficiency and plotted in arbitrary units.

Figure 3. Fragments of the kappa immunoglobulin light chain intron enhancer. Fragments were generated by PCR using primers that were engineered to contain *HindIII* sites. Amplified products were cloned into the *HindIII* sites of pBluescript, and pGEM-7. Luciferase reporter gene vectors, were made by cloning these fragments into the expression vector, KP.LUC. Restriction sites *HindIII*, *HinfI*, *DdeI*, and *AluI* are germline encoded sequences that define the 5' and 3' boundaries of fragments shown. The template for the PCR amplification was the genomic subclone, pSPECK (35).

examined. We do note, however, that at the pre-B cell stage, B-cell, and plasma cell stage, the activity of 3'aE.u700.TK.LUC construct is remarkably similar to the activity of the K3'E.IM.TK.LUC (Fig. 1).

To determine whether either of the heavy chain enhancers might have an inherent capacity for synergizing, we tested dimers of each enhancer for greater than additive transcriptional

Figure 4. Contribution of individual enhancer binding sites to the synergistic activity. A) Transfection of the myeloma cell line, S194. KB-, E2-, and E3- designations indicate mutations to those sites within the K3'E.212.KP.LUC vector. B) Transfection of the plasma cell line, S107. Multiple preparations were transfected, and representative results are shown. Transfections were performed in triplicate, and error bars represent the standard error of the mean. C) Co-transfection with the Id expression vector, E:Id, into the myeloma cell line, S194. E:Id, stippled bar, was added in an 8 μ g:1 μ g ratio to the transfection with K3'E.IM.KP.LUC. To keep DNA concentrations equal, the vector alone, solid bar, also contained 8 μ g pBluescript. D) Transfection of the myeloma cell line, S194. K3'E(KB-) designation indicates mutation to the κ B site within the 3' enhancer in the K3'E.IM.KP.LUC vector. Luminescence was corrected for transfection efficiency and plotted in arbitrary units.

activation. We transfected the constructs u700.u700.TK.LUC and 3'aE.3'aE.TK.LUC into S194 cells. As is shown in figure 2, the combination of two enhancers was approximately additive in the case of the 3'aE and somewhat less than additive for the μ enhancer. These results are consistent with the report of Kiledjian *et al.*, who observed proportional increases in CAT reporter gene expressions with the addition of 1 to 24 μ enhancers (16). We conclude that neither the heavy chain μ or 3'aE enhancers interact synergistically when multimerized.

Various intron fragments containing the kappa intron enhancer can synergize with the kappa 3' enhancer

Previously, it was reported that the intron enhancer of the kappa locus could synergize with the kappa 3' enhancer to effect high levels of transcription (6, 7). We sought to determine which sequences in the intron enhancer participate in the synergistic activation. The intron enhancer fragment used in previous studies spans at least 1 kbp (7), and 870 bp (6). In order to identify the

minimal sequences within the kappa intron that are required for high level activation, we examined two smaller fragments of the enhancer: A 512 bp *Hinf*I fragment, and a 212 bp *Dde*I fragment corresponding to the DNase hypersensitive region previously defined (18) (Fig. 3). The 212 bp fragment contains the κ A, κ B, E2, and E3 sites which have been characterized functionally and in binding studies (13, 19). Other less well characterized sites, including a matrix attachment region (20), a silencer region (21, 22), a non-consensus octamer binding site (23), and an AP-1 binding site (J.Schanke and BVN, submitted), lie 5' of this core.

Transient transfections were performed in the myeloma cell line S194. Levels of luciferase activity measured in extracts of these cells transfected with the dual enhancer constructs were much greater than the additive activities of either enhancer alone (Fig. 4A). Thus, the 212 bp intron enhancer is able to synergize with the κ 3'E. This result indicates that regions outside this 212 bp fragment are dispensable for synergistic activation with the κ 3'E, although these flanking regions may also contribute to higher level expression (see below).

