A Ternary Complex Factor-Dependent Mechanism Mediates Induction of egr-1 through Selective Serum Response Elements following Antigen Receptor Cross-Linking in B Lymphocytes

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Received 13 June 1994/Returned for modification 17 August 1994/Accepted 17 November 1994

Induction of the primary response gene egr-1 occurs rapidly following antigen receptor cross-linking in B lymphocytes. Antisense studies have demonstrated that this induction is necessary for their subsequent activation to this signal. The present study examines the molecular mechanism whereby the receptor-generated signals interact with the egr-1 promoter to elicit transcription. Deletion mapping and point mutations have indicated that two of the five serum response elements (SREs) in the egr-1 promoter can mediate induction. Of the two critical SREs, both are capable of mediating maximal induction even in the absence of the other SRE. Our results also indicate that adjacent Ets motifs are necessary for induction. Like the c-fos SRE, the egr-1 SRE/Ets sites are occupied by a multiprotein (ternary) complex containing a homodimer of serum response factor and an unidentified member of the Ets family of transcription factors. The identification of a ternary complex-dependent mechanism of egr-1 induction, along with selective utilization of SREs in B lymphocytes, suggests that a complicated array of signaling cascades interacts with unique combinations of regulatory elements in the egr-1 promoter in different cell types.

The primary response gene egr-1 (45), which is also known as Krox-24, zif/268, NGFI-A, and TIS8 (7, 24, 25, 30), encodes a transcription factor (EGR1) that has a molecular mass of approximately 82 kDa and contains three C2H2 zinc fingers. Transcription of egr-1 is induced within minutes of mitogenic stimulation in cells of most lineages and generally parallels the pattern observed for c-fos (45). The coregulation of c-fos and egr-1 can be explained by the elements common to the promoters of both genes (for a review, see reference 29). c-fos promoter activity has been extensively studied and has served as a paradigm of primary response gene expression. Induction of c-fos is mediated by distinct promoter elements, depending on the cell type and stimuli (16, 44, 49).

In contrast to the detailed studies regarding the regulation of c-fos promoter activity, regulation of egr-1 expression is poorly understood. Of the studies which have examined egr-1 promoter activity, most have mapped inducibility to a region containing five serum response elements (SREs) (1, 6, 8, 10, 21, 23, 33, 35, 36, 39). The majority of these studies conclude that the individual SREs contribute approximately equally to egr-1 induction. Recently, in three systems, preferential utilization of SREs following stimulation has been suggested. The first two studies demonstrated that the two TATA-proximal SREs and an adjacent cyclic AMP response element (CRE)/AP-1-like site mediate induction in response to nerve growth factor (NGF) in PC12 cells and to cytokine treatment in myeloid cells (9, 38). In the final group of studies, myeloid cells were shown to utilize the two 5′ SREs, either alone or in concert, during egr-1 induction in response to a variety of pharmacological stimuli (21, 22). These studies suggest that tissue-specific and stimulus-dependent utilization of promoter elements may be important for egr-1 induction. Although in each case SREs appear to be necessary for induction, the reason for the preferential utilization of specific SREs is unknown. The possibility exists that preferential usage may reflect the need for additional elements which cooperate either directly or indirectly with the SREs. SRE-cooperating elements might include the CRE/AP-1 site (9, 38) or Ets motifs, as is the case for the c-fos SRE (43).

Activation of B lymphocytes results from engagement of the antigen receptor (BCR) (3) and is dependent on egr-1 expression (31). The biochemical events resulting from BCR cross-linking include activation of receptor-associated tyrosine kinases, phospholipid hydrolysis (with subsequent Ca2+ mobilization and protein kinase C activation), activation of the ras/mitogen-activated protein kinase (MAPK) pathway, and an increase in phosphatidylinositol 3-kinase activity (for a review, see reference 3). In recognition of the complexity of both the BCR-generated signaling cascade and the egr-1 promoter region (52), the present study was undertaken to assess whether a unique combination of regulatory elements mediates induction of this gene in B lymphocytes. In contrast to previous reports, this study implicates a specific pair of SREs in egr-1 induction. Unexpectedly, either of these two SREs is sufficient to mediate maximal induction, and the presence of both has no added effect. This is of interest for studies of the human egr-1 promoter, in which one of these critical SREs is absent (37). Furthermore, point mutations have demonstrated that induction requires Ets motifs adjacent to the tandem SREs. The unique configuration of regulatory elements which mediated egr-1 induction in B lymphocytes suggests a mechanism whereby a variety of stimuli in different cell lineages elicit induction of this gene through distinct combinations of promoter elements.

