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π, a Pre-B-Cell-Specific Enhancer Element in the Immunoglobulin Heavy-Chain Enhancer

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We have identified a new immunoglobulin heavy-chain enhancer element, designated π, between the μE2 and μE3 elements. The π enhancer element is transcriptionally active primarily during early stages of B-cell development but becomes virtually inactive during B-cell maturation at about the stage of immunoglobulin κ light-chain gene rearrangement. Mutational analysis suggests that the π element is crucial for immunoglobulin heavy-chain enhancer activity at the pre-B-cell stage but is almost irrelevant for enhancer activity at the mature B-cell or plasma-cell stage. The activity of the π enhancer element correlates with the presence of an apparently pre-B-cell-specific protein-DNA complex. The similarity of the π site to recognition sequences for members of the *ets* gene family suggests that the protein(s) interacting with the π site most likely are *ets*-related transcription factors.

The intronic immunoglobulin heavy-chain (IgH) enhancer is located between the last joining-region exon (J_μ) and the first constant-region exon (C_μ) (8, 21). This enhancer has been shown to be of utmost importance for both B-cell-specific and developmental-stage-specific expression of the IgH gene as well as for the recombinational process (17, 42). Additional enhancers 3′ of the IgH gene have been identified but have not been characterized in more detail (13, 45).

A 700-bp *Xba*I-*Eco*RI fragment (μ700) contains most of the activity of the intronic IgH enhancer and functions during all stages of B-cell development but is inactive in nonlymphoid cells (39). The IgH enhancer shows a complex modular arrangement of several different overlapping stimulatory and negative elements (see Fig. 1) (21, 39, 42). The B-cell specificity of the IgH enhancer is apparently regulated by positively acting B-cell-specific enhancer elements, including octamer and μB, negatively acting non-B-cell-specific silencer elements, and protein-protein interactions between these B-cell-specific factors and ubiquitously expressed transcription factors (4, 5, 11, 20, 31, 33, 39, 42, 44, 52, 55, 65, 73).

We have characterized the B-cell-specific enhancer element, μB, in more detail and have shown that the μB element is a critical component of the IgH enhancer in lymphoid cell-specific expression (44, 52). Both the octamer and the μB elements are active during all stages of B-cell development, correlating with the transcriptional activity of IgH genes (39, 44, 52), even though the octamer element appears to be less active at the pre-B-cell stage.

We now report the identification of a novel enhancer element, designated π, between the μE2 and μE3 elements of the IgH enhancer (see Fig. 1). We show that this highly conserved enhancer element is pre-B-cell specific and of crucial importance for the activity of the IgH enhancer at the pre-B-cell stage only. The similarity of the π site to binding sites for members of the *ets* gene family suggests that the protein(s) interacting with the π site belong to the *ets* gene family.

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MATERIALS AND METHODS

Cell cultures. The following cells were grown as described previously (2, 3, 30, 41, 43, 51, 56): S194 (murine myeloma), NIH 3T3 (murine fibroblasts), HeLa (human cervical carcinoma), U-343 Mg (human glioma), U-251 Mg Ag Cl1 (human glioma), C127 (murine mammary cells), L929 (murine fibroblasts), U-706T (human glioma), U-Cl 2:6 (human glioma), U-1240 Mg (human glioma), P19 (murine teratocarcinoma), EJ (human bladder carcinoma), U-937 (human monocytic cells), Jurkat (mature human T-cell line), PD31 (Abelson murine leukemia virus-transformed pre-B-cell line), 230-238 (murine pre-B-cell line), 38B9 (murine pre-B-cell line), K40B.1 (murine pre-B-cell line), K40B.2 (murine pre-B-cell line), NFS5.3 (murine late pre-B-cell line), 70Z/3 (murine late pre-B-cell line), BASC 6C2 (murine pre-B-cell line), WEHI 231 (murine mature B-cell line), X63 Ag8 (murine myeloma), Namalwa (human mature B-cell line), MOLT 3 (mature human T-cell line), MOLT 4 (mature human T-cell line), HAFTL (murine Harvey v-*ras*-transformed early pre-B-cell line), and 32D (murine early myeloid cell line).

Nuclear extracts. Nuclear extracts were prepared by the method of Dignam et al. (14). All buffers included leupeptin at 0.3 μg/ml, 5 mM phenylmethylsulfonyl fluoride (PMSF), antipain at 0.3 μg/ml, and aprotinin at 2 μg/ml.

DNase I footprinting analysis. μ700 WT/Δ56 and μ700 π^-/Δ56 (see Fig. 1 and 6 and Results) were digested with *Hin*dIII and *Bam*HI; the restriction sites were located 5′ and 3′ of the enhancer in the Δ56 plasmid, respectively (22, 39). The 700-bp IgH enhancer fragments were gel purified, digested with *Hin*fI, dephosphorylated, and end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase at the 5′ end of the noncoding strand. After digestion with *Dde*I, end-labeled *Hin*fI-*Dde*I fragments were gel purified. DNase I footprinting was performed as described by Jones et al. (32) with 0.3 to 0.8 ng of end-labeled IgH enhancer fragments (8,000 cpm), 20 μg of total nuclear extract or no protein, 1 μg of poly(dI-dC) (Pharmacia), and 2% polyvinyl alcohol in a 50-μl volume. A G+A Maxam-Gilbert (50) sequencing reaction of the end-labeled IgH enhancer fragments was run as a marker.

EMSA. DNA-binding reactions and electrophoretic mobility shift assays (EMSA) were performed as described previ-

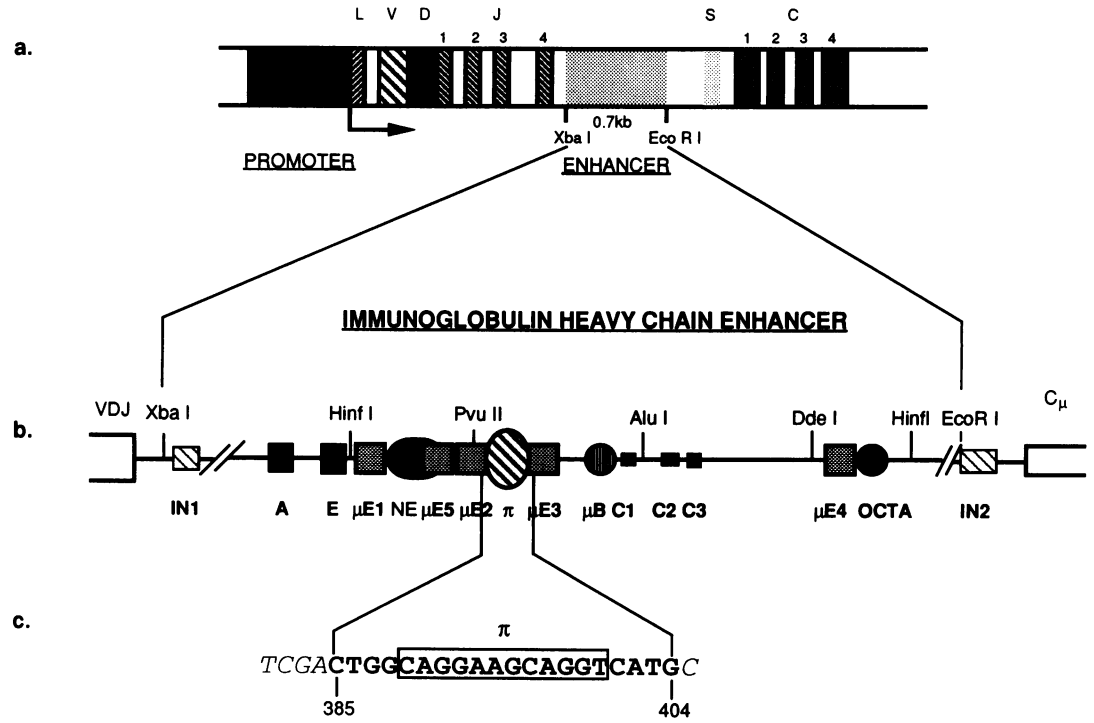


FIG. 1. Schematic diagram of regulatory regions in the murine IgH enhancer and location of the π enhancer element. (a) IgH gene structure. The transcription initiation site is indicated by the arrow. The leader (L)-, variable (V)-, diversity (D)-, joining (J)-, and constant (C)-region exons are shown as hatched boxes. Promoter regions are in black. Enhancers are indicated by shaded areas in the introns. S indicates a switch region in the IgH gene. (b) Schematic diagram of regulatory regions in the murine IgH enhancer and location of the π enhancer element. Filled symbols represent the various transcription factor-binding sites as indicated below the diagram and described in the text. Open boxes represent exons for the constant and variable regions, and the line indicates the intron. The sequence of π is indicated by the expansion below the diagram. The enhancer fragment referred to as μ700 extends between the *Xba*I and *Eco*RI sites. μ300 extends between the *Pvu*II and *Eco*RI sites. (c) Sequence of the π site and flanking nucleotides used for oligonucleotide constructs. The box indicates the sequence of the π site. Boldface nucleotides were derived from the IgH enhancer sequence. Italic nucleotides flank the oligonucleotide to create a restriction site.

