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Immunology

A promoter element that exerts positive and negative control of the interleukin 2-responsive J-chain gene

(B-cell immune response/interleukin 2 nuclear signal/transcriptional activation/DNA binding)

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ABSTRACT **In a primary immune response a signal from interleukin 2 (IL-2) induces B lymphocytes to express the gene for the IgM joining component, the J chain. The signaling mechanism was pursued in this study by examining the J-chain gene 5′ flanking region for regulatory sequences and interacting nuclear factors. The analyses identified a major control region located between −75 and −45 that encodes two adjacent elements: a T-rich sequence (JA) containing a single positive regulatory motif and an A+G-rich sequence (JB) containing overlapping positive and negative regulatory motifs. Dissection of the two elements indicated that the bifunctional JB sequence is the likely target of the IL-2 signal. The evidence was based on findings that (*i*) JB activity correlated with J-chain gene transcription—i.e., JB acts as a repressor in J-chain-silent B cells and as an activator in J-chain-expressing cells, and (*ii*) JB activator function is mediated by a B-cell-specific nuclear protein, NF-JB, that exhibits an IL-2-responsive binding pattern.**

The synthesis of J chain by B lymphocytes has several features that make it an attractive model for analyzing the mechanism of lymphokine signaling. First, the expression of J chain is developmentally regulated at the transcriptional level (1). The gene remains silent during the early antigen-independent stages of B-lymphocyte differentiation and becomes activated only during a primary immune response when the J-chain product is used for the assembly and secretion of pentamer IgM antibody (2). Second, the extracellular cues for J-chain gene activation are known; they are provided by either of two lymphokines—interleukin 2 (IL-2) or interleukin 5 (IL-5) (3, 4)—that are secreted by antigen-activated T-helper cells. Finally, and most importantly, the activation events can be reproduced under defined conditions *in vitro*; a cloned murine B-cell line, BCL_1, can be stimulated to transcribe the J-chain gene by treatment with physiological doses of recombinant IL-2 (5) and/or recombinant IL-5 (4).

By taking advantage of these features, some progress has been made in defining the mechanism of IL-2 signaling. Analyses of signal input have established that the J-chain response is initiated by IL-2 binding to high-affinity IL-2 receptors expressed by antigen-activated B cells (6) and their counterpart BCL_1 cells (7). The 75-kDa component of the bimolecular receptor then delivers the IL-2 message to a relay system within the cell (7, 8). Although the relay system has yet to be completely defined, two candidates for early events in the pathway have recently been described. One is a tyrosine kinase that is associated with the IL-2 receptor (9–11) and the other is a glycosylated form of phosphatidylinositol that is rapidly hydrolyzed in response to IL-2 to generate two potential second messengers (12, 13).

Analyses of IL-2 signal outcome have shown that transcription of the J-chain gene correlates with chromatin changes in the 5′ region. A single nuclease hypersensitive site is coinduced with J-chain gene expression either in normal B lymphocytes stimulated with mitogen or in BCL_1 cells stimulated with recombinant IL-2 (14). This finding suggested that the lymphokine signal alters the 5′ structure of the J-chain gene to allow access of the transcriptional machinery. To pursue the mechanism(s) involved, we examined the hypersensitive site for regulatory sequences and for sites of interaction with nuclear proteins. Here we present evidence for an IL-2-responsive element that contains overlapping positive and negative regulatory motifs and binds a nuclear factor, NF-JB, in a B-cell- and development-specific pattern.

MATERIALS AND METHODS

Cell Culture. B-cell lines were maintained in RPMI 1640 medium containing L-glutamine, penicillin/streptomycin, 50 mM 2-mercaptoethanol, and 10% (vol/vol) fetal calf serum. The myeloma, hybridoma, T-, and nonlymphoid cell lines were cultured in Dulbecco's modified Eagle's medium supplemented as described above except that the 2-mercaptoethanol was omitted.

