Identification of an Sp1-like Element within the Immunoglobulin κ 3′ Enhancer Necessary for Maximal Enhancer Activity†

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ABSTRACT: A number of functional DNA sequences have been identified within the murine immunoglobulin κ 3′ enhancer (κE3′). These DNA sequences were identified using plasmid reporter constructs in which the centrally active core region (or mutants of that region) of the enhancer was placed directly adjacent to the promoter of a reporter construct. Functional DNA sequences thus identified were found to bind to the transcription factors PU.1, NF-EM5, E2A, ATF-1, or CREM. In the studies reported here, we show that additional enhancer sequences that lie outside of the core region are necessary for maximal enhancer activity when the core region is not directly adjacent to the promoter. A series of progressive and internal deletion constructs shows that enhancer sequences between nucleotides 275 and 329 are important for enhancer activity. Progressive deletion to nucleotide position 329 resulted in a 4-fold reduction in enhancer activity. Using electrophoretic mobility shift assays, we show that this segment of the enhancer binds to ubiquitously expressed nuclear factors. Dimethyl sulfate methylation interference assays indicated protein-DNA interactions within a G-rich sequence between positions 302 and 306 and an A-rich sequence between positions 319 and 329. Ultraviolet light protein−DNA cross-linking studies revealed nuclear factors of approximately 85 and 105 kDa that bind to the newly identified enhancer region. Oligonucleotide competition studies and binding studies with purified Sp1 or Sp1 antibodies indicate that Sp1 can bind to this sequence. These studies show that functional sequences within the κE3′ enhancer include an Sp1-like site approximately 90 bp 5′ of the central 132 bp region originally believed to account for most of the enhancer activity.

Immunoglobulin κ (Igκ) gene expression is controlled by the activity of two enhancers. The initially identified enhancer (Eκ) lies within the intron that separates the joining from constant region exons (Queen & Baltimore, 1983; Queen & Stafford, 1984; Picard & Schaffner, 1984). This enhancer is developmentally controlled, being silent at the pre-B cell stage, but active at the B cell and plasma cell stages. The activity of this enhancer is controlled to a large extent by the availability of the active form of the transcription factor NF-κB (Atchison & Perry, 1987; Lenardo et al., 1987; Sen & Baltimore, 1986a,b). The second Igκ enhancer lies 8.5 kb downstream of the constant region exon (Meyer & Neuberger, 1989) and is referred to as the 3′ enhancer (κE3′). This enhancer is similar to the intron enhancer in its developmental regulation. That is, both enhancers are active only in B cells and plasma cells (Picard & Schaffner, 1984; Meyer & Neuberger, 1989; Stafford & Queen, 1983). However, the 3′ enhancer does not bind to NF-κB and shows activity in plasmacytoma cells that are deficient in NF-κB (Hagman et al., 1990; Meyer et al., 1990; Pongubala & Atchison, 1991). Therefore, the mechanism of control of the κE3′ enhancer is distinct from that of the intron enhancer.

Several reports have characterized DNA sequences important for activity of the murine κE3′ enhancer. Meyer and Neuberger (1989) originally narrowed the 3′ enhancer region to a 1.1 kb DNA fragment. Subsequently, Meyer et al. (1990) identified a 145 bp region of the enhancer that possessed essentially full activity. Several internal deletions within this region significantly lowered enhancer activity. Our laboratory has also characterized sequences important for activity of the κE3′ enhancer. These studies identified a central core region of 132 bp that exhibits high enhancer activity (Pongubala & Atchison, 1991). Within this central core, we have identified positive-acting DNA sequences that bind to the transcription factors PU.1, NF-EM5 (PIP), E2A, ATF-1, and CREM (Pongubala et al., 1992, 1993; Pongubala & Atchison, 1991, 1995). A study of sequences within the human 3′ enhancer revealed similar functional segments (Judde & Max, 1992).

Nearly all of the studies characterizing sequences important for murine κE3′ enhancer activity have utilized plasmid constructs in which enhancer DNA segments were placed immediately upstream of either the β-globin promoter or the herpes thymidine kinase promoter. These studies have been extremely useful for identifying positive-acting sequences within the enhancer. However, these studies may not have detected DNA elements necessary for maximal activity when the core segment of the enhancer is positioned more distal to the promoter.

In the studies reported here, we show that a DNA sequence located approximately 90 bp 5′ of the enhancer core is necessary for maximal enhancer activity when the enhancer

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Table 1

pUC reverse	CAGGAAACAGCTATGAC
TK reverse	TTCGAATTCGCAATGA
145−162	GCGAAGCTTGCAGTAAGGATCTAAGT
195−211	GCGAAGCTTCTGCAACAACAGAGAGT
246−262	GCGAAGCTTACATCTTCTACCATTTC
275−295	GCGAAGCTTAGTACCCACCCATATCTCCCC
301−317	GAGAAGCTTTCCCCCATACCAGACTG
312−330	GCGAAGCTTAGACTGGTTATTGATTTTC
LS329−345	CGGGTCGACTCATGGTGACTGGCCTG
LS302−281	CGGGTCGACATGGGTGGGGAGATATG
LS387−392	GCGGTCGACCCAAGATAGCAACTGTC
LS380−375	GCGGTCGACTAAGGTAGCATTACTTT
275−300	AATTCAGTACCCAC CCATATC TCC CCACCC AG GTCATGGGTGGGTATAGAGGGGTGGGTCTTAA
290−309	AATTCCTCC CCACCC ATCCC CCATAG GGAGGGGTGGGTAGGGGGTATCTTAA
301−329	GATCCTCC CCCATACCAGACTGGTTATTGATT TTA GAGGGGGTATGGTCTGACCAATAACTAAAATCTAG
307−329	AATTCATACCAGACTGGTTATTGATT TTG GTATGGTCTGACCAATAACTAAAACTTAA
361−393	GATCCGTAATGCTA CCTTATTGGGAGTGTCCCATGGACA GCATTACGATGGAATAACCCTCACAGGGTACCTGTCTAG
Sp1	ATTCGATCGGGGCGGGGCGAG TAAGCTAGCCCC GCCCCGCTC

core is not directly adjacent to the promoter. This segment of the enhancer binds to a nuclear factor either very similar to or identical to the transcription factor Sp1.