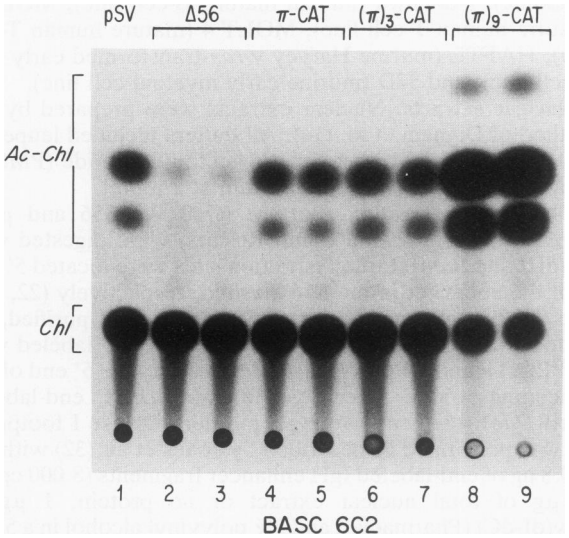


FIG. 2. The π site acts as a strong enhancer element in BASC 6C2 pre-B cells. Shown are the CAT activities of π oligonucleotide constructs, the enhancerless Δ56 background plasmid (Δ56), and the SV40 enhancer (pSV) in BASC 6C2 pre-B cells. *Ac-Chl*, acetylated chloramphenicol; *Chl*, nonacetylated chloramphenicol.

ously (6, 7). Samples of 20 μl containing 5 μg of nuclear extract, 0.5 μg of heparin-agarose-purified nuclear factors, or 4 μl of affinity-purified nuclear factor π were incubated with 0.1 to 0.2 ng of a ^{32}P-labeled immunoglobulin (Ig) π wild-type DNA fragment (5,000 cpm)–10 mM Tris-Cl (pH 7.5)–50 mM NaCl–1 mM dithiothreitol (DTT)–1 mM EDTA–1.75% polyvinyl alcohol–5% glycerol–2 μg of bovine serum albumin (Boehringer) and with 2 μg of poly(dI-dC), 1 μg of poly(dI-dC), or 0.4 μg of poly(dI-dC) for total nuclear extract, heparin-agarose-purified nuclear factors, or affinity-purified nuclear factors, respectively. Samples were incubated in the presence or absence of competitor oligonucleotides for 15 min at room temperature and run on 4% polyacrylamide gels containing as a buffer 25 mM Tris-HCl (pH 8.5), 190 mM glycine, and 1 mM EDTA.

Oligonucleotides used for competition studies were as follows:

(i) Ig π wild-type oligonucleotide

5′-TCGACTGGCAGGAAGCAGGTCATGC-3′
3′-GACCGTCCTTCGTCCAGTACGAGCT-5′

(ii) Ig π M1 oligonucleotide

5′-TCGACTGGCATTAAGCAGGTCATGC-3′
3′-GACCGTAATTCGTCCAGTACGAGCT-5′

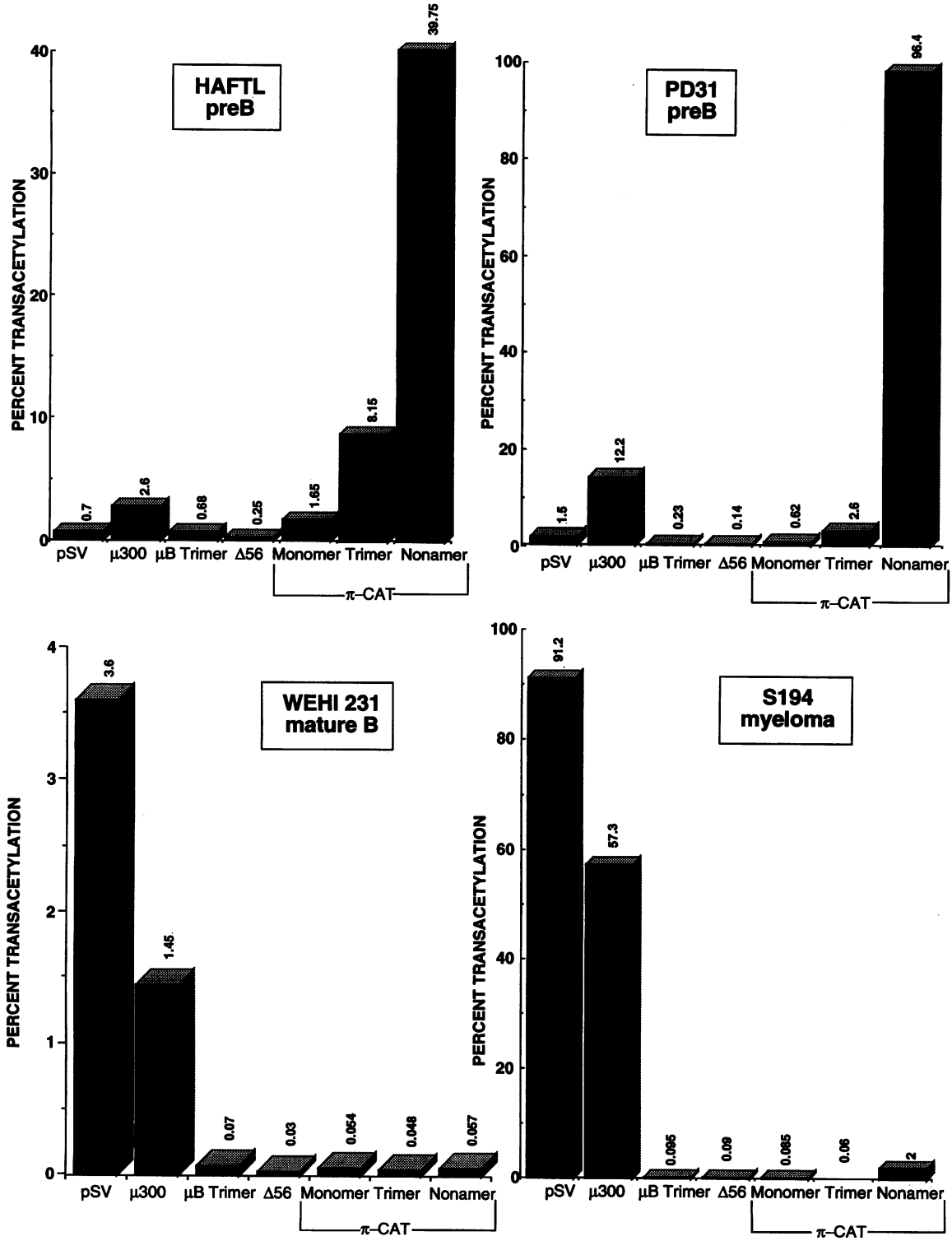


FIG. 3. The π enhancer element is active only in pre-B cells. Shown are the relative CAT activities of π (π-CAT) and μB (μB) oligonucleotide constructs, the IgH enhancer (μ300), and the SV40 enhancer (pSV) in B cells of different developmental stages. The CAT activity of each construct transfected into S194 myeloma, WEHI 231 mature B, HAFTL early pre-B, or PD31 pre-B cells is compared with that of the enhancerless Δ56 background plasmid. μ300 extends between the *Pvu*II and *Eco*RI sites (Fig. 1) (39). CAT values are averages derived from one representative experiment involving two independent transfections. Repeated experiments produced similar results, with standard deviations of less than 15% compared with the values given.

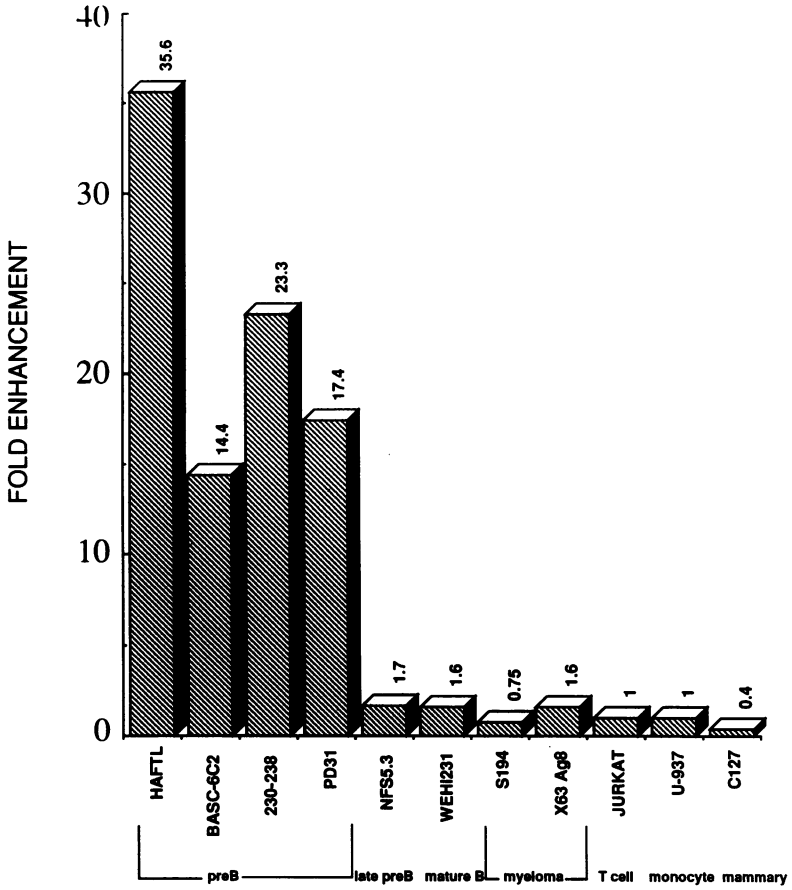


FIG. 4. Relative CAT activity of the π oligonucleotide trimer in different cell types. Shown is the relative CAT activity of a π oligonucleotide trimer construct in B cells of different developmental stages and non-B cells. The CAT activity of each construct transfected into S194 or X63 Ag8 myeloma, WEHI 231 mature B, NFS5.3 late pre-B, HAFTL early pre-B, PD31, BASC 6C2, or 230-238 pre-B, Jurkat mature T, U-937 monocytic, or C127 mammary cells is compared with that of the enhancerless Δ56 background plasmid. CAT values are averages derived from two representative experiments involving two independent transfections. Repeated experiments produced similar results, with standard deviations of less than 20% compared with the values given.