Figure 5. Enhancer activity of immunoglobulin light chain gene enhancer combinations. A) Transfection of myeloma cell line, S194. B) Transfection of the pre-B cell line 1-8. Solid bars represent un-induced activity, and stippled bars are induced activity with 10 μ g/ml of LPS.

Mutation of the κ B or E2, but not E3, site within the kappa intron enhancer severely impairs enhancer synergy

We sought to determine the crucial binding sites within the kappa intron involved in the synergistic effect. We therefore examined the effects of mutating binding sites within the kappa intron enhancer in the context of the both kappa enhancers. It has been reported that deleterious effects of mutation of certain E box sites within the μ enhancer can only be detected in the context of fragments that contain other binding sites as well (16, 24). Accordingly, we constructed dual enhancer vectors containing mutations, or deletions, within the kappa intron enhancer to help identify the contribution of known binding sites to the synergistic transcriptional activity.

Disruption of the NF- κ B site was performed by PCR mediated site-directed mutagenesis. This mutation was created in the context of two dual enhancer constructs, K3'E.IM(KB-).KP.LUC, and K3'E.212(KB-).KP.LUC. In both cases, transcriptional activity was reduced to about the activity of κ 3'E

Figure 6. Enhancer activity of immunoglobulin light chain gene enhancer combinations, after transfection of myeloma cell line, S194. Luminescence was corrected for transfection efficiency and plotted in arbitrary units.

construct alone (Fig. 4A and data not shown). Mutations which disrupt the binding of the NF- κ B were previously shown to reduce the activity of the intron enhancer (13). These data are also in agreement with observations from transfection of S107 plasma cells, which lack active NF- κ B binding activity (25). Similarly, the dual enhancer vector displays about 20% of wild type activity in S107 cells (Fig. 4B). We conclude that NF- κ B binding is a necessary component of the synergy between the kappa enhancers.

Mutations that disrupt the E2 binding site were engineered in the K3'E.212(E2-).KP.LUC vector. This mutation resulted in a similar reduction to about 20% of wild type activity in S194 cells (Fig. 4A). This finding is supported by the observation that co-transfection of Id, into S194 cells, results in activity of the dual enhancer vector that is about 20% of uninhibited value (Fig. 4C). Id is a helix-loop-helix (HLH) heterodimerization partner for products of the E2A gene which bind to the intron enhancer at the E2 site and appear to bind to the κ 3'E enhancer as well (26). Id lacks a basic DNA-binding domain and the heterodimers it forms with products of the E2A locus cannot bind to the target site in the enhancer. Co-transfection of Id has been shown to reduce by five-fold the expression of reporter gene vectors driven by either the kappa intron or 3' enhancer alone. Our results demonstrate that a parallel decrease occurs in the synergistic activity upon co-transfection of Id.

The E3 site of the intron enhancer has been shown to bind proteins (27) that are common to the kappa and μ enhancers (μ E3 site). This site is functionally activating in the context of the heavy chain intron enhancer (16). Mutation of this site in the context of the kappa intron enhancer alone resulted in up to a 3-fold reduction in enhancer activity (13). Mutation of E3 site in the K3'E.212(E3-).KP.LUC vector has little effect on synergistic expression relative to wild type levels (Fig. 4A). Taken together, these results indicate that NF- κ B and E2 binding factors are critical for both the synergistic expression observed in the dual enhancer context, as well as the intron enhancer in isolation. In addition, we note that the contribution of E3 binding factors may

be negligible to the activity of this enhancer combination. Disruption of the E1 site was not performed because factors binding to this site have not been observed (14).

Mutation of the consensus κ B site within the kappa 3' enhancer does not eliminate the synergistic interaction with the intron enhancer

An additional NF- κ B consensus binding site exists within the κ 3'E (28). This site has not been implicated in the activity of the 'core' of the 3' enhancer (28), but factors binding NF- κ B sites have been shown to be involved in synergistic activation in a variety of contexts. Two κ B sites synergize when placed upstream of a c-fos promoter driving a CAT reporter (29). Dual κ B sites exist within the HIV-LTR and may synergistically activate HIV transcription (30). κ B binding factors have also been observed to synergize in the human interferon-B promoter (31). We tested the possibility that the κ B site in the 3' enhancer participated in the synergistic interaction with the intron enhancer. We found, however, that mutations in this site have no adverse affect on the synergistic interaction with the intron enhancer, compared with a wild-type 3' enhancer (Fig. 4D).

