 κ B elements strongly activate gene expression in non-lymphoid cells and function synergistically with NF1 elements

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ABSTRACT

κ B elements have been described as lymphoid-specific transcriptional activators. Here we show that κ B elements are able to stimulate expression from test promoters more than 100-fold in T47D and 3T3 non-lymphoid cells. We also demonstrate that nuclear proteins from T47D cells form two prominent complexes with HIV κ B sites. Since the complexes formed in nuclear extracts from T47D and PHA/PMA stimulated Jurkat cells comigrate in polyacrylamide gels, we suggest that the respective binding protein in T47D cells is either NF- κ B or a closely related family member. In addition we provide evidence that NF1 and κ B elements can act synergistically to further increase transcriptional activity.

INTRODUCTION

Protein binding to the sequence GGGACTTTCC in the immunoglobulin κ -chain gene enhancer (1) and the HIV-LTR (2) has been detected in mature B-cells (3) and phorbol ester activated T cells (2) and was correlated with transcriptional stimulation (1, 2). Therefore this sequence and its cognate binding factor(s) (NF- κ B or related proteins) are important to the transcriptional activation of κ -chain genes and the HIV-LTR in their respective target cells. In support of this, a restriction fragment (4) and oligonucleotides (5, 6) containing the κ B sequence activate expression from heterologous promoters only in lymphoid cells. However, the functional presence of the sequence GGGACTTTCC in enhancers of other viral (7–9) and cellular genes (10, 11) and the presence of NF- κ B like DNA-binding activity in activated fibroblasts (12) as well as in other non-activated non-lymphoid cells (13–17) suggests that this regulatory element and its cognate binding factors may be used more broadly in transcriptional regulatory circuits.

Despite data from *in vivo* (1, 2, 4–7, 18, 19) and *in vitro* (8, 9, 13, 15, 20, 21) studies there is no consensus to which extent κ B elements alone are capable of activating gene expression in non-lymphoid cells. Since these studies employed a number of different κ B sites, enhancers and promoters, it seems possible that distinct transcription elements adjacent to κ B sites within a given construct may contribute qualitatively and quantitatively to its activity. For example, the very potent enhancer of the HCMV IE1 gene, which is active in numerous cell types, contains several κ B sites embedded in binding sites for several nuclear proteins, including transcription factor NF1 (8, 9). To better understand the potential role of κ B elements in non-lymphoid cells we studied the transcriptional activities of κ B sites from the HIV-LTR in the context of the promoter of the mouse whey acidic protein (WAP) gene (22). This gene is expressed *in vivo* only in functional mammary cells (23, 24) and contains an array of protein binding sites including some for NF1-like transcription

factors (25, 26). Furthermore, we analyzed the functional interaction of κ B and NF1 elements.

MATERIALS AND METHODS

T4 DNA ligase, T4 polynucleotide kinase, the large fragment of DNA polymerase I (Klenow enzyme) and restriction enzymes were obtained from New England Biolabs, Beverly, MA. Radioisotopes were obtained from Amersham.

Recombinant plasmids

The XhoI-KpnI (−420 to +24), XbaI-KpnI (−88 to +24) and AvalI-KpnI (−34 to +24) fragments spanning the promoter of the mouse WAP gene (22) were linked to the structural gene encoding the bacterial enzyme chloramphenicol acetyl transferase (CAT). Synthetic oligonucleotides containing wild-type or mutant κ B sequences from the HIV-LTR (−104 to −79) (ref. 2 and Fig. 1A) were purchased from Midland Certified Reagent, Midland TX. The synthetic, high affinity binding site for NF1 (TGGCACTGTGCCAA) was described earlier (27). Complementary oligonucleotides were treated with T4 polynucleotide kinase, hybridized, and ligated into recombinant plasmids. κ B sites were cloned into the SstI site at position −354, the XbaI site at position −88 or at position −34 of the WAP gene promoter (Fig. 1B).