MATERIALS AND METHODS

B-lymphocyte isolation and culture. Spleens were removed aseptically from BALB/c mice (The Jackson Laboratory). For these studies, 8- to 12-week-old animals were used. Following mincing, 1 lymphocytes were removed by treatment with anti-Thy-1.2 (HO13.4) antisera and rabbit complement (Pel-Freez).
Erythrocytes were removed by hypotonic lysis, and the remaining cells were spun over a 75% Percoll cushion to remove debris. B lymphocytes were approximately 85% pure at this stage and were placed in culture for 72 h in B-cell assay medium (RPMI 1640 with 10% fetal calf serum, 2 mM l-glutamine, nonessential amino acids, 100 μg/ml of penicillin and streptomycin per ml, 5 x 10^-5 M 2-mercaptoethanol) with lipopolysaccharide (LPS) from Salmonella typhi (Sigma) at 50 μg/ml. The B-cell blasts obtained after 72 h of culture in LPS were used in the transcription assay described below.

**Plasmid construction.** For construction of plasmids containing egr-1 promoter fragments linked to the reporter gene encoding chloramphenicol acetyltransferase (CAT), the following protocol was used. egr-1 promoter fragments were generated by PCR using the genomic egr-1 clone (p357) (52) as a template. PCR-generated fragments were cloned into pBLCAT2.3, a promoterless CAT vector created by removal of the thymidine kinase promoter region from pCATpoly. These plasmids were used in place of pE359, and pE342 were the generous gifts of V. Sukhatme (Beth Israel Hospital, Boston, Mass.), and their construction was described elsewhere (5, 54). Probes consisted of double-stranded DNA from the region named, and the DNA fragments containing point mutations were generated by PCR with Vent (Exo -) polymerase (New England Biolabs). pE395, pE359, and pE342 were the generous gifts of V. Sukhatme (Beth Israel Hospital, Boston, Mass.), and their construction has been described in detail elsewhere (10). Briefly, they contain egr-1 promoter sequences from -395 to +65 in the case of pE395, -359 to +65 in the case of pE359, and -342 to +65 in the case of pE342. The sequences were cloned into the CAT vector pCATpoly. These plasmids were used in place of their pBLCAT2.3-based counterparts in Fig. 1B. Plasmids containing internal deletions in the egr-1 promoter (pBLCAT2.3, pBLCAT2.3, and pBLCAT2.3) were constructed as follows. The plasmids were linearized at -250 bp by Sall digestion. Bal31 treatment was used to remove promoter sequences. Following Bal31 treatment, the plasmids were digested with Apal to generate a uniform 3′ boundary for all deletions (-45). Blunt-ended ligations were then carried out. All other egr-1 promoter-containing plasmids were generated by PCR by using standard techniques. Plasmid DNA was isolated by the anion-exchange method (QIA-GEN). DNA from multiple preps were assessed for most of the constructs, and the results were uniformly consistent. All plasmids were sequenced by the double-stranded method (Sequenase; U.S. Biochemical).

**Transient transfection of B lymphocytes.** After 72 h of culture in LPS, B lymphocytes were transiently transfected with constructs containing various portions of the egr-1 promoter directing expression of the CAT reporter gene. The construct pBL903 contains egr-1 sequences from -903 to +65. pBL395 (and pE395) contains sequences from -395 to +65. pBL359 (and pE359) contains sequences from -359 to +65. pE342 contains sequences from -342 to +65, and pBLCAT2 contains the egr-1 TATA region from -45 to +65. Following transfection, cells were rested for 36 h and then stimulated with PMA at 10 ng/ml. Twelve hours poststimulation, the cells were harvested and lysates were assayed for CAT activity as described in Materials and Methods. CAT activity was quantitated, and the results are expressed as fold induction (as defined by the ratio of activity in PMA-stimulated over unstimulated cells). Locations of selected promoter elements are indicated. Open squares, SREs; closed circles, Ets motif; exp., experiment; n.d., not done.