The kappa intron and 3' enhancers can each synergize when dimerized

In an effort to address the question of whether a specific interaction were operative between the intron and 3' enhancers, we chose to multimerize individual enhancers and compare their activity with the K3'E.IM.KP.LUC construct. This would test whether synergy is an inherent property of each enhancer, or whether both enhancers are required. Tandem dimers were made using the approximately 800 bp fragments of both enhancers. We also tested smaller fragments of the intron enhancer. We found that the activity of the IM.IM.KP.LUC, K3'E.K3'E.-KP.LUC, and K3'E.IM.KP.LUC vectors were remarkably very similar after transfections of the S194 cell line (Fig. 5A). We conclude that synergy between kappa enhancers can be achieved by simple tandem duplication of either enhancer. Therefore, the synergy does not seem to require a special interaction between the two different enhancers. The similar activities of the dual enhancer constructs was independent of the particular promoter used (data not shown). Notably, the dimer synergy does not appear to be a general property of all Ig enhancers, as multimers of the heavy chain μ (16), or 3' α enhancers do not show synergy (Fig. 1A). Interestingly, the addition of a third kappa intron enhancer, IM.IM.IM.KP.LUC and IM.IM.IM.TK.LUC, resulted in only small increases in activity with respect to the dual enhancer constructs (Fig. 6). Thus, greater than additive increases in activity are observed when we increase the number of enhancers from one to two, but not from two to three.

The IM.IM.KP.LUC and K3'E.K3'E.KP.LUC constructs were also transfected into pre-B cell line 1-8 (Fig. 5B). Based on the observation that the intron enhancer is functional in LPS induced pre-B cells, we predicted synergistic activation by the dual intron enhancer construct in these experiments. We also predicted that the dual 3' enhancer vector would not synergistically activate transcription at this stage. As shown in Fig. 5B, the IM.IM.KP.LUC vector is indeed synergistically active with respect to the IM.KP.LUC vector in LPS induced 1-8 cells, but not in the uninduced cells. Also, in accordance with our predictions, the K3'E.K3'E.KP.LUC was not synergistically activated in these cells. We conclude that synergy

Figure 7. Synergistic enhancer activity of immunoglobulin light chain gene enhancer fragments in combination. **A)** Transfection of myeloma cell line, S194. **B)** Transfection of myeloma cell line, S194. Luminescence was corrected for transfection efficiency and plotted in arbitrary units.

is dependent on the developmental stage in which each enhancer is activated.

Tandem duplication of the 212 bp kappa intron enhancer fragment does not result in synergy

We have observed that two copies of the large fragment of the intron enhancer are capable of synergizing with each other. We were interested in determining the minimum sequence requirements for this synergistic activity. We expected that, since the 212 bp fragment is able to synergize with the κ 3'E, we would observe synergy between two copies of this fragment, and possibly no synergy between smaller fragments. We constructed tandem repeats of two fragments: 194.194, 212.212. These vectors were transfected into S194 cells (Fig. 7A). Remarkably, the 194.194 and 212.212 dual enhancer constructs are unable to activate transcription synergistically, even though the 212 bp

intron enhancer fragment contains the positive regulatory sequence elements that have been characterized functionally and by protein binding studies. However, the 5' end of the 212 bp fragment lies within a region that was recently implicated in positive regulation of the locus, the κ BS sequence (22). This has not been precisely footprinted, and it is not known whether binding to the κ BS site would be abrogated in this fragment. The 194 bp fragment lacks the binding site for the KA factor which appears to bind only in pre-B cells. We conclude that cooperative activation of transcription by dimerized intron enhancer fragments requires additional sequences beyond the 212 bp core, though these sequences are seemingly not required for synergy with the κ 3'E.