Preparation and Assay of Nuclear Protein Extracts. Large-scale extracts were prepared from 10^9 cells by the detergent lysis method of Peterson *et al.* (15). All buffers contained the following protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.5 mM $Na_2S_2O_5$, aprotinin (10 units/ml), leupeptin (5 mg/ml), and pepstatin A (5 mg/ml). Miniextracts were prepared from 10^7 cells as described (16). For gel mobility-shift assays, 8 μg of the nuclear protein extract was incubated 30 min at 22°C with 10^4 cpm of end-labeled DNA probe (0.1–0.2 ng), 6 μg of poly(dI-dC), and 1× binding buffer [10 mM Tris·HCl, pH 7.5/50 mM NaCl/1 mM EDTA/5% (vol/vol) glycerol/1 mM dithiothreitol] in a 15-μl reaction volume. The samples were electrophoresed at 11 V/cm on a 4% polyacrylamide gel (19:1) containing 5% glycerol in 0.25× TBE buffer (1× TBE buffer = 90 mM Tris/90 mM boric acid/2.0 mM EDTA, pH 8.3).

Footprinting Assays. Methylation protection assays were performed by a standard method (17). Each preparative scale mobility-shift binding reaction was 5-fold in extract [poly(dI-dC)] and volume and 20-fold in probe. The probe was a 225-base-pair (bp) *Hin*dIII/*Asp*718 fragment containing the J-chain sequence −118 to +106 that was end-labeled on the top or bottom strand.

For 1,10-phenanthroline–copper protection footprinting, mobility-shift binding reaction mixtures were scaled up 40-fold and electrophoresed under standard conditions. The whole gel was treated with 1,10-phenanthroline–copper re-

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Abbreviations: IL-2, interleukin 2; CAT, chloramphenicol acetyltransferase.

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agent, and the protected bases were assayed as described (18). The probe in these assays was a 91-bp *Hin*dIII/*Xba* I fragment containing the J-chain sequence −83 to −8 that was end-labeled on either strand.

Plasmid Constructions for Deletion and Mutational Analyses. A wild-type reference plasmid was constructed by inserting a *Bam*HI fragment spanning −1150 to +88 of the J-chain promoter into the *Bgl* II site of the pCAT3M plasmid. To detect promoter activity, a 1.0-kilobase (kb) *Xba* I restriction fragment encoding the μ heavy-chain enhancer (19) was inserted with *Bam*HI linkers into the pCAT3M plasmid at the *Bam*HI site 3′ to the chloramphenicol acetyltransferase (CAT) gene. Progressive 5′ deletions were made in the promoter region by cleaving at an *Sac* I site at position −268 in the J-chain sequence, digesting with BAL-31 for various lengths of time, adding *Xba* I linkers, and ligating the 5′ ends generated to an *Xba* I site in the upstream polylinker. Single- and double-base changes were introduced into the translational start site and the JB binding site by subjecting the J-chain promoter to oligonucleotide-directed site-specific mutagenesis (20). The deletion end points and base substitutions were determined by sequence analyses (21) with Sequenase (United States Biochemical).

Plasmid Constructions for Linked Promoter Analyses. In the pγ42Cass1 vector (22) the CAT gene is under control of a truncated γ-fibrinogen promoter (−54 to +36) that includes a TATA box and a single SP1 binding site. The J1 fragment (−83 to −8) and oligonucleotides representing the JA element, the JB element, and various mutant JB sequences were synthesized with *Xba* I linkers and inserted either singly or in multiple copies into the polylinker upstream of the γ-fibrinogen promoter. All constructs were sequenced to determine oligomer copy number and orientation.

Transfection and CAT Assays. Transfections were performed by the DEAE-dextran technique (23) with 10 μg of supercoiled test plasmid for 10^7 cells. Cell extracts were prepared after 48 hr and assayed for CAT activity (24).

RESULTS

Identification of a Major Control Region in the 5′ Flanking Sequence of the J-Chain Gene. The control sequences upstream of the J-chain gene were defined by generating a series of 5′ deletions and measuring the effects of the deletions in transient transfection assays. For these experiments, a reference plasmid was constructed from pCAT3M by inserting 1.24 kb of 5′ J-chain sequence in the polylinker upstream of the CAT gene and 1.0 kb of μ heavy-chain enhancer sequence (19) at a *Bam*HI site downstream. The J-chain insert spanned base pairs −1150 to +88 and included the region (−170 to +88) that becomes DNase I hypersensitive upon transcription (14). The translational start site encoded within the J-chain insert was altered by site-specific mutagenesis to ensure correct initiation of CAT translation. The downstream μ enhancer was included to stimulate detectable promoter activity by the J-chain insert. Control experiments established that the stimulatory effect of the enhancer was independent of its orientation and location in the construct (data not shown).

