MOLECULAR AND CELLULAR BIOLOGY, Jan. 1989, p. 303–311
0270-7306/89/010303-09$02.00/0

Vol. 9, No. 1

Anatomy of a New B-Cell-Specific Enhancer

W. KOCH, C. BENOIST, AND D. MATHIS*

Laboratoire de Génétique Moléculaire des Eucaryotes du Centre National de la Recherche Scientifique and Unité 184 de Biologie Moléculaire et de Génie Génétique de l'Institut National de la Santé et de la Recherche Médicale, Institut de Chimie Biologique, Faculté de Médecine, Strasbourg, France

Received 23 June 1988/Accepted 19 October 1988

The major histocompatibility complex class II molecules, like the immunoglobulins, are prominent B-lymphocyte markers. Herein, we describe a B-cell-specific enhancer associated with the murine class II gene, E_α^k. This enhancer has a complex anatomy that suggests interactions between remotely spaced elements. Of particular interest is the finding that two CCAAT boxes spaced one kilobase apart are important for enhancer activity. Somewhat surprisingly, the E_α and immunoglobulin enhancers seem to show little resemblance.

Many eucaryotic genes rely on enhancer elements for high-level expression (for reviews, see 16, 25; A. K. Hatzopoulos, U. Schlokat, and P. Gruss, *in* B. D. Hames and D. M. Glover, ed., *Eukaryotic RNA Synthesis and Processing*, in press; B. Wasylyk, Biochim. Biophys. Acta, in press). These constitute a rather diverse class of regulatory elements differing significantly in the magnitude, specificity, and reach of their stimulatory activity. Some enhancers are capable of increasing transcription a fewfold, others, a few hundredfold. Enhancers can occur in association with genes that are ubiquitously expressed or with loci that are transcribed under strict cell-type-specific control. They may act constitutively or may show inducible or repressible activity. Enhancers can reside within the 5′-flanking region, the 3′-flanking region, or even within the transcribed portion of a gene. They may be capable of stimulating transcription from an RNA initiation site located kilobases (kb) away or may operate efficiently only when closely juxtaposed to the start site.

Probably the best characterized cell-type-specific enhancers are those associated with the heavy (μ) and light (κ) chain immunoglobulin loci (for reviews and references, see 1, 6, 16; Hatzopoulos, Schlokat, and Gruss, in press). These elements are largely responsible for the marked B-cell specificity of immunoglobulin gene transcription. The μ and κ enhancers are both located within an intron that is brought near the normal RNA initiation site only after locus rearrangement during differentiation. Both consist of multiple 6- to 12-base-pair (bp) motifs that are recognized by sequence-specific DNA-binding proteins: the octamer motif, multiple Ephrussi sequences, and the NF-κB binding site. The different motifs exert diverse influences on transcription; some enhance constitutively (the octamer [5, 7, 15, 33]), some appear to be inducible (the NF-κB site [15, 22]), and others appear to repress (μE2 [10, 31]). Interplay between the motifs, or more accurately, between the proteins that bind to them, results in a strong B-cell-specific stimulatory activity. The μ and κ elements have served as prototypes for the cell-type-specific enhancer in general and for the B-cell-specific enhancer in particular. But it is not known whether enhancers associated with other B-cell-restricted genes fit this prototype.

Next to the immunoglobulins, the most prominent set of B-cell surface proteins are the major histocompatibility complex (MHC) class II molecules. The precise pattern of immunoglobulin and class II molecule expression within the B-lymphocyte lineage does differ, however (for reviews, see 6, 27). The latter are displayed prominently on B cells but are generally absent from the surface of immature pre-B and terminally differentiated plasma cells. In addition, class II antigens can be found on a few non-B cells that do not express immunoglobulin, most notably macrophages, interdigitating (dendritic) cells, and thymic epithelial cells. Finally, the MHC class II and immunoglobulin genes respond differentially to a variety of effectors, e.g., lipopolysaccharide, gamma interferon, and interleukin-4.

Because of these similarities and differences, we set out to compare the mechanisms that control immunoglobulin and MHC class II gene expression. In this report, we describe a B-cell-specific enhancer located in the 5′-flanking region of the murine class II gene, E_α. This enhancer has a complex and interesting anatomy which suggests interplay between widely spaced DNA sequence motifs. Surprisingly, there seems to be little resemblance between the E_α and immunoglobulin gene enhancers.

MATERIALS AND METHODS

Cells. The cells used in this study were: M12, WEHI 231, and A20 (murine B-lymphoma lines); PD31 and 18.81 (murine pre-B lines); AG501.X63 (a murine plasmacytoma line referred to in the text and figures as X63); BW5147 (a murine T-lymphoma line); and NIH 3T3 and LMTK (murine fibroblast lines). All cell lines were grown in Dulbecco medium supplemented with 10% fetal calf serum. A20, PD31, and 18.81 cells were grown in the presence of 50 μM 2-mercaptoethanol.

Plasmids. The control plasmids pA0 and pA101 were obtained from M. Zenke and P. Chambon (34). To test for enhancer activity, various fragments of the E_α gene and mutants thereof were inserted at the *Bam*HI site of pA101. The basic plasmids were pX3 and pS3. pX3 was created by inserting a 230-bp *Xho*I-*Alu*I fragment from the plasmid pEA3CATXL (position −215 to position +12 of the E_α gene) into the *Xho*I and *Bam*HI sites of the pA101 polylinker. The *Xho*I and *Bam*HI sites were preserved. pS3 was made by cloning the 2-kb *Xba*I-*Xho*I fragment from pEA3CATXL (positions −2172 to −215) between the *Xba*I and *Xho*I sites of the pX3 polylinker region. All of the other plasmids were primary or secondary derivatives of pX3 or pS3. (i) pXD21, pXD301, pXD10, and pXD6 were produced by oligonucleotide-directed mutagenesis of a single-stranded M13 template

* Corresponding author.

bearing the −215 to +12 region of E_α. They have the deletions −93 to −80 (X box), −61 to −48 (Y box), −93 to −48 (X and Y boxes), and −186 to −142, respectively. (ii) pXYC and pXXC were also created by oligonucleotide-directed mutagenesis in M13. For the former, the Y box was replaced by the sequence ACGTTCTGGCTACA; for the latter, the X box was replaced by CATCTGTTGCTAGG. (iii) pXD19 carries only the −97 to −40 fragment of E_α. The inserted fragment was made by exonuclease *Bal* 31 digestions of pX3. (iv) pA14 and pA5 resulted from deletions obtained with *Bal* 31; their 5′ endpoints are −168 and −97, respectively. (v) pSD21 and pSD302 are pS3 derivatives that carry deletions of the X and Y boxes. They were obtained by replacing the *Xho*I-*Bam*HI fragment of pS3 with that of pXD21 or pXD301. (vi) pSD212 and pSD213 were produced from pS3 by religation after cutting with *Eco*RV-*Bam*HI (removing −1148 to +12) or *Sma*I (deleting −2172 to −1180). pSD216 resulted from religation of pSD213 after digesting with *Xho*I-*Bam*HI; thus, only the fragment −1148 to −215 remains. (vii) pSD31, pSD32, and pSD33 have deletions of the X′ box (−1359 to −1346), Y′ box (−1392 to −1379), and W box (−1202 to −1193), respectively. They are on the pS3 background and were obtained by oligonucleotide-directed mutagenesis of an M13 template carrying the −2172 to −1148 fragment. (viii) pSD21/31, pSD302/32, pSD21/33, and pSD302/33 have double deletions of the X and X′, Y and Y′, X and W, and Y and W boxes, respectively. They were all constructed by replacing the 1-kb *Sma*I fragment (pA101 polylinker to E_α position −1180) of a single-mutated pS3 clone with that of a single-mutated pSD212 derivative (see below). (ix) pSD312 was obtained by replacement of the *Eco*RV-*Bam*HI fragment of pS3 (1,162 bp) with the *Sca*I-*Bam*HI fragment of pBR322 (890 bp). (x) pSD2121, pSD2122, and pSD2123 carry the X′, Y′, and W deletions (respectively) on a pSD212 background. They were created by oligonucleotide-directed mutagenesis as above, but were recloned into pA101 rather than pS3. (xi) p1, p21, and pSph were derived from pSD212 by religation after cutting with *Aat*II, *Aat*II-*Sal*I, and *Aat*II-*Sph*I. Thus, their 5′ endpoints are −1760, −1663, and −1214, respectively. (xii) p24, p16, p49, p37, p22, p59, and p69 also derive from pSD212. Progressive 5′-end deletions were created by cutting with *Aat*II-*Sal*I, digesting with exonuclease III for increasing times, treating with mung bean nuclease, and religating. (xiii) pSD221, pSD222, and pSD223 were derived from pS3 by deletion of the *Sph*I-*Bam*HI, *Kpn*I-*Bam*HI, and *Sal*I-*Bam*HI fragments. Thus, their 3′ endpoints are −1214, −1322, and −1663, respectively.