Tandem duplication of a 350 bp intron enhancer fragment restores the synergistic effect

A longer intron enhancer fragment containing an additional 140 bp 5' of the 212 bp core, was multimerized and inserted into the KP.LUC vector. This 350.350.KP.LUC vector does display activity that is nearly equivalent to the IM.IM vector (Fig. 7B). We conclude that the 350 bp fragment mediates high level activation when multimerized, but the 212 bp fragment does not. A longer fragment, encompassing the 212 bp core and 184 bp downstream, 394.394.KP.LUC was more active than the 212.212.KP.LUC vector (Fig. 7B). We conclude that sequences 5' of the kappa intron enhancer core are required for synergistic activation of tandem copies of this enhancer, but are not crucial for synergistic activation with the κ 3'E.

DISCUSSION

We previously reported that the kappa intron and 3' enhancers interact in a developmentally controlled manner, with synergy in later B-cell stages (6). Our results were in contrast to results presented by Pongubala and Atchison (25) who suggested the 3' enhancer showed some inducibility in pre-B cells and showed significantly higher activity than the intron enhancer in mature B cells. Their results have now been explained by the fact that the 3' enhancer construct they used inadvertently contained two inserts of the 3' enhancer (M. Atchison, personal communication). Thus, our results showing the 3' enhancer is only stronger in mature B cells when paired with the intron enhancer or itself, are entirely consistent with their observations.

The identification of additional enhancers 3' of the heavy chain in the mouse (8) and rat (32) raises the possibility that these enhancers may also cooperate. We have now tested the interaction of the μ and 3' α enhancers of the mouse immunoglobulin heavy chain locus. The heavy chain μ and 3' α enhancers were not observed to synergize with each other; only modest additive effects were seen in transient transfection experiments. Further, dimers of either heavy chain enhancer failed to synergize while dimers of large fragments of the kappa enhancers do synergize. Thus, both kappa enhancers appear to be capable of synergism, while the heavy chain enhancers are not. Our results are in general agreement with the report of Dariavach *et al.*, who saw only a modest increase in the transcription of a CAT reporter gene in stable transfections when the mouse μ enhancer was paired with the rat 3' α E (a significantly stronger enhancer than the mouse counterpart) (9).

The phenomenon of synergistic interaction of the two enhancers might be expected to be confined to the light chain given the

following considerations. First, heavy and light chain transcription rates have been observed to be approximately equal in run-off assays using primary lymphocytes (12). Second, the heavy chain intron enhancer appears to be at least ten-times stronger than either kappa enhancer, when compared directly in transfection assays (11). Thus, the combined effect of the two kappa enhancers may be sufficient to match heavy chain expression driven by the μ enhancer, but would not be able to match heavy chain expression if the μ enhancer were to synergize with another enhancer. The great distance of the heavy chain from the intron enhancer, about 100 kb, raises a question about its role in V(D)J transcription. It has been suggested that the primary role of the 3' α E may be to drive nearby germline promoters prior to switch recombination (F. Alt, personal communication). We have further characterized the developmental activation of the 3' α E and find that it is active in the B-lymphoma line A20. Its activity in this stage is consistent with a putative role in driving germline promoters supporting switch recombination during later B-cell stages.

Since synergistic enhancer activation appears to be specific to the kappa locus, we were interested in further analyzing this property. Mutational and deletional analyses were performed in the context of reporter gene constructs driven by both enhancers. The 212 bp core, the 512 bp, and 800 bp (IM) fragment were each observed to synergize with the κ 3'E. Mutations of κ B, E2, and E3 were performed in the K3'E.212.KP.LUC context. The κ B and E2 site disruption decreased transcription to less than 10% of wild type, while E3 site disruption had little effect. These results are directly parallel to those of Lenardo *et al.* (13), who examined mutations of these sites within the context of the intron enhancer alone. We conclude that the same sites which activate the intron enhancer in isolation are necessary for the synergistic interaction. It is interesting to note that only the κ B site is unique to the kappa enhancers. A κ E2 site is also found in the μ enhancer (μ E5), although it is not clear whether these sites bind the same factors (1).