Using this plasmid, progressive 5′ deletions were made in the J-chain insert starting at an *Sac* I site at position −268. The reference and deleted constructs were then assayed for their ability to drive CAT expression by transfection into S194 mouse myeloma cells that express high levels of J chain (2). The analyses identified a major control region positioned in the middle of the hypersensitive region directly upstream of a TATA sequence (Fig. 1). Thus, deletion of base pairs −75 to −45 resulted in a 10-fold decrease in CAT expression, whereas deletions from base pairs −1150 to −75 reduced CAT expression <2-fold.

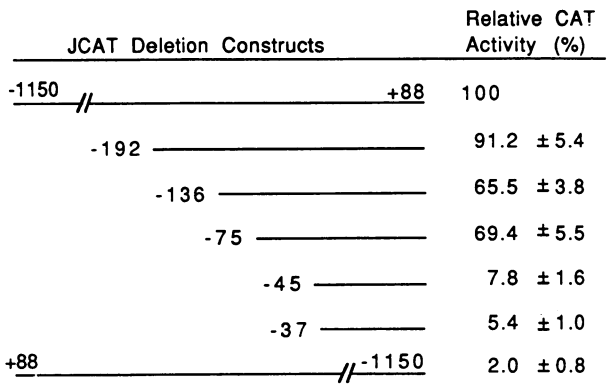

FIG. 1. Effect of deletions on promoter activity of the J-chain gene 5′ flanking region. Deletion constructs were transfected into J-chain-expressing S194 cells and compared for CAT activity with the reference construct. Mean percentages (±SEM) were calculated from the average values of four independent, duplicate determinations. Absolute percentage conversion of chloramphenicol to acetylated forms by the reference construct was 6.48 ± 1.64 per μg of protein per 0.1 mCi of [^{14}C]chloramphenicol (1 Ci = 37 GBq).

The Major Control Region Contains Both Positive and Negative Regulatory Elements. The function of the major control region was further evaluated by examining its activity in the context of a heterologous promoter. A J-chain promoter fragment spanning base pairs −83 to −8 (designated J1) was cloned into the γ42Cass1 vector (22) upstream of a truncated rat γ-fibrinogen promoter and the CAT gene (Fig. 2), and the effect of the insert on CAT expression was assayed both in J-chain-positive S194 cells and in J-chain-negative Jurkat human T cells and PD31 pre-B cells. Control experiments showed that the truncated γ-fibrinogen promoter induced a low level of CAT synthesis in S194 and Jurkat cells but an essentially undetectable level in PD31 cells. For the PD31 experiments, therefore, a 1.0-kb μ enhancer sequence was introduced into the plasmid vector 3′

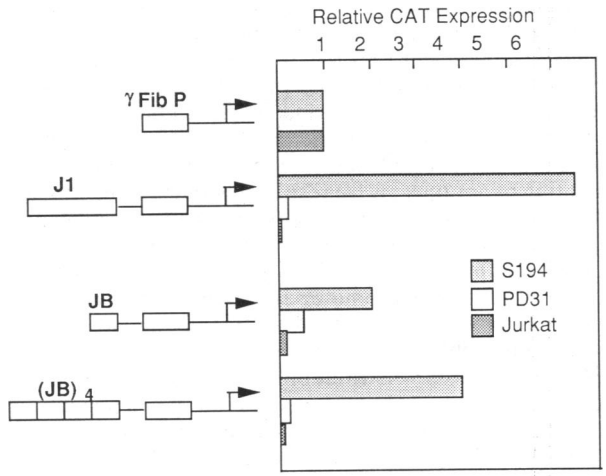

FIG. 2. Positive and negative regulatory activity by the JB element. Constructs containing the J1 fragment (base pairs −83 to −8) or single or four copies of the JB oligomer were assayed for their effects on promoter-driven CAT expression in J-chain-positive S194 myeloma cells and J-chain-negative PD31 pre-B cells and Jurkat T cells. Relative CAT activities were determined from the average of at least three independent duplicate determinations; in all cases the SEM was less than ±20%. Absolute percentage conversions of chloramphenicol to acetylated forms were 0.0837 ± 0.041 and 0.095 ± 0.017 μg of protein per 0.1 mCi of [^{14}C]chloramphenicol for the γ42Cass1 construct in S194 and Jurkat cells, respectively, and 0.127 ± 0.016 per μg of protein per 0.1 mCi of [^{14}C]chloramphenicol for the γ42Cass1-Eμ construct in PD31 cells.