MATERIALS AND METHODS

Plasmid Constructions. Enhancer DNA fragments were cloned upstream of the TKCAT vector containing sequences −109 to +51 of the herpes virus thymidine kinase promoter driving expression of the bacterial chloramphenicol acetyltransferase gene. Enhancer sequences 80−808 were isolated as a *Bgl*II-*Xba*I DNA fragment and cloned by blunt end ligation in both orientations into the blunted *Bam*HI site of TKCAT. Enhancer sequences 80−520 were isolated as a *Bgl*II-*Ava*II DNA fragment and blunt end cloned into the *Hin*cII site of pUC18. This fragment was isolated by digestion with *Bam*HI and *Hin*dIII and blunt end ligated into the blunted *Sal*I site of TKCAT. Plasmids 145−808, 195−808, 246−808, 312−808, and 301−808 were prepared by polymerase chain reaction (PCR) using the 80−808 plasmid as a template with appropriate oligonucleotides (see below) containing a 5′ *Hin*dIII site and the pUC reverse primer. PCR products were extracted with chloroform, precipitated with ethanol, digested with *Bam*HI and *Hin*dIII, and then cloned into *Bam*HI-*Hin*dIII-cut TKCAT. Plasmids 329−808, 347−808, and 362−808 were prepared by Bal31 progressive deletion as previously described (Park & Atchison, 1991). Internal enhancer deletions were prepared by PCR amplification of the 80−808 template plasmid with divergent oligonucleotide primers (see below) containing a 5′ *Sal*I site and either the pUC forward primer or the TK reverse primer. After PCR amplification, 5′ and 3′ DNA fragments were cut with either *Sal*I-*Bam*HI or *Sal*I-*Hin*dIII and cloned into *Hin*dIII-*Bam*HI cut TKCAT. Plasmids containing 5′ deletions in the context of the 301−329 deletion were prepared by PCR with the appropriate oligonucleotide primers using the Δ301−329 plasmid as a template. Plasmid 520−390TKCAT was previously described (Pongubala & Atchison, 1991). Plasmids with pUC18 DNA insertions were prepared by placing a 322 bp *Pvu*II pUC18 DNA fragment by blunt end ligation into the blunted *Bam*HI site of the appropriate plasmids. The integrity of all clones was confirmed by dideoxynucleotide DNA sequence analysis. The oligonucleotides used in this study are listed in Table 1.

Cell Culture and Transfection. S194 plasmacytoma cells were grown in Dulbecco's Modified Eagle Medium containing 10% horse serum (GIBCO) to a density of 5×10^5 cells/mL. Cells were transfected by the DEAE-dextran (Pharmacia) procedure according to Grosschedl and Baltimore (1985) except that chloroquine treatment was omitted. Each transfection included 4 μg of the reporter plasmid and 1 μg of β-galactosidase expression plasmid pCH110 (Hall et al., 1983) to normalize transfection efficiencies. Cells were fed 20 h later and were harvested 40−44 h after transfection. Cell extracts were prepared by freeze−thaw cycles, and chloramphenicol acetyltransferase assays and thin-layer chromatography were performed according to Gorman et al. (1982).

Electrophoretic Mobility Shift Assays (EMSAs). Nuclear extracts were prepared by the method of Dignam et al. (1983). EMSA reactions contained 4−8 μg of nuclear extract protein, 5000 cpm of ^{32}P-labeled DNA fragment or oligonucleotide probe, 3.2 μg of poly(dI/dC)·poly(dI/dC), 10 mM Tris-HCl, 50 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, and 5% glycerol at pH 7.4 in a 20 μL reaction mixture. Incubation was for 30 min at room temperature. Reaction mixtures with Sp1 (Promega) contained 0.1, 0.5, or 1.0 footprint units (fpu) of purified Sp1. One fpu is defined as the amount of Sp1 needed to give full protection against DNase 1 digestion on the SV40 early promoter (Promega). Oligonucleotide competitors were added in the amounts indicated in the figures. Antibodies (Sp1 and CREM; Santa Cruz Biotechnology Inc.) were added 15 min prior to addition of the labeled DNA probe. Either 1 or 2 μL of antibody (0.1−0.2 μg) was added to supershift reaction mixtures. The Sp1 antibody was raised against Sp1 sequences 436−454 and was affinity purified (Santa Cruz). Reactions were resolved on 4% nondenaturing polyacrylamide gels (6.7 mM Tris-HCl, 3.3 mM sodium acetate, and 1 mM EDTA at pH 7.5) prerun for 30 min at room

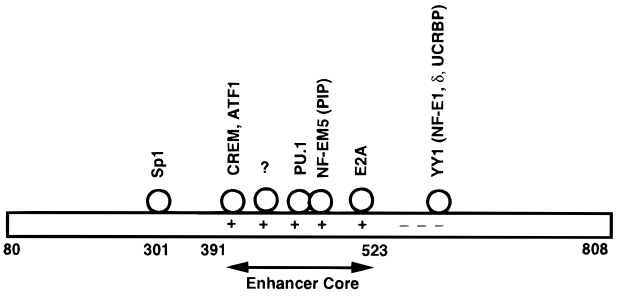

FIGURE 1: Summary of the κE3′ enhancer. The sequence from 80 to 808 of the κE3′ enhancer is represented as a rectangle. The relative positions of positive- and negative-acting DNA sequences within the enhancer are indicated by positive or negative signs, respectively. The proteins that bind to these sequences are shown above each sequence element. The relative position of the enhancer core is shown by the double-headed arrow below the enhancer. The Sp1 site was identified by the studies presented here.

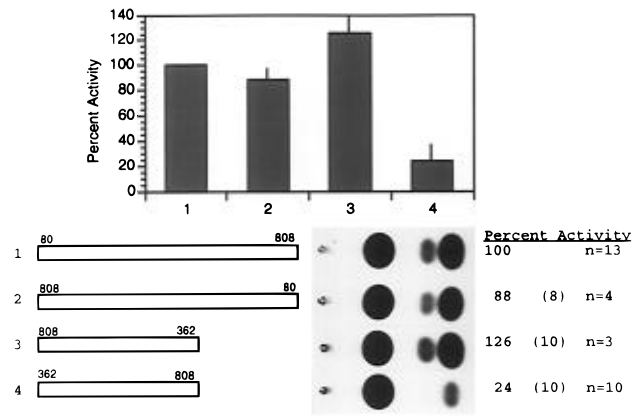

FIGURE 2: Deletion of the sequence from 80 to 361 reduces enhancer activity when the core element is distal to the promoter. The DNA sequences present in each construct are shown on the left with the CAT data shown in the middle panel. Transfections were performed in S194 plasmacytoma cells. Percent CAT activity is shown on the right with the number in parentheses indicating the standard deviation. *n* values indicate the number of times each transfection was performed. The top panel shows the data in histogram form with standard deviations represented by thin vertical lines.

temperature. Oligonucleotide probes were labeled by a fill-in reaction with Klenow polymerase in the presence of [^{32}P]αdATP.