Cell culture, transfections and enzymatic assays

BALB/c 3T3 and HeLa cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum and T47D cells were grown in RPMI 1640. Ten μ g of each test plasmid were transfected into HeLa cells and into the mammary epithelial cell line T47D using DEAE-Dextran, and transient expression of the CAT enzyme was used as a measure of transcriptional activity. Acetylated and non-acetylated forms of chloramphenicol were separated using thin-layer chromatography followed by scintillation counting. Transfection into 3T3 cells was accomplished with calcium phosphate. Activities of the recombinant plasmids in T47D and 3T3 cells were expressed in either of two ways; the absolute activity in pmol conversion per mg of cytoplasmic protein per min of incubation at 37°C; or the level of activation of a promoter in the presence of transcription elements. This was possible since the baseline activities of the test promoters in the absence of enhancer elements were at least 5 times over background. Each experiment was performed at least four times.

Mobility shift assays

Nuclear proteins were prepared as describe previously (25, 26). A 30 bp double stranded oligonucleotide (Fig. 1A) which spans HIV enhancer sequences from −104 to −79 and contains SstI sticky ends, and a mutated oligonucleotide in which the G residues necessary for NF- κ B binding (2, 5) had been altered (Fig. 1A) were cloned into the SstI site of pBS (Stratagene). The probes were excised from the plasmids as EcoRI-SstI fragments and labeled with [³²P] α -dATP and the Klenow enzyme. As an NF1 binding site a consensus sequence (TGGCACTGTGCCAA) which binds NF1-like proteins with high affinity (27) was cloned into pBS and excised with EcoRI and HindIII. Probes were incubated with 5 μ g nuclear protein in the presence of 10 μ g poly (dI-dC) as competitor DNA. Both the wild type and mutant NF- κ B oligonucleotides were also used as sequence specific competitor DNAs.

RESULTS

Transcriptional activity of κ B elements

A 30 bp oligonucleotide (Fig. 1A) containing the two κ B sites from the HIV-LTR (2) was synthesized, ligated into a test plasmid (Fig. 1B) containing the promoter of the mouse

Figure 1 Expression plasmids and κ B elements. A) Oligonucleotides containing two wild type (A1) κ B sites from the HIV-LTR and two mutant sites (A2) which do not bind NF- κ B (2). The arrows represent the wild type κ B repeats and the crossed out arrows the mutant repeats. B) Expression plasmids. 1) WAP gene promoter (–450 to +24) linked to the bacterial CAT gene. 2) Oligonucleotide A1 was inserted into the SstI site of the WAP gene promoter. 3) Oligonucleotide A2 was inserted. 4) HIV-LTR from –65 to +80 was linked to the CAT gene. 5) HIV-LTR from –103 to +80.

WAP gene (22), and analyzed for its ability to stimulate gene expression in non-lymphoid cells. This promoter was chosen because it is active *in vivo* only in the functional mammary gland (23, 24) and does not appear to contain elements specifically associated with transcriptional stimulation in the cell types tested. We initially analyzed expression of this hybrid gene in BALB/c 3T3 cells, because previous experiments stated that this cell type was unable to support transcriptional stimulation of the *c-fos* (5) or β -globin promoters (6) by an oligonucleotide containing a κ B sequence (5, 6). Very little conversion of chloramphenicol to its acetylated forms was observed with the plasmid containing the WAP promoter with no inserted oligonucleotide (Fig. 2, lane 1). However, two κ B elements inserted at position –354 of the WAP promoter sharply activated gene expression (Fig. 2, lane 3). A mutant oligonucleotide which does not bind NF- κ B (1, 2, 5) did not activate the WAP promoter significantly (Fig. 2, lane 2), indicating that sequences required for NF- κ B binding are also required for transcriptional stimulation in non-lymphoid cells. The strong activation of the WAP promoter by the κ B elements was also observed in T47D cells (data not shown).

We analyzed the effect of the κ B sites in the context of one of its natural environments, the HIV-LTR. In contrast to the strong stimulatory effect of NF- κ B sites in the WAP promoter, the HIV-LTR deletion mutant –103 containing κ B and Sp1 elements was expressed only two-fold better than the deletion mutant –65 containing two functional

Figure 2 Expression of test plasmids in 3T3 cells. Plasmids containing the WAP and HIV promoters (Fig. 1) and the *c-fos* promoter (5) were transfected into 3T3 cells and CAT activities were assayed after 48 hours followed by thin layer chromatography. Lanes 1–3) CAT activities from plasmids shown in Fig. 1, lanes B1–3. Lanes 4–6) CAT activities from plasmids containing the *c-fos* promoter alone or with κ B elements (5). Lanes 5 and 6) CAT activities from plasmids shown on Fig. 1, lanes B4, 5. The arrow points to the top of the thin layer chromatography plate.