Transfections. DNA was transfected into tissue culture cells by using the DEAE-dextran protocol of McCutchan and Pagano (18).

For lines M12, A20, NIH 3T3, and LMTK (which remain attached to the culture dish throughout the transfection and washing procedures) 2×10^6 cells were seeded onto 10-cm dishes 24 h prior to transfection in 10 ml of medium–10% fetal calf serum (FCS). Just prior to transfection, the medium was removed, and the cells were washed once with 5 ml of serum-free medium. The cells were then incubated in 800 μl of transfection mix (serum-free medium containing 10 μg of plasmid DNA and 0.25 mg of DEAE-dextran per ml) for exactly 5 min at 25°C. The incubation was stopped by removing the transfection mix and washing the cells with 5 ml of medium–10% FCS. Finally, the cells were incubated with 15 ml of medium–10% FCS at 37°C for 48 h.

For lines WEHI 231, PD31, 18.81, X63, and BW5147 (which do not maintain contact with the culture dish throughout the procedure) 10^7 cells were suspended in 800 μl of transfection mix for 5 min at 25°C in 15-ml polystyrene screw-cap tubes. Uptake of DNA into the cells was arrested by adding 14 ml of medium–10% FCS. The cells were then spun for 5 min at 1,000 rpm, suspended in 15 ml of medium–10% FCS, transferred onto 10-cm dishes, and incubated at 37°C for 48 h. With some lines (M12, NIH 3T3, LMTK), the amount of RNA produced from the transfected plasmids was increased two- to fivefold by adding chloroquine (Sigma Chemical Co.) to a final concentration of 0.1 mM during the first 4 h of the 48-h incubation period in medium–10% FCS.

RNA analysis. Cells were rinsed twice in sodium phosphate buffer (pH 7.5)–140 mM NaCl, suspended in 300 μl of 10 mM Tris hydrochloride (pH 7.9)–10 mM NaCl–2 mM $MgCl_2$, and lysed with 10 μl of 10% Nonidet P-40. Nuclei were pelleted, and the RNA-containing supernatant was added to 300 μl of 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–2% sodium dodecyl sulfate. RNA was extracted once with 600 μl of phenol-chloroform, precipitated twice with ethanol, dried, and dissolved in water.

Amounts of RNA transcribed from the various transfected plasmids were quantitated by S1 nuclease analysis. Five to 50 μg of total cytoplasmic RNA was hybridized with a specific 5′-end-^{32}P-labeled DNA probe, as described by Zenke et al. (34). The hybridization of RNA and probe was performed in 50% deionized formamide–10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES, pH 6.5)–0.4 M NaCl for 12 h at 37°C. To trim the protected probe, the hybridization mix (usually 20 μl) was incubated for 30 min at 37°C with 50 U of S1 nuclease (Appligene, France) contained in 200 μl of 300 mM sodium chloride–30 mM sodium acetate (pH 4.5)–4.5 mM zinc acetate. The reaction was stopped by adding 50 μl of 2.5 M ammonium acetate–50 mM EDTA–10 μg of *E. coli* tRNA (Sigma). Nucleic acids were precipitated with 2-propanol. After centrifugation, the samples were dissolved, denatured, and loaded onto an 8% denaturing polyacrylamide gel.

RESULTS

B-cell-specific enhancer activity associated with the −2172 to +12 fragment of the murine class II gene, E_α^k. Only 1,906 bp of 5′-flanking DNA is required for efficient expression of the E_α gene in B cells of transgenic mice (4, 14, 30, 32). Moreover, when the −2,172 to +12 fragment of the E_α gene is ligated to a reporter gene, this chimeric construct can also be effectively transcribed in transgenic-mouse B cells (H. J. Fehling et al., manuscript in preparation). Hence, in our search for B-cell-specific enhancer activity associated with the E_α locus, we have concentrated on the −2,172 to +12 region.

Our strategy had been to measure the capacity of various fragments in this region to activate transcription from a heterologous promoter. We have taken a reductionist view of the definition of an enhancer, deemphasizing the importance of activity at a distance and activity in both orientations. This view is in line with contemporary opinion that enhancers constitute a diverse set of regulatory elements with a spectrum of properties and that enhancers are not really fundamentally different from upstream regulatory elements (16, 25).

Figure 1 diagrams the enhancer test construct we employed; there is a rabbit β-globin reporter gene and the simian virus 40 (SV40) early promoter, including the TATA box and 21-bp repeats but lacking the 72-bp repeats. The enhancerless plasmid (pA101) is not transcribed in most cell