to the CAT gene. The presence of the enhancer stimulated the γ-fibrinogen promoter activity in PD31 cells to a level equivalent to that obtained without the enhancer in S194 cells.

The assays in J-chain-expressing S194 cells confirmed the presence of a positive-acting element or elements in the major control region (Fig. 2); the insertion of the J1 fragment upstream of the heterologous promoter induced a 6- to 7-fold increase in the basal level of CAT expression. The assays in J-chain-negative cells, on the other hand, revealed that the major control region also contained negative regulatory elements (Fig. 2); the J1 insert reduced CAT expression 9-fold in Jurkat cells and 4- to 5-fold in PD31 cells. In the latter cells, the repressive activity of the insert may have been partially overridden by a contribution from the μ enhancer that was present in the PD31 constructs.

Identification of a Monofunctional JA and a Bifunctional JB Element. The positive and negative regulatory elements were more precisely located by functional analyses of subfragments of the major control region. Oligonucleotides representing −74 to −57 (designated JA) and −59 to −41 (designated JB) were cloned into the γ42Cass1 vector with and without the μ enhancer. Those plasmids containing a single copy or four copies of each oligomer, all in 5′ to 3′ orientation, were selected and their activity was assayed after transfection into J-chain-silent and J-chain-expressing cells. The JA sequence was found to exert only positive regulatory activity. In both PD31 and S194 cells, constructs containing a JA monomer increased CAT expression 1.5-fold and those containing a tetramer produced a 6-fold increase (S. Karray, J.S.C., and M.E.K., unpublished data). By contrast, the JB sequence was found to be bifunctional (Fig. 2). In S194 cells, it enhanced the activity of the heterologous promoter; a single JB oligonucleotide induced a 2-fold increase in the basal level of CAT expression and four copies resulted in a 4-fold increase. In J-chain-negative cells, however, the same JB sequence acted as a repressor; one to four copies caused an 8-fold reduction in CAT activity in Jurkat cells and a 2- to 5-fold reduction in PD31 cells.

Identification of Nuclear Factors Binding to the Major Control Region. The interaction of regulatory proteins with the JA and JB elements was examined by gel mobility-shift assays using nuclear extracts from J-chain-negative B-lymphoma cells. The binding patterns obtained were consistent with the functions of the JA and JB elements. Thus, a single complex was formed with the positive-acting JA sequence, whereas several complexes, one major and two more slowly migrating minor ones, were resolved with the bifunctional JB sequence (Fig. 3*A*). Each complex was shown by competition experiments to represent a specific protein–DNA interaction; binding was maintained in the presence of a 100-fold molar excess of unrelated promoter subfragment and completely competed in the presence of a 100-fold molar excess of homologous subfragment (Fig. 3*A*).

When the tissue distribution of the various JA and JB factors was examined, two exhibited a B-cell-specific pattern. Binding of the major JB protein (Fig. 3*B*) and the JA protein (data not shown) was observed only with extracts from B-cell lines; no comparable gel-shift bands were detected with extracts from helper or cytotoxic T cells or from fibroblast or epithelioid cell lines. In addition, binding of the major JB factor (NF-JB) was significantly reduced in extracts from fully differentiated myeloma lines expressing J chain. The amounts of complex formed were 5- to 10-fold less than those obtained with extracts from lymphoma lines representative of earlier J-chain-negative stages in B-cell development (Fig. 3*B*). The decrease in NF-JB binding could not be attributed to nonspecific factors, such as protein loading or protein degradation, since no losses were observed in the DNA-binding activities of other known components in the myeloma extracts (16). The decreased binding appeared, therefore, to represent a development-specific change associated with transcription of the J-chain gene.