DMS Methylation Interference Assays. The oligonucleotide containing sequence 301−329 was cloned into the *Bam*HI site of pUC18. This plasmid was linearized with either *Bam*HI or *Hin*dIII, treated with calf intestine alkaline phosphatase (Boehringer), end labeled with [^{32}P]γATP by polynucleotide kinase (NEB), and digested with the second enzyme to release the oligonucleotide insert. Labeled DNA was purified by polyacrylamide gel electrophoresis, and dimethyl sulfate methylation interference assays were performed according to Atchison et al. (1990).

UV Cross-Linking. The upper strand of oligonucleotide 301−329 was annealed at its 3′ end with a complementary 10 bp oligonucleotide. The DNA was made double-stranded with Klenow polymerase in the presence of BUdr, [^{32}P]-αdATP, 2 mM dCTP, 2 mM dGTP, and 1 mM dTTP. Binding reactions were performed as described above using the BUdr-substituted probe. After polyacrylamide gel electrophoresis, the gel was irradiated for 15 min with UV light (254 nm). Protein−DNA complexes were cut out of the gel, electroeluted, precipitated with 4 volumes of acetone, and then resolved on an SDS−polyacrylamide gel.

RESULTS

5′ Sequences within the Enhancer Are Required for Maximal Activity. Previously, we characterized sequences within the immunoglobulin κ 3′ enhancer important for positive or negative transcriptional regulation (Pongubala & Atchison, 1991; Park & Atchison, 1991). These studies showed that a centrally located 132 bp core region of the enhancer [sequence 391−523 according to Meyer and Neuberger (1989)] is responsible for most of the enhancer activity observed in plasmacytoma cells, whereas sequences flanking the 3′ side of this core region are necessary for repression of enhancer activity in pre-B cells. A diagram of the κE3′ enhancer and its binding proteins is shown in Figure 1. Our early studies that demonstrated the importance of the central 132 bp core segment used a variety of plasmid constructs that generally contained the enhancer sequences in only one orientation (Pongubala & Atchison, 1991). In the present study, we have assayed enhancer constructs in both orientations and have identified a segment of the enhancer necessary for maximal function when the core region of the enhancer is moved to a position distal to the promoter.

Enhancer constructs were prepared that contain various segments of the κE3′ enhancer upstream of the thymidine kinase promoter driving expression of the chloramphenicol acetyltransferase gene (TKCAT). Consistent with our previous work (Pongubala & Atchison, 1991, 1995; Pongubala et al., 1992), the TKCAT reporter was inactive in S194 plasmacytoma cells (data not shown). However, the entire κE3′ enhancer which spans nucleotide sequences 80−808 stimulated expression over 30-fold and was found to be equally active when assayed in both orientations (Figure 2, lanes 1 and 2). An enhancer construct that contains sequences 362−808 was equally active when the core region (sequences 391−523) was positioned proximal to the promoter (lane 3). However, when the same sequences were assayed in the opposite orientation, activity was greatly reduced (lane 4). This was surprising because activity of the intact enhancer (sequences 80−808) was unaffected by altering its orientation. The construct in lane 4 lacks sequence 80−361, suggesting that an element within this region may be necessary for maximal activity when the enhancer core is distal to the promoter. In this construct, the core region is positioned approximately 300 bp further upstream from the promoter.

If a positive-acting element resides between nucleotides 80 and 362, one would expect that a construct containing sequence 80−523 would be more active than one containing sequence 391−523 when the core segment is positioned distal to the promoter. Plasmids containing sequence 391−523 or 80−523 showed activity comparable to that of the intact enhancer when the core region was adjacent to the promoter (Figure 3, lanes 1−3). However, when a 300 bp DNA fragment derived from pUC18 was inserted between the promoter and the core sequences, construct 80−523 showed 6−7-fold higher activity than the construct containing only sequence 391−523 (lanes 4 and 5). Therefore, sequences between 80 and 391 contribute to enhancer activity when the core region is placed distal to the promoter. It

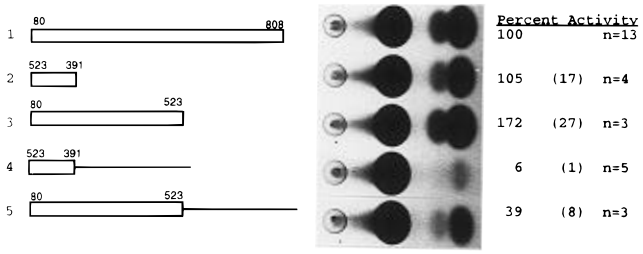

FIGURE 3: Reduced activity of the enhancer core when positioned distal to the promoter. Enhancer constructs are shown on the left with enhancer DNA sequences represented by open rectangles and pUC18 DNA sequences by thin lines. CAT data from transfections in S194 plasmacytoma cells are shown in the middle panel. Percent CAT activities of each construct are shown in the right panel. Numbers in parentheses indicate the standard deviations, and *n* values indicate the number of times each transfection was performed.

should also be noted that sequences between 523 and 808 must also contribute to enhancer activity (compare lanes 1 and 5). However, the function of these sequences was not explored further.

Identification of Sequences between Nucleotides 80 and 391 Important for Enhancer Activity. Our results indicated the presence of functional enhancer sequences between positions 80 and 391. Functional assays were therefore performed to more precisely identify the important enhancer sequences. Progressive enhancer deletions were prepared, linked to TKCAT, and assayed for enhancer activity in S194 plasmacytoma cells (Figure 4A,B). Each deletion construct contained nucleotide position 808 adjacent to the promoter in order to maintain the enhancer core at the same relative position. Enhancer activity of each deletion construct was compared to the activity of the intact enhancer defined as 100%.