**RESULTS**

Deletional analysis of the egr-1 promoter implicates sequences between -395 and -359 as necessary but not sufficient for induction. The egr-1 gene contains multiple potential regulatory elements within its promoter region (52). These include CRE/AP-1-like motifs, EGR-1-binding sites, and five SREs (Fig. 1) (52). The egr-1 promoter also contains several potential binding sites for Ets family members. The physiological relevance of these sites is difficult to assess, because the consensus binding sequence for Ets family members is highly variable outside the three-nucleotide core GGA (14). Because cooperation between an Ets motif and the SRE in the c-fos...
promoter has been well documented, only Ets motifs within 50 bp of the egr-1 SREs are shown in Fig. 1A. Using 5' deletions of the egr-1 promoter region (Fig. 1), we initiated studies to determine which of the potential transcriptional regulatory elements were necessary for induction following stimulation of nontransformed B lymphocytes. Following a 72-h culture with the polyclonal B-cell mitogen LPS, B cells from murine spleens were transiently transfected with a reporter construct (pBL903) in which 903 bp of the egr-1 5' flanking region was linked to the CAT reporter gene. After transfection, the cells were stimulated with the phorbol ester PMA (10 ng/ml). Enzymatic assay for CAT activity revealed a strong induction following PMA treatment (Fig. 1A, experiments 1 and 2). The maximal response to stimulation varies between experiments, presumably because of variations in the condition of the primary B cells used in these studies. The response seen with pBL903 is similar to what we have previously reported regarding expression of the endogenous egr-1 promoter following PMA treatment or BCR cross-linking (41).

Previous studies have also established that PMA can substitute for the BCR-generated signal with respect to induction of egr-1 transcription (40). Because PMA treatment results in a greater level of egr-1 induction than does BCR cross-linking (40), it was used in many of the studies presented here.

To determine the 5' boundary of the egr-1 promoter region mediating induction in B cells, constructs containing progressive deletions were evaluated for activity. Transfection of a construct containing 395 bp of flanking region (pBL395) yielded activity equal to that seen with pBL903 in both of the experiments shown (Fig. 1A), indicating that sequences 5' to −395 are unnecessary for egr-1 induction in B cells. In contrast, transfection of a construct containing only the egr-1 TATA box region (pBL-ET) revealed no induction. In Fig. 1B, transfections were performed with constructs containing further 5' deletions. Deletion of sequences between −395 and −359 resulted in an 80% inhibition of egr-1 induction (compare pE395 and pE359 in Fig. 1B). A construct containing a promoter fragment extending to only −342 (pBL342) was also inducible (Fig. 1B). The region identified by these deletions (−395 to −359) contains a single SRE (i.e., SRE no. 4) and several potential Ets motifs.

Having implicated egr-1 promoter sequences between −395 and −359 as necessary for induction, studies were carried out to assess whether this region was also sufficient for induction. For this purpose, a CAT construct containing sequences from −395 to −355 linked to the egr-1 TATA region (pBL355) was transiently transfected, and its activity was compared with that of pBL395 (Fig. 2). While pBL355 was marginally inducible, its level of induction was only a fraction (10 to 20%) of that obtained with the most active construct, pBL395. This finding applies to cells stimulated with either PMA (left panel) or by cross-linking of the antigen receptor (right panel). These results imply that, although they are necessary, sequences between −395 and −355 are not sufficient to mediate egr-1 induction.

**Implication of SRE no. 4 in egr-1 induction.** Because constructs containing 5' deletions suggested that SRE no. 4 might play a role in egr-1 induction, a construct carrying point mutations in this element was evaluated for inducibility. Mutations previously demonstrated to inhibit function of the c-fos SRE (18) were introduced into this element (pBL395mSRE4). In experiments comparing the activity of this construct with that of the wild-type construct pBL395, the mutations decreased promoter activity by an average of 84% over the course of several experiments (Fig. 3A and data not shown) when PMA was used as a stimulus. Induction in response to BCR cross-linking was also inhibited to an equivalent degree by this mutation (data not shown), confirming that PMA and receptor-generated signals utilize the same promoter elements.

