MOLECULAR AND CELLULAR BIOLOGY, Mar. 1993, p. 1796–1804
0270-7306/93/031796-09$02.00/0

Vol. 13, No. 3

A Mitogen-Responsive Promoter Region That Is Synergistically Activated through Multiple Signalling Pathways

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Received 29 October 1992/Returned for modification 24 November 1992/Accepted 15 December 1992

A regulatory region of the human transferrin receptor gene promoter was found to be required for increased expression in response to serum or growth factors. This region contains two elements that appear to cooperate for full responsiveness. We found that sodium orthovanadate treatment of cells significantly activated expression of promoter constructs containing these elements. 12-*O*-Tetradecanoylphorbol-13-acetate alone induced a twofold increase in expression but acted synergistically with vanadate to generate a highly elevated level of expression. Dibutyryl cyclic AMP alone had no effect on expression, but when added together with vanadate and 12-*O*-tetradecanoylphorbol-13-acetate, led to superinduction of the promoter construct. Induction of expression by these reagents was delayed several hours, and the kinetics were identical to those observed for serum induction.

Transferrin receptors (TR) mediate uptake of iron into cells by binding and endocytosis of transferrin. Expression of TR is coupled to cell proliferation, and blocking of receptor function prevents cells from entering the S phase. This is most likely the result of an increased need for iron during the DNA-synthetic phase of the cell cycle. Rapidly proliferating cells have elevated levels of TR compared with quiescent, noncycling cells (13, 22). When quiescent cells are activated by growth factors or mitogens, the level of cell surface TR increases. Increased expression of TR in mitogen-activated cells involves increased transcription of the TR-encoding gene (21). This is a delayed response that occurs several hours after mitogen stimulation and reaches a maximum just prior to entry into the S phase.

Previously we have characterized the promoter region of the TR-encoding gene and analyzed the DNA-protein interactions within this region. We found two elements that are protected in DNase I footprinting experiments (3, 26, 28). These protein recognition sites are indicated by the boxes labelled A and B in Fig. 1A. Element A is GC rich and contains a consensus binding sequence for transcription factor Sp1. However, we have demonstrated that multiple factors can interact within this region (26). Element B contains a sequence that is similar to the sequences recognized by both the Ap1 and cyclic AMP (cAMP) response element-binding protein (CREB) families of transcription factors. Microinjection of oligonucleotides that span both elements A and B was able to block serum induction of DNA synthesis (27). However, oligonucleotides for either site alone had no effect. These results suggest that the factors that recognize these sequences in the TR promoter have cooperative effects that are critical for the signalling pathways involved in transition to the S phase.

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In this study, we mapped this region of the TR promoter by deletion analysis and characterized the elements required for growth factor and mitogen responsiveness in further detail. In addition, we examined the effects of reagents known to activate specific intracellular signalling pathways on TR promoter usage. We found that some of these reagents act synergistically to superactivate expression from the TR promoter constructs to extremely high levels.

MATERIALS AND METHODS

DNA constructs. The TR promoter region from positions −114 to +251 was subcloned in the *Hin*dIII site in the polylinker of plasmid pSAF. The chloramphenicol acetyltransferase (CAT)-encoding gene was excised from pSV0CAT by using *Hin*dIII and *Bam*HI and inserted immediately downstream of the TR insert, creating the construct pTRSAFCAT. This construct was digested with *Kpn*I and *Sal*I, and then unidirectional deletions from the 5′ end of the promoter insert were created by the method of Henikoff (14), by using a kit supplied by Promega. The endpoints of the deletions were determined by DNA sequencing, and the appropriate constructs were selected for further analysis.

Transfection and selection of stable transformants. Deletion mutation constructs pTR14 (−78), pTR20 (−55), and pTR28 (−24) were cotransfected with pSV2Neo into Swiss 3T3 cells by the calcium phosphate precipitation method of Chen and Okayama (7). Stable transformants were selected by using 400 μg of G418 per ml. Colonies appeared after 2 to 3 weeks in selective medium, and all of the colonies for each construct were pooled to establish a stable cell line. The average number of copies of the CAT constructs integrated into the genome of each cell line was estimated by the dot blot method of Bresser et al. (5), by using 10^6 cells. The blots were probed with a ^{32}P-labelled fragment containing the CAT-encoding gene. Control blots contained a known number of copies of a CAT-encoding gene construct, and the copy number per cell was estimated by excision of the nitrocellulose dots and counting in a scintillation counter.