Deletion to nucleotide positions 145, 195, 246, or 301 had little deleterious effect on enhancer activity (Figure 4A,B, lanes 1−6). However, deletion of an additional 11 nucleotides to position 312 resulted in a lowering of enhancer activity to a level of about 40% of that of the intact enhancer (lane 7). Further deletion to position 329 resulted in only about 25% activity (lane 8). Similar levels of enhancer activity were observed with constructs deleted to positions 347 or 362 (lanes 9 and 10). Therefore, a positive enhancer element clearly resides within the region between nucleotides 301 and 329.

To determine whether sequences 301−329 were completely responsible for the loss in enhancer activity, these sequences were deleted in the context of the entire enhancer. While the deletion of sequences 80−329 resulted in a 25% drop in enhancer activity (Figure 4), internal deletion of sequences 301−329 resulted in a more modest drop to 74% activity (Figure 5, lanes 1 and 2). Thus, additional functional elements must exist in the region between 80 and 301. To identify the region of this additional element, progressive enhancer deletions were prepared in the context of the 301−329 internal deletion. These studies showed that deletion to 145, 195, 246, or 275 did not significantly lower enhancer activity in the context of the 301−329 internal deletion (lanes 3−6). Internal deletion of sequences 386−392 also had no effect on enhancer activity (lane 7). The above results suggest that the additional functional segment lies between sequences 275 and 301 and the most dramatic loss of

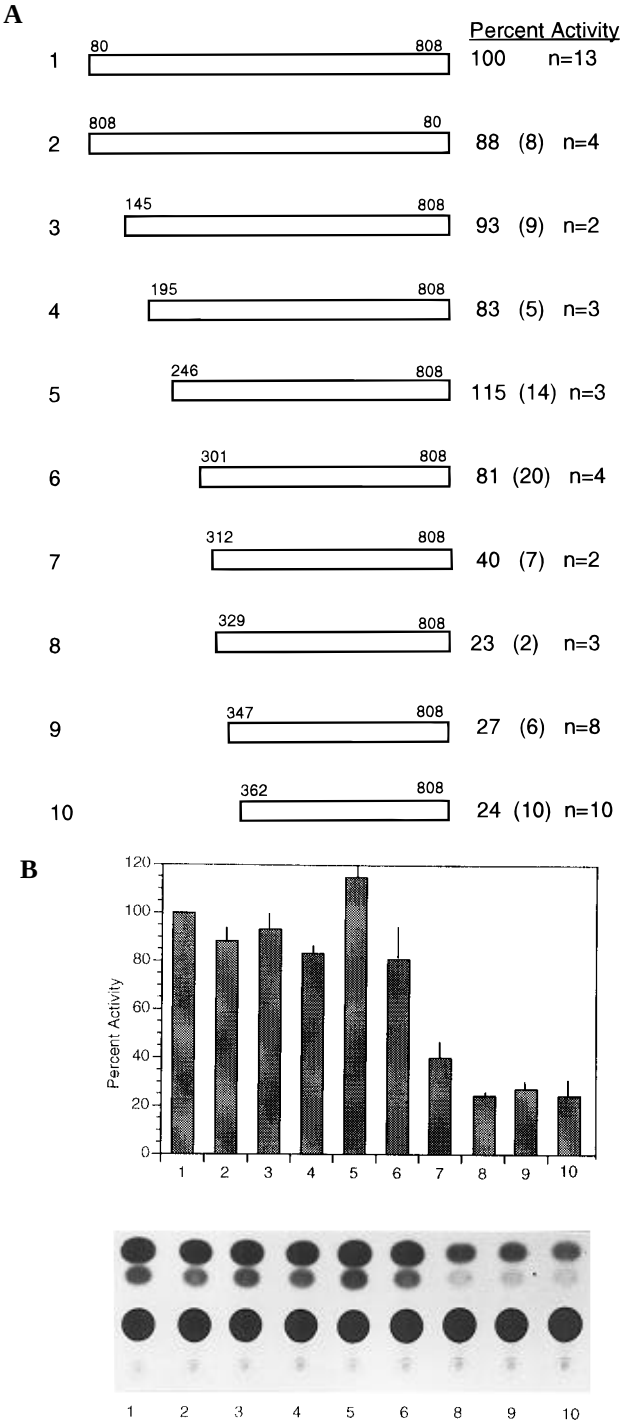

FIGURE 4: Sequences between nucleotides 301 and 329 are important for maximal enhancer activity. (A) Enhancer sequences present in each construct are represented by the open rectangles. Percent CAT activities of each construct are shown in the right panel. Numbers in parentheses indicate standard deviations, and *n* values indicate the number of times each transfection was performed. (B) Representative CAT data obtained with constructs 1−6 and 8−10 are shown. In the top panel, all data are represented in histogram fashion with the thin lines representing standard deviations. Numbers below each lane indicate the construct number.

enhancer activity requires further deletion of sequences 301−329.

Identification of Nuclear Factors That Bind to the Functional Region. Our transfection data indicated that a positive-acting sequence is present between enhancer residues 301 and 329. To search for nuclear factors that might bind to this region of the enhancer, EMSA was performed with a