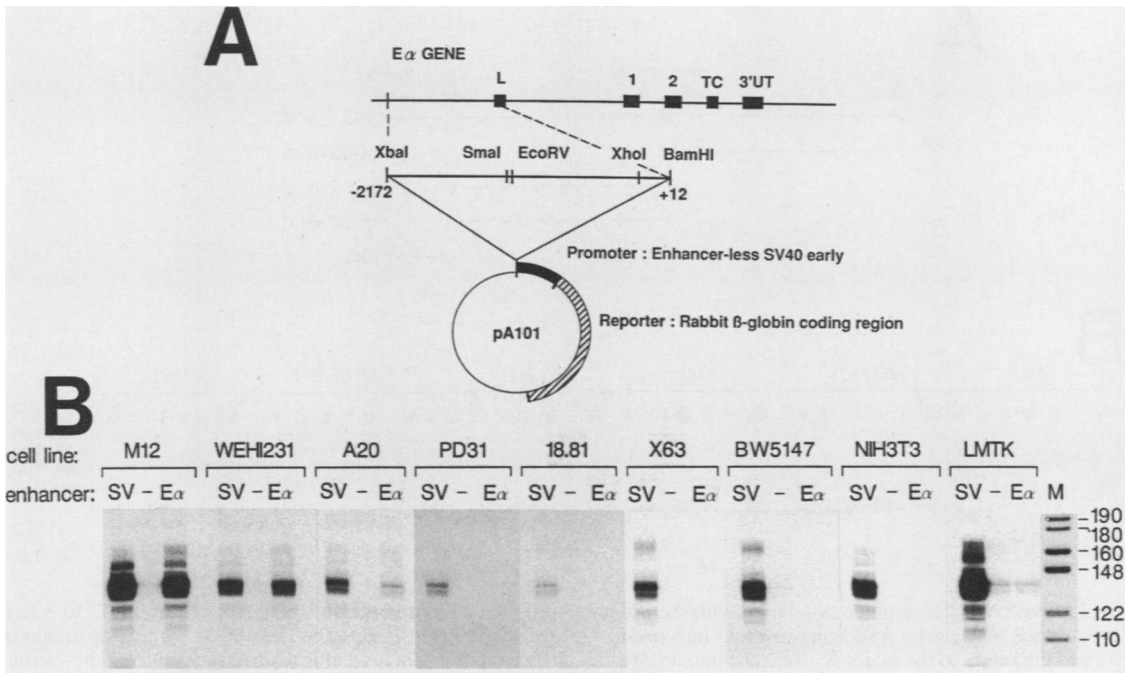

FIG. 1. Demonstration of B-cell-specific enhancer activity associated with the E_α 5′ end. (A) Construct used to test enhancer activity. The E^k_α gene (top) consists of five exons (■) which encode the leader peptide (L), the extracellular domains (1 and 2), the transmembrane segment and cytoplasmic tail (TC), and the 3′ untranslated region (3′UT). pA101 (bottom) consists of a reporter (the rabbit β-globin-coding region) and the SV40 early promoter (minus the 72-bp repeats) (for further details, see reference 34). The −2172 to +12 segment (relative to the transcription start site) of the E_α gene was inserted into pA101 at the polylinker immediately upstream of the SV40 promoter; the resulting plasmid is called pS3. Another construct (not diagrammed) has the SV40 enhancer (coordinates 179 to 346) inserted at the polylinker of pA101 and is called pA0 (34). (B) Quantitative S1 nuclease analysis of RNA transcribed from pA0, pA101, and pS3. The three plasmids were transfected into M12, WEHI 231, and A20 (mature B cells); PD31 and 18.81 (pre-B cells); X63 (a plasmacytoma); BW5147 (a T-lymphoma line); and NIH 3T3 and LMTK (fibroblasts). RNA was isolated 48 h later and analyzed by S1 nuclease mapping. The signals represent single-stranded probe fragments protected against digestion by RNA initiating at a few sites under the control of the SV40 promoter. Leftmost lane of each panel, pA0; center lane, pA101; rightmost lane, pS3. Lane M, size marker (in base pairs) ^{32}P-labeled *Hpa*II digest of pBR322.

types and thus serves as a negative control. A plasmid bearing the SV40 enhancer (pA0) is quite efficiently transcribed in most cell types and can be used as a positive control. As an initial test of E_α enhancer activity, we created a plasmid (pS3) carrying the −2172 to +12 fragment. pA101, pA0, and pS3 were individually transfected into several lymphoid and nonlymphoid cell lines, and transcription from each construct was evaluated 48 h later by quantitative S1 nuclease mapping of cytoplasmic RNA. All of the experiments were repeated at least three times with at least two independent plasmid preparations.

As shown in Fig. 1, the enhancerless plasmid is not expressed or is only very feebly expressed in all cell lines tested (center lanes of each panel). In contrast, the SV40 enhancer greatly activates transcription in all the lines (left lanes of each panel). The variable degree of activation reflects some true differences in SV40 enhancer function as well as a repeatable variation in transfection efficiency of the different cell lines. The 2-kb E_α fragment clearly shows enhancer activity but only in B cells that normally express MHC class II genes: M12, WEHI 231, and A20. This B-cell-specific stimulatory activity is quite potent, ranging from as strong as that of the SV40 enhancer in M12 and WEHI 231 cells to about 20% as strong as that in A20 cells. Class II-negative cells of the B lineage, the pre-B cells PD31 and 18.81 and the plasmacytoma X63, do not support E_α enhancer function, nor do a T cell (BW5147) and two fibroblast lines (NIH 3T3 and LMTK).

Thus, we conclude that the −2172 to +12 fragment of the E_α gene bears sequences that are capable of enhancing transcription from a heterologous promoter. This stimulatory activity is restricted to mature B cells that normally express MHC class II molecules.

Both halves of the −2172 to +12 fragment show enhancer activity. As a first step in localizing the sequences responsible for enhancer function, we bisected the −2172 to +12 fragment and cloned each half into the enhancer test construct. The two plasmids (pSD212 and pSD213) were transfected into several B and non-B cells, and transcription from each construct was measured after 48 h. Transcription levels from pA0 and pS3 served as positive standards, and levels from pA101 represented the negative standard.

Both halves of the E_α 2-kb fragment quite clearly show enhancer activity (Fig. 2). The promoter-distal half (pSD212, lane b in each panel) operates to various degrees in all cell types tested; the promoter-proximal half (pSD213, lane c in each panel) exhibits pronounced B-cell specificity. The strength of each half relative to the uncleaved −2172 to +12 fragment varies in the different B-cell lines (compare lanes a, b, and c). For A20 and WEHI 231, transcription is about equal from pSD212, pSD213, and pS3. For M12, transcription from pS3 is greater than the sum of that from pSD212 and pSD213. This was also observed for the human B-lymphoblast line Raji (11; data not shown) and may suggest cooperative effects. Both halves of the enhancer appear to be largely orientation independent, as would have been predicted from the opposite orientation of the X-Y and X′-Y′ motifs; that is, when the inserted fragments are flipped

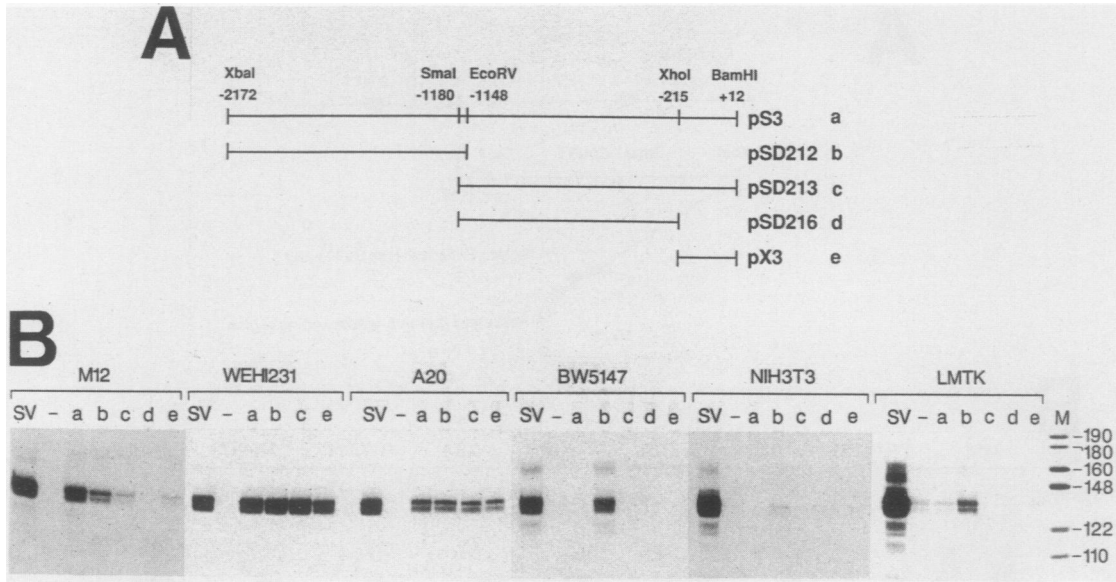

FIG. 2. Analysis of enhancer activity associated with the promoter-proximal and promoter-distal halves of the −2172 to +12 fragment of the E_α gene. (A) Maps of E_α gene fragments inserted into pA101. All fragments were inserted at the polylinker just upstream of the SV40 promoter. Numbering refers to the major E_α transcriptional start site. (B) S1 nuclease analysis of RNA transcribed from the various plasmids. The set of pA101-derived plasmids was transfected into B (M12, WEHI 231, A20) and non-B (BW5147, NIH 3T3, LMTK) cells. Two days later, RNA was isolated and quantitated by S1 nuclease analysis, probing for transcripts driven by the SV40 promoter. In each panel: SV, pA0; −, pA101; a, pS3; b, pSD212; c, pSD213; d, pSD216; e, pX3. Lane M, size marker, as in Fig. 1B.