Sp1 sites (Fig. 2, lanes 7 and 8). Locating κ B sites upstream of another heterologous promoter, *c-fos*, resulted in a 5- to 10-fold transcriptional induction (Fig. 2, lanes 3–6).

The magnitude of κ B mediated transcriptional stimulation of the WAP promoter in non-lymphoid cells was unanticipated since κ B binding sites upstream of the *c-fos* (5), β -globin

Figure 3 Expression from the WAP gene promoter in the presence or absence of κ B elements in 3T3 and T47D cells. 48 hours after transfection of test plasmids into cells, Cat activities were determined from 10 μ g of cytoplasmic protein, analyzed by thin layer chromatography and quantitated by excising converted chloramphenicol from thin layer chromatography plates followed by scintillation counting. Compiled data from three experiments (one representative experiment is shown in Fig. 4) are presented as pmol conversion of chloramphenicol per min. per mg of assayed cellular protein. a) WAP promoter from -88 to +24 linked to the CAT gene. b) Oligo A1 (Fig. 1) was linked to the WAP promoter at -88. c) Oligo A2 (Fig. 1). d) the HCMV promoter/enhancer from -1145 to +7 was linked to the CAT gene. The TATA sequence motifs are boxed and the TGGCA motif in the WAP promoter in its inverse orientation is indicated.

Figure 4 Expression of the WAP gene promoter in the presence or absence of κ B elements in 3T3 (A) and T47D (B) cells. The autoradiograph of one experiment outlined in Fig. 3 is shown. The arrows point to the major acetylated forms of chloramphenicol. The activities shown are from the following constructs: a) WAP gene promoter from -88 to $+24$ linked to the CAT gene. b) Oligo A1 (Fig. 1) was linked to the WAP promoter at -88 . c) Oligo A2 (Fig. 1).

(6) or SV40 early promoter (4) were reported as not activating gene expression in non-lymphoid cells. Our experiments in conjunction with published data (2–6) raises the question of whether the position of κ B sites within a given promoter and/or the presence of additional transcription elements modify its activity. Since the WAP promoter is a target for multiple proteins (25), including the transcription factor NF1 (26), we tested whether surrounding sequences contributed to the κ B mediated activity. The WAP promoter was truncated at -88 , ligated to either the wild type or mutated κ B sites and analyzed in 3T3 and T47D cells (Fig. 3 and 4). Most of the high affinity binding sites for nuclear proteins including all but one NF1-like sites were deleted from this construct. In the presence of wild type κ B sites adjacent to one NF1-like element, expression in T47D and 3T3 cells was stimulated at least 200-fold (Fig. 3 and 4).

Cooperativity between κ B and NF1 elements

Since the WAP promoter contains an NF1 element at -80 adjacent to the cloned κ B sites, it seemed possible that the two elements could have acted synergistically to give rise to the high level of transcription. To test the possibility of κ B and NF1 elements acting synergistically, we fused the corresponding binding sites to a WAP gene core promoter (-34 to $+24$) containing a TTTAAA box as the only identified transcription element. Therefore, cooperative interactions of κ B sites with other general transcription elements were excluded to the best of our knowledge. κ B elements alone stimulated transcription in 3T3 and T47D cells when located in proximity of the core promoter at least 100-fold (Fig. 5c). In combination with NF1 elements, which by themselves did not stimulate transcription, an additional 3-fold increase in gene expression was observed (Fig. 5d). Although neither NF1 binding sites nor κ B elements alone stimulated transcription when separated from the WAP promoter by the entire sequence of pBS (3000 bp) (Fig. 5e and f), in combination they stimulated gene expression 9- and 15-fold in 3T3 and T47D cells, respectively (Fig. 5h). This supports the concept that cooperativity between elements is important for gene expression.