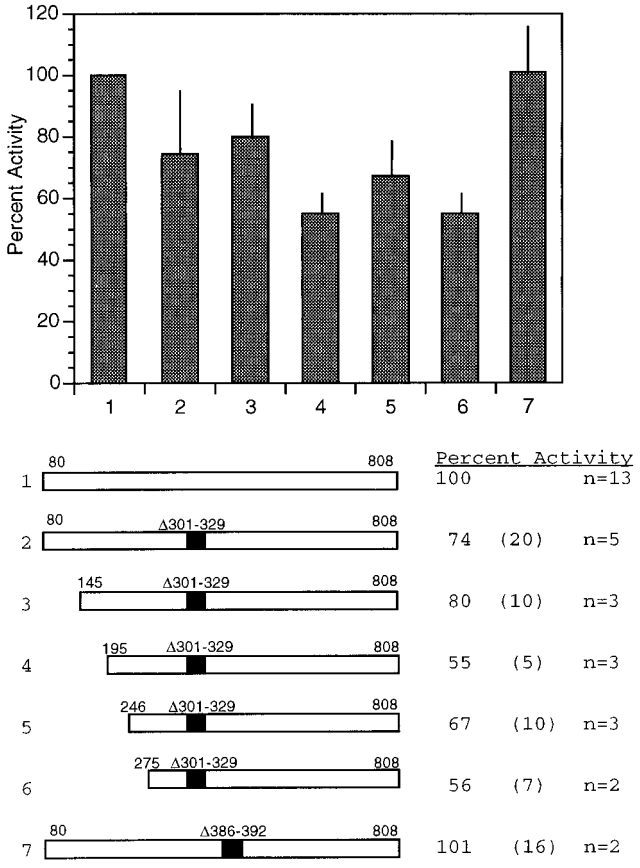

FIGURE 5: Additional DNA sequences contribute to enhancer activity. The lower left panel represents the various enhancer constructs with numbers indicating the end points of each construct. The filled rectangles represent internal deletions of either sequences 301−329 or 386−392. Percent CAT activities of each construct are shown in the right panel. Numbers in parentheses indicate standard deviations and *n* values indicate the number of times each transfection was performed. In the top panel, all data are represented in histogram fashion with the thin lines representing standard deviations. Numbers below each lane indicate the construct number.

301−329 oligonucleotide probe in the presence of specific and nonspecific oligonucleotide competitors. A protein−DNA complex was indeed observed with this probe (Figure 6A, lane 1). Addition of increasing quantities of the self oligonucleotide sequence resulted in competition of the protein−DNA complex (Figure 6A, lanes 1−4), while no competition was observed with an unrelated oligonucleotide (lanes 5−7).

EMSA was performed with nuclear extracts isolated from a variety of cell types to determine the tissue specificity of the nuclear factors that bind to the 301−329 DNA probe. Complex formation was observed with all nuclear extracts tested, including those isolated from a pre-B cell line (3−1), a B cell lymphoma (38C), two plasmacytoma lines (MPC11 and S194), a cervical carcinoma line (HeLa), and a kidney fibroblast line (Cos; data not shown).

To more precisely identify the DNA sequences necessary for protein−DNA interaction, dimethyl sulfate (DMS) methylation interference assays were performed. While assays with the top DNA strand failed to show protein−DNA contact sites (data not shown), numerous contact sites were identified on the lower strand (Figure 6B). These assays indicated that methylation of G residues at positions 302−306 on the bottom strand inhibited protein−DNA interaction. Similarly, methylation of A residues 319, 320, 322, 323, and 326−329 inhibited interaction. Therefore, two regions within the 301−329 segment are capable of interacting with nuclear factors.

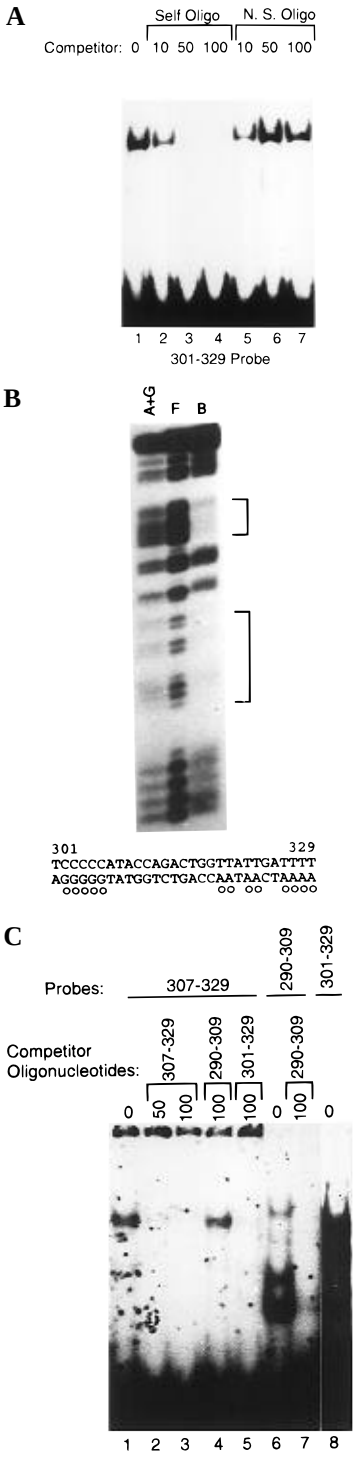

FIGURE 6: Identification of nuclear factors that bind to enhancer sequences 301−329. (A) EMSA was performed with sequences 301−329 as probe and S194 nuclear extract. The amount in nanograms of competitor oligonucleotides is indicated above each lane. (B) Protein−DNA contact sites were determined by DMS methylation interference assay. An A + G sequencing reaction is shown in the left lane with F and B representing free and bound fractions, respectively. Brackets to the right of the gel indicate regions of protein contact. In the lower panel, the identities of the nucleotides involved in protein interaction are indicated by the open circles. (C) EMSA was performed with a variety of DNA probes and oligonucleotide competitors. The identities of the probes and competitors used in each assay are indicated above each lane. The amount of each competitor in nanograms is also indicated.

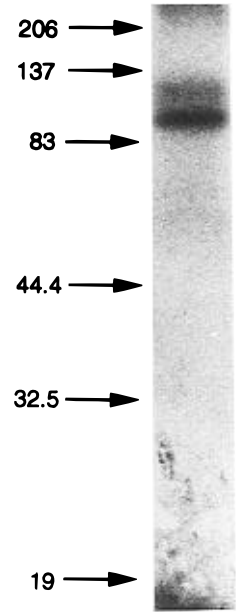

FIGURE 7: Proteins of 105 and 85 kDa bind to enhancer sequences 301−329. UV cross-linking experiments were performed with enhancer sequences 301−329 and S194 nuclear extract proteins. Cross-linked proteins were resolved by SDS−PAGE and visualized by autoradiography. The positions of molecular mass size markers are shown to the left of the gel.