around in pSD212 and pSD213, both constructs are efficiently transcribed in B-lymphoma cells (data not shown).

The ubiquitous activity of the promoter-distal enhancer is somewhat surprising. One possible explanation is that in our enhancer test construct this element was brought in close proximity to the promoter and ubiquitously acting motifs which could not exert their influence over long distances now functioned efficiently. To address this possibility, we created a plasmid (pSD312) that has 890 bp of pBR322 sequences ($ScaI_{3846}$ - $BamHI_{375}$) inserted into pSD212 between the E_α −2172 to −1148 fragment and the SV40 promoter. This construct was as actively transcribed as pSD212 in the mature B cells M12 and A20 but showed no transcription in the non-B cells BW5147 and X63 (data not shown). Apparently then, the cell-type nonspecific transcription of pSD212 is caused by the juxtaposition of the distal element with the promoter.

Because of sequence considerations (see below), we suspected that the promoter-proximal enhancer actually resides on the −215 to +12 fragment. To test this proposition, we cloned the −1148 to −215 and −215 to +12 fragments into the enhancer test construct to create plasmids pSD216 and pX3. As illustrated in Fig. 2, the former is not detectably transcribed in any cell line tested (lane d); the latter is transcribed almost as effectively as pSD213 (carrying the entire 1-kb promoter-proximal fragment) in all three B-cell lines (compare lanes c and e of each panel).

We conclude then that the E_α enhancer is bipartite; there is a B-cell-specific element in the −215 to +12 region and another element on the −2172 to −1148 fragment that is B-cell specific in its proper context but that acts ubiquitously when brought close to the promoter.

Stimulatory activity of the −215 to +12 fragment dependent largely on the X and Y boxes. The 230 bp of E_α sequence in pX3 include two motifs that are conserved in all MHC class II genes examined to date: X (−93 to −80) and Y (−61 to −48) (3, 4). Studies with transgenic mice have shown that X and Y are critical for accurate and efficient initiation of transcription on the E_α gene (4). In addition, both motifs serve as recognition sites for sequence-specific DNA-binding proteins (3, 4, 11, 19). Of particular interest is the finding that Y actually includes a CCAAT box and is recognized by a CCAAT-box-binding protein.

On the basis of these considerations, we constructed plasmids pXD21 and pXD301 (carrying clean deletions of the X and Y boxes) and transfected them into the B-cell line M12. Transcription from each construct was compared with that from pX3 (positive standard) and pA101 (negative standard). Neither pXD21 nor pXD301 was transcribed above background levels (Fig. 3). The same result was obtained with pXD10, which bears a deletion of X, Y, and the sequences between. To rule out the possibility that the deletions are detrimental because they interfere with spacing rather than because they eliminate a particular DNA sequence, we made and tested pXXC and pXYC, which carry random sequences replacing the X and Y boxes. Again, neither plasmid was significantly transcribed in M12 cells. Thus, X and Y are crucial for promoter-proximal enhancer activity.

To test whether X and Y are sufficient for enhancer function, we inserted into the enhancer test construct a 57-bp fragment consisting essentially of X, Y, and the sequences between them. This plasmid (pXD19) is clearly transcribed in M12, although only about 30% as efficiently as pX3 is. This result is consistent with the observation that certain deletions in the −215 to +12 fragment that do not touch the X and Y boxes lead to somewhat reduced enhancer activity (pXD6, pA14, pA5).

It may be worth noting that deletion of the E_α TATA box and transcription initiation site has very little influence on enhancer activity as measured by transcription from the

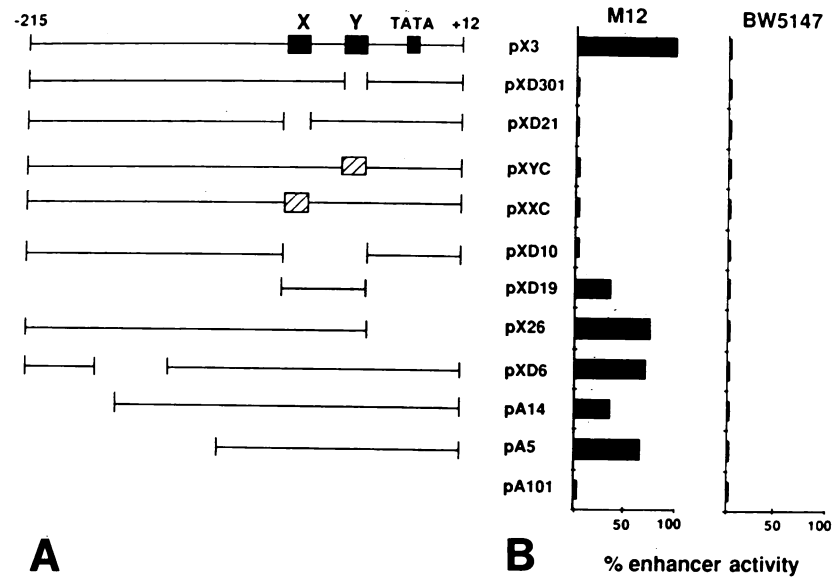

FIG. 3. Delineation of sequences important for activity of the −215 to +12 enhancer. (A) Maps of wild-type and mutant −215 to +12 fragments cloned into pA101. The X and Y boxes are conserved sequence motifs discussed in the text. Our assay does not measure transcription directed from the E_α TATA box, which in any case is quite low. Construction of the various mutants is described in Materials and Methods, as are the exact coordinates of the deletions. pXYC and pXXC have random sequences replacing the Y or X box. (B) Bar graph representations of S1 nuclease mapping results. The various plasmids were transfected into the MHC class II-positive B-cell line M12 and the class II-negative T-cell line BW5147. After two days, RNA was isolated and analyzed by S1 nuclease mapping, probing for transcripts initiated at the SV40 promoter. The signals were quantitated by densitometric tracing. The values obtained with M12 cells were normalized, taking as 100% the value obtained from pX3-transfected cells. With all constructs, the signals obtained for enhancer activity in BW5147 cells had, even on overexposed autoradiographs, a very low intensity, below the sensitivity of the densitometer, similar to that observed with the enhancerless plasmid pA101. The bar graphs are plots of averages from three independent experiments with two different plasmid preparations. Standard errors ± 10% of the means.

SV40 promoter. This point is best substantiated by comparing the pX26 and pX3 values in Fig. 3.