κ B elements are recognized by nuclear proteins from T47D cells

We attempted to correlate the transcriptional activity of the HIV κ B elements with the

Figure 5 Activity of the WAP gene core promoter in the absence or presence of NF1 and κ B elements in 3T3 and T47D cells. Cat activities were determined from 50 μ g of cytoplasmic protein and quantitated by excising converted chloramphenicol from thin layer chromatography plates followed by scintillation counting. Compiled data from three experiments are presented as pmol conversion of chloramphenicol per min. per mg of assayed cellular protein. a) the WAP gene core promoter from -34 to +24 was linked to the CAT gene. b-d) the NF1 and κ B elements and a combination of both were linked to position -34 of the WAP gene promoter. e, f, h) the NF1 and κ B elements and a combination of both were linked behind the CAT gene. g) the NF1 site is linked to position -34 of the WAP gene and the κ B elements were linked behind the CAT gene. The three asterisks represent the entire pBS sequence. The activity of the WAP gene core promoter was set arbitrarily as 1 and the induction levels are shown. The autoradiographs show the result of one transfection experiment into T47D cells.

binding of transcription factors and searched for κ B binding proteins in nuclear extracts from T47D cells. Nuclear proteins from T47D cells grown to confluency formed two major complexes with a fragment containing 2 HIV κ B repeats (Fig. 6A, lane f). Unlabeled wild type but not mutant κ B sites (Fig. 1A) competed with the probe for the formation of protein-DNA complexes (Fig. 6A, lanes g and h) indicating that the nuclear proteins from T47D cells contain NF- κ B binding specificity. The two major complexes probably represent binding either to one or to both κ B sites on the probe. NF- κ B binding activity has been shown to be inducible in Jurkat cells by PMA/PHA (2). Similarly, nuclear proteins from PMA/PHA stimulated but not from unstimulated Jurkat cells recognized the HIV κ B sites used in this study (Fig. 6A, lanes b and c). Since the complexes formed in nuclear extract from T47D and stimulated Jurkat cells were of similar, if not identical, mobility it is likely that the binding protein in T47D cells is either NF- κ B or a closely related family member.

Our transfection studies had clearly shown that NF1 and κ B elements can function synergistically suggesting some interaction between the respective transcription factors. In extension to the functional data we also detected strong NF1 binding activity in nuclear extract from T47D cells. The activity, as measured by the binding to a high affinity NF1 binding site (27), was equally strong in unstimulated and stimulated Jurkat cells but most pronounced in T47D cells (Fig. 6B, lanes b to d). In addition to two major complexes, minor species were observed suggesting a certain heterogeneity among NF1 binding proteins.

Figure 6 Binding of nuclear proteins from T47D and Jurkat cells to HIV κ B (A) and NF1 (B) sites. A) Two HIV κ B repeats were incubated with 10 μ g of nuclear proteins from unstimulated (lane b) or PMA/PHA stimulated Jurkat cells (lanes c to e) or T47D cells (lanes f to h) followed by electrophoresis in a 5% low ionic strength polyacrylamide gel. In the competition assays shown in lanes c, d, g and h the extracts were incubated with a 50-fold excess of a wild type (lanes d and g) or mutant κ B element (lanes e and h) prior to adding the radioactively labeled probe. The upper arrows point to the PMA/PHA inducible complexes in Jurkat cells which represents NF- κ B activity (2, 3, 12). The lower arrow indicates the free probe. Lane a shows the probe in the absence of nuclear proteins. Panel A is composed of 2 parts from the same gel. B) A radioactively labeled fragment containing synthetic NF1 binding sites (27) was incubated with nuclear proteins from unstimulated (lane b) or PMA/PHA stimulated (lane c) Jurkat cells or T47D cells followed by electrophoresis. Lane a) probe in the absence of nuclear proteins. The upper arrows point to the major complexes and the lower arrow indicate the free probe.

DISCUSSION

The sequence GGGACTTTCC was described as a κ B element (2, 3) and has been implicated in B-cell specific and phorbol ester inducible gene expression in T-cells and non-lymphoid cells (2–6). Although κ B elements alone function in a lymphoid-specific fashion in the context of some promoters (4–6), the studies presented here demonstrate that they strongly activate gene expression in combination with the WAP promoter in non-lymphoid cells and in the absence of phorbol esters. We suggest that contextual sequences, in particular NF1-like binding sites, and probably additional transcription elements are critical for the strong activity in non-lymphoid cells. Furthermore, in the light of all available information (this paper, 2, 12, 29–32), it is likely that the transcription factor NF- κ B is utilized as a constitutive and/or inducible regulatory element by a large number of lymphoid and non-lymphoid cell types.