While this study has not examined the role of individual binding sites in the κ 3'E, we do note that co-transfections with Id abrogate synergy. Id is known to interfere with bHLH protein binding to the 3' enhancer. Furthermore, we noted that the κ 3'E fragment we used contains a consensus κ B site. Because κ B sites have been shown to show dramatic synergistic effects (28–30) in isolation or in context of other enhancer elements, there appeared to be a strong possibility that the kappa synergy was mediated through the κ B sites in each of the individual kappa enhancers. However, we found that the κ 3'E κ B site does not play a critical role in the synergistic interaction with the intron enhancer.

Our data does not address whether the synergism involves a direct physical interaction between the two enhancers. Synergistic activation of transcription by the combination of multiple enhancer factor binding sites may be observed with or without a direct interaction between the factors (33, 34). Thus, the cumulative effect of multiple transcriptional activators arrayed along an enhancer may, in some cases, result in a greater than additive increase in transcription in the absence of cooperative DNA binding. However, as our results show, this is not a general property, as the cumulative factor binding provided by combining heavy chain enhancers does not result in synergistic activity.

The mechanism of synergistic activation does not appear to require the specific combination of the two different kappa enhancers, as tandem copies of IM or κ 3'E are also

synergistically active. This indicates that, with respect to transcriptional activation, these enhancers are not performing complementary functions; each can substitute for the other. This is also not a general property, as the IgH enhancers do not synergize when multimerized.

We speculate that the presence of two enhancers in the kappa locus, that are independently regulated, provides for fine control of transcription at various stages of B-cell development. This combination of separate kappa enhancers is able to match transcriptional levels driven by the μ enhancer at various stages of development. Our data suggests that the capacity for the low level transcription early, and high level transcription later in development would not have been afforded by simple duplication of either enhancer element.

We have seen that the 212 bp core intron enhancer fragment is capable of synergizing with the κ 3'E enhancer but not with itself; while, larger fragments containing additional sequences upstream of the core restore the 'self-synergy'. We envisioned two possible roles for these flanking sequences: 1) they may provide factor binding sites important for the 'self-synergy', or 2) a spacing requirement exists which allows synergy only when the core elements are sufficiently far apart. These upstream sequences have been identified by others as containing binding sites for octamer factors and other positive and negative sequences. Since the 350 bp fragment synergized while the 394 bp fragment did not, the data seem to favor a functional role of the 5' flanking sequence. This region was not required for synergy with the κ 3'E. This suggests that whatever role this 5' region may be playing in the tandem dimer experiments, that function is either unnecessary in the K3'E.IM context, or it is replaced by sequences in κ 3'E.

Synergistic activity appears to reflect the developmental activation of each enhancer, in that synergy between duplicated kappa intron enhancers, but not kappa 3' enhancers, occurs in the pre-B cell line, 1-8. A curious exception to this occurred upon transfecting 3-1 pre-B cells. These cells show a 5-fold activation of the kappa intron enhancer, but tandem intron enhancers showed no additional activity (data not shown). Thus, two active enhancers are not always sufficient for synergistic enhancer activity.

A few rules have emerged from our investigations into the requirements for synergistic activation by kappa enhancers: both κ B and E2 sites seem required in the intron enhancer; two active enhancers are necessary but not always sufficient for synergism; and, greater than additive increases occur upon moving from one to two kappa enhancers, but not from two to three.

These experiments provide insight into the coordinated regulation of immunoglobulin gene transcription which appears to be orchestrated by multiple separate enhancers. The kappa enhancers are differentially regulated, but are capable of synergizing, while the enhancers of the heavy chain locus do not synergize. Significantly, the cumulative enhancer activity of each locus is nearly equivalent.

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