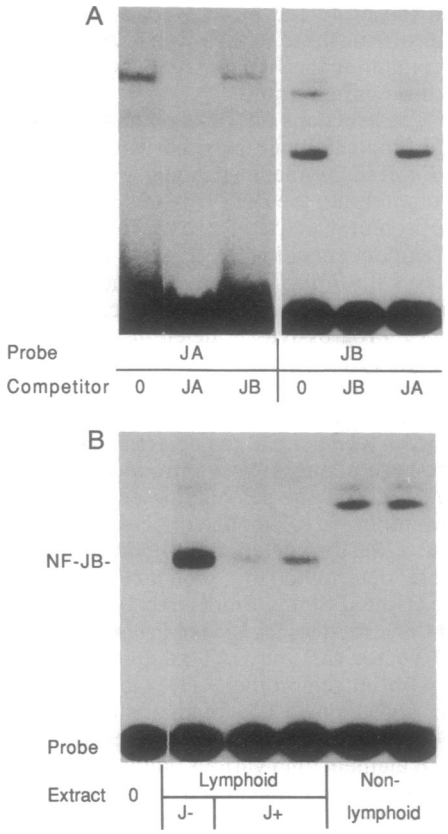

FIG. 3. Gel mobility-shift analyses of the binding of nuclear factors to JA and JB elements in the J-chain gene promoter. (*A*) Specificity of factor binding. For the binding reactions, nuclear extract from K46R B-lymphoma cells was incubated with labeled JA or JB oligonucleotide in the presence or absence of 100-fold molar excess of unlabeled JA and JB oligomers. JA, CTCTGGGGTTATTTTAAG; JB, AAGAAAGCAGAAGCAGCAT. (*B*) Tissue- and stage-specific binding of JB factor. Gel mobility-shift assays of nuclear extracts from the following representative cell lines: J-chain-negative mature B cell, K46R; J-chain-positive myeloma S194 and IgM-secreting hybridoma, D2; J-chain-negative nonlymphoid lines, L929 and HeLa. The probe was the JB oligonucleotide described above.

Functional Analyses of the JB Factor. The binding properties of the JB factor suggested that it might mediate the repressor rather than the promoter activity of the bifunctional JB element. To resolve the role of NF-JB, we examined the effect of mutations in the NF-JB recognition site on the functions of the JB element. Synthetic oligonucleotides were used to (*i*) introduce single- and double-base substitutions into the JB binding site of the wild-type CAT plasmid (Fig. 1) and (*ii*) insert mutated JB elements into the γ42Cass1 vector. The changes were directed to nucleotides at positions −53 and −51 to −50 that were known from footprinting assays to be located near the center of the site. As shown in Fig. 4*A*, the JB protein protected a 9-bp A+G-rich region (−56 to −48) on the coding strand and a corresponding 12-bp region (−58 to −47) on the noncoding strand. Gel mobility-shift assays (Fig. 4*B*) confirmed that substitutions made selectively abolished NF-JB binding, leaving the two more slowly migrating JB complexes intact.

Contrary to the expectations from the NF-JB binding pattern, the mutations were found to affect only the positive control element in the JB site. Thus, in S194 cells the activity of the 1.24-kb J-chain promoter was reduced almost 3-fold by the introduction of a single base change and 8-fold by a 2-base