To determine whether SRE no. 4 was actually unique in its ability to mediate induction or whether the loss of any of the SREs present in pBL395 would result in a loss of induction, further studies were performed. In Fig. 3B, the results indicate that in contrast to SRE no. 4, point mutations in SRE no. 3 fail to decrease maximal induction to either PMA or BCR cross-linking. Because early reports of the egr-1 promoter sequence identified an element at −331 as SRE-like (52), we examined the contribution of this pseudo-SRE (SREα) to induction of egr-1 promoter activity. Like SRE no. 3, mutation of the SREα element had no effect on inducibility to either stimulus (Fig. 3B). This is in keeping with published reports showing failure of this element to bind SRF at detectable levels in fibroblasts (6).

**Internal deletions implic ate sequences between −335 and −274 as necessary but not sufficient for induction of egr-1 promoter.** In order to delineate the 3' boundary of the response element, internal deletions of the egr-1 promoter were made. These constructs were based on pBL395 and had truncations beginning at −45 and extending in the 5' direction as indicated (Fig. 4A). Deletion of sequences between −45 and −253 had little effect on promoter activity (compare pBL395 and pBLΔ253). Similarly, deletion to −274 (pBLΔ274) failed to significantly decrease inducibility. In contrast, deletion of sequences from −45 to −335 (pBLΔ335) reduced activity by approximately 85%, suggesting that elements between −274 and −335 are necessary for egr-1 induction in B cells.

Similar to the results presented above for the region from −395 to −359, the region from −274 to −335 is insufficient to mediate induction when it is cloned upstream of the TATA region. This is evident in the experiment shown in Fig. 4B, in
which two constructs containing overlapping regions of this area were found to be unable to confer inducibility above background levels.

**Analysis of Ets site requirement for egr-1 induction.** Because the results presented above indicate that the region between −335 and −274 is necessary for induction, as is SRE no. 4, we examined this region for elements which might cooperate with SRE no. 4 to confer inducibility. In the c-fos promoter, some stimuli mediate induction through the SRE only when the adjacent Ets motif is functional as well (13). The possibility exists that the loss of an Ets motif at −315 might explain the inhibition seen by internal deletions between −335 and −274. To assess the role of this site in induction, constructs carrying either wild-type (pBLΔ340wtETS) or mutated (pBLΔ340mETS) versions of the site were compared with a parental construct (pBLΔ340), which contained only SRE no. 3 and no. 4 (and the Ets sites upstream of SRE no. 4) (Fig. 5). While the parental construct was only marginally inducible by either PMA (left panel) of BCR cross-linking (right panel), the addition of a wild-type version of the −315 Ets site restored inducibility to both stimuli. In contrast, addition of a mutated version of the −315 Ets motif had no effect.

The region directly 5′ of SRE no. 4 contains an Ets motif at −375 and two overlapping Ets motifs at −385 (52). These elements are in a position similar to the configuration exhibited by the c-fos SRE and Ets motif (50). To assess the role of these elements in mediating egr-1 induction, a construct in which the overlapping sites at −385 were deleted (pBL382)
was compared with the parental construct pBL395 (Fig. 6). The loss of the two Ets motifs had no significant effect on induction by either PMA or BCR cross-linking. In contrast, mutation of the remaining Ets motif at −375 (pBL382mETS) inhibited inducibility by approximately 65% for both stimuli.

SRE no. 5 can mediate induction of egr-1 in the absence of SRE no. 4. The deletional analysis which identified SRE no. 4 as critical for induction was performed in the context of promoter sequences extending only to −395. Because SRE no. 5 is not included in these sequences and because it contains an Ets-binding site immediately 5' of its core (52), we reasoned that in the absence of SRE no. 4, SRE no. 5 might be capable of conferring inducibility. To assess this possibility, the full-length promoter construct pBL903 was modified to include the point mutations introduced in SRE no. 4 as described in the legend to Fig. 3. This construct (pBL903mSRE4) was transfected, and its activity was compared with that of the wild-type, pBL903. In two experiments (Fig. 7), no effect of the mutation was evident. The lack of inhibition was true for both PMA treatment (left panel) and BCR cross-linking (right panel). These results suggest that either SRE no. 4 or SRE no. 5 is sufficient with respect to the SRE components necessary for induction in the context of the full-length egr-1 promoter.