The two regions of protein−DNA contact identified in the DMS methylation interference assays suggested that at least two factors are responsible for the complex observed in our EMSA studies. To test this, EMSA studies were performed with oligonucleotide probes that contained only one of the DNA sequences identified by our DMS methylation interference studies. A probe containing sequences 307−329 yielded a slowly migrating complex with which self DNA sequences and a 301−329 competitor but not a 290−309 competitor effectively competed (Figure 6C, lanes 1−5). Similarly, a probe containing sequences 290−309 yielded a complex with a slightly slower mobility as well as complexes with faster mobilities with which self sequences (Figure 6C, lanes 6 and 7) but not sequences 307−329 competed (data not shown). The slower mobility complexes observed with the 290−309 and the 307−329 probes migrated at a position similar to that of the complex observed with the 301−329 probe. Indeed, the 301−329 complex can be resolved into these two complexes (see below). The origin of the faster mobility complexes observed with the 290−309 probe is unclear and may be due to sequences 290−300. Our data strongly suggest that two distinct nuclear factors give rise to the EMSA complex observed with the 301−329 probe. It should be noted that these factors may bind to DNA cooperatively because complex formation is much more efficient with the 301−329 probe than with the 290−309 or the 307−329 probes (compare lanes 1, 6, and 8).

Identification of Interacting Proteins by UV Cross-Linking. UV cross-linking experiments were performed to further characterize the factors that bind to the 301−329 probe. These experiments revealed two high-molecular mass nuclear factors that bound to the 301−329 probe (Figure 7). Bands of 115 and 95 kDa were observed by this procedure. Subtraction of approximately 10 kDa for the mass of the oligonucleotide probe indicated that factors of 105 and 85 kDa bind to this portion of the κE3′ enhancer. Whether these are distinct proteins, modified versions of the same protein,

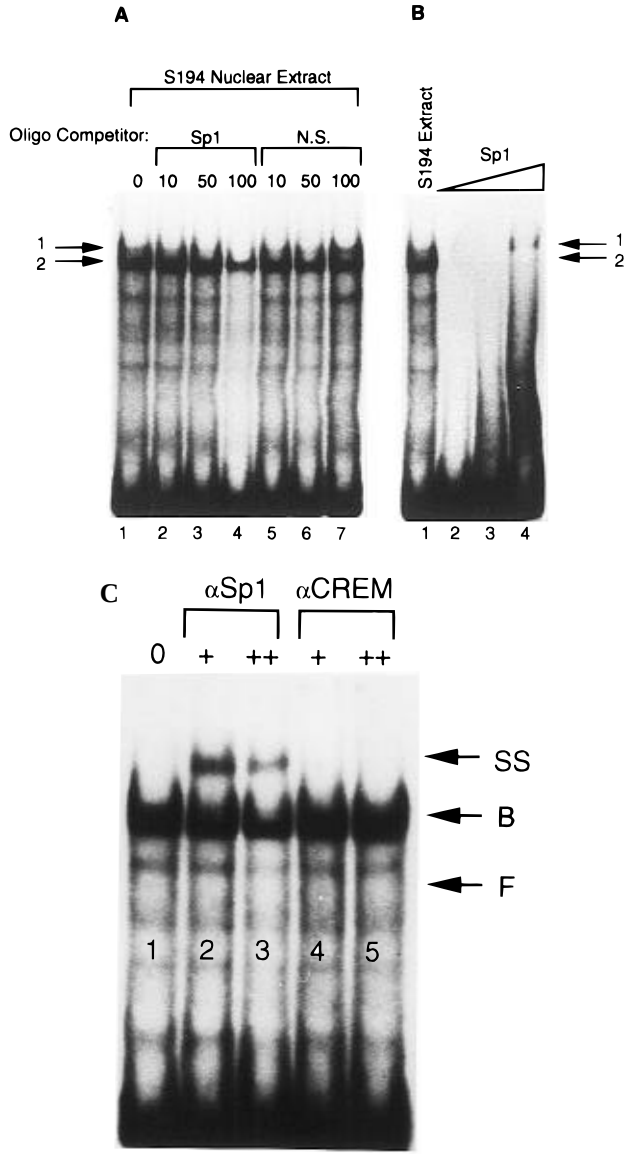

FIGURE 8: Sp1 binds to the 301−329 probe. (A−C) EMSA was performed with the 301−329 probe and either S194 nuclear extract proteins or purified Sp1 (0.1, 0.5, and 1.0 fpu; see Materials and Methods). Sp1 or CREM antibodies (0.1 and 0.2 μg) were added 15 min prior to addition of the labeled DNA probe. The position of the supershifted complex is indicated by the arrow labeled SS. Complexes 1 and 2 are indicated by the arrows. Above each lane are indicated the source of protein and the quantity of oligonucleotide competitors in nanograms.

or proteolytic fragments of the same protein cannot be determined for certain. However, our EMSA data are most consistent with two distinct proteins binding to the 301−329 probe (see Figure 6C).

Sp1 Binds to the 301−329 Probe. Because of the G-rich nature of one of the protein−DNA contacts identified in Figure 6B and the large size of the factors identified by UV cross-linking (Figure 7), we sought to determine whether Sp1 could bind to this region of the κE3′ enhancer. Using longer electrophoretic runs, we separated the EMSA complex with the 301−329 probe into two closely migrating complexes (Figure 8A, lane 1, complexes 1 and 2). Addition of increasing quantities of an unlabeled Sp1 oligonucleotide competitor abolished the more slowly migrating complex (lanes 2−4), whereas an unrelated oligonucleotide had no effect (lanes 5−7). Purified Sp1 protein yielded a complex with a mobility very similar to that of the slower mobility

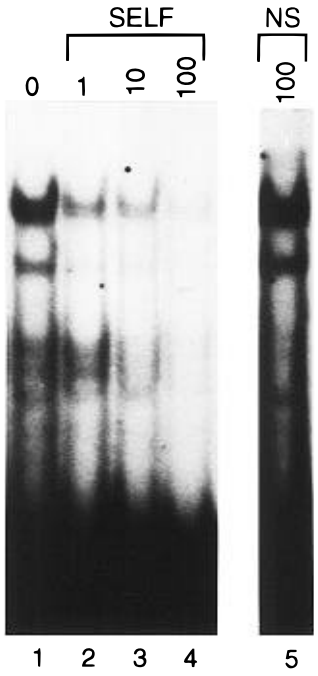

FIGURE 9: Nuclear factors bind between residues 275 and 300. EMSA was performed with the 275−300 oligonucleotide probe and S194 nuclear extract. The amounts in nanograms of specific (SELF) or nonspecific (NS) oligonucleotides added to the reaction mixtures are indicated above the lanes.

complex (Figure 8B, lanes 1−4). In addition, inclusion of Sp1 antibodies but not CREM antibodies resulted in a supershift of the more slowly migrating complex (Figure 8C). Therefore, Sp1 can bind to the region of the κE3′ enhancer that we have determined to be of functional importance.