(iii) Ig π M2 oligonucleotide

5′-TCGACTGGCAGGAAGCCTTTCATGC-3′
3′-GACCGTCCTTCGGAAAGTACGAGCT-5′

(iv) Ig π M3 oligonucleotide

5′-TCGACTGGCAGGAAGCAGGTTTTGC-3′
3′-GACCGTCCTTCGTCCAAAACGAGCT-5′

(v) JC virus enhancer (18) oligonucleotide

5′-TCGACATGCTTGGCTGGCAAGCCATCC-3′
3′-GTACGAACCGACCGTTCGGTAGGAGCT-5′

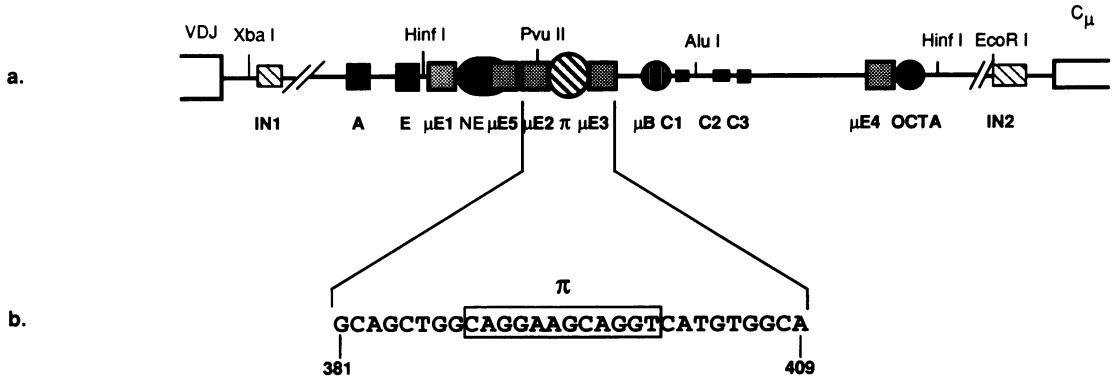
Chromatographic purification. Heparin-agarose (0.5 ml) was equilibrated with buffer D (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] [pH 7.9], 0.2 mM EDTA, 0.5 mM DTT, 0.1 M KCl, 20% glycerol). Nuclear extract (10 mg) in buffer D was applied five times to a heparin-agarose column and washed with 10 column volumes of buffer D. Nuclear proteins were step eluted from the heparin-agarose column with 2.5 column volumes of buffer D containing, successively, 150, 250, 350, 500, and 900 mM KCl and dialyzed against buffer D. Activity was measured by EMSA and eluted primarily in the 250 and 350 mM KCl fractions.

DNA affinity chromatography was performed as described by Kadonaga and Tjian (34) with a double-stranded 25-bp π oligonucleotide

5′-TCGACTGGCAGGAAGCAGGTCATGC-3′
3′-GACCGTCCTTCGTCCAGTACGAGCT-5′

from the IgH enhancer with TCGA 5′-overhanging ends. Chromatography was carried out with buffer F (20 mM KH_2PO_4 [pH 7.2], 0.1 M KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1 mM PMSF). The sample was mixed with 20 μg of poly(dI-dC) per ml on ice for 30 min, mixed with the affinity matrix for 1 h at 4°C, and loaded into a column. The column was washed with five column volumes of buffer F and step eluted with buffer F containing, successively, 200, 300, 500, and 1,000 mM KCl. Activity as measured by EMSA eluted primarily in the 200 and 300 mM fractions.

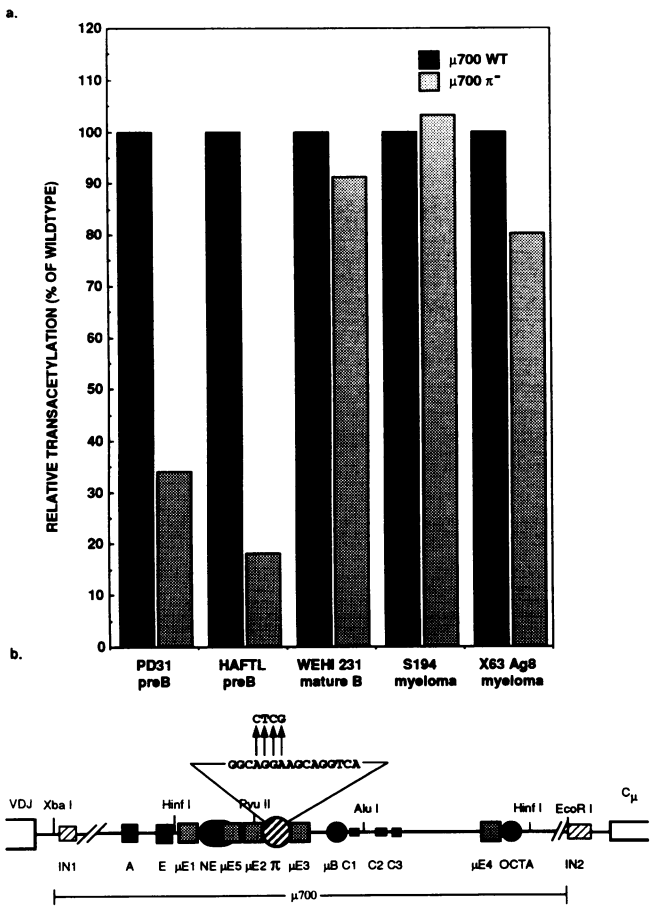
Constructs and mutants. For enhancer-chloramphenicol acetyltransferase (CAT) plasmid construction, the *Xho*I-linked 700-bp IgH enhancer was inserted into the *Sal*I site of the Δ56–c-*fos*–CAT plasmid in the B orientation (see Fig. 1b and 6) as previously described by Gilman et al. (22) and Lenardo et al. (39). A synthetic 25-bp π oligonucleotide



c.

π-CAT CONSTRUCT	DNA SEQUENCE	ENHANCER ACTIVITY (% OF WILDTYPE)
WILDTYPE (WT)	TCGA**CTGGCAGGAAGCAGGTCATG**C	100
MUTANT 1 (M1)	TCGA**CTGGCA<u>TT</u>AAGCAGGTCATG**C	6
MUTANT 2 (M2)	TCGA**CTGGCAGGAAGC<u>CTT</u>TCATG**C	53
MUTANT 3 (M3)	TCGA**CTGGCAGGAAGCAGGT<u>TT</u>TG**C	118

FIG. 5. Relative CAT activities of π oligonucleotide mutants. (a) Schematic diagram of the IgH enhancer regulatory elements. (b) Sequence of the π site and flanking regions. (c) Sequences of wild-type and mutant π oligonucleotides and CAT assay results obtained in PD31 pre-B cells. CAT values are averages derived from one representative experiment involving two independent transfections. Repeated experiments produced similar results, with standard deviations of less than 20% compared with the values given. Boldface nucleotides are derived from the IgH enhancer sequence. Lightface nucleotides indicate flanking sequences creating a restriction site for insertion into plasmids. Underlined sequences indicate nucleotides introduced by mutations.



5′-TCGACTGGCAGGAAGCAGGTCATGC-3′
3′-GACCGTCCTTCGTCCAGTACGAGCT-5′

containing *Sal*I and *Xho*I ends was inserted as a monomer, a trimer, and a nonamer into the *Sal*I site of the Δ56–c-*fos*–CAT plasmid (22, 39). Similarly, trimers of mutant oligonucleotides Ig π M1, Ig π M2, and Ig π M3 (see above) were inserted into the *Sal*I site in the Δ56–c-*fos*–CAT plasmid.

Site-directed mutagenesis. Specific site-directed mutagenesis of four nucleotides in the π DNA motif of the murine IgH enhancer were introduced by the gap-heteroduplex method (66) with oligonucleotide 5′-CTGCAGCAGCTGG CCTCGAGCAGGTCATGTGG-3′ (see Fig. 6b). Mutant IgH enhancer constructs were partially sequenced. At least two independent isolates of each mutant construct were grown, analyzed, and used for the experiments.

DNA transfection assays. Cells were transfected with 10 μg of DNA by the DEAE-dextran method (39, 56), except that C127 cells were transfected by the calcium phosphate coprecipitation method (39, 57). The cells were harvested 48 h after transfection, and 30 to 100 μg of heat-inactivated (39)

FIG. 6. Mutation of the π site affects 700-bp IgH enhancer transcriptional activity only in pre-B cells. (a) Relative CAT activities of μ700 WT and μ700 π⁻ IgH enhancers in B cells of different developmental stages. CAT values are averages derived from two representative experiments involving two independent transfections. Repeated experiments produced similar results, with standard deviations of less than 10% compared with the values given. (b) Regulatory elements in the IgH enhancer. The wild-type sequence of π is indicated by the expansion above the diagram. Arrows point to the nucleotides introduced by mutations into the wild-type sequence.