A feature of sequences that bind NF- κ B with high affinity is that they are asymmetric in nucleotide sequence (GGGACTTTCC in the HIV-LTR) while the related transcription factor H2TF1 recognizes palindromic sequences (GGGATTCCC in the H-2K promoter) with higher affinity (17, 30). The nuclear protein(s) in T47D cells forms complexes with HIV κ B elements similar to those found with extracts from PMA/PHA stimulated but not from unstimulated Jurkat cells. Since we have not purified the binding protein(s) from T47D cells nor have we analyzed the contact points with the κ B element we cannot exclude the possibility that proteins other than NF- κ B, like H2TF1, KBF1, EBP1 or HIVEN86 may be responsible for the binding and activity observed. However, NF- κ B but not H2TF1 is induced by PHA/PMA suggesting that the binding protein in T47D cells is either NF-

κ B or a member of a protein family with similar DNA binding features. In addition, recent biochemical evidence suggests that there is probably only one NF- κ B protein which can be found in many lymphoid and non-lymphoid cells (30, 31). NF- κ B can exist as an inactive form in non-B-cells (e.g. 12, 30) and its binding activity is inducible by a number of stimuli, including phorbol esters (12), viral infection (30) and cell density. Thus, the high levels of κ B activity in T47D nuclei in the absence of phorbol esters could in part be due to cell density.

There is growing evidence that the sequence context in which regulatory elements are embedded contributes to their activities. For example, dependent on their location NF1 binding sites can support transcription or replication or do not contribute to any measurable activity (33). The magnitude of κ B mediated transcriptional stimulation in 3T3 and T47D cells is also influenced by adjacent promoter sequences. Strong transcriptional stimulation is seen in the context of the WAP and human cytomegalovirus (HCMV) IE1 core promoters (unpublished) but not with the HIV or *c-fos* promoters. Since the HCMV IE1 and the WAP promoters contain high intrinsic activities *in vitro*, it is possible that they respond more vigorously to the activity of control elements than does the HIV promoter which has a lower basic activity. Similarly, transcriptional activity but not binding of the κ B element within the IL-2Ra is susceptible to its sequence environment (32). Moreover, in the context of the WAP promoter κ B sites can function as upstream elements at positions -34, -88 and -354 whereas in the *c-fos* and β -globin promoters κ B sites had to be close to the TATA box in order to activate transcription in myeloma cells (5, 6).

Cooperativity between different transcription elements emerges as a means to modify gene expression (34, 35). Similarly, κ B mediated transcriptional stimulation from the WAP gene core promoter was further increased in the presence of NF1 binding sites, suggesting that these two elements can function synergistically. In particular, when the κ B elements were separated from the WAP gene core promoter by several thousand bp they stimulated gene expression in non-lymphoid cells only in the concomitant presence of adjacent NF1 sites. One operational definition of enhancers, which commonly consist of more than one transcription element (7, 9, 28), is that they stimulate gene expression over large distances. The κ B element can stimulate transcription over a distance of several hundred bp in the context of the IG κ -chain gene enhancer (1) but fails to do so by itself (5, 6). Therefore it appears possible that the long range action of enhancers can be attributed to the combination of individual enhancer elements as exemplified here for κ B and NF1. Support for the concept that NF1-like proteins can naturally function synergistically with κ B elements comes from the HCMV enhancer/promoter in which the two elements are located next to each other (9) and act cooperatively in B-cells and phorbol ester activated T-cells (H. H. Niller and L.H., unpublished). Since we detected exquisitely strong NF1-like binding activity in T47D cells future studies will address the question whether synergism between regulatory elements is the consequence of cooperative protein-DNA interaction or the recruitment of additional transcription factors. For instance, NF1-like proteins may have a general role in transcriptional activation as they can act synergistically with several inducible transcription elements (34-38).

In conjunction with recent work by Lenardo et al. (30) our results shed additional light on the role of NF- κ B in non-lymphoid cells. NF- κ B cannot longer be considered a transcription factor which primarily targets lymphoid-specific genes but rather serves multiple roles in many, if not all, cell types as exemplified by its role in β -interferon gene regulation (30). Specifically its interaction with additional elements within the transcription

complex, as shown here for NF1, may be critical for the diversity of transcription events in non-B cells (reviewed in 31). Finally, our study shows that a de novo arrangement of transcription elements can be useful in identifying functional capabilities of individual elements.

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Note in Proof

After our manuscript had been submitted to *Nucleic Acids Research* on 4/21/89, Lenardo et al. (30, 31) published data demonstrating the functional presence of NF- κ B in mouse fibroblasts.

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