Our internal deletion studies (Figure 5) also suggested that a positive-acting sequence may lie between residues 275 and 300. We performed EMSA with these sequences as probe and detected two complexes (Figure 9, lane 1). The unlabeled self oligonucleotide (lanes 2−4) but not by nonspecific oligonucleotide competed with these complexes (lane 5). The slow mobility of these complexes and the presence of a G-rich sequence between nucleotides 290 and 299 prompted us to determine whether Sp1 could also bind to this region. However, Sp1 does not bind to this region because an Sp1-specific oligonucleotide did not compete for complex formation, purified Sp1 did not bind, and Sp1 antibodies did not alter the EMSA pattern (data not shown).

DISCUSSION

Identification of a New Enhancer Element in the κE3′ Enhancer. Our previous studies on characterizing functional sequences within the κE3′ enhancer showed that a centrally located core segment (nucleotides 391−523) contained most of the sequences necessary for activity within plasmacytoma cells (Pongubala & Atchison, 1991). These studies showed that reporter constructs containing enhancer sequences 80−523, 391−808, or 391−523 showed comparable enhancer activity. Interestingly, all of these constructs contain the core segment (sequences 391−523) adjacent to the TK promoter in the reporter plasmid. However, our studies presented here show that, in the absence of sequences that flank the core segment, enhancer activity is significantly reduced when the core segment is placed several hundred base pairs distal to the promoter. This distance effect is not observed with the entire enhancer, indicating the presence of positive-acting sequences outside of the core segment of the enhancer. Indeed, our progressive deletion studies identified a positive-acting segment between sequences 301 and 329. Deletion of enhancer sequences 80−329 reduced enhancer activity to 25% of that of wild type when the core segment was positioned distal to the promoter. Our results show that the core segment of the enhancer can support maximal levels of enhancer activity when it is positioned directly adjacent to the promoter, but additional enhancer sequences are required for maximal activity when the core is positioned 300 bp distal to the promoter.

Internal deletion of the sequences 301−329 in the context of the entire enhancer had a much less deleterious effect on enhancer activity than was observed with the progressive deletion constructs. Therefore, additional sequences between positions 80 and 301 contribute to enhancer activity. By linking progressive deletions in the context of the 301−329 internal deletion, we demonstrated that these additional sequences most likely reside between positions 275 and 301. However, additional studies will be required to more definitively localize functional sequences within this region.

Nuclear Factors Bind to the Functionally Defined Enhancer Segment. EMSA and DMS methylation interference assays were used to search for nuclear factors that bind to enhancer sequences 301−329. These studies identified ubiquitously expressed proteins that contact enhancer sequences 302−306 and 319−329. The former sequence consists of a consecutive stretch of G residues, whereas the later region is A-T-rich. We also demonstrated that nuclear factors bind to DNA sequences between residues 275 and 300. We currently do not know the identities of the proteins that bind between sequences 275−300 and 319−329. We have screened a B cell cDNA expression library by the method of Vinson et al. (1988) with these sequences and have failed to isolate cDNA clones encoding proteins that bind to these sequences. However, EMSA studies with oligonucleotide competitors, with purified Sp1, and with Sp1 antibodies indicated that Sp1 can bind to the 301−329 region of the enhancer. Therefore, at least one of the proteins that binds to this functionally important region of the enhancer is very similar to or identical to Sp1.

The sequence of the Sp1 binding site in the κE3′ enhancer (5′-GGGGG-3′) is unlike the canonical Sp1 binding sequence (5′-GGGGCGGGG-3′). The κE3′ Sp1 sequence would be predicted to have relatively low affinity for Sp1 (Desjarlais & Berg, 1992). Indeed, our EMSA studies indicate that purified Sp1 binds relatively poorly to this sequence (Figure 8B). Perhaps Sp1 is assisted in binding to the κE3′ enhancer by interactions with other transcription factors. In support of this, our EMSA studies in Figure 6C suggest that Sp1 binds more efficiently in the presence of another DNA binding protein. Sp1 can cooperatively bind to DNA with Oct1, NF-κB, RelA, AP-1, or AP-2 (Jansen & Pettersson, 1990; Perkins et al., 1993, 1994; Majello et al., 1994; Andersson et al., 1994). Understanding the potential cooperative binding of Sp1 to the κE3′ enhancer will require further studies. The identity of the protein that binds to the A-rich sequence in the 319−329 region of the κE3′ enhancer is presently unknown. It will be interesting to determine whether this protein interacts with Sp1 to increase DNA binding efficiency.

Our transfection studies indicated that the functional region of the enhancer we identified here contributes about 4-fold to enhancer activity when the core is positioned distal to promoter sequences. Sp1 activates transcription of a variety of genes, and activation can range from as low as 2-fold to as high as 500-fold [see, for example, Courey and Tjian (1988), Hariharan et al. (1989), Line et al. (1992), Guerrini et al. (1993), Boisclair et al. (1993), Chen et al. (1993), Zhang et al. (1994), Wu et al. (1994), Yang et al. (1995), Tamaki et al. (1995), and Mutero et al. (1995)]. The 4-fold activation we observed here for the κE3′ enhancer is a common activation range for Sp1. Similar to the case with the κE3′ enhancer, in other systems, Sp1 has been observed to contribute to transcriptional activation over distances (Su et al., 1991; Mastrangelo et al., 1991).

Complex Regulation of the κE3′ Enhancer. The identification of additional functional sequences within the κE3′ enhancer emphasizes the complex nature of this regulatory element. Activity of this enhancer is developmentally controlled and is B cell lineage-specific. The only tissue-restricted factors known to regulate κE3′ enhancer activity are the factors PU.1 and NF-EM5 (Pongubala et al., 1992, 1993; Pongubala & Atchison, 1995). Sp1 is ubiquitously expressed and therefore does not appear to play a direct role in controlling tissue specificity of enhancer function. Other factors appear to be necessary for activation of enhancer activity during the transition from the pre-B cell to the B cell stage. Thus far, no κE3′ enhancer binding factors have been identified that are absent in pre-B cells but present in B cells and plasma cells when the enhancer is active. However, a cDNA clone encoding a protein with the properties of NF-EM5 (PIP; Eisenbeis et al., 1995) was recently cloned. Levels of the mRNA encoding this protein are higher at later stages of B cell development when the enhancer is maximally active. Therefore, quantitative changes in protein levels or post-translational modifications may contribute significantly to developmental control of enhancer activity. In addition, relief of repression exerted by enhancer sequences 523−808 must also contribute to activation of the κE3′ enhancer (Park & Atchison, 1991). With many of the proteins known to control enhancer activity now identified, it may be possible to determine the key regulatory steps in the developmental control of Igκ transcription.