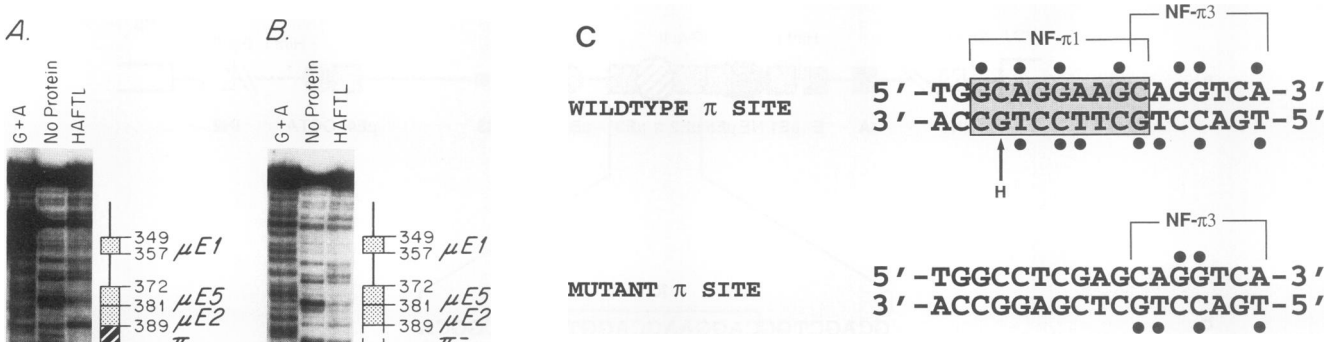


FIG. 7. Binding of nuclear factors to wild-type and mutant π sites in the IgH enhancer. (A and B) DNase I footprinting assays with wild-type (A) and mutant (B) noncoding strands of the IgH enhancer fragment. Labeled enhancer fragments were incubated with 20 μg of total nuclear extract from HAFTL pre-B cells (lane 3). Lane 2 was a control reaction without protein. A G+A Maxam-Gilbert sequencing reaction of the same DNA fragments was run as a marker (lane 1). The positions and names of enhancer elements in the IgH enhancer are indicated by boxes together with the coordinates of each site as described by Ephrussi et al. (15). The open box in panel B indicates the position of the altered π site. (C) Summary of apparent interactions at the π site. The DNA sequences containing the wild-type or mutant π site are shown. Hypersensitive sites, seen in DNase I footprinting assays, are indicated by an arrow and an H. Nucleotides protected in the DNase I footprinting assays are indicated by solid circles. Minimal binding sites for protein-DNA complexes 1 and 3, based on the results of these DNase I footprinting assays and EMSA (see Fig. 11 and 12), are indicated above the DNA sequences. The putative binding site for pre-B-cell-specific activity is indicated by the shaded box.

cell extract was assayed for CAT activity as described previously (24) in a 1- or 2-h incubation at 37°C. Transfections for every construct were performed independently in duplicate and repeated two to four times. Samples were analyzed by thin-layer chromatography (24). The percentage of acetylated chloramphenicol was determined by cutting out samples of nonacetylated and acetylated forms of chloramphenicol and measuring the amount of radioactivity by liquid scintillation counting.

RESULTS

Identification of a novel enhancer element, π, in the IgH enhancer. While analyzing the murine IgH enhancer by DNase footprinting for protein-DNA interactions, we recently (44) observed between the μE2 and μE3 enhancer elements a putative lymphoid cell-specific footprint that had not been previously described (Fig. 1). The sequence of this putative enhancer element, 5′-CAGGAAGCAGGT-3′, is depicted in Fig. 1b, and we refer to this site as the π site.

To determine whether the π site indeed acts as an enhancer element, we generated a synthetic 25-bp oligonucleotide containing the π DNA motif (Fig. 1) (see Materials and Methods). We inserted one, three, or nine copies of this oligonucleotide upstream of the truncated c-*fos* promoter in the Δ56 plasmid (22). Enhancer activity was measured as the ability of the different constructs to induce transcription of the CAT gene after transient expression in the murine pre-B-cell line BASC 6C2 (30, 56). The transcriptional activities of these constructs were compared with that of the parental enhancerless Δ56 plasmid itself. pSV2CAT, which contains the simian virus 40 (SV40) enhancer (24), served as a positive control and was very active in BASC 6C2 cells. As shown in Fig. 2, very little CAT activity was detectable with the Δ56 plasmid itself, whereas one copy of the π element stimulated transcription almost 10-fold. Enhancer activity increased with increased copy number, such that nine copies of the π element stimulated transcription by more than 400-fold. These results indicate that the π site is a very powerful enhancing element.

The π enhancer element is active only in pre-B cells. To determine whether the π enhancer element is regulated during B-cell development, we transfected murine cell lines representing different stages of B-cell development with the Δ56 plasmid containing one, three, or nine copies of the π enhancer element or containing three copies of another, lymphoid cell-specific IgH enhancer element, μB (44). pSV2CAT, μ300 (the region between the *Pvu*II and *Eco*RI sites shown in Fig. 1), and μ700 (the region between the *Xba*I and *Eco*RI sites shown in Fig. 1) (39) served as positive controls. The SV40 enhancer and IgH enhancer fragments μ300 and μ700 (data not shown) stimulated transcription in all cell lines. However, the π enhancer element was active only in pre-B-cell lines HAFTL, PD31, and BASC 6C2 and virtually inactive in mature B-cell line WEHI 231 or myeloma cell line S194 (Fig. 3). Only with nine copies of the π enhancer element was some activity detected in the myeloma cell line, but no activity was seen in the mature B-cell line. Three copies of the μB enhancer, in contrast, did not express any intrinsic enhancer activity in any cell line.

To test in more detail the stage and tissue specificity of the

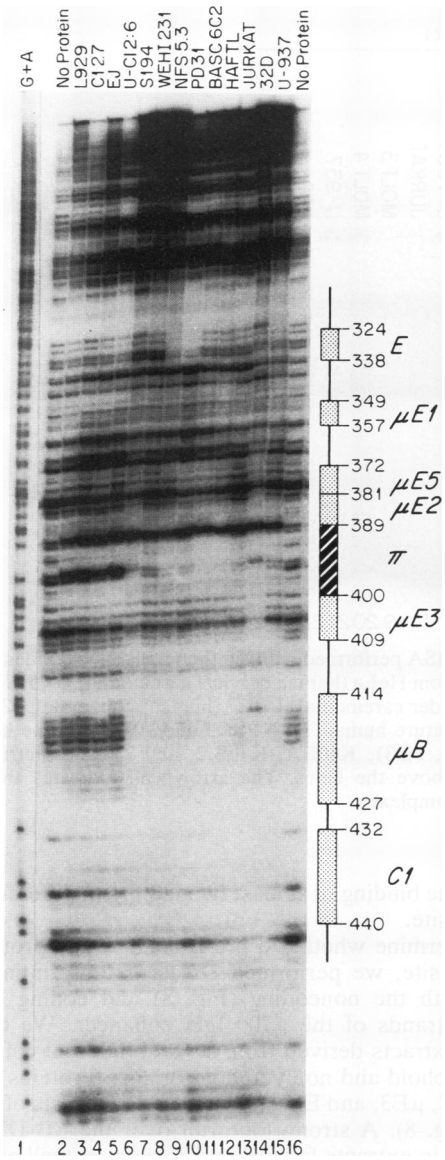


FIG. 8. Binding of nuclear factors from various cell types to the π site in the IgH enhancer. Shown is a DNase I footprinting assay done with the noncoding strand of the IgH enhancer fragment. The labeled enhancer fragment was incubated with 20 μg of total nuclear extract from L929 (murine fibroblastic), C127 (murine mammary), EJ (human bladder carcinoma), U-Cl 2:6 (human glioma), S194 (murine myeloma), WEHI 231 (murine mature B), NFS5.3 (murine late pre-B), PD31 (murine pre-B), BASC 6C2 (murine pre-B), HAFTL (murine early pre-B), Jurkat (mature human T), 32D (murine early myeloid), and U-937 (human monocytic) cells as indicated above the lanes. Lanes 2 and 16 were control reactions without protein. A G+A Maxam-Gilbert sequencing reaction of the same DNA fragments was run as a marker (lane 1). The positions and names of enhancer elements in the IgH enhancer are indicated by boxes together with the coordinates of each site as described by Ephrussi et al. (15).

π enhancer element, we transfected a wider range of cell lines with the Δ56 plasmid containing three copies of the π enhancer element and compared its activity in these cell lines with that of the parental Δ56 plasmid. Again, the π enhancer element was a potent enhancer only in pre-B-cell lines, increasing transcription 14- to 36-fold, depending on the cell line (Fig. 4). Practically no enhancer activity was detected in late pre-B, mature B, or myeloma cells. Furthermore, the π enhancer element was also inactive in Jurkat T cells, U-937 monocytic cells, and C127 mammary cells. These results suggest that the IgH enhancer contains in addition to the lymphoid cell-specific enhancer elements μB and octamer, which are active during all stages of B-cell development, a stage-specific enhancer element, π.

Effect of mutations on the activity of an isolated π enhancer element. To further define the sequence requirements for π enhancer activity, we synthesized several oligonucleotides (M1, M2, and M3) that spanned the π element and that contained mutations of either two or three nucleotides in the π site (Fig. 5). Three copies of these mutant oligonucleotides were inserted into the Δ56 plasmid. The transcriptional activities of mutant π enhancers were compared with those of the wild-type π enhancer trimer and the parental Δ56 plasmid (Fig. 5). The mutation in M1, in the 5′ half of the π site, almost completely abrogated all enhancer activity (Fig. 5). The mutation in M2 reduced the enhancer activity of the π element only slightly, and the mutation in M3, at the 3′ end of the π site, had no effect on transcriptional activity (Fig. 5). These data suggest that the 5′ half of the π site contains most, if not all, of the sequence required for enhancer activity.

Mutation of the π site reduces the activity of the IgH enhancer specifically in pre-B cells. To test whether the π enhancer element contributes to the activity of the IgH enhancer in the context of the whole 700-bp enhancer, we introduced a mutation affecting the same region of π as the M1 mutation, which inhibits the function of an isolated π element, into the π site of μ700 (Fig. 6b). The full-length 700-bp wild-type (μ700 WT) and mutant (μ700 π^-) IgH enhancers, cloned into the Δ56 plasmid (22), were transiently expressed in B-cell lines of different developmental stages, and the transcriptional activity of the mutant enhancer was compared with that of the wild-type enhancer. Mutation of the π site within μ700 drastically reduced the activity of the IgH enhancer in the pre-B-cell lines HAFTL and PD31 (Fig. 6a) but had only a slight effect in the mature B-cell line WEHI 231 or in the myeloma cell lines S194 and X63 Ag8. CAT activity was reduced by ~65% in PD31 and by more than 80% in HAFTL pre-B cells but at most 20% in mature B or myeloma cells (Fig. 6a). These data most vividly illustrate the pre-B-cell specificity of the π enhancer element and strongly suggest that the π site is an important element for transcriptional control of the IgH enhancer at the pre-B-cell stage but not during later stages of B-cell development.