Ternary complex formation at SRE no. 4 in B lymphocytes. At the c-fos SRE, a ternary complex forms between a homodimer of SRF and a second protein, p62TCF (43). p62TCF, an Ets family member, also contacts DNA directly at an Ets motif located 2 bp 5' of the c-fos SRE (43). In most cell types which have been examined, formation of this complex is constitutive rather than inducible (55). However, inducible ternary complex formation has been described for astrocytes, for example (27). Because deletional analysis and point mutations suggested that a combination of SREs and Ets motifs was required for induction of egr-1, studies were designed to assess the nature of the complex(es) formed at these elements by nuclear proteins in B lymphocytes. Nuclear extracts from B lymphocytes were used in an electrophoretic mobility shift assay (Fig. 8). By using the c-fos SRE with its adjacent Ets site for comparison (48), a ternary complex was observed at SRE no. 4 (egr-1 probe [from −395 to −359] in Fig. 8). When the region containing the Ets site was deleted (egr-1 SRE no. 4 probe), only the lower complex, corresponding to SRF homodimer binding, was observed (42, 43). To ensure that the upper complex was actually due to cooperative binding of an Ets family member, competitions with unlabeled oligonucleotides were conducted. The oligonucleotide used for this purpose contained the Drosophila E74A-binding site (2). This site has been shown to compete for p62TCF binding thereby inhibiting ternary complex formation at the c-fos SRE (20, 34).

As is evident in Fig. 8, the upper complex is inhibited by the addition of an unlabeled E74A site, while SRF homodimer binding is unaffected. These results suggest that the upper...
our results implicate an Ets site at −315 as necessary for induction in certain instances.

The reason for preferential utilization of SREs no. 4 and 5 may be inferred from our data demonstrating the importance of the Ets motifs adjacent to these SREs. Using a PCR-based site selection technique, Treisman et al. recently developed a consensus sequence for p62TCF-binding sites which cooperate with SREs to allow ternary complex formation (51). The most preferred consensus contained CGGAA as a nearly invariable core. Only a subset of Ets motifs can, therefore, serve as high-affinity p62TCF-binding sites. When the egr-1 promoter region was examined (52), the site was found to occur in three locations, two of which are the sites immediately 5' of SREs no. 4 and 5. Although several Ets motifs flank SREs no. 1 and 2, these do not match the p62TCF consensus site. The third p62TCF consensus site in the egr-1 promoter is the −315 site which has been identified as critical in this study.

The location of the −315 p62TCF site is unique among the sites identified to date in that it is not contiguous with an SRE. We initially considered the possibility that the −315 site cooperates with the SRE-like element (SRE\(\psi\)) in Fig. 3), centered at −331. In the nur77 promoter, studies have suggested that the interaction of SRF with a degenerate SRE-like element might be stabilized by ternary complex formation (53). However, the SRE-like element in the nur77 promoter contains mutations which are considerably different from that in the SRE\(\psi\) element in the egr-1 promoter. In addition, we have not been able to detect ternary complex formation in our in vitro binding assay using a probe encompassing the SRE\(\psi\) and the −315 Ets element (data not shown). In fact, using anti-SRF antisera, we have been unable to detect binding of any SRF-related proteins to SRE\(\psi\) (data not shown). In support of this finding, Christy and Nathans reported that the SRE\(\psi\) element at −331 was unable to compete for SRF binding to the c-fos SRE (6).

On the basis of these observations, it appears unlikely that the −315 p62TCF motif cooperates with the SRE\(\psi\) element to mediate activation of egr-1. As final proof that SRE\(\psi\) plays no role in egr-1 induction in B cells, point mutation in this element was found to have no effect on inducibility (Fig. 3).

The closest bona fide SRE to the −315 p62TCF site is SRE no. 3, located 27 bp upstream. However, SRE no. 3 does not contribute to induction of egr-1 in B cells, as is evident in Fig. 3B. The reason for the lack of activity by SRE no. 3 remains puzzling, since preliminary in vitro binding studies suggest that it can form a ternary complex with the p62TCF motifs at either −375 or −315 when SRE no. 4 is mutated, as in pBL395SRE4 (29a). It may be that the lack of a p62TCF motif immediately 5' of SRE no. 3 is responsible for its lack of transcriptional activity in vivo.