All of the aforementioned constructs were also transfected into the T hybridoma BW5147, which does not support the stimulatory activity of the intact −215 to +12 fragment. This experiment was aimed at detecting enhancer activity that might be uncovered by removal of a repressor sequence. Since none of the plasmids are transcribed above background levels in BW5147 (or in X63, data not shown), we surmise that the promoter-proximal 230-bp stretch does not carry a repressor of activity in non-B cells.

In summary, the X and Y boxes are crucial determinants of the B-cell-specific enhancer activity associated with the −215 to +12 fragment of the E_α gene. Other sequences in the region serve to optimize enhancer function.

Stimulatory activity of the −2172 to −1148 fragment dependent primarily on the W sequence. To delineate the sequence elements required for activity of the −2172 to −1148 fragment in a promoter-adjacent position, we created a series of deletions in the plasmid pSD212, which carries the −2172 to −1148 fragment. The mutant plasmids were transfected into the MHC class II-positive B-cell line M12 and into the class II-negative plasmacytoma X63. Transcription from the mutants was compared with that from pSD212 (positive standard) and pA101 (negative standard). The results are summarized in Fig. 4.

The first set of mutants has progressively longer deletions proceeding in a 3′ direction from position −2172. A deletion of up to 750 bp (p37) does not result in any significant loss of transcriptional activity whether assayed in M12 or X63 cells. When 870 (p22) or 930 (pSph) bp are removed, there is a 50% loss of activity but only in M12 cells. The different effects of these deletions in M12 and X63 do not simply reflect a dichotomy between class II molecule expressors and nonexpressors, because WEHI 231 values resemble those of X63 (data not shown). Finally, deletion of 980 bp (p69) or more (p59) reduces transcription to background levels in both cell lines. Seemingly then, the most important sequence element of this enhancer resides between positions −1240 and −1190; there is another element located between positions −1420 and −1300 that is quantitatively influential in some cell lines.

To support this conclusion, we created a complementary set of deletions in pSD212 that proceed in a 5′ direction from position −1148. The first deletion (to position −1214) resulted in complete loss of transcriptional activity in both M12 and X63 cells (pSD221). Longer deletions did not restore transcriptional activity (pSD222 and pSD223). These results emphasize the importance of the element located between −1240 and −1190 and suggest that the secondary element between positions −1420 and −1300 cannot act autonomously.

The region between −1400 and −1150 of the E_α^k gene contains several potentially important DNA sequence motifs (2, 4a). Most strikingly, a second copy of the X-Y pair occurs in reverse orientation; X′ and Y′, as they have been termed, reside at positions −1359 to −1346 and −1392 to 1379, respectively. It may be worth noting that Y′ (like Y) includes a CCAAT box. There is in addition a sequence termed W at positions −1202 to −1193 that is known to bind a B-cell-specific and ubiquitously occurring nuclear protein (2).

To directly test the contribution of X′, Y′, and W to enhancer function, we made a clean deletion of each in pSD212 and measured the transcriptional activity of the mutant plasmids in M12 and X63. Deletion of the Y′ box (pSD2122) had little or no effect in either cell line, while

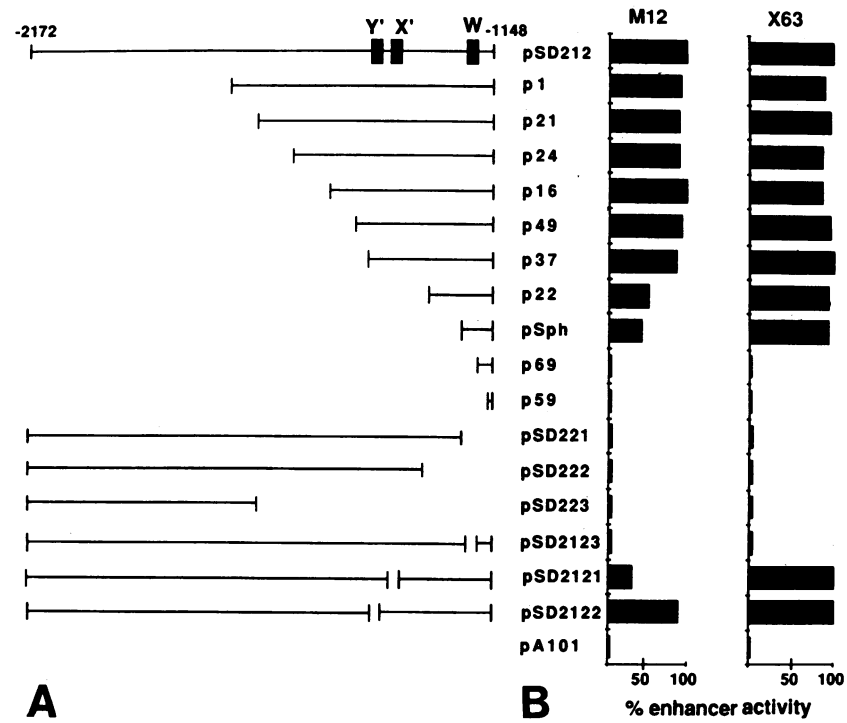

FIG. 4. Delineation of sequences important for enhancer activity of the −2172 to −1148 fragment when placed in a promoter-adjacent position. (A) Maps of wild-type and mutant −2172 to −1148 fragments cloned into pA101. The X′, Y′, and W boxes are sequence motifs discussed in the text. Derivation of the mutant plasmids and a list of deletion coordinates is given in Materials and Methods. (B) Bar graphs of S1 mapping results. The various plasmids were transfected into the class II-positive B-cell line M12 and the class II-negative plasmacytoma X63. After two days, RNA was isolated and transcripts initiated from the SV40 promoter were quantitated by S1 nuclease mapping. Densitometric tracing of the bands representing S1-resistant probe molecules was performed and the values were normalized, taking as 100% for each line the value obtained for RNA from cells transfected with pSD212. The bar graphs are plots of averages from four (M12) or three (X63) independent experiments with two different plasmid preparations. Standard errors ± 10% of the means.

deletion of the X′ box (pSD2121) resulted in a fourfold reduction of transcription in M12 but not in X63 cells. The difference between the X63 and M12 values with pSD2121 resembles that previously observed with the p22 and pSph mutants (see above). Finally, a clean deletion of the W box (pSD2123) resulted in essentially complete abrogation of enhancer activity.

Clearly then, the most critical element for the enhancer activity of the −2172 to −1148 fragment in a promoter-adjacent position is the W motif. This is true whether we measure stimulation in class-II-molecule-expressing cells or in nonexpressers. The X′ box seems to modulate the activity of the enhancer up to fourfold in some cell types.

Function of both the promoter-proximal and promoter-distal enhancers in the context of the −2172 to +12 fragment. Thus far, we have described the properties of the promoter-proximal and promoter-distal E_α elements as they operate in isolation at a promoter-adjacent position. Obviously, it is also important to study the enhancers in context, juxtaposed on the −2172 to +12 fragment. Hence, we created a series of pS3-based plasmids that bear single- or double-motif deletions. The mutant plasmids were transfected into M12 and X63 cells, and transcription from each compared with that from pS3 (positive standard) and pA101 (negative standard).

Both the promoter-distal and promoter-proximal elements function in the context of the −2172 to +12 fragment (Fig. 5). Deletions of the X or Y box destroy the stimulatory activity of the −215 to +12 fragment in isolation (Fig. 3), but in the context of the entire 2-kb fragment they result in only a two- or threefold reduction in stimulation (Fig. 5, pSD21 and pSD302). Similarly, deletion of the W motif would critically impede activity of the −2172 to −1148 fragment in isolation (Fig. 4), but in the context of the 2-kb fragment it has essentially no effect on stimulatory capacity (Fig. 5, pSD33). X′ and Y′ deletions are also incapable of abolishing the activity of the −2172 to +12 fragment (Fig. 5, pSD31 and pSD32).