Mutation of the π site inhibits the binding of a nuclear factor to the IgH enhancer. To correlate the functional data described above with the binding of a specific protein to the π site and to confirm that mutation of the π site decreases the binding of a specific nuclear factor, we carried out DNase I footprinting experiments with the noncoding strand of the μ700 WT or μ700 π^- IgH enhancer. We compared a *Hin*fI-*Dde*I fragment of μ700 WT with that of μ700 π^- by using a nuclear extract derived from the pre-B-cell line HAFTL. Proteins bound to μE2, μE3, μE5, and μB were detected in both wild-type and mutant enhancers (Fig. 7A and B). The HAFTL extract gave rise to a strong footprint over the wild-type π site (Fig. 7A and C). In contrast, no footprint was seen over the functionally important 5′ half of the mutant π site (Fig. 7B and C). Interestingly, a footprint over the functionally inactive 3′ half of the mutant π site was

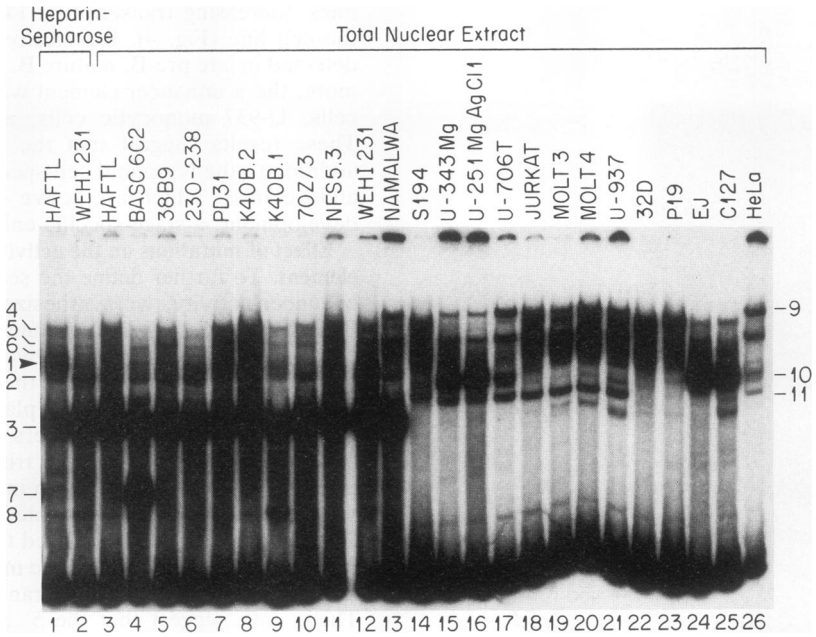


FIG. 9. A variety of nuclear factors interact with the IgH π site. Shown is an EMSA performed with synthetic oligonucleotides coding for the π site. Labeled π oligonucleotides were incubated with 5 μg of nuclear extract from HeLa (human cervical carcinoma), U-251 Mg Ag Cl1, U-343 Mg, and U-706T (human glioma), C127 (murine mammary), EJ (human bladder carcinoma), U-937 (human monocytic), 32D (murine early myeloid), P19 (murine teratocarcinoma), Jurkat, MOLT 3, and MOLT 4 (mature human T), NFS5.3 and 70Z/3 (murine late pre-B), WEHI 231 (murine mature B), HAFTL (murine early pre-B), 38B9, BASC 6C2, PD31, K40B.1, K40B.2, and 230-238 (murine pre-B), Namalwa (human mature B), and S194 (murine myeloma) cells as indicated above the lanes. The arrowhead indicates the putative pre-B-cell-specific factor NF-π; the numbers indicate the different DNA-protein complexes.

still present (Fig. 7B), suggesting the presence of two separate binding sites for two different factors in the π site (Fig. 7C). Similar results were obtained with the coding strand (data not shown). Apparent protein-DNA interactions over the π site, based on the results of these DNase I footprinting assays and EMSA (see Fig. 11 and 12) are shown in Fig. 7C. Our results confirm that mutation of the π site indeed decreases the affinity for a specific π-binding factor and also suggest the binding of at least two factors to different regions of the π site.

To determine whether a pre-B-cell-specific protein binds to the π site, we performed DNase I footprinting experiments with the noncoding (Fig. 8) and coding (data not shown) strands of the μ700 IgH enhancer. We compared nuclear extracts derived from several different cell types of both lymphoid and nonlymphoid origins. Proteins bound to μE1, μE2, μE3, and E were detected in extracts of most cell types (Fig. 8). A strong footprint over the μB DNA motif was seen in extracts from B and T cells, as well as myeloid cells, but was absent in extracts from nonhematopoietic glioma, fibroblastic, bladder carcinoma, and mammary cells (Fig. 8). Protein-DNA interactions over the π site appeared to be more complex. A footprint over the π DNA motif was detected in every nuclear extract (Fig. 8) that had a prominent hypersensitive site at the 5′ end of the π site. Extracts from B and T cells, as well as from myeloid cells, gave rise to a footprint that extended over the full π site, whereas a

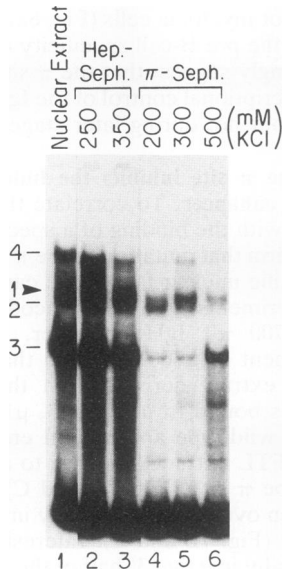


FIG. 10. DNA affinity purification of π-binding proteins. Shown is an EMSA performed with synthetic oligonucleotides coding for the π site. Labeled π oligonucleotides were incubated with equivalent amounts of total nuclear extract or heparin-Sepharose (Hep.-Seph.)-purified or π oligonucleotide-Sepharose (π-Seph.) affinity-purified nuclear proteins eluted at different KCl salt concentrations. A total nuclear extract from HAFTL cells was first purified over heparin-Sepharose, and the 350 mM KCl eluate was further purified over π oligonucleotide-Sepharose. The arrowhead indicates the pre-B-cell-specific factor NF-π; the numbers indicate the different DNA-protein complexes.

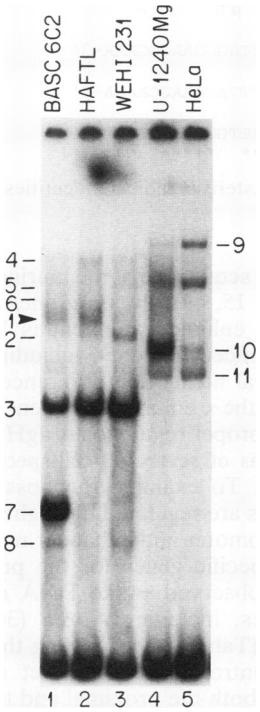


FIG. 11. A pre-B-cell-specific nuclear protein, NF-π, binds to the IgH π site. Shown is an EMSA performed with synthetic oligonucleotides coding for the π site. Labeled π oligonucleotides were incubated with 1 μg of heparin-Sepharose-purified nuclear extract from HeLa (human cervical carcinoma), U-1240 Mg (human glioma), BASC 6C2 (murine pre-B), WEHI 231 (murine mature B), and HAFTL (murine early pre-B) cells as indicated above the lanes. The arrowhead indicates the pre-B-cell-specific factor NF-π; the numbers indicate the different DNA-protein complexes.

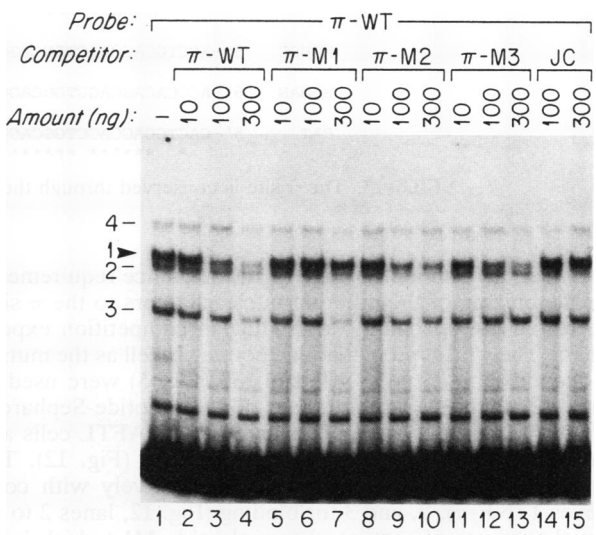


FIG. 12. Point mutation M1 but not M2 or M3 abolishes the binding of NF-π to the π site. Shown is an EMSA performed with synthetic oligonucleotides coding for the π site. Assays with affinity-purified nuclear proteins from HAFTL cells were carried out with no competitor (lane 1), with 10, 100, or 300 ng of the wild-type π oligonucleotide (lanes 2 to 4) or the mutant π oligonucleotide M1 (lanes 5 to 7), M2 (lanes 8 to 10), or M3 (lanes 11 to 13), or with a nonspecific JC virus enhancer oligonucleotide (lanes 14 and 15).

second hypersensitive site was induced in the middle of the π site by extracts from nonhematopoietic cells. However, no difference was revealed between footprints caused by extracts from B cells of different developmental stages, suggesting a more subtle difference between the π-binding factors in pre-B cells and mature B or myeloma cells.

