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Identification of a Novel Enhancer Element Mediating Calcium-Dependent Induction of Gene Expression in Response to either Epidermal Growth Factor or Activation of Protein Kinase C

PHILIPPE LENORMAND,[1] DAVID PRIBNOW,[2] KARIN D. RODLAND,[2] AND BRUCE E. MAGUN[2]*

Centre de Biochimie, Centre National de la Recherche Scientifique, Université de Nice, 06034 Nice, France,[1] and Department of Cell Biology and Anatomy, Oregon Health Sciences University, Portland, Oregon 97201-3098[2]

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The VL30 family of defective murine retroviruses consists of 100 to 200 members, of which fewer than 5% appear to be transcriptionally active. A genomic clone of the transcriptionally active VL30 element RVL-3 was identified and sequenced. Genetic analysis indicated that a triple-repeat sequence within the RVL-3 long terminal repeat is capable of functioning as an inducible enhancer element responding to a variety of agonists. In Rat-1 fibroblasts, the ability of the RVL-3 enhancer to mediate induction of gene expression from a heterologous promoter in response to either epidermal growth factor (EGF) or phorbol ester treatment required coelevation of intracellular calcium. Two CArG boxes present in the triple-repeat sequence appeared to exert a negative effect on gene expression, as mutation of these sequences elevated the basal level of expression observed without altering the fold induction in response to either EGF or protein kinase C activation. In the presence of these CArG elements, mutation of AP-1-like sites adjacent to the CArG elements significantly inhibited the ability of either EGF or phorbol esters to induce gene expression. The effect of mutating these AP-1-like sites was relieved by simultaneous mutation of the CArG sites, indicating that interactions among these sites modulate RVL-3 expression. Mutational analysis and gel mobility shift experiments have identified a third sequence within the VL30 triple-repeat element that is required for the induction of gene expression and serves as a binding site for nuclear proteins. Sequence comparisons indicate that this enhancer element has not been described previously.

VL30 elements comprise a family of defective endogenous retroviruslike sequences in the mouse genome. While VL30 elements lack functional *gag* or *env* genes necessary for infectivity, they appear to function as mobile elements capable of inserting into other chromosomal loci (23, 44, 48). These elements frequently contain an array of potent regulatory sequences that mediate positive transcriptional responses to a variety of stimuli (5). VL30 expression in response to either transformation or proliferative stimuli is extremely robust, as indicated by the abundant representation of VL30 clones in subtractive cDNA libraries constructed to identify either transformation-induced or growth-associated genes (7, 12, 43). Transformation of murine fibroblasts in vitro by viruses such as simian virus 40 or by chemical carcinogens such as 3-methylcholanthrene consistently produces a marked elevation in the expression of VL30 mRNA (9, 12, 43). VL30 expression is also inducible by epidermal growth factor (EGF) (15, 36), serum (2, 13), and activation of protein kinase C (PKC) (7, 36) and by combined treatment with glucocorticoids and cyclic AMP (19). Transcription of VL30 elements occurs within 5 min of stimulation by either EGF or 12-*O*-tetradecanoyl-13-phorbol acetate (TPA) and does not require protein synthesis (36).

To simplify the analysis of VL30 regulation, we have transferred single mouse VL30 elements into the rat genome, using a pseudotype-mediated transfection process (35). The single mouse VL30 element introduced into the Rat-1 line (called RVL-3) displays transcriptional induction of VL30 RNA within 3 min of treatment with either EGF or the tumor promoter TPA. Induction by EGF is unaffected by depletion of PKC activity, indicating that both PKC-dependent and PKC-independent pathways are capable of inducing VL30 (37). We have also shown that this VL30 element can be induced in RVL-3 cells by elevated intracellular Ca^{2+}, either in response to the calcium ionophore A23187 (38) or in response to Ca^{2+}-elevating agonists such as endothelin-1 (28). This induction of VL30 by intracellular Ca^{2+} displays a threshold effect; at intracellular [Ca^{2+}] below 165 nM, VL30 expression is minimal, while at concentrations above 200 nM, VL30 is strongly induced (38).

To identify the sequence elements in the VL30 long terminal repeat (LTR) responsible for regulating transcription in response to these signaling pathways, we have conducted a structure-function analysis of the RVL-3 LTR. In this report, we have identified a triple-repeat sequence within the RVL-3 LTR that mediates responses to serum, EGF, and TPA (24). The ability of TPA to induce expression from the VL30 triple repeat element is weak compared with that of serum, but induction by TPA is greatly augmented by simultaneous exposure of the cells to thapsigargin, an elevator of intracellular Ca^{2+} (24). This synergistic interaction between two distinct signaling pathways activated by tumor-promoting agents suggests that the VL30 LTR may serve as a useful model system for addressing how multiple signal transduction pathways mediate the transcriptional events associated with tumor promotion. Moreover, a similar synergistic interaction is observed between EGF and elevated Ca^{2+}. In this report, we have analyzed the respective contributions of three subsequences within the VL30 enhancer to the induction of VL30 expression in response to either EGF stimulation or activation of PKC by TPA.

* Corresponding author.