In addition, the enhancer activity displayed by the 2-kb fragment is not the simple sum of its two halves (Fig. 5). Two points are most illustrative. (i) A deletion of X or Y in the −215 to +12 fragment or a deletion of W in the −2172 to −1148 fragment abolishes the enhancer activity of both moieties (Fig. 3 and 4). Yet the double deletions X+W and Y+W in the −2172 to +12 fragment result in only a three- to fivefold reduction in transcription levels (Fig. 5). (ii) A deletion of Y′ in the −2172 to −1148 fragment has little effect on stimulatory activity, appearing much less detrimental than an X′ or W deletion (Fig. 4). But in the context of the entire 2-kb fragment, a Y′ mutation is more drastic than either an X′ or W mutation (Fig. 5). This is true with both single and double deletions (compare pSD32 with pSD31 and pSD33 or pSD302/32 with pSD302/33). Comparison of pSD302 with pSD302/33 and pSD21 with pSD21/33 or pSD21/31 indicates that X′ and W still play some role, however.

These discrepancies could reflect the fact that in the context of the entire 2-kb fragment, a particular sequence motif has the opportunity to interact with both adjacent and remote companion motifs. Alternatively, different motifs in the promoter-distal element may take precedence when it is

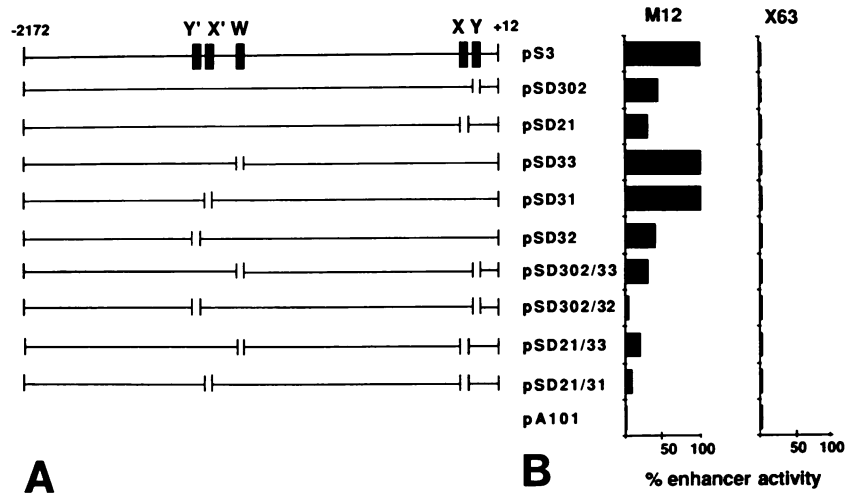

FIG. 5. Determination of motifs critical for the enhancer activity of the entire −2172 to +12 fragment. (A) Maps of the wild-type and mutant −2172 to +12 fragments cloned into pA101. The X, Y, X′, Y′, and W boxes are sequence elements discussed in the text. Details of the production and precise location of the deletions can be found in Materials and Methods. (B) Bar graphs of S1 nuclease mapping results. The plasmids were transfected into M12 (B-cell) and X63 (plasmacytoma cell) lines. RNA was isolated after two days, and transcription initiated from the SV40 promoter was quantitated by S1 nuclease mapping. The values obtained for specific RNA transcribed from the constructs in M12 cells were related to the value obtained from cells transfected with pS3, which was set to 100%. In X63 cells, only very little activity was observed, and none of the constructs exhibited any activity over that detected with the enhancerless plasmid pA101. The quantitations are based on three (M12) or two (X63) independent experiments with two different plasmid preparations. Standard errors ± 15% of the means.

located hundreds of bases away from the SV40 promoter. Motif deletions in the construct which intercalates pBR322 sequences between the E_α enhancer and the SV40 promoter (pSD312) should resolve this issue.

It may be worth pointing out that all of the stimulatory activity displayed by the −2172 to +12 fragment, whether attributable to the promoter-proximal or promoter-distal component, is B cell specific. None of the mutants are transcribed above background levels in either X63 or BW5147 cells (Fig. 5; data not shown).

From these results, we conclude that both halves of the −2172 to +12 fragment contribute to its B-cell-specific enhancer activity. Yet, the compound enhancer is not the simple sum of its two components.

DISCUSSION

B-cell-specific enhancer. The murine E_α gene, like other MHC class II loci, is transcribed constitutively in mature B cells. As far as is known, this is the only cell type in mice that shows constitutive expression of class II genes. In this report, we describe an enhancer that no doubt contributes to the transcriptional activity of the E_α gene in B cells. The enhancer extends over a 2-kb stretch at the 5′ end of the gene and has a complex anatomy. There is a promoter-proximal element that is B cell specific and relies principally on the conserved X and Y sequences. There is also a promoter-distal element that is B cell specific when located at a distance from the promoter but is ubiquitously acting in a promoter-adjacent position. The activity of this element depends on the X′, Y′, and W sequences, with their relative contributions differing greatly according to the context of the element.

So far, the cell-type specificity of the E_α enhancer correlates perfectly with expression of the endogenous class II genes; it is active in several mature B-cell lines but not in pre-B or plasmacytoma cells nor in several nonlymphoid class II-negative cell lines. We do not know yet whether the activity of this enhancer is restricted purely to B cells or if it will prove operative in other cells that express E_α, in particular in gamma-interferon-inducible cells. (Preliminary results do suggest that it is inactive in thymic epithelial cells).

An enhancer with a few of these features has recently been described (28). A 1.8-kb fragment at the 5′ end of the human class II gene DQα is capable of stimulating transcription from the thymidine kinase promoter. The stimulatory activity also centered around the X and Y boxes. The DQα enhancer seems similar to the E_α promoter-proximal element although we do not have information on its cell-type specificity. We are also ignorant of whether the DQα gene carries an element analogous to the E_α promoter-distal element.

Our findings should be considered in conjunction with two results obtained with transgenic mice. First, mouse cells carrying an E_α gene with a clean deletion of either the X or Y box still express E_α mRNA in a cell-type-specific fashion (4). But the transcripts are less abundant than with the wild-type gene and are initiated at multiple sites as far upstream as the X′-Y′ region. This phenotype could occur if the X and Y deletions abrogate promoter-proximal enhancer activity, leaving transcription to be stimulated (less efficiently) by the promoter-distal element. It would thus be similar to the phenotype displayed by the pSD21 and pSD302 constructs (Fig. 5). The fact that aberrant initiation does not occur on pSD21 and pSD302 (data not shown) may be attributable to the strong influence of the SV40 promoter.

Second, mouse cells that carry an E_α gene with only 1,180 bp of 5′-flanking sequence fail to express E_α mRNA in B lymphocytes although not in other class II-positive cells (e.g., gamma-interferon-treated macrophages) (4a, 30, 32). This result appears inconsistent with the observation that the −1180 to +12 fragment shows significant B-cell-specific enhancer activity in transfection experiments (Fig. 2). The discrepancy is probably related to the different assay sys-

tems; the E_α gene in transgenic mice is subject to developmental influences and chromosomal position effects which do not affect the E_α gene in transfected cells.