B-cell- and pre-B-cell-specific proteins bind to the π enhancer element. To determine the tissue specificity of the π site and to characterize in more detail the proteins that interact specifically with the π enhancer element, we performed EMSA with a π oligonucleotide as a probe. We compared the abilities of nuclear extracts from a variety of different cell types to form complexes with the π DNA motif. The π oligonucleotide formed several complexes with nuclear proteins present in extracts from different cell types (Fig. 9). We could distinguish at least 11 different complexes, some of them showing a strikingly tissue-specific expression pattern. Complex 3 was observed only in B cells, whereas complex 10 was only formed by extracts from nonhematopoietic cells. Complex 7 was observed only in pre-B-cell line BASC 6C2. Interestingly, complex 3 was not present in nuclear extracts from S194 myeloma cells but was present in extracts from both pre-B and mature B cells. Complex 2 was also present in extracts from both pre-B and mature B cells. Complex 1 was very weak but appeared to be present mainly in pre-B cells and probably in T cells. Overall, the complexity of the electrophoretic mobility shift pattern did not enable us to determine which protein-DNA complex might be relevant for the activity of the π site and, like the DNase I footprinting experiments, these experiments suggested that nuclear extracts from a variety of different cell types contain proteins that have similar or identical binding specificities for the π site in vitro.

To characterize further the proteins interacting with the π enhancer element, we fractionated nuclear extracts over heparin-Sepharose (Fig. 10). We performed EMSA with heparin-Sepharose-purified nuclear proteins from pre-B-cell lines BASC 6C2 and HAFTL, mature B-cell line WEHI 231, glioma cell line U-1240 Mg, and cervical carcinoma cell line HeLa (Fig. 11). As shown in Fig. 11, complex 1 appeared to be present in extracts from the two pre-B-cell lines but not in extracts from mature B, glioma, or cervical carcinoma cells. We refer to complex 1 as NF-π. Complexes 2 and 3 were present in both pre-B and mature B cells but not in glioma or cervical carcinoma cells. Complexes 10 and 11 were formed only by proteins present in glioma and cervical carcinoma cells.

These results indicate that a variety of different tissue-restricted proteins interact with the π enhancer element. The data also suggest that NF-π may be a pre-B-cell-specific nuclear factor that correlates with the pre-B-cell-specific activity of the π enhancer element, although additional experiments will be needed to prove this suggestion.

Further purification of π-binding factors was achieved by affinity chromatography of a heparin-Sepharose-fractionated nuclear extract from the pre-B-cell line HAFTL over a π oligonucleotide-Sepharose column. Complexes 1 to 4 were the major proteins eluting from the π-Sepharose column (Fig. 10). Complexes 1 and 2 were the major components of the 200 and 300 mM KCl eluates.

Mutations affecting the activity of the π enhancer element inhibit the binding of pre-B-cell-specific nuclear factor NF-π. To test whether the presence of the pre-B-cell-specific factor NF-π (complex 1) correlates with the function of the π

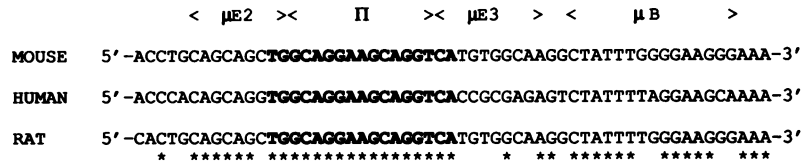


FIG. 13. The π site is conserved through the evolution of the IgH gene. Asterisks indicate identities.

enhancer element and to analyze the sequence requirements for the binding of the different nuclear factors to the π site, we tested the specificity of binding in competition experiments. The wild-type π oligonucleotide as well as the mutant π oligonucleotides M1, M2, and M3 (Fig. 5) were used as competitors in EMSA with the π oligonucleotide-Sepharose affinity-purified 200 mM KCl eluate from HAFTL cells and the wild-type π oligonucleotide as a probe (Fig. 12). The wild-type oligonucleotide competed effectively with complexes 1 (NF-π), 2, and 3 for binding (Fig. 12, lanes 2 to 4). Equal amounts of mutant oligonucleotide M1, which lacks enhancer activity, still competed efficiently with complexes 2 and 3 but were unable to inhibit the binding of NF-π (Fig. 12, lanes 5 to 7). Mutant oligonucleotide M2, with some enhancer activity, competed slightly with NF-π but less efficiently than the wild-type oligonucleotide (Fig. 12, lanes 8 to 10). Furthermore, mutant oligonucleotide M2 competed very efficiently with complex 2 but very little with complex 3. Mutant oligonucleotide M3, with full enhancer activity, competed effectively with NF-π, much less effectively with complex 2, and not at all with complex 3 (Fig. 12, lanes 11 to 13).

These data demonstrate that mutations of the π enhancer element that abrogate its activity (Fig. 5) also eliminate the binding of NF-π (Fig. 12) but have no effect or only a marginal effect on the formation of complexes 2 and 3. On the other hand, mutations that have no effect on π enhancer activity have no effect on the binding of NF-π but result in a significant reduction in the formation of complexes 2 and 3. These findings confirm that binding specificity and the presence of NF-π strictly correlate with the activity of the π enhancer element in pre-B cells. On the basis of these results, together with the DNase I footprinting results, we can conclude that NF-π binds to the 5′ part of the π site, to the minimal sequence GCAGGAAGC. Complexes 2 and 3 appear to interact with the 3′ part of the π site but have very little effect on the activity of the π enhancer element.

The π enhancer element is conserved through the evolution of the IgH gene. The importance of particular enhancer elements for the transcriptional regulation of a gene is often reflected in their conservation through evolution. When we compared the DNA sequences of the murine, human, and rat IgH enhancers (10, 15, 49), the only ones available, it was evident that the π enhancer element is 100% conserved, whereas other enhancer elements, including μB, μE2, and μE3, contain several nucleotide differences (Fig. 13). This result suggests that the π enhancer element may be of crucial importance for the proper regulation of IgH gene expression.

Regulatory regions of several B-cell-specific genes contain π-like DNA motifs. To examine the possibility that other B-cell-specific genes are regulated through π-like enhancers, we analyzed the promoter and enhancer regions of a variety of lymphoid cell-specific genes for the presence of π-like DNA motifs. We observed π-like DNA motifs in several B-cell-specific genes, including v-*preB* (36), λ5 (37), TdT (61), and B29 (27) (Table 1), suggesting that NF-π may be involved in the control of a whole set of B-cell-specific genes. In addition, both the proximal and the distal promoters of the T-cell-specific *lck* gene (19, 67) contain a highly homologous DNA motif that is 100% conserved. This fact suggests that a T-cell-specific NF-π-related protein may be involved in T-cell-specific gene expression. Purified NF-π protein will enable us to determine whether these related DNA motifs bind NF-π as well.

The π enhancer element shows a high degree of similarity to binding sites for members of the *ets* transcription factor-oncogene family. To determine whether previously described transcription factors interact with the π enhancer element, we compared the NF-π-binding site with DNA motifs recognized by known transcription factors. We observed a striking homology between the DNA-binding sites for members of the *ets* gene family (48, 53, 64, 70) and that for NF-π, especially in the core GGAA motif (Table 2). In particular, binding sites for PEA3 (71, 74) and Ets-1 and Ets-2 (23, 25, 29, 40, 53, 72) are remarkably similar to those for NF-π. Furthermore, the μB site of the IgH enhancer, which is located downstream of π, contains a similar GGAA motif, and we recently found that the Pu.1 transcription factor (35), a member of the *ets* family, binds specifically to the μB site and that its binding pattern correlates with the functional activity of this site (1). We are now in the process of evaluating the interaction of several members of the *ets*

TABLE 1. π-related DNA motifs

Gene	Species	DNA sequence	Location	Expression	Homology[a]	Reference(s)
IgH (π site)	Mouse, human, and rat	GGCAGGAAGCAGG	Enhancer	B cells	13	10, 15, 49
π-Related DNA motifs						
v-*preB*	Mouse	GGGAGGAAGCACC	Promoter	Pre-B cells	10	36
λ5	Mouse	GGGAGGAAGCATA	First intron	Pre-B cells	10	37
TdT	Mouse	AGCAGGAAGTTGT	Promoter	Pre-B cells	9	61
B29	Mouse	GGCAGGAAGGGGC	Promoter	B cells	10	27
lck	Mouse and human	GGCAGGAAGCTTG	Promoter 1	T cells	11	19, 67
lck	Mouse and human	GGCAGGAAGCTTG	Promoter 2	T cells	11	19, 67

[a] Number of homologous residues of a total of 13 residues.

TABLE 2. Transcription factors with π-related DNA-binding sites

Transcription factor	DNA-binding site[a]	Gene[b]	Reference(s)
NF-π1	5'-GGCAGGAAGCAGG-3'	IgH	10, 15, 49
NF-μB	5'-TATTGGGGAAGGG-3'	IgH (mouse)	15
	5'-TATTTAGGAAGCA-3'	IgH (human)	49
ets related			
ETS-1, ETS-2, ELK-1, ERG	5'-CAGGAAGTG-3'	Polyomavirus	40, 59, 60, 71, 74
ETS-1	5'-CAGAGGATGTG-3'	TCR-α	29
ETS-1, ETS-2	5'-AGAGCGGAAGCGCG-3'	MSV	25
GABP-α	5'-CGGAAA-3'	ICP4	69
SAP-1, ELK-1	5'-CACAGGATGTC-3'	c-*fos*	12, 28
Pu.1	5'-AAAGAGGAACTTGG-3'	MHCII I-Aβ	35
E74, ELK-1, SAP-1	5'-TAACCGGAAGTAAC-3'	E74	53, 59
Elf-1	5'-AGGAGGAAAAA-3'	IL-2	68
Elf-1	5'-GACAGGAACAG-3'	HIV-2	38
ETS-1, ETS-2	5'-GGAGGAAAT-3'	HTLV-I	23

[a] Boldfacing indicates consensus sequence.
[b] TCR-α, alpha T-cell receptor; MSV, murine sarcoma virus; MHCII I-Aβ, major histocompatibility class II antigen; IL-2, interleukin-2; HIV-2, human immunodeficiency virus type 2; HTLV-I, human T-cell lymphotropic virus type I.

family with the π site. So far, antibodies against Pu.1 and Ets-1 have failed to either prevent binding or to supershift NF-π in EMSA (data not shown). Furthermore, we recently isolated a new member of the *ets* transcription factor family from pre-B cells (47).