The results presented here demonstrate that two molecules of p62TCF must be recruited to the egr-1 promoter for efficient induction in B cells. In addition, only three of the Ets motifs in the promoter can serve this high-affinity p62TCF-binding function. The presence of any one of these p62TCF-binding sites alone (with one of the appropriate SREs) is not sufficient to mediate induction. This is demonstrated by the lack of induction with the pE359, pA355, and pBL382mETS constructs. The presence of all three p62TCF sites confers no increase in inducibility beyond that observed with only two sites (compare pBL903 and pBL395 in Fig. 1). The requirement for two p62TCF sites reflects a cooperative rather than an additive effect, since single sites confer negligible inducibility. However, the cooperativity is not at the level of SRF recruitment, on the basis of our in vitro binding studies (29a). Recruitment of two p62TCF molecules into a complex with a single dimer of SRF also appears to be possible (pBL395mSRE3 in Fig. 3B and our

FIG. 8. Electrophoretic mobility shift assay demonstrates the presence of nuclear proteins from B lymphocytes capable of forming ternary complexes at SRE no. 4 in the egr-1 promoter. Nuclear extracts prepare from B lymphocytes which were either unstimulated, treated with anti-\(\mu\), or treated with PMA were tested for binding to three SRE-containing probes. (A) Results obtained with a probe representing egr-1 promoter sequences from −395 to −359. This region contains SRE no. 4 as well as its adjacent Ets motifs. (B) Results in which the probe contained only the core SRE no. 4 sequence from the egr-1 promoter, without any adjacent Ets motifs. (C) Results obtained with a murine c-fos SRE/Ets-containing probe. Competing, unlabeled oligonucleotides corresponding to the E74A-binding site were added to reactions mixtures in 100-fold molar excess (lanes labeled E74) prior to the addition of labeled probe. Complexes corresponding to the reported migration of SRF homodimer (SRF) or ternary complex (SRF/Ets) bound to the c-fos SRE are indicated at the left.

In this study, deleitional analysis and point mutations localize induction of egr-1 in B cells to SRE no. 5 at −400 and SRE no. 4 at −360. Either SRE alone is sufficient for maximal induction by BCR or PMA stimulation. (In the human egr-1 promoter, SRE no. 5 is not conserved [37]; SREs no. 3, 2, and 1 are incapable of mediating induction when they are present either alone or in combination (pE359 and pE342 in Fig. 1, for example). These results are in marked contrast to those of several previous studies of the egr-1 promoter in other cell types which demonstrated relatively equal contributions by all five SREs (1, 6, 8, 10, 33, 35, 36, 39).

The results presented here also implicate Ets motifs in the vicinity of the relevant SREs as critical for induction. This combination of SREs and Ets motifs is similar to that required by many stimuli which induce expression of the c-fos promoter (50). As at the c-fos SRE/Ets site, the egr-1 SRE/Ets sites function as binding sites for a multiprotein complex consisting of an Ets family member and a homodimer of SRF (50). In addition to the Ets motifs immediately 5' of SREs no. 4 and 5,

DISCUSSION

Induction of egr-1 is a ubiquitous correlate of mitogenic stimulation and in many cases also accompanies differentiation (32, 46). Antisense studies with B lymphocytes have established that an activation response to antigen receptor-generated signals requires egr-1 expression (31). The present study was designed to determine the molecular mechanism by which these signals lead to an increase in egr-1 transcription. Although the egr-1 promoter contains five SREs (52), the results presented here indicate that egr-1 induction in B lymphocytes can be mediated by either of two of these elements. In addition, induction requires two adjacent Ets motifs. Our data indicate that the SRE/Ets elements serve as a binding site for a multiprotein complex that is similar to the ternary complex described for the c-fos SRE.