Enhancer-binding proteins. It is of interest to consider the proteins known to bind to the E_α enhancer to ascertain whether their distribution or properties can account for the B-cell specificity of enhancer function.

The promoter-proximal enhancer depends primarily on the X and Y boxes; nuclear proteins that specifically recognize these sequences have recently been described. NF-Y is a large (200- to 300-kilodalton) complex that binds to the Y box (3, 4, 9, 11, 19, 26). It is, in fact, a CCAAT-box-binding protein and, as such, occurs in all cell types (3, 9). NF-X appears to be an even larger complex that binds to the X box (4, 11, 19). It is also detected in all cell types so far examined.

The promoter-distal enhancer depends variably on the X′, Y′, and W boxes, depending on its context with respect to the promoter. It has been demonstrated quite clearly that X′ and Y′ are recognized by NF-X and NF-Y just as the promoter-proximal X and Y are (4a). The W motif binds at least two nuclear proteins (termed NF-W1 and NF-W2) characterized in detail elsewhere (2).

These results have several interesting implications that require further investigation. (i) Y is critical for enhancer activity of the promoter-proximal element (Fig. 3, pXD301) and Y′ is crucial for promoter-distal enhancer function (Fig. 5, cf. pSD302 and pSD302/32). Both sequences contain a CCAAT box and are recognized by the CCAAT-box-binding protein, NF-Y. As far as we know, this represents the first functional evidence that a CCAAT box is implicated in enhancer activity. This would indicate a potential analogous to that displayed by the immunoglobulin octamer (ATTTG CAT) known to function as both a promoter and enhancer element (16). One should also bear in mind the binding duality of CBP/EBP, which recognizes CCAAT boxes as well as elements in the SV40 and possibly immunoglobulin enhancers (13, 21). These observations support the notion that promoter and enhancer elements may not be fundamentally different (16, 25). (ii) The W motif is recognized by both a B-cell-specific and ubiquitous nuclear protein (2). NF-W1 and NF-W2 have different affinities for and make distinct contacts on the recognition sequence. In theory, they could also interact differentially with other regulatory proteins involved in E_α expression. We suggest that the ability of the promoter-distal enhancer to act ubiquitously or with B-cell specificity might depend on which of these proteins is bound.

Model. The placement and orientation of the X-Y and X′-Y′ homologs and the sensitivity of the promoter-distal element to its position prompts us to propose the model illustrated in Fig. 6. The essential feature of the model is a dimerization of the proteins binding to X and X′ and Y and Y′, or both, resulting in a loop-out of the intervening DNA and a rapprochement of the promoter-proximal and promoter-distal regions and consequently of any proteins binding to them. Thus, for example, NF-W1 and NF-W2 might be brought into contact with RNA polymerase at the RNA initiation site.

At least two observations are consistent with this model. First, the distance but not the sequence between the X and Y boxes is conserved among all MHC class II genes, and this distance is also precisely conserved between all X′ and Y′ boxes (4a). Second, X and X′ bind the same protein, as do Y and Y′, suggesting a possibility for the formation of homodimers. Indeed, preliminary cross-linking studies suggest that purified NF-Y can form homodimers (X.-J. Li and R. Hooft van Huijsduijnen, unpublished data). However, no experimental evidence exists yet to support the model. If the E_α enhancer proves to rely on a looping mechanism, it will be reminiscent of other procaryotic (8, 12, 17, 20) and eucaryotic (29) regulatory elements. That such a mechanism may be used generally for gene regulation has been discussed at length (23, 24).

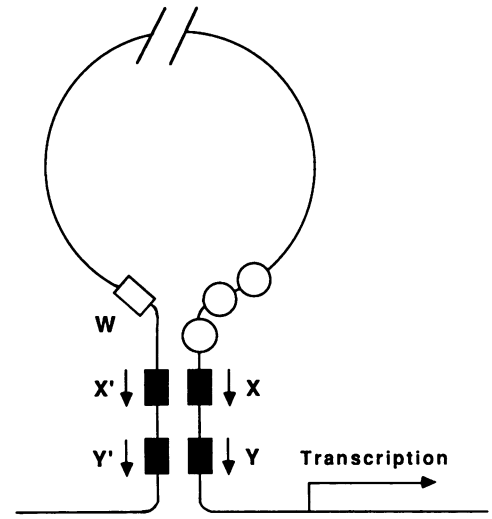

FIG. 6. A model of E_α enhancer structure. A model proposed to explain some of the features of E_α enhancer function invokes the formation of a DNA loop, produced by the dimerization of proteins binding to the X, Y, X′, and Y′ motifs (▬) as described in the text. Protein binding to the W motif (▭) is also described in the text. Proteins recognizing as-yet-unidentified sequence motifs (○); start site of RNA transcription (↓).

E_α and immunoglobulin enhancers. One goal of these studies was to compare the mechanisms controlling immunoglobulin and MHC class II gene expression in B cells. Both gene classes seem to rely on a strong cell-type-specific enhancer, but there are two striking dissimilarities. First of all, the B-lineage cells that support immunoglobulin and E_α enhancer activity differ markedly. Immunoglobulin enhancers can function in pre-B, mature B, and plasma cells (although the κ enhancer is active only in the latter two); the E_α enhancer operates only in mature B-cell lines. Second, the sequence motifs and consequently the DNA-binding proteins implicated in enhancer function seem to be quite distinct. The immunoglobulin enhancers are composed of multiple stimulatory 6- to 12-bp motifs, most notably the octamer motif, the NF-κB binding site, and the Ephrussi sequences. As discussed elsewhere (4a), the −2172 to +12 E_α fragment carries a sequence (positions −1703 to −1693) that is highly homologous to the NF-κB binding site on the immunoglobulin κ enhancer as well as multiple Ephrussi-like sequences (positions −1821 to −1807, −1758 to −1744, and −1546 to −1532). There is no stretch with a perfect match to the immunoglobulin octamer motif on this fragment, but there is one with a single-base mismatch (position −1917 to −1910). That none of these sequences play a significant role in E_α enhancer function is evidenced by results from several constructs presented in Fig. 4 and 5. Conversely, the E_α enhancer depends on multiple short DNA segments (X, Y, X′, Y′, W), and none of these has been detected along the immunoglobulin enhancers. In addition, competition experiments show that the proteins binding to these E_α motifs are distinct from those that recognize immunoglobulin motifs (2).

We are led to conclude, then, that immunoglobulin and MHC class II enhancers rely on largely independent mechanisms to achieve B-cell specificity.

ACKNOWLEDGMENTS

We thank Jacques Bollekens for providing two of the mutant constructs, M. Zenke and P. Chambon for plasmids, F. Ruffenach and A. Staub for oligonucleotides, P. Gerber and P. Reiter for excellent technical assistance, and M. Pierres, C. Paige, and S. Carson for cell lines.

This work was supported by institutional grants from the Centre National de la Recherche Scientifique and Institut National de la Santé et de la Recherche Médicale and by a grant to D.M. and C.B. from the Association pour la Recherche sur le Cancer. W.K. was supported by fellowships from the Centre National de la Recherche Scientifique and the Deutscher Akademischer Austauschdienst (Sonderprogramm Gentechnologie).

LITERATURE CITED

1. **Calame, K. L.** 1985. Mechanisms that regulate immunoglobulin gene expression. Annu. Rev. Immunol. **3**:159–195.
2. **Dorn, A., C. Benoist, and D. Mathis.** 1989. New B-lymphocyte-specific enhancer-binding protein. Mol. Cell. Biol. **9**:312–320.
3. **Dorn, A., J. Bollekens, A. Staub, C. Benoist, and D. Mathis.** 1987. A multiplicity of CCAAT box-binding proteins. Cell **50**: 863–872.
4. **Dorn, A., B. Durand, C. Marfing, M. Le Meur, C. Benoist, and D. Mathis.** 1987. Conserved MHC class II boxes -X and Y- are transcriptional control elements and specifically bind nuclear proteins. Proc. Natl. Acad. Sci. USA **84**:6249–6253.

4a. **Dorn, A., H. Fehling, W. Koch, M. LeMeur, P. Gerlinger, C. Benoist, and D. Mathis.** 1988. B-cell control region at the 5′ end of a major histocompatibility complex II gene: sequences and factors. Mol. Cell. Biol. **8**:3975–3987.

5. **Dreyfus, M., N. Doyen, and F. Rougeon.** 1987. The conserved decanucleotide from the immunoglobulin heavy chain promoter induces a very high transcriptional activity in B-cells when introduced into an heterologous promoter. EMBO J. **6**:1685–1690.
6. **Flavell, R. A., H. Allen, B. Huber, C. Wake, and G. Widera.** 1985. Organization and expression of the MHC of the C57 black/10 mouse. Immunol. Rev. **84**:29–50.
7. **Gerster, T., P. Matthias, M. Thali, J. Jiricny, and W. Schaffner.** 1987. Cell type-specific elements of the immunoglobulin heavy chain gene enhancer. EMBO J. **6**:1323–1330.
8. **Griffith, J., A. Hochschild, and M. Ptashne.** 1986. DNA loops induced by cooperative binding of lambda repressor. Nature (London) **322**:750–752.
9. **Hooft van Huijsduijnen, R., J. Bollekens, A. Dorn, C. Benoist, and D. Mathis.** 1987. Properties of a CCAAT box-binding protein. Nucleic Acids Res. **15**:7265–7281.
10. **Imler, J. L., C. Lemaire, C. Wasylyk, and B. Wasylyk.** 1987. Negative regulation contributes to tissue specificity of the immunoglobulin heavy-chain enhancer. Mol. Cell. Biol. **7**:2558–2567.
11. **Koch, W., S. Candeias, J. Guardiola, R. Accolla, C. Benoist, and D. Mathis.** 1988. An enhancer factor defect in a mutant Burkitt lymphoma cell line. J. Exp. Med. **167**:1781–1790.
12. **Krämer, H., M. Amouyal, A. Nordheim, and B. Müller-Hill.** 1988. DNA supercoiling changes the spacing requirement of two lac operators for DNA loop formation with lac repressor. EMBO J. **7**:547–556.
13. **Landschulz, W. H., P. F. Johnson, E. Y. Adashi, B. J. Graves, and S. L. McKnight.** 1988. Isolation of a recombinant copy of the gene encoding c/EBP. Genes Dev. **2**:786–800.
14. **Le Meur, M., P. Gerlinger, C. Benoist, and D. Mathis.** 1985. Correcting an immune-response deficiency by creating E_α gene transgenic mice. Nature (London) **316**:38–42.
15. **Lenardo, M., J. W. Pierce, and D. Baltimore.** 1987. Protein-binding sites in Ig gene enhancers determine transcriptional activity and inducibility. Science **236**:1573–1577.
16. **Maniatis, T., S. Goodbourn, and J. A. Fischer.** 1987. Regulation of inducible and tissue-specific gene expression. Science **236**: 1237–1245.
17. **Martin, K., L. Huo, and R. F. Schleif.** 1986. The DNA loop model for ara repression: AraC protein occupies the proposed loop sites in vivo and repression-negative mutations lie in these same sites. Proc. Natl. Acad. Sci. USA **83**:3654–3658.
18. **McCutchan, J. H., and J. S. Pagano.** 1968. Enhancement of the infectivity of SV40 DNA with DEAE-dextran. J. Natl. Cancer Inst. **41**:351–356.
19. **Miwa, K., C. Doyle, and J. L. Strominger.** 1987. Sequence-specific interactions of nuclear factors with conserved sequences of human class II major histocompatibility complex genes. Proc. Natl. Acad. Sci. USA **84**:4939–4943.
20. **Mukherjee, S., H. Erickson, and D. Bastia.** 1988. Enhancer-origin interaction in plasmid R6K involves a DNA loop mediated by initiator protein. Cell **52**:375–383.
21. **Peterson, C. L., and K. L. Calame.** 1987. Complex protein binding within the mouse immunoglobulin heavy-chain enhancer. Mol. Cell. Biol. **7**:4194–4203.
22. **Pierce, J. W., M. Lenardo, and D. Baltimore.** 1988. Oligonucleotide that binds nuclear factor NF-κB acts as a lymphoid-specific and inducible enhancer element. Proc. Natl. Acad. Sci. USA **85**:1482–1486.
23. **Ptashne, M.** 1986. Gene regulation by proteins acting nearby and at a distance. Nature (London) **322**:697–701.
24. **Schleif, R.** 1988. DNA looping. Science **240**:127–128.
25. **Serfling, E., M. Jasin, and W. Schaffner.** 1985. Enhancers and eukaryotic gene transcription. Trends Genet. **1**:224–330.
26. **Sherman, P. A., P. V. Basta, and J. P. Y. Ting.** 1987. Upstream DNA sequences required for tissue-specific expression of the HLA-DRα gene. Proc. Natl. Acad. Sci. USA **84**:4254–4258.
27. **Sullivan, K. E., A. F. Calman, M. Nakanishi, S. Y. Tsang, Y. Wang, and B. M. Peterlin.** 1987. A model for the transcriptional regulation of MHC class II genes. Immunol. Today **8**:289–293.
28. **Sullivan, K. E., and B. M. Peterlin.** 1987. Transcriptional enhancers in the HLA-DQ subregion. Mol. Cell. Biol. **7**:3315–3319.
29. **Théveny, B., A. Bailly, C. Rauch, M. Rauch, E. Delain, and E. Milgrom.** 1987. Association of DNA-bound progesterone receptors. Nature (London) **329**:79–81.
30. **van Ewijk, W., Y. Ron, J. Monaco, J. Kappler, P. Marrack, M. Le Meur, P. Gerlinger, B. Durand, C. Benoist, and D. Mathis.** 1988. Compartmentalization of MHC class II gene expression in transgenic mice. Cell **53**:357–370.
31. **Weinberger, J., P. S. Jat, and P. A. Sharp.** 1988. Localization of a repressive sequence contributing to B-cell specificity in the immunoglobulin heavy-chain enhancer. Mol. Cell. Biol. **8**:988–992.
32. **Widera, G., L. C. Burkly, C. A. Pinkert, E. C. Böttger, C. Cowing, R. D. Palmiter, R. L. Brinster, and R. A. Flavell.** 1987. Transgenic mice selectively lacking MHC class II (I-E) antigen expression on B cells: an in vivo approach to investigate Ia gene function. Cell **51**:175–187.
33. **Wirth, T., L. Staudt, and D. Baltimore.** 1987. An octamer oligonucleotide upstream of a TATA motif is sufficient for lymphoid-specific promoter activity. Nature (London) **329**:174–178.
34. **Zenke, M., T. Grundström, H. Matthes, M. Wintzerith, C. Schatz, A. Wildeman, and P. Chambon.** 1986. Multiple sequence motifs are involved in SV40 enhancer function. EMBO J. **5**:387–397.