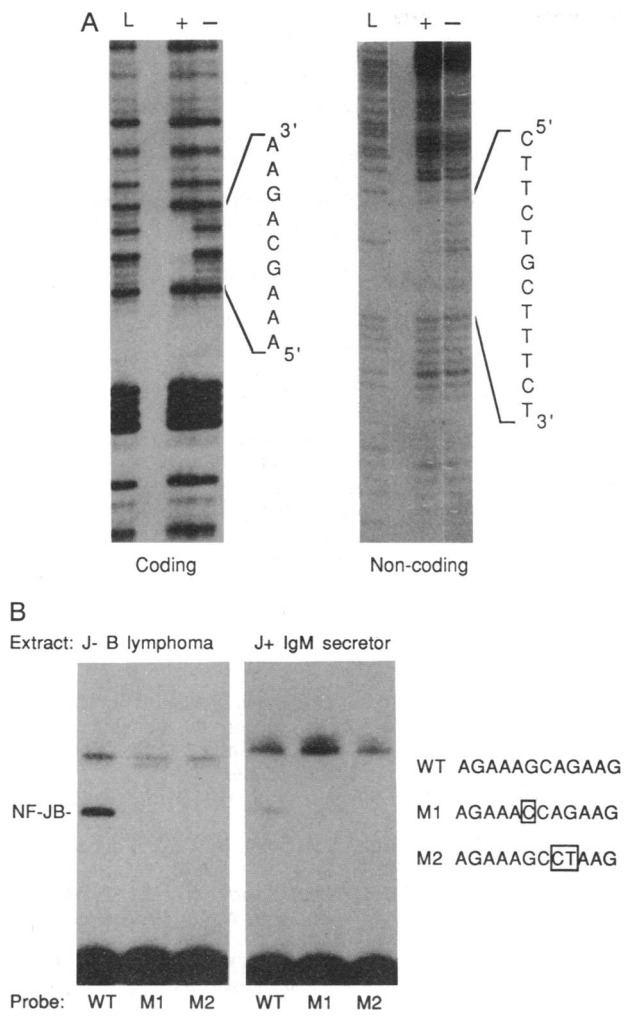

FIG. 4. Site-specific mutagenesis of the JB element. (*A*) Sequence of the JB binding site. Footprint on the coding strand was determined by methylation protection and that on the noncoding strand was determined by 1,10-phenanthroline–copper protection as described. (*B*) Effect of mutations on NF-JB binding. Gel mobility-shift assays of nuclear extracts from the J-chain-negative K46R B lymphoma and the J-chain-positive MxW 231 hybridoma. The probes were synthetic oligonucleotides representing the wild-type (WT) JB sequence (see legend to Fig. 3), the single (M1) mutant, or the double (M2) mutant.

change in the JB binding site (Fig. 5). These results were supported by assays of the mutant-linked promoter constructs. In J-chain-positive S194 cells, the 2-base change in the JB sequence not only nullified the stimulatory activity of the oligomers (compare Fig. 5 with Fig. 2), but it also significantly reduced the activity of the truncated γ-fibrinogen promoter. A single copy of the M2 oligomer decreased the basal level of CAT expression by one-half and four copies produced a 12-fold decrease (Fig. 5). In J-chain-negative PD31 and Jurkat cells, on the other hand, the 2-base change in the JB sequence failed to relieve the repressive effect of the oligomers; in no case did the levels of CAT expression exceed that of the wild-type constructs (compare Fig. 5 with Fig. 2).

These findings provided clear evidence that NF-JB serves as an activator of J-chain gene transcription despite the fact that its binding decreases with J-chain gene expression. In addition, the linked promoter analyses provided evidence that a second nuclear factor recognizes the JB sequence and serves as a repressor of J-chain gene transcription. Constructs with base changes that prevented JB binding suppressed the basal level of γ-fibrinogen promoter-driven transcription in both J-chain-negative and J-chain-positive cells.