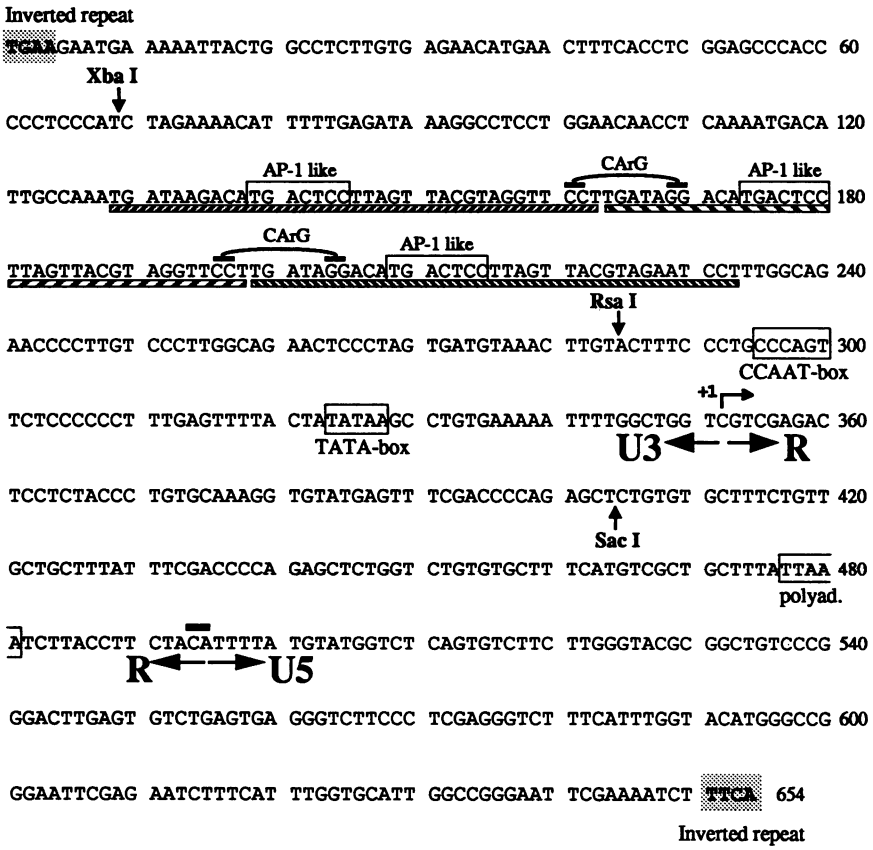

FIG. 1. Sequence of the RVL-3 LTR. The sequence of each 35-bp repeat is underlined by a unique hatched box. The inverted repeat at each end of the LTR is highlighted, and the sequences of the putative polyadenylation site, CCAAT box, TATA box, and AP-1-like site are enclosed in boxes. The sequence of the CArG box is indicated by the two linked black boxes. The recognition sites for the restriction enzymes *Xba*I, *Rsa*I, and *Sac*I are indicated by vertical arrows. The junctions between the structural parts of the LTR are indicated by horizontal opposing arrows. The start point of transcription (+1) is located at the boundary between the U3 and R segments at position 352.

MATERIALS AND METHODS

Cell lines. Rat-1 cells (32) and stably transformed Rat-1 derivatives were maintained in Dulbecco's modified Eagle medium (DMEM) plus 10% defined calf serum (HyClone) in a 37°C, 95% air–5% CO_2 incubator. Cultures were supplemented with 10 μg each of gentamicin and amphotericin B (Fungizone; Sigma) per ml. Stably transfected clonal cell lines were selected and cultured in the presence of 750 μg of G418 (GIBCO) per ml.

Isolation and sequencing of a genomic clone containing the RVL-3 LTR. A genomic clone of the RVL-3 VL30 element was obtained from high-molecular-weight DNA prepared from the RVL-3 cell line subjected to partial restriction endonuclease digestion with *Bam*HI. Fragments of approximately 12 to 15 kbp were isolated and ligated to EMBL3 arms (Stratagene) and packaged by using Gigapak Gold extracts (Stratagene). The primary library contained approximately 1.2×10^6 independent clones, about half of which were actually recombinant. VL30-containing clones were identified by hybridization of plaque lifts against an 833-bp fragment of the BVL-1 VL30 LTR (20) labeled with [^{32}P]dCTP by nick translation to a specific activity of 8×10^7 dpm/μg. A 5.75-kb *Pst*I fragment which hybridized strongly to the BVL-1 LTR fragment used for plaque selection was subcloned into the *Pst*I site of pGEM4Z (Promega) to create plasmid pRVL3-B6. Sequence data were generated from both strands of the entire LTR according to the method of Sanger et al. (39) and assembled into an unambiguous continuous sequence comprising 2,362 bp, using the GENSEQ program available on the BIONET time share network (Intelligenetics). Regions of sequence similarity were identified by using the FASTA paradigm of Pearson and Lipman (31).

Use of PCR for cloning the VL30 triple repeat. To produce DNA segments containing the desired promoter and enhancer sequences with flanking restriction sites suitable for cloning into the pCAT-BASIC vector, the polymerase chain reaction (PCR) was performed with synthetic oligonucleotide primers, using standard PCR conditions. Sequencing of the resulting fragments after cloning into the chloramphenicol acetyltransferase (CAT) expression vectors was performed to verify that the constructs contained the expected sequences. Primers used were as follows: for the thymidine kinase (TK) promoter (126 bp from positions −109 to +17 [10]), 5′-GTATATCCTGCAGCAAACCCCGCCCAGCGTC TTGT-3′ (upstream) and 5′-GTATACTCTAGAGCAGGGT CGCTCGGTGTTCGA-3′ (downstream); for the VL30 promoter (107 bp from positions 257 to 343 [Fig. 1]), 5′-GTATA CCTGCAGAACTCCCTAGTGATGT-3′ (upstream) and 5′-GTATACACTAGTGGAGTCTCGACGACCAGC-3′ (downstream); and for the VL30 enhancer, 5′-GTCAACAAGC

TTGCCAAATGATAA-3′ (upstream) and 5′-GTTGACGTC GACCTGCAGTTCTGCCAAAGGATT-3′ (downstream).

Plasmids and synthetic oligonucleotides. The expression vectors pTES and pTEN were generously donated by P. Mellon (pTES was referred to as *tkcat* 3 in reference 10). Plasmids pGEM3Z, pGEM4Z, pCAT-BASIC, and pGLCONTROL were purchased from Promega. Because efficient site-directed mutagenesis of repeated subregions of the enhancer would have been problematic, we chose to synthesize large oligonucleotides which encompassed each mutated triple-repeat sequence and to introduce these into the vector pCBTK (pCAT-BASIC, with the addition of the herpes simplex TK promoter). Oligonucleotides were assembled on a Milligen/Biosearch Cyclone DNA synthesizer. The sequences of the synthetic oligonucleotides used to produce mutant triple repeats are illustrated in Fig. 4; the A, B, and C domains were each mutated at three nucleotide positions as indicated in the figure. After cloning into pCBTK, the mutant sequences were verified by direct sequencing.

Transfection of Rat-1 cells. (i) Transient transfections. Rat-1 cells grown to 80% confluency were transfected by two methods: the $CaPO_4$ method of Gorman et al. (17) and the Lipofectin method of Felgner et al. (14). Cells transfected with $CaPO_4$ were exposed to DNA-$CaPO_4$ for 4 h, subjected to glycerol shock for 2 min, and allowed to recover in 10% calf serum (HyClone) for 12 h. Following recovery, the cells were serum deprived for 3 days before exposure to experimental agonists for 4 h. Cells transfected with Lipofectin were exposed to 1 ml of the DNA-Lipofectin mixture (at a ratio of 5 μg of DNA to 20 μl of Lipofectin) for 4 h. Five milliliters of DMEM was then added to the plate, and the cells were exposed to agonists either immediately or 24 h later as described in the figure legends.

(ii) Stable transfections. The CAT expression vectors were mixed with the selective plasmid pk0-neo (47) at a 100-fold molar excess of the CAT expression vector. The cells were transfected by the $CaPO_4$ precipitation method described above. The cells were grown to near confluency in DMEM supplemented with 10% defined calf serum and split at a ratio of 1 to 10, and the selective antibiotic G418 (GIBCO) was added to the culture medium at a concentration of 750 μg/ml for approximately 2 weeks. Independent clonal colonies were isolated; approximately 40% of these expressed the CAT protein at high levels upon growth in 10% defined calf serum.

Measurement of CAT activity. Cellular proteins were extracted essentially as described by Gorman et al. (17), and the cell extracts were incubated for 10 min at 70°C to inhibit cellular acetylases. CAT activity was measured by the two-phase CAT assay of Neumann et al. (29). When the basal-level CAT expression of several plasmids was compared, plasmid pGLCONTROL (Promega) was added during the transfection and luciferase expression was used to normalize the transfection efficiency. Luciferase expression was measured in a Packard Luminometer essentially as described previously (1).

Mobility shift assays. Nuclear extracts from Rat-1 fibroblasts were prepared essentially as described by Dignam et al. (11) and frozen in aliquots. DNAs used in binding experiments were either synthetic oligonucleotides (30J and consensus AP-1) or appropriate restriction fragments purified by electrophoresis from agarose gels onto NA-45 membranes and recovered by elution and ethanol precipitation as described previously (4). DNA concentrations were estimated from relative ethidium bromide intensity following agarose gel electrophoresis. Sequences of the 30J, 2RJ, 1R, and 3R DNA fragments and their mutant derivatives are indicated in Fig. 4. The AP-1 and 30J synthetic oligonucleotides were annealed in vitro to produce >90% duplex form as indicated by comparative acrylamide gel electrophoresis of native and denatured samples. DNA fragments used as probes were end labeled to >90% efficiency by Klenow polymerase-directed fill-in reactions including [^{32}P]dCTP. Electrophoretic mobility shift assays were conducted essentially as described by Singh et al. (42), with the following modifications. Binding of nuclear extracts (5 μg) was conducted for 10 min at 0°C in 20 μl of binding buffer (10 mM Tris-HCl [pH 7.9], 15 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, 10% glycerol) in the presence of 1 μg of poly(dI-dC), 0.5 μg of denatured salmon sperm DNA, and 200 μg of bovine serum albumin per ml, with or without added competitor DNA. Labeled probe DNA was then added at a final concentration of 0.2 nM, and incubation at 0°C continued for 20 min. Dye was added, and samples were loaded immediately onto 5% (80:1) acrylamide–0.5× Tris-borate-EDTA gels containing 3% glycerol. Electrophoresis was conducted at 4°C with a field strength of 8 V/cm for 3 to 4 h. DNA-protein bands were visualized by autoradiography of dried gels.

Reagents. Thapsigargin (LC Services, Woburn, Mass.) was dissolved in dimethyl sulfoxide and stored at −20°C prior to use. TPA and A23187 were obtained from Sigma and were dissolved in dimethyl sulfoxide. EGF was purified in our laboratory as previously described (25, 40).

RESULTS

Sequence analysis of the RVL-3 VL30 LTR. Alignment of the nucleotide sequence of the RVL-3 LTR with those of previously sequenced VL30 LTRs (e.g., those described in references 7, 20, 21, and 30) showed clustered regions of sequence identity throughout the LTR. On the basis of these sequence comparisons, we located the boundaries of the U3, R, and U5 segments of the RVL-3 LTR within the 654-bp RVL-3 LTR, as diagrammed in Fig. 1.

The promoter region of LTRs is generally associated with the U3 segment immediately upstream of the R segment, which begins at the site of transcriptional initiation. We assigned putative CCAAT, TATAA, and start sites by comparison with the NVL-3 LTR (30); the putative TATA box (TATAA [22]) is located between nucleotides (nt) 322 and 328 (Fig. 1). A sequence homologous to CTF/NF-1 binding sites (GCCCAGT [22]) is located 21 nt upstream from the TATA box. Interestingly, 60 nt upstream of the putative CCAAT-box there are three tandemly repeated 35-bp sequences. The repeat sequences represent the most prominent feature of the U3 region of the RVL-3 LTR and share sequence similarities with other known enhancer sequences: each 35-bp element contains a heptad nucleotide sequence, TGACTCC, which is nearly identical to the consensus AP-1 binding site TGA(G/C)TCA (3). AP-1 binding sites have been shown to convey transcriptional induction elicited by pkC activation in several other genes (3, 33). Additionally, the boundaries between the adjacent repeats engender two decanucleotide sequences CCTTGATAGG which resemble the core sequence of a dyad symmetry element present within the c-*fos* gene (CCATATTAGG [16]). This core sequence, referred to as a CArG element, was initially identified in the promoter region of actin genes (26). Although the VL30 CArG element (CCTTGATAGG) differs from the consensus sequence of CArG elements in that a single G is present within the A/T-rich central se-

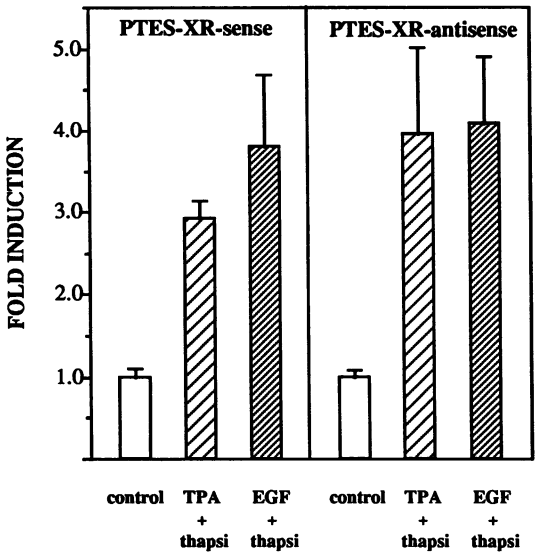

FIG. 2. CAT expression in Rat-1 cells transfected transiently with the vectors pTES-XR-sense and pTES-XR-antisense. Rat-1 cells were transfected by the $CaPO_4$ method as described in Materials and Methods (5 μg of plasmid DNA was transfected per 6-cm plate). Following the glycerol shock, cells were allowed to recover in DMEM plus 10% HyClone bovine calf serum for 12 h before being serum deprived for 72 h. Cells were then treated with the agonists for 4 h prior to harvesting. The cells were stimulated either with no addition (control), thapsigargin (thapsi; 0.5 μM) plus EGF (10 ng/ml), or thapsigargin (0.5 μM) plus TPA (100 ng/ml) for 4 h prior to harvesting. Cells were harvested, and CAT activity was measured as described in Materials and Methods. Each column represents the mean ± standard deviation of CAT activity from four independent samples, expressed as fold induction over the unstimulated controls. The results shown are representative of at least three qualitatively similar experiments. CAT activity was calculated from the slope observed during the linear phase of the conversion reaction, expressed as counts per minute of [^{3}H]acetyl coenzyme A converted per hour per 40 μg of protein.

quence, the distal CArG box of the cardiac actin gene also contains a single G and was shown to have activity comparable to that of canonical CArG elements (18, 27).

The triple repeat of the RVL-3 LTR acts in an orientation-independent fashion. We have previously demonstrated that an *Xba*I-*Sac*I fragment of the VL30 LTR (comprising nt 69 to 404; Fig. 1) can mediate induction in response to EGF, TPA, or serum when introduced into a promoterless expression vector containing a CAT reporter gene (24). This 335-bp *Xba*I-*Sac*I restriction fragment encompasses the three 35-bp repeats, as well as the putative CCAAT and TATAA boxes and transcriptional start point of the VL30 promoter.

We have previously described the responsiveness of a 224-bp *Xba*I-*Rsa*I fragment of VL30 (nt 69 to 283) cloned in the sense orientation into the pTES vector (10), which contains a CAT reporter gene driven by a TK promoter. This pTES-XR-sense construct showed an induction of CAT activity in response to both EGF and TPA in the presence of elevated intracellular Ca^{2+} (Fig. 2) (24). Interestingly, while either EGF or TPA alone was sufficient to induce CAT expression from the 335-bp *Xba*I-*Sac*I fragment bearing the RVL-3 CCAAT, TATAA, and start point sequences (24), coelevation of intracellular Ca^{2+} was required for substantial induction from the smaller *Xba*I-*Rsa*I fragment fused to the TK promoter. In this experiment and in other experiments described in this report, we have used the sesqueterpene lactone thapsigargin to elevate intracellular Ca^{2+} levels. Thapsigargin acts as an inhibitor of the Ca^{2+} ATPase responsible for reuptake of Ca^{2+} into intracellular vesicles, so that thapsigargin treatment results in the release of Ca^{2+} from inositol trisphosphate-sensitive and -insensitive stores (45).

To test whether DNA fragments containing the triple-repeat sequence can function in an orientation-independent manner, we cloned the *Xba*I-*Rsa*I fragment into the pTES vector in the reverse orientation (pTES-XR-antisense). Induction of CAT expression in response to either EGF plus thapsigargin or TPA plus thapsigargin was obtained from cells transfected by either the pTES-XR-sense or pTES-XR-antisense vectors (Fig. 2). When these agonists were tested singly, neither EGF, TPA, nor thapsigargin was able to induce significant increases in CAT expression from these plasmids (reference 24 and data not shown).

The enhancer activity of the triple repeat is promoter independent. To determine whether the Ca^{2+}-dependent gene expression observed with pTES-XR-sense was a result of substituting the TK promoter for the VL30 promoter, we subcloned 123 bp containing the entire triple-repeat region (nt 121 to 243, Fig. 1) into the multiple cloning site of the vector pCAT-BASIC as described in Materials and Methods. The triple-repeat sequence was placed immediately adjacent to and upstream of the appropriate promoter from either VL30 (nt 257 to 343; Fig. 1) or TK (nt −109 to +17 [10]). The vector containing both the triple-repeat sequence and the VL30 promoter is referred to as pCBVL-3R; the analogous vector containing the TK promoter is called pCBTK-3R. Plasmid pCBVL-3R differs from the *Xba*I-*Sac*I LTR plasmid described in reference 24 in the omission of the 52 nt adjacent to the *Xba*I site at the 5′ end and the 33 bp adjacent to the *Sac*I site at the 3′ end.

Since stably transfected cells could be serum deprived for prolonged periods of time to yield very low endogenous levels of second messengers characteristic of quiescent cells, we established stable Rat-1-derived cell lines containing the plasmids of interest. The results presented in Fig. 3 are representative of those obtained with 12 independently isolated cell lines stably transfected by either pCBVL-3R or pCBTK-3R. These results indicate that the triple-repeat sequence was able to confer induction of CAT expression when cloned upstream of either the TK promoter or the VL promoter. Furthermore, these results demonstrated that for constructs containing either the TK or VL promoter, the elevation of intracellular Ca^{2+} by thapsigargin was required in order for cells to respond abundantly to either EGF or TPA.

Mutational analysis. We originally chose to test the effects of mutations within the A and C domains indicated in Fig. 4 because of the significant sequence similarity between these domains and two well-characterized enhancer elements. The A domain contains a sequence nearly identical to that of the TRE/AP-1 site described by Angel et al. (3); furthermore, the TGACTCC sequence present in the RVL-3 LTR has been implicated as contributing to basal transcription in the JE gene (46). The C domains contain a CArG box similar in sequence to the most distal CArG element present in the cardiac actin gene (26). As described in Materials and Methods, we used synthetic oligonucleotides amplified by PCR and introduced into the vector pCBTK to produce the CAT expression vectors for these experiments. The plasmids harboring each of the mutant oligonucleotides (designated Am and Cm for A-domain and C-domain mutants) differed from the plasmid harboring the wild-type triple-

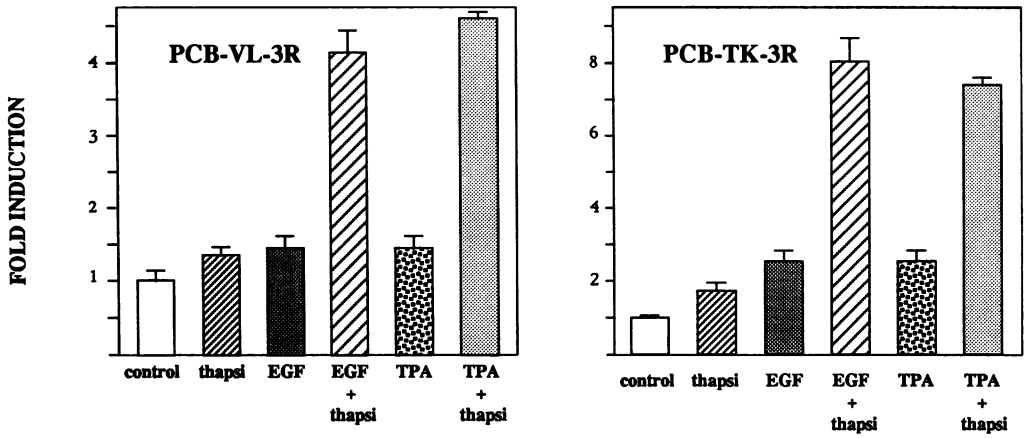

FIG. 3. Induction of CAT expression from the plasmids pCBVL-3R and pCBTK-3R following stable transfection. Cell lines were stably transfected by plasmids pCBVL-3R and pCBTK-3R as described in Materials and Methods. Columns display the induction of CAT expression from one clonal cell line transfected by each plasmid. Similar results were obtained for a great majority of the 15 clonal lines examined for each transfection. The stable clones grown to confluency were serum deprived for 72 h and then treated with the agonists for 4 h prior to harvesting. The cells were stimulated either with no addition (control), with thapsigargin (thapsi; 0.5 μM), with EGF (10 ng/ml) alone or plus thapsigargin (0.5 μM), or with TPA (100 ng/ml) alone or plus thapsigargin (0.5 μM) for 4 h prior to harvesting. Cells were harvested, and CAT activity was measured as described in Materials and Methods. CAT activity was calculated from the slope observed during the linear phase of the conversion reaction, expressed as counts per minute of [^{3}H]acetyl coenzyme A converted per hour per 40 μg of protein. Each column represents the mean ± standard deviation of the fold induction observed in three independent samples, such that 1 = the mean CAT activity observed with each promoter in unstimulated cells.

repeat sequence only at the indicated sites (Fig. 4), as verified by direct sequencing. The A and C domains were each mutated at three nucleotide positions that have been deemed likely to disrupt the function of consensus AP-1 and CArG sites (8, 26).

Figure 5A illustrates the unstimulated and induced levels of CAT expression observed following transient transfection of Rat-1 cells with either the control or mutated plasmids depicted in Fig. 4. When serum-deprived cells containing the wild-type triple repeat (pCBTK-3R) were stimulated by treatment with EGF and thapsigargin, a consistent 3.5- to 6-fold increase in CAT expression was observed. Mutation of the A domain in pCBTK-3R-Am inhibited induction of CAT expression. While mutation of the C domain in pCBTK-3R-Cm appeared to elevate the basal level of CAT expression in unstimulated cells, the CAT activity observed following agonist stimulation was correspondingly increased, resulting in the same fold induction as was observed with the wild-type enhancer. When both the A and C domains were mutated in plasmid pCBTK-3R-AmCm, a dramatic increase in CAT activity was still observed following cotreatment with EGF and thapsigargin. This result suggests that the inhibitory effect of mutations in the A domain of pCBTK-3R-Am could be relieved by simultaneous mutation of the C domain in plasmid pCBTK-3R-AmCm. Similar results were obtained when TPA was used as an agonist instead of EGF (data not shown; see Fig. 8). Neither TPA alone, EGF alone, nor thapsigargin alone was able to induce significant CAT expression from any of the wild-type or mutant 3R plasmids (data not shown).

To extend and verify the results obtained with mutant enhancers in transient transfection experiments, we tested cell lines produced by stable transfection of the mutated plasmids for the ability to induce CAT expression following EGF and thapsigargin cotreatment. At least 12 independent cell lines were tested for each construct. Figure 5B illustrates the wide range in fold induction of CAT expression observed in independent cell lines transfected by a given

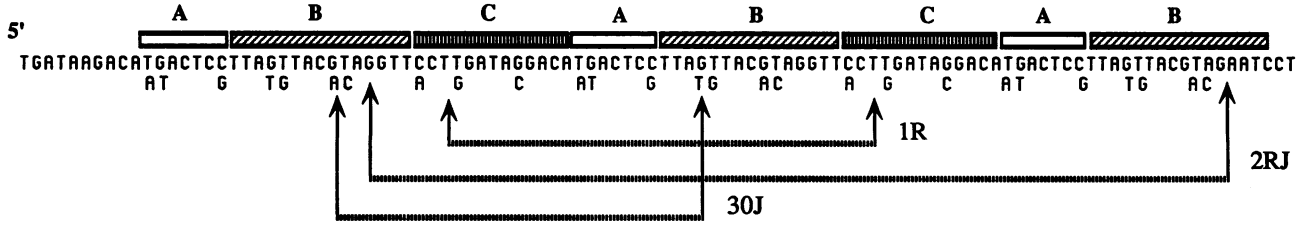

FIG. 4. Diagram of the VL30 triple repeat and associated mutations. The sequence of the wild-type triple-repeat oligonucleotide is shown, with the nucleotide substitution present in each mutation displayed beneath. The three subsequences identified as the A, B, and C domains are indicated by boxes above the sequence. Four long oligonucleotides were made: one with mutations in the A domain (TK-3R-Am), a second with mutations in the B domain (TK-3R-Bm), a third with mutations in the C domain (TK-3R-Cm), and a fourth with mutations in both the A and C domains (TK-3R-AmCm). Arrows beneath the nucleotide sequence represent the oligonucleotides used as competitors in the gel mobility shift assays (1R, 2RJ, and 30J).

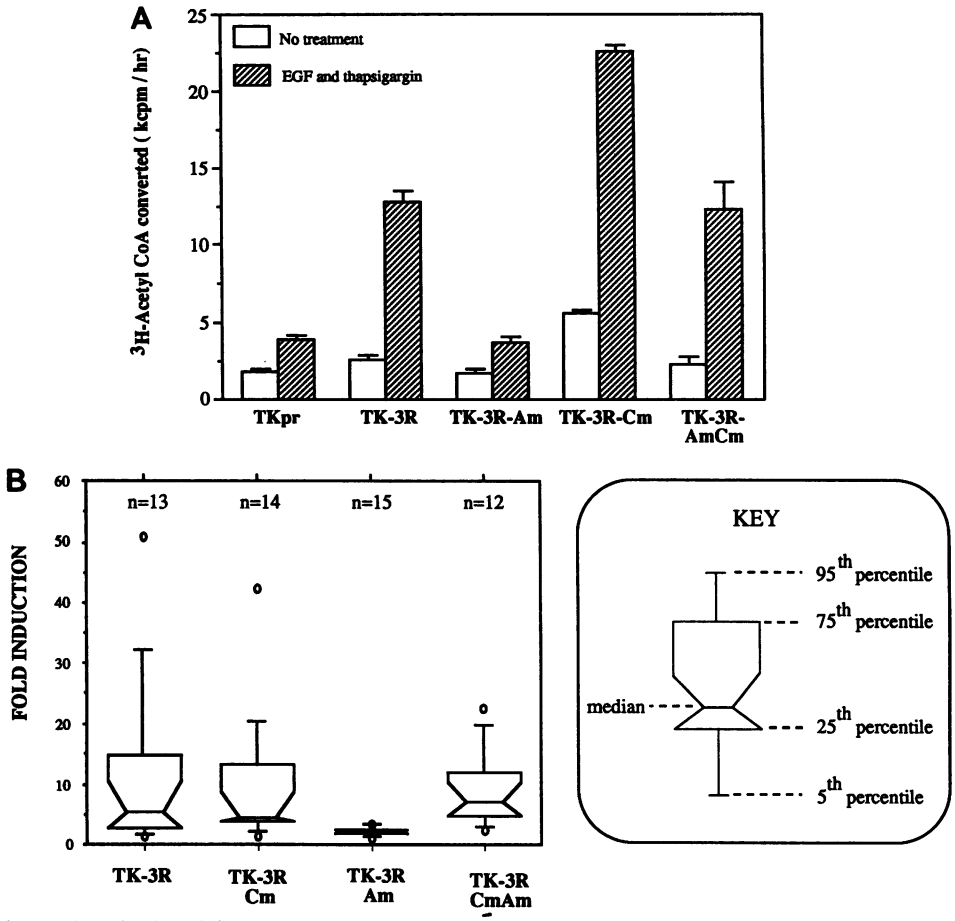

FIG. 5. Effects of mutations in the triple repeat on induction of CAT expression in response to EGF plus thapsigargin. (A) Transient transfections. Rat-1 cells were transfected by the $CaPO_4$ method as described in Materials and Methods (5 μg of plasmid DNA was transfected per 6-cm plate). Cells were allowed to recover in DMEM with 10% HyClone bovine calf serum for 12 h before being serum deprived for 72 h; cells were then treated either with no additions or with EGF (10 ng/ml) and thapsigargin (0.5 μM) for 4 h prior to harvesting. The plasmids transfected were pCBTKpr (enhancerless), pCBTK-3R, pCBTK-3R-Am, pCBTK-3R-Cm, and pCBTK-3R-AmCm. Cells were harvested, and CAT activity was measured as described for Fig. 2. Each column represents the mean ± standard deviation of CAT activity of four independent samples. CoA, coenzyme A. (B) Stable clones. Stable clones were obtained by cotransfection of the CAT expression vector with the selective plasmid pk0-neo as described in Materials and Methods, followed by selection in G418 (750 μg/ml). Independent colonies were isolated by trypsinization in cloning rings. The stimulation of CAT expression following EGF (10 ng/ml) plus thapsigargin (0.5 μM) was measured for a number of clones stably transfected by plasmids pCBTK-3R, pCBTK-3R-Am, pCBTK-3R-Cm, and pCBTK-3R-AmCm. Above each column is displayed the number of independently isolated clones examined. Results are expressed as a histogram of the fold induction in the presence of EGF plus thapsigargin compared with results for unstimulated cells. The histograms indicate the percentiles of the distribution as described in the adjacent key. The open circles represent the full range of observed inductions.

plasmid. For example, cell clones stably transfected with plasmid pCBTK-3R showed induction of CAT expression ranging from 2- to 51-fold. Nonetheless, significant differences in the distribution pattern of CAT induction were observed between the groups of clones transfected with each plasmid. The cell lines transfected with the plasmid harboring mutations in the A domain (TK-3R Am) displayed a distribution clustered around twofold induction, with no values exceeding fourfold. In contrast, the median of the distribution was fivefold induction for the plasmid with the wild-type triple repeat, fourfold for the plasmid with the C domain mutated (TK-3R Cm), and sevenfold for the plasmid with both the A and C domains mutated (TK-3R AmCm). Furthermore, cell lines transfected with the plasmid harboring the AP-1-like mutated sites (pCBTK-3R-Am) never showed induction of CAT expression exceeding 4-fold, whereas 25% of the cell lines transfected with the wild-type triple repeat displayed induction over 15-fold, and 25% of the cell lines transfected with plasmids pCBTK-3R-Cm and pCBTK-3R-AmCm were induced over 13- and 12-fold, respectively. Thus, the results of the stable transfection assays confirmed the results obtained with transient transfections. Furthermore, the observed induction of CAT expression from TK-3R AmCm in response to both EGF-thapsigargin and TPA-thapsigargin provides strong evidence for the presence of a unique inducible enhancer element whose function is unaffected by either the A or C mutations.

Protein binding to the VL30 triple repeat. As a further step in identifying the sequence elements responsible for modulating the level of CAT expression in response to treatment with either EGF-thapsigargin or TPA-thapsigargin, we performed a series of electrophoretic mobility shift assays using

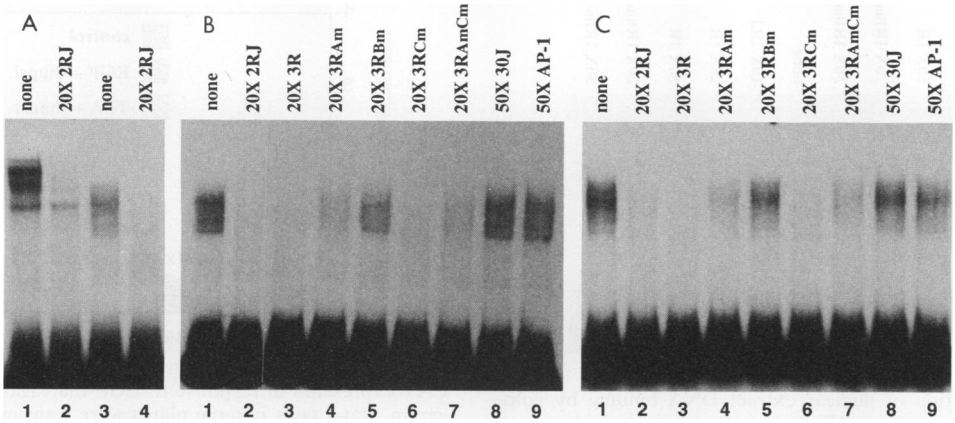

FIG. 6. Binding of nuclear proteins from Rat-1 fibroblasts to subregions of the VL30 enhancer. (A) Specific enhancer complexes from bovine calf serum (lanes 1 and 2)-treated or from serum-deprived (lanes 3 and 4) cells were revealed by DNA mobility shift analysis performed as described in Materials and Methods. Five micrograms of crude nuclear extract was incubated with ^{32}P-end-labeled 2RJ probe for 20 min at 0°C, with or without prior addition of unlabeled competitor at a molar excess over probe as indicated. (B and C) Specific complexes from cells treated for 30 min with EGF and thapsigargin (B) or with TPA and thapsigargin (C) were competed for by the indicated DNAs, including wild-type and mutant versions of the triple-repeat sequence (lanes 3 to 7). Mobility shifts were conducted as for panel A. The TRE/AP-1 sequence is 5′-GACATGACTCATTAG-3′ (plus its complement); nucleotide sequences of the probe and other competitors are shown in Fig. 4.

nuclear protein extracts from serum-deprived and stimulated Rat-1 cells. The DNA probe used for these experiments, designated 2RJ, was chosen to contain two repeats of a 35-bp sequence beginning immediately before the CArG sequence as depicted in Fig. 4.

Nuclear extracts obtained from Rat-1 cells which had been either grown continuously in the presence of serum or serum deprived for 72 h contained proteins capable of binding to the 2RJ fragment (Fig. 6A). As expected, both the 2RJ fragment itself and the wild-type triple repeat (3R) were effective competitors, virtually eliminating protein binding to radiolabeled 2RJ fragments when added at a 20-fold excess (Fig. 6A, lanes 2 and 4; Fig. 6B and C, lanes 2 and 3). Interestingly, the apparent size of the DNA-protein complexes differed significantly and reproducibly as a function of cell treatment.

The specificity of the protein-DNA binding interaction was used to assist in the identification of sequence elements contributing to the *trans* activation of gene expression by comparing the abilities of various mutant sequences to compete for binding to the 2RJ fragment. Triple repeats containing mutations only in the CArG sites (3R-Cm) competed approximately as well as did the wild type (Fig. 6B and C, lanes 6), indicating that the CArG mutations did not prevent protein binding. Triple repeats containing mutations in the three AP-1-like sequences (3R-Am and 3R-AmCm) were slightly less effective as competitors (Fig. 6B and C, lanes 4 and 7). Interestingly, a synthetic oligonucleotide containing a consensus AP-1 sequence (TGAGTCA) which was capable of binding six different familial Fos-Jun heterodimers produced by in vitro translation (data not shown) failed to compete with 2RJ for protein binding (Fig. 6B and C, lanes 9). This result suggests that members of the Fos-Jun family of transcription factors were not responsible for the observed binding. Fos-Jun proteins appeared to be present in the serum-treated Rat-1 extracts, as a DNA-protein complex of appropriate mobility was formed when the consensus AP-1 oligonucleotide was incubated with Rat-1 extracts (data not shown). Furthermore, 30J, a 30-bp synthetic oligonucleotide designed to contain one iteration of the C and A domains (CArG box and AP-1-like sequences; Fig. 4) and little else, also failed to compete measurably for protein binding to the 2RJ probe, suggesting that neither of these subsequences is sufficient for complex formation. By elimination, the region between the A and C domains (designated as the B domain) was suspected of harboring base pairs critical for complex formation and, by inference, conferring inducibility.

To test this possibility, we constructed a synthetic triple repeat containing clustered mutations within each B domain (3R-Bm; Fig. 4). The ability of the 3R-Bm triple repeat to compete effectively for complex formation was tested in comparison with the other mutated versions of the triple repeat. 3R-Bm DNA failed to compete with 2RJ DNA for protein binding whether extracts from EGF-thapsigargin- or TPA-thapsigargin-treated cells were used (Fig. 6B and C, lanes 5). Similar results were obtained in mobility shift experiments using nuclear extracts from serum-treated and serum-deprived cells (data not shown).

The complexity of the VL30 triple repeat provides the opportunity for cooperative interactions among both distinct enhancer sequences and repeated copies of the same sequence. We therefore felt that it was important to determine the binding properties of a single-repeat element that contained the B domain, whose integrity was necessary for protein binding, as demonstrated in the preceding experiments. As shown in Fig. 7, the wild-type single-repeat element (1R; Fig. 4) was able to compete effectively for protein binding to 2RJ, but only at much higher concentrations: 50× 1R was approximately as effective as 10× 2RJ, while 20× 1R was much less effective than 10× 2RJ (Fig. 7, lanes 3, 4, 9, and 10). A 1R oligonucleotide mutated in the B region was ineffective as a competitor (Fig. 7, lanes 5, 6, 11, and 12), indicating that some of the altered base pairs played an important role in protein binding.

Functional importance of the B-sequence element. Since mutations in the B domain of the VL30 repeat element clearly affected the ability of the repeat to bind nuclear proteins, we introduced these mutations into the pCBTK-3R expression vector to determine their effects on the inducible

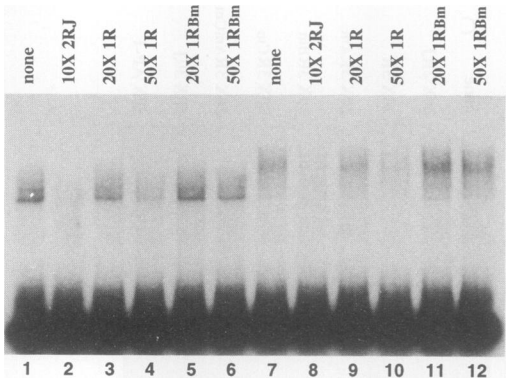

FIG. 7. Competition of nuclear extract DNA binding by wild-type single-repeat sequences (1R) but not by mutant single-repeat sequences (1R-Bm). Nuclear extracts from Rat-1 fibroblasts treated with either EGF plus thapsigargin (lanes 1 to 6) or TPA plus thapsigargin (lanes 7 to 12) were used in gel mobility shift assays as described for Fig. 6. ^{32}P-end-labeled 2RJ was used as the probe, and the indicated molar excess of unlabeled competitor DNA was added during the binding reaction as described in Materials and Methods. The nucleotide sequences of 2RJ and the competitor oligonucleotides are shown in Fig. 4.

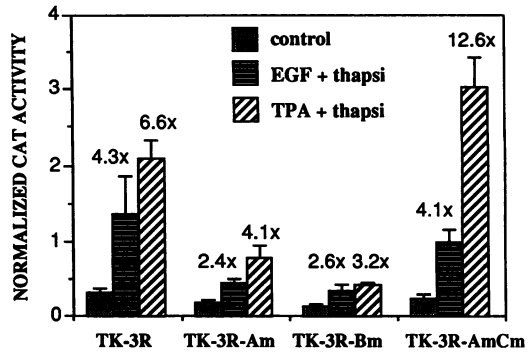

FIG. 8. Effect of mutations in the B domain on the induction of CAT expression in response to EGF-thapsigargin and TPA-thapsigargin. Rat-1 cells in 6-cm plates were transiently transfected with 2.5 μg of pGLCONTROL plus 2.5 μg of plasmids pCBTK-3R, pCBTK-3R-Am, pCBTK-3R-Bm, and pCBTK-3R-AmCm as described for Fig. 5A. Following 72 h of serum deprivation, cells were exposed to either no additions (control), EGF at 10 ng/ml plus thapsigargin (thapsi) at 1 μM, or TPA at 100 ng/ml plus thapsigargin at 1 μM. Cell extracts were harvested, and CAT and luciferase assays were performed as described in Materials and Methods. CAT activity (10^3 cpm of [^{3}H]acetyl coenzyme A incorporated per h measured during the linear phase of the reaction) was normalized to luciferase activity in arbitrary luminometer units. Luciferase activity was also within the linear limits of the assay, as determined by the luciferase dose-response curve. Results are expressed as the mean ± standard deviation for quadruplicate samples, and the fold induction observed relative to the control (no additions) level is noted for each plasmid and agonist combination.

expression of CAT activity. When tested in transient transfection assays including cotransfection with the pGLCONTROL luciferase expression vector (Promega) for normalization of transfection efficiency, plasmid pCBTK-3R-Bm consistently failed to respond significantly to treatment with either EGF-thapsigargin or TPA-thapsigargin (Fig. 8). CAT activities measured from cells transfected with pCBTK-3R-Bm were consistently equal to or less than those observed from the enhancerless plasmid pCBTKpr. These results suggested that sequences in the B domain were responsible for the induction of CAT activity observed in pCBTK-3R-AmCm and that the protein-DNA complexes observed in the mobility shift assays were functionally important.

DISCUSSION

Enhancer properties of the triple repeat. The triple-repeat sequence of the RVL-3 LTR displays the three defining characteristics of classical enhancer elements: promoter independence, position independence, and orientation independence. The results presented above have demonstrated that the triple-repeat sequence of the RVL-3 LTR from nt 121 to 243 (Fig. 1) is effective in promoting induction of CAT expression following either TPA or EGF treatment of Rat-1 cells in conjunction with Ca^{2+} elevation via thapsigargin treatment. This effect was observed whether the triple-repeat sequence was installed upstream of the RVL-3 promoter region or the TK promoter (plasmid pCBVL-3R or pCBTK-3R, respectively). Induction was also observed when the repeat sequence was placed either 330 bp upstream of the TK promoter in plasmid pTES-XR-sense or 11 bp upstream of the TK promoter in the plasmid pCBTK. The triple-repeat sequence was also able to confer induction of CAT expression when placed in either orientation (plasmids pTES-XR-sense and pTES-XR-antisense). Therefore, the triple-repeat sequence of the RVL-3 LTR functions as an inducible enhancer. It should be noted that the TPA-responsive element (VLTRE) from the B10 VL30 element described by Bohm (6) was totally absent from both pCBTK-3R and pCBVL-3R.

Mutational analysis reveals new enhancer element and functional antagonism between CArG and AP-1-like sites. The results of the mutational analysis demonstrated clearly that neither the AP-1-like sites nor the CArG elements of the triple-repeat sequence were necessary to confer induction of CAT expression to enhancerless plasmids, since plasmid pCBTK-3R-AmCm containing mutations in both of these sequence elements was still inducible. A search of the AmCm nucleotide sequence against a data base of known enhancer sequences (using the MacVector software; IBI) indicated that no known enhancer sequences were present outside of the AP-1-like site and the CArG box, nor had any been created inadvertently as a result of the point mutations. This result suggested that an additional, as yet uncharacterized growth factor-responsive element was present in the VL30 triple repeat. Gel mobility shift experiments indicated that alteration of a 15-bp sequence between the AP-1-like site and the CArG box abrogated the ability of the VL30 repeat to bind nuclear proteins. Functional tests of mutations covering 8 bp of this B domain of the VL30 repeat indicated that the B domain played an essential role in mediating the induction of CAT expression in response to both EGF and TPA treatment. DNase footprinting experiments are currently in progress to define the precise boundaries of the protein-binding region involving the B domain. Sequence analysis suggests that a novel DNA sequence is involved in protein binding to the B domain, as regions of significant similarity to known enhancer elements are not apparent within this region.

Although the mutations in the A and C domains were originally chosen on the basis of the presence of substantial sequence similarity between these domains and consensus

AP-1 and CArG elements, respectively, it is quite clear that the A domain of the RVL-3 LTR does not function as a conventional AP-1 binding site. First, the A domain was not required for induction, as the pcBTK-3R-AmCm expression vector was capable of responding positively to either EGF-thapsigargin or TPA-thapsigargin treatment, despite the presence of mutations known to inactivate conventional AP-1 sites. Second, the A domain of VL30 did not appear to bind members of the Fos-Jun family of *trans*-activating proteins, since an oligonucleotide containing a consensus AP-1 sequence did not compete for protein binding to the VL30 repeat element. This same AP-1 oligonucleotide was bound efficiently by six different heterodimers of the Fos-Jun family produced by in vitro translation; it is also bound efficiently by proteins present in nuclear extracts from treated and control Rat-1 cells (32a). Furthermore, the 30J oligonucleotide, which contains an intact A domain, also failed to compete for binding to 2RJ. Although the VL30 A domain is clearly not functioning as a conventional AP-1 site, sequences in the A domain may function to overcome repression mediated by the CArG sites, since mutations in the A domain significantly inhibited the inductive response when the C domain was intact. When both the C and A domains were mutated, sequence elements present in the B domain were capable of mediating induction in response to either EGF or TPA treatment in the presence of elevated intracellular Ca^{2+} levels.

The apparent antagonism between negative effects mediated by the CArG sites and the positive effects mediated by the A and B domains suggests that basal-level transcription in unstimulated cells reflects a balance of regulatory interactions in which the CArG sites prevail. Reversible repression mediated by CArG elements has also been observed in the c-*fos* promoter region, where the CArG element appears to mediate both the inductive response to serum treatment and the autoinhibitory effects of elevated c-Fos protein (34, 41). In the VL30 enhancer, however, the negative effects of the CArG element appear to act as a volume control, reducing the magnitude of gene expression under both basal and stimulated conditions. When both the A and B domains were intact, agonist stimulation produced the same fold induction whether or not the CArG elements had been mutated. However, when the CArG elements were intact, neither the A domain nor the B domain was sufficient for induction of gene expression, as both the 3R-Am and 3R-Bm mutants were markedly inhibited in CAT assays. This observation could indicate that interactions between multiple positive enhancer elements are required to overcome the repressive effects of the CArG element. Alternatively, a single positive enhancer element may be responsible for Ca^{2+}-dependent induction of gene expression in response to EGF and TPA. As we have not yet defined the boundaries of the protein-binding region identified in the gel mobility shift assays, it is possible that the functional enhancer element overlaps mutations present in both 3R-Am and 3R-Bm, so that either set of mutations could impede the enhancer's ability to overcome repression from the CArG element.

Need for repeated elements. One obvious and probably important feature of the RVL-3 enhancer is its tandemly repeated structure which engenders multiple transcriptional response elements. To assess whether effective binding to the enhancer required the presence of multiple binding sites, we compared the ability of double-repeat and single-repeat elements to compete for binding to nuclear proteins, as measured in gel mobility shift assays. These results clearly indicated that DNA fragments containing multiple-repeat units were more effective competitors than fragments containing single-repeat units, although single-repeat units were capable of competing. Interestingly, we have been unable to detect significant direct binding of nuclear proteins to the end-labeled single repeat (1R oligonucleotide [32a]). This suggestion of cooperative binding interactions between repeat units is currently under further investigation in our laboratory.

Bohm and coworkers have described the identification of a TPA-responsive sequence element present in the B10 VL30 element which is highly expressed in mouse skin following topical application of TPA (6, 7). The VLTRE identified by Bohm and coworkers contributes to both the basal and TPA-induced expression of VL30 in mouse keratinocytes. However, this VLTRE is not included in either pcBTK-3R or pcBVL-3R, nor was Bohm's VLTRE responsive to EGF treatment (6). Therefore, it appears that the B domain described in this report contains a unique Ca^{2+}-dependent enhancer element capable of mediating gene expression in response to either EGF treatment or PKC activation.

In summary, results presented here clearly indicate that positive and negative regulatory elements present in the RVL-3 enhancer interact with each other to modulate transcriptional induction. The CArG elements in this enhancer appear to exert primarily negative effects, as their mutation elevated basal levels without altering the extent of induction. The loss of induction following mutation of the A domain indicates that at least some base pairs within this domain can contribute positively to transcriptional induction. Furthermore, the observation of inducible expression following concurrent mutation of both the A and C domains indicates that an additional inducible response element resides within the RVL-3 triple-repeat sequence. The experiments described in this report indicate that sequences within the B domain of the VL30 repeat function as inducible enhancer elements responsive to EGF, TPA, and elevated intracellular Ca^{2+}. Furthermore, nuclear proteins present in agonist-treated Rat-1 cells are capable of binding specifically to the VL30 enhancer; these proteins do not appear to be members of the Fos-Jun family. DNase footprinting and methylation interference experiments are currently in progress to define more precisely the structure of this multiple agonist response element. We are also actively engaged in the purification and characterization of the proteins binding to this element.

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