In this study, deleitional analysis and point mutations localize induction of egr-1 in B cells to SRE no. 5 at −400 and SRE no. 4 at −360. Either SRE alone is sufficient for maximal induction by BCR or PMA stimulation. (In the human egr-1 promoter, SRE no. 5 is not conserved [37]; SREs no. 3, 2, and 1 are incapable of mediating induction when they are present either alone or in combination (pE359 and pE342 in Fig. 1, for example). These results are in marked contrast to those of several previous studies of the egr-1 promoter in other cell types which demonstrated relatively equal contributions by all five SREs (1, 6, 8, 10, 33, 35, 36, 39).

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unpublished data). This higher-order complex has not previously been reported for any SRE, perhaps because no other SREs have been identified which have p62TCF-binding sites located both upstream and downstream. However, the appearance of a higher-order complex is not entirely unexpected, since a single SRF dimer does contain two p62TCF interaction domains. The need for two p62TCF molecules may reflect the maximal potential for interaction with the basal transcriptional machinery, which may have a limited number of surfaces available for upstream activating molecules.

Whether transactivation of egr-1 in vivo is mediated by two p62TCF molecules recruited into separate ternary complexes at SREs no. 4 and 5 or whether it is mediated by a higher-order complex involving both upstream and downstream p62TCF molecules at SRE no. 4 is currently unknown and will require the development of more sensitive techniques for examining in vivo complex formation.

It should be noted that the mapping strategy utilized here may have biased our results in favor of the importance of the −315 p62TCF site. By using constructs lacking SRE no. 5 and its adjacent p62TCF motif to define the importance of the −315 site (pBLΔ340, for example), we may have forced the egr-1 promoter to utilize a nonphysiological configuration for recruiting two p62TCF molecules. Further experiments are necessary to determine whether this is the case. However, physiological situations exist which result in egr-1 promoter configurations similar to the pBL395 truncation. For example, as noted above, in the human egr-1 promoter, SRE no. 5 is not conserved (37), perhaps making the presence of the −315 Ets site more important.

The finding that a ternary complex-dependent mechanism mediates induction of egr-1 in B cells has several implications for BCR signal transduction. Regulation of gene expression through the c-fos SRE is potentially controlled by at least three mechanisms (50). One mechanism involves regulation by modification of SRF, a second relies on regulation of p62TCF binding, and the third mechanism involves regulation that is dependent on the postbinding modification of p62TCF by phosphorylation. Induction of egr-1 in B lymphocytes appears to be mediated by the last of these mechanisms. Our results suggest that a ternary complex is present at egr-1 SREs, even in unstimulated cells.

In fibroblasts, the p62TCF component of this constitutively bound complex is inducibly phosphorylated by MAPK, an event which is correlated with increased transcription of c-fos (19, 28). Consistent with this model, BCR cross-linking has been shown to activate the ras/MAPK pathway (4, 11, 15). Furthermore, our results using ras and raf dominant negative expression vectors have indicated that egr-1 induction following BCR cross-linking is indeed dependent on activation of the ras/raf/MAPK pathway (29b). Although this hypothesis predicts that p62TCF should be inducibly phosphorylated in B cells following BCR cross-linking, to date we have not been able to detect this event. These experiments have been hampered by the fact that p62TCF is actually a family of Ets-related proteins and that the identity of the p62TCF expressed in B lymphocytes is currently unknown.

An overview of the findings in this study and others points to a complex network of interactions between different signal transduction pathways and distinct combinations of regulatory elements in the egr-1 promoter. Many stimuli appear to utilize all five SREs and may function through the core motifs, without ternary complex formation (1, 6, 8, 21, 23, 33, 35, 36, 39). Induction through the core SRE sequences without any contribution by p62TCF has been clearly demonstrated for the c-fos SRE (13). In contrast to most studies of egr-1 promoter activity, stimulation of human myeloid leukemia cells (22, 38), T cells (9), and in these reports, B lymphocytes, each requires a unique combination of regulatory elements within the egr-1 promoter. Considered together, these studies establish a mechanism whereby multiple signal transduction pathways converge on a complex promoter to induce transcription of the ubiquitously expressed primary response gene egr-1.

ACKNOWLEDGMENTS

We thank T. Kadesch and V. P. Sukhatme for generously providing plasmids and J. Carman, M. Maltzman, and A. Norvell for helpful comments regarding these studies. S.B.M. was supported by a predoctoral training grant from the NCI. J.G.M. was supported by NIH grant AI25568 and is a scholar of the Leukemia Society of America.

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