DISCUSSION

The IgH gene becomes rearranged during early stages of B-cell differentiation and remains transcriptionally active during all stages of B-cell differentiation (9, 63). We have identified a new enhancer element, designated π, in the IgH enhancer. We demonstrate that the ability to respond to the π element occurs primarily during early stages of B-cell development and is not evident in more mature B cells, even those entering the stage of Ig κ light-chain gene rearrangement. Mutational analysis suggests that the π element is crucial for IgH enhancer activity at the pre-B-cell stage but is irrelevant for enhancer activity at the mature B-cell or plasma-cell stage. The activity of the π enhancer element correlates with the presence of an apparently pre-B-cell-specific protein-DNA complex, NF-π, which might be related to the *ets* gene family (48, 54, 64). Because transcription of the unrearranged IgH gene occurs prior to rearrangement and appears to be essential for recombination (9, 62, 63), the π element might be a critical activator of IgH sterile transcription as well. A variety of other B-cell-specific genes contain DNA motifs similar to π; thus, NF-π might be an important regulatory factor in the control of B-cell-specific gene expression and B-cell differentiation.

The π site represents a novel pre-B-cell-specific regulatory element in the IgH enhancer. Its similarity to binding sites for *ets*-related transcription factors suggests that a member of the *ets* gene family might interact with the π site. Indeed, the IgH enhancer contains another *ets*-related enhancer element, μB (42, 44, 52), with the typical 5'-GGAA-3' sequence of *ets*-related binding sites (48, 53) occurring at its center. We (1) have shown that μB binds a B-cell- or macrophage-specific member of the *ets* family, Pu.1 (35). Pu.1 appears, however, not to interact with π (1). There are several other striking differences between the π site and the μB site, making it unlikely that the same factors interact with both sites. Whereas μB is active throughout B-cell development (44, 52), π appears to act primarily at the pre-B-cell stage. Furthermore, an isolated π site, but not an isolated μB site, functions as an autonomous enhancer. In addition, gel mobility shift assays for π and μB have revealed different protein-DNA complexes and a distinct cell type distribution (1). Therefore, we can assume that the IgH enhancer contains at least two distinct *ets*-related enhancer elements with strikingly different activities. This feature is very similar to those of a whole set of T-cell-specific genes, including the interleukin-2, alpha and beta T-cell receptor, CD2, and CD3 genes, all of which have at least two critical *ets*-related enhancer elements (29, 68, 70). Several other B-cell-specific genes, including the Ig κ, TdT, and mb-1 genes, apparently also contain *ets*-related binding sites in their regulatory regions (16, 26, 46, 58). We and others have shown elsewhere that μB is important for the B-cell-specific activity of the IgH enhancer (44, 52), and we now show the crucial role of the π site for the activity of the IgH enhancer at the pre-B-cell stage. Therefore, it is likely that *ets*-related transcription factors play similar crucial roles in B-cell-specific gene regulation and potentially B-cell differentiation, as has been proposed for T cells.

We do not know the identity of NF-π yet. However, in an attempt to isolate members of the *ets* gene family that are expressed in pre-B cells, we recently cloned and characterized the gene for a novel *ets*-related transcription factor, ERP (for Ets-related protein) (47). The expression of the ERP factor in the B-cell lineage appears to be elevated at the pre-B-cell stage and to decline upon B-cell maturation. We are now in the process of determining whether ERP is able to interact with the π site.

Several other *ets*-related enhancer elements, including LyF-1 and BLyF, have been identified for other B-cell-specific genes with binding sites similar to π (16, 46). On the basis of the activities and expression patterns of LyF-1 and BLyF, NF-π seems to be distinct from these factors.

We have noted the presence of π-related DNA sequences in at least three pre-B-cell-specific genes, the TdT (61), v-*preB* (36), and λ5 (33) genes. We have no evidence as to their function or whether they interact with NF-π. Nevertheless, the presence of potential π sites in other genes expressed at the pre-B-cell stage enhances the possibility that NF-π plays an important role in early B-cell gene regulation and B-cell differentiation.

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REFERENCES

1. **Akbarali, Y., P. Oettgen, L. Binder, and T. A. Libermann.** Unpublished data.
2. **Alt, F. W., M. G. Reth, T. K. Blackwell, and G. D. Yancopoulos.** 1986. Regulation of immunoglobulin variable-region gene assembly. Mt. Sinai J. Med. **53**:166–169.
3. **Alt, F. W., N. Rosenberg, R. J. Casanova, E. Thomas, and D. Baltimore.** 1982. Immunoglobulin heavy-chain expression and class switching in a murine leukaemia cell line. Nature (London) **296**:325–331.
4. **Araki, K., H. Maeda, J. Wang, D. Kitamura, and T. Watanabe.** 1988. Purification of a nuclear trans-acting factor involved in the regulated transcription of a human immunoglobulin heavy chain gene. Cell **53**:723–730.
5. **Augereau, P., and P. Chambon.** 1986. The mouse immunoglobulin heavy-chain enhancer: effect on transcription in vitro and binding of proteins present in HeLa and lymphoid B cell extracts. EMBO J. **5**:1791–1797.
6. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53**:211–217.
7. **Baeuerle, P. A., and D. Baltimore.** 1988. IκB: a specific inhibitor of the NF-κB transcription factor. Science **242**:540–546.
8. **Banerji, J., L. Olson, and W. Schaffner.** 1983. A lymphocyte-specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. Cell **33**:729–740.
9. **Blackwell, T. K., M. W. Moore, G. D. Yancopoulos, H. Suh, S. Lutzker, E. Selsing, and F. W. Alt.** 1986. Recombination between immunoglobulin variable region gene segments is enhanced by transcription. Nature (London) **324**:585–589.
10. **Brueggemann, M., J. Free, A. Diamond, J. Howard, S. Cobbold, and H. Waldmann.** 1986. Immunoglobulin heavy chain locus of the rat: striking homology to mouse antibody genes. Proc. Natl. Acad. Sci. USA **83**:6075–6079.
11. **Calame, K. L.** 1989. Immunoglobulin gene transcription: molecular mechanisms. Trends Genet. **5**:395–399.
12. **Dalton, S., and R. Treisman.** 1992. Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. Cell **68**:597–612.
13. **Dariavach, P., G. T. Williams, K. Campbell, S. Pettersson, and M. S. Neuberger.** 1991. The mouse IgH 3′-enhancer. Eur. J. Immunol. **21**:1499–1504.
14. **Dignam, J. P., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
15. **Ephrussi, A., G. M. Church, S. Tonegawa, and W. Gilbert.** 1985. B lineage-specific interactions of an immunoglobulin enhancer with cellular factors in vivo. Science **227**:134–138.
16. **Feldhaus, A. L., D. Mbangkollo, K. L. Arvin, C. A. Klug, and H. Singh.** 1992. BLyF, a novel cell-type- and stage-specific regulator of the B-lymphocyte gene *mb-1*. Mol. Cell. Biol. **12**:1126–1133.
17. **Ferrier, P., B. Krippl, T. K. Blackwell, A. J. Furley, H. Suh, A. Winoto, W. D. Cook, L. Hood, F. Costantini, and F. W. Alt.** 1990. Separate elements control DJ and VDJ rearrangement in a transgenic recombination substrate. EMBO J. **9**:117–125.
18. **Frisque, R. J., G. L. Bream, and M. T. Cannella.** 1984. Human polyomavirus JC virus genome. J. Virol. **51**:458–469.
19. **Garvin, A. M., S. Pawar, J. D. Marth, and R. M. Perlmutter.** 1988. Structure of the murine *lck* gene and its rearrangement in a murine lymphoma cell line. Mol. Cell. Biol. **8**:3058–3064.
20. **Gerster, T., P. Matthias, M. Thali, J. Jiricny, and W. Schaffner.** 1987. Cell type-specificity elements of the immunoglobulin heavy chain gene enhancer. EMBO J. **6**:1323–1330.
21. **Gillies, S. D., S. L. Morrison, V. T. Oi, and S. Tonegawa.** 1983. A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. Cell **33**:717–728.
22. **Gilman, M. Z., R. N. Wilson, and R. A. Weinberg.** 1986. Multiple protein-binding sites in the 5′-flanking region regulate c-*fos* expression. Mol. Cell. Biol. **6**:4305–4316.
23. **Gitlin, S. D., R. Bosselut, A. Gegonne, J. Ghysdael, and J. N. Brady.** 1991. Sequence-specific interaction of the Ets1 protein with the long terminal repeat of the human T-lymphotropic virus type I. J. Virol. **65**:5513–5523.
24. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
25. **Gunther, C. V., J. A. Nye, R. S. Bryner, and B. J. Graves.** 1990. Sequence-specific DNA binding of the proto-oncoprotein ets-1 defines a transcriptional activator sequence within the long terminal repeat of the Moloney murine sarcoma virus. Genes Dev. **4**:667–679.
26. **Hagman, J., and R. Grosschedl.** 1992. An inhibitory carboxyl-terminal domain in Ets-1 and Ets-2 mediates differential binding of ETS family factors to promoter sequences of the mb-1 gene. Proc. Natl. Acad. Sci. USA **89**:8889–8893.
27. **Hermanson, G. G., M. Briskin, D. Sigman, and R. Wall.** 1989. Immunoglobulin enhancer and promoter motifs 5′ of the B29 B-cell-specific gene. Proc. Natl. Acad. Sci. USA **86**:7341–7345.
28. **Hipskind, R. A., V. N. Rao, C. G. Mueller, E. S. Reddy, and A. Nordheim.** 1991. Ets-related protein Elk-1 is homologous to the c-fos regulatory factor p62TCF. Nature (London) **354**:531–534.
29. **Ho, I. C., N. K. Bhat, L. R. Gottschalk, T. Lindsten, C. B. Thompson, T. S. Papas, and J. M. Leiden.** 1990. Sequence-specific binding of human Ets-1 to the T cell receptor α gene enhancer. Science **250**:814–818.
30. **Holmes, K. L., J. H. Pierce, W. F. Davidson, and H. R. Morse.** 1986. Murine hematopoietic cells with pre-B or pre-B/myeloid characteristics are generated by in vitro transformation with retroviruses containing fes, ras, abl, and src oncogenes. J. Exp. Med. **164**:443–457.
31. **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.
32. **Jones, K. A., K. R. Yamamoto, and R. Tjian.** 1985. Two distinct transcription factors bind to the HSV thymidine kinase promoter in vitro. Cell **42**:559–572.
33. **Kadesch, T.** 1992. Helix-loop-helix proteins in the regulation of immunoglobulin gene transcription. Immunol. Today **13**:31–36.
34. **Kadonaga, J. R., and R. Tjian.** 1986. Affinity purification of sequence-specific DNA binding proteins. Proc. Natl. Acad. Sci. USA **83**:5889–5893.
35. **Klemsz, M. J., S. R. McKercher, A. Celada, B. C. Van, and R. A. Maki.** 1990. The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. Cell **61**:113–124.
36. **Kudo, A., and F. Melchers.** 1987. A second gene, VpreB in the λ5 locus of the mouse, which appears to be selectively expressed in pre-B lymphocytes. EMBO J. **6**:2267–2272.
37. **Kudo, A., N. Sakaguchi, and F. Melchers.** 1987. Organization of the murine Ig-related λ5 gene transcribed selectively in pre-B lymphocytes. EMBO J. **6**:103–107.
38. **Leiden, J. M., C. Y. Wang, B. Petryniak, D. M. Markovitz, G. J. Nabel, and C. B. Thompson.** 1992. A novel Ets-related transcription factor, Elf-1, binds to human immunodeficiency virus type 2 regulatory elements that are required for inducible *trans*-activation in T cells. J. Virol. **66**:5890–5897.
39. **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.
40. **Leprince, D., P. Crepieux, and D. Stehelin.** 1992. c-ets-1 DNA binding to the PEA3 motif is differentially inhibited by all the mutations found in v-ets. Oncogene **7**:9–17.
41. **Lewis, S., N. Rosenberg, F. Alt, and D. Baltimore.** 1982. Continuing κ-gene rearrangement in a cell line transformed by Abelson murine leukemia virus. Cell **30**:807–816.
42. **Libermann, T. A., and D. Baltimore.** 1990. Transcriptional