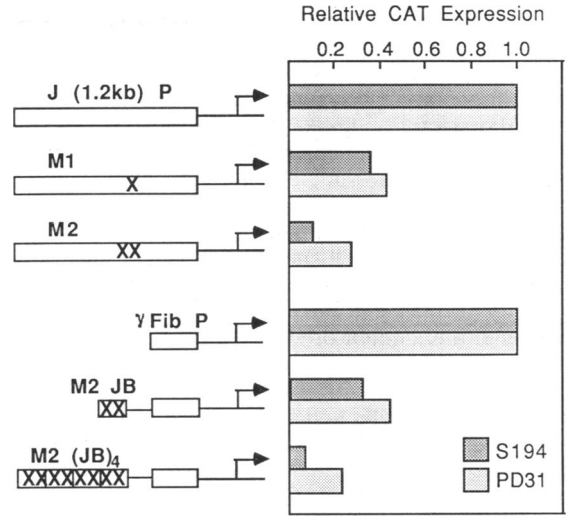

FIG. 5. Effect of mutations in the JB element on J-chain and heterologous promoter activity. Constructs containing mutant or wild-type J-chain promoter inserts and constructs containing a single or four copies of the M2 JB oligomer were assayed for CAT production in J-chain-expressing S194 cells and J-chain-silent PD31 cells. Values given represent the average of at least three independent duplicate determinations; in all cases, the SEM was less than ±25%. Absolute percentage conversion of chloramphenicol to acetylated forms by the wild-type J-chain promoter construct was 6.60 ± 0.48 per μg of protein per 0.1 mCi of [^{14}C]chloramphenicol in S194 cells and 0.0112 ± 0.0028 per μg of protein per 0.1 mCi of [^{14}C]chloramphenicol in PD31 cells. Absolute percentage conversion of chloramphenicol to acetylated forms for the γ42Cass1 construct in S194 cells and the γ42Cass1-Eμ construct in PD31 cells is given in the legend to Fig. 2.

DISCUSSION

The IL-2 signaling system plays a critical role in development of an immune response. IL-2 is one of a battery of lymphokines secreted by antigen-activated T-helper cells (25). Unlike other lymphokines, however, IL-2 delivers its signal selectively to activated lymphocytes since B and T cells express high-affinity IL-2 receptors only after antigen stimulation (26, 27). As a result, autocrine stimulation of responding T-helper cells expands the population and maintains the production of lymphokines including IL-2 (28). Stimulation of responding B cells and potentially cytotoxic T cells induces their proliferation and differentiation into mature antibody-secreting and killer populations, respectively (ref. 8 and references therein). Information on the biochemical action of IL-2, therefore, provides insights into both the mechanism of lymphokine signaling and the events required for maturation of an effector immune population.

Our previous studies have shown that one of the IL-2-signaled events in a B-cell immune response is the activation of J-chain gene transcription (5). These studies have now been extended to identify and characterize the major control region in the J-chain gene promoter. It is a sequence located between −75 and −45 that encodes two control elements: a T-rich sequence (JA) containing a single positive regulatory motif and an A+G-rich sequence (JB) containing overlapping positive and negative regulatory motifs. A search of the MACVECTOR program for transcription factor binding sites revealed that the JA sequence has no significant homology with any of the known control sequences. The JB sequence, on the other hand, resembles the purine-rich target site of the

ets oncogene family but lacks the GGAA core reported to be essential for *ets* domain binding (29).

Linked promoter analyses of the two elements indicated that the bifunctional JB sequence is the likely target of the IL-2 signal; its activity, unlike that of the JA sequence, was found to correlate with J-chain gene transcription—i.e., JB functions as a repressor in J-chain-negative cells and as an activator in J-chain-expressing cells. Evidence for the IL-2 responsiveness of the JB element was also obtained from its interaction with the B-cell-specific nuclear protein NF-JB. Gel mobility-shift assays showed that NF-JB binding is significantly decreased in extracts from cells transcribing the J-chain gene; the amount of complex formed is 1/5 to 1/10 that obtained with extracts from J-chain-silent cells. Moreover, decreases in NF-JB binding are induced by IL-2 treatment of BCL_1 cells under conditions in which J-chain gene transcription is the only detectable outcome of the IL-2 signal (16).

The properties of the JB factor, its IL-2 responsive binding pattern and B-cell-specific expression, indicated that it has a central role in regulating the activity of the major control region and thus expression of the J-chain gene. Clues to that role were obtained from linked promoter analyses of the JA and JB elements in J-chain-expressing cells. First, mutations that prevent NF-JB binding were found to change the JB element from an activator to a strong repressor. This conversion suggests that one function of NF-JB is to regulate, probably by direct competition, the binding of a repressor to the JB sequence. Second, sequences containing both the JB and JA elements were found to be potent transcriptional activators (J1 data, Fig. 2; S. Karray, J.S.C. and M.E.K., unpublished observations), whereas the individual elements had relatively weak activity. This synergism suggests that a second function of NF-JB is to positively regulate J-chain gene expression through interactions with the upstream JA activator. How NF-JB is induced by an IL-2 signal to perform such functions and how that performance results in decreased DNA binding have yet to be determined. Resolution of the role of NF-JB must, therefore, await not only further analyses of NF-JB interactions with other factors, but also a definition of the IL-2-induced modification responsible for the binding change.

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