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REFERENCES

Andersson, G., Phalman, S., Parrow, V., Johansson, I., & Hammerling, U. (1994) *Cell Growth Differ. 5*, 27−36.
Atchison, M. L., & Perry, R. P. (1987) *Cell 48*, 121−128.
Atchison, M. L., Delmas, V., & Perry, R. P. (1990) *EMBO J. 9*, 3109−3117.
Boisclair, Y. R., Brown, A. L., Casola, S., & Rechler, M. M. (1993) *J. Biol. Chem. 268*, 24892−24901.
Chen, H.-M., Pahl, H. L., Scheibe, R. J., Zhang, D.-E., & Tenen, D. G. (1993) *J. Biol. Chem. 268*, 8230−8239.
Courey, A. J., & Tjian, R. (1988) *Cell 55*, 887−898.
Desjarlais, J. R., & Berg, J. M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 7345−7349.
Dignam, J. D., Lebovitz, R. M., & Roeder, R. G. (1983) *Nucleic Acids Res. 11*, 1475−1489.
Eisenbeis, C. F., Singh, H., & Storb, U. (1995) *Genes Dev. 9*, 1377−1387.
Gorman, C. M., Moffat, L. F., & Howard, B. H. (1982) *Mol. Cell. Biol. 2*, 1044−1051.
Grosschedl, R., & Baltimore, D. (1985) *Cell 41*, 885−897.
Guerrini, L., Gong, S. S., Mangasarian, K., & Basilico, C. (1993) *Mol. Cell. Biol. 13*, 3202−3212.
Hagman, J., Rudin, C. M., Haasch, D., Chaplin, D., & Storb, U. (1990) *Genes Dev. 4*, 978−992.
Hall, C. V., Jacob, P. E., Ringold, G. M., & Lee, F. (1983) *J. Mol. Appl. Genet. 2*, 101−110.
Hariharan, N., Kelley, D. E., & Perry, R. P. (1989) *Genes Dev. 3*, 1789−1800.
Janson, L., & Pettersson, U. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4732−4736.
Judde, J.-G., & Max, E. E. (1992) *Mol. Cell. Biol. 12*, 5206−5216.
Lenardo, M., Pierce, J. W., & Baltimore, D. (1987) *Science 236*, 1573−1577.
Lin, X., Wang, Z., Gu, L., & Deuel, T. F. (1992) *J. Biol. Chem. 267*, 25614−25619.
Majello, B., DeLuca, P., Hagen, G., Suske, G., & Lania, L. (1994) *Nucleic Acids Res. 22*, 4914−4921.
Mastrangelo, I. A., Courey, A. J., Wall, J. S., Jackson, S. P., & Hough, P. V. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5670−5674.
Meyer, K. B., & Neuberger, M. S. (1989) *EMBO J. 8*, 1959−1964.
Meyer, K. B., Sharpe, M. J., Surani, M. A., & Neuberger, M. S. (1990) *Nucleic Acids Res. 18*, 5609−5615.
Mutero, A., Camp, S., & Tayor, P. (1995) *J. Biol. Chem. 270*, 1866−1872.
Park, K., & Atchison, M. L. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9804−9808.
Perkins, N. D., Edwards, N. L., Duckett, C. S., Agranoff, A. B., Schmid, R. M., & Nabel, G. J. (1993) *EMBO J. 12*, 3551−3558.
Perkins, N. D., Agranoff, A. B., Pascal, E., & Nabel, G. J. (1994) *Mol. Cell. Biol. 14*, 6570−6583.
Picard, D., & Schaffner, W. (1984) *Nature 307*, 80−82.
Pongubala, J. M. R., & Atchison, M. L. (1991) *Mol. Cell. Biol. 11*, 1040−1047.
Pongubala, J. M. R., & Atchison, M. L. (1995) *J. Biol. Chem. 270*, 10304−10313.
Pongubala, J. M. R., Nagulapalli, S., Klemsz, M. J., McKercher, S. R., Maki, R. A., & Atchison, M. L. (1992) *Mol. Cell. Biol. 12*, 368−378.
Pongubala, J. M. R., Van Beveren, C., Nagulapalli, S., Klemsz, M. J., McKercher, S. R., Maki, R. A., & Atchison, M. L. (1993) *Science 259*, 1622−1625.
Queen, C., & Baltimore, D. (1983) *Cell 33*, 741−748.
Queen, C., & Stafford, J. (1984) *Mol. Cell. Biol. 4*, 1042−1049.
Sen, R., & Baltimore, D. (1986a) *Cell 46*, 705−716.
Sen, R., & Baltimore, D. (1986b) *Cell 47*, 912−928.
Stafford, J., & Queen, C. (1983) *Nature 306*, 77−79.
Su, W., Jackson, S., Tjian, R., & Echols, H. (1991) *Genes Dev. 5*, 820−826.
Tamaki, T., Ohnishi, K., Hartl, C., LeRoy, E. C., & Trojanowska, M. (1995) *J. Biol. Chem. 270*, 4299−4304.
Vinson, C. R., LaMarco, K. L., Johnson, P. F., Landschulz, W. H., & McKnight, S. L. (1988) *Genes Dev. 2*, 801−806.
Wu, R.-L., Chen, T.-T., & Sun, T.-T. (1994) *J. Biol. Chem. 269*, 28450−28459.
Yang, X., Fyodorov, D., & Deneris, E. S. (1995) *J. Biol. Chem. 270*, 8514−8520.
Zhang, D.-E., Hetherington, C. J., Tan, S., Dziennis, S. E., Gonzalez, D. A., Chen, H.-M., & Tenen, D. G. (1994) *J. Biol. Chem. 269*, 11425−11434.

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