regulation of immunoglobulin gene expression. Mol. Aspects Cell Regul. **6**:399–421.

43. **Libermann, T. A., M. Jaye, R. M. Lyall, B. Westermark, W. Drohan, A. Schmidt, T. Maciag, and J. Schlessinger.** 1987. An angiogenic growth factor is expressed in human glioma cells. EMBO J. **6**:1627–1632.
44. **Libermann, T. A., M. Lenardo, and D. Baltimore.** 1990. Involvement of a second lymphoid-specific enhancer element in the regulation of immunoglobulin heavy-chain gene expression. Mol. Cell. Biol. **10**:3155–3162.
45. **Lieberson, R., S. L. Giannini, B. K. Birshtein, and L. A. Eckhardt.** 1991. An enhancer at the 3′ end of the mouse immunoglobulin heavy chain locus. Nucleic Acids Res. **19**:933–937.
46. **Lo, K., N. R. Landau, and S. T. Smale.** 1991. LyF-1, a transcriptional regulator that interacts with a novel class of promoters for lymphocyte-specific genes. Mol. Cell. Biol. **11**: 5229–5243.
47. **Lopez, M., P. Oettgen, U. Dendorfer, and T. A. Libermann.** Submitted for publication.
48. **Macleod, K., D. Leprince, and D. Stehelin.** 1992. The ets gene family. Trends Biochem. Sci. **17**:251–256.
49. **Maeda, H., K. Araki, D. Kitamura, J. Wang, and T. Watanabe.** 1987. Nuclear factors binding to the human immunoglobulin heavy-chain gene enhancer. Nucleic Acids Res. **15**:2851–2869.
50. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
51. **McKearn, J. P., and N. Rosenberg.** 1985. Mapping cell surface antigens on mouse pre-B cell lines. Eur. J. Immunol. **15**:295–298.
52. **Nelsen, B., T. Kadesch, and R. Sen.** 1990. Complex regulation of the immunoglobulin μ heavy-chain gene enhancer: μB, a new determinant of enhancer function. Mol. Cell. Biol. **10**:3145–3154.
53. **Nye, J. A., J. M. Petersen, C. V. Gunther, M. D. Jonsen, and B. J. Graves.** 1992. Interaction of murine ets-1 with GGA-binding sites establishes the ETS domain as a new DNA-binding motif. Genes Dev. **6**:975–990.
54. **Papas, T. S.** 1992. Oncogenes as probes for cellular signal processes: the family of ETS genes. AIDS Res. Hum. Retroviruses **8**:824–833.
55. **Peterson, C. L., and K. Calame.** 1989. Proteins binding to site C2 (μE3) in the immunoglobulin heavy-chain enhancer exist in multiple oligomeric forms. Mol. Cell. Biol. **9**:776–786.
56. **Pierce, J. H., and S. A. Aaronson.** 1985. Myeloid cell transformation by *ras*-containing murine sarcoma viruses. Mol. Cell. Biol. **5**:667–674.
57. **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.
58. **Pongubala, J. M., S. Nagulapalli, M. J. Klemsz, S. R. McKercher, R. A. Maki, and M. L. Atchison.** 1992. PU.1 recruits a second nuclear factor to a site important for immunoglobulin κ 3′ enhancer activity. Mol. Cell. Biol. **12**:368–378.
59. **Rao, V. N., and E. S. Reddy.** 1992. A divergent ets-related protein, elk-1, recognizes similar c-ets-1 proto-oncogene target sequences and acts as a transcriptional activator. Oncogene **7**:65–70.
60. **Reddy, E. S., and V. N. Rao.** 1991. erg, an ets-related gene, codes for sequence-specific transcriptional activators. Oncogene **6**:2285–2289.
61. **Riley, L. K., J. K. Morrow, M. J. Danton, and M. S. Coleman.** 1988. Human terminal deoxyribonucleotidyltransferase: molecular cloning and structural analysis of the gene and 5′ flanking region. Proc. Natl. Acad. Sci. USA **85**:2489–2493.
62. **Schlissel, M. S., and D. Baltimore.** 1989. Activation of immunoglobulin κ gene rearrangement correlates with induction of germline κ gene transcription. Cell **58**:1001–1007.
63. **Schlissel, M. S., L. M. Corcoran, and D. Baltimore.** 1991. Virus-transformed pre-B cells show ordered activation but not inactivation of immunoglobulin gene rearrangement and transcription. J. Exp. Med. **173**:711–720.
64. **Seth, A., R. Ascione, R. J. Fisher, G. J. Mavrothalassitis, N. K. Bhat, and T. S. Papas.** 1992. The ets gene family. Cell Growth Differ. **3**:327–334.
65. **Singh, H., R. Sen, D. Baltimore, and P. A. Sharp.** 1986. A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. Nature (London) **319**:154–158.
66. **Stewart, G. S. A. B., S. Dovey, and D. M. O'Rourke.** 1988. A rapid method for site directed mutagenesis of plasmid DNA. BioTechniques **6**:511–518.
67. **Takadera, T., S. Leung, A. Gernone, Y. Koga, Y. Takihara, N. G. Migamoto, and T. W. Mak.** 1989. Structure of the two promoters of the human *lck* gene: differential accumulation of two classes of *lck* transcripts in T cells. Mol. Cell. Biol. **9**:2173–2180.
68. **Thompson, C. B., C.-Y. Wang, I.-C. Ho, P. R. Bohjanen, B. Petryniak, C. H. June, S. Miesfeldt, L. Zhang, G. J. Nabel, B. Karpinski, and J. M. Leiden.** 1992. *cis*-Acting sequences required for inducible interleukin-2 enhancer function bind a novel Ets-related protein, Elf-1. Mol. Cell. Biol. **12**:1043–1053.
69. **Thompson, C. C., T. A. Brown, and S. L. McKnight.** 1991. Convergence of Ets- and notch-related structural motifs in a heteromeric DNA binding complex. Science **253**:762–768.
70. **Wang, C. Y., B. Petryniak, I. C. Ho, C. B. Thompson, and J. M. Leiden.** 1992. Evolutionarily conserved Ets family members display distinct DNA binding specificities. J. Exp. Med. **175**: 1391–1399.
71. **Wasylyk, C., P. Flores, A. Gutman, and B. Wasylyk.** 1989. PEA3 is a nuclear target for transcription activation by non-nuclear oncogenes. EMBO J. **8**:3371–3378.
72. **Wasylyk, C., A. Gutman, R. Nicholson, and B. Wasylyk.** 1991. The c-Ets oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins. EMBO J. **10**:1127–1134.
73. **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.
74. **Xin, J. H., A. Cowie, P. Lachance, and J. A. Hassell.** 1992. Molecular cloning and characterization of PEA3, a new member of the Ets oncogene family that is differentially expressed in mouse embryonic cells. Genes Dev. **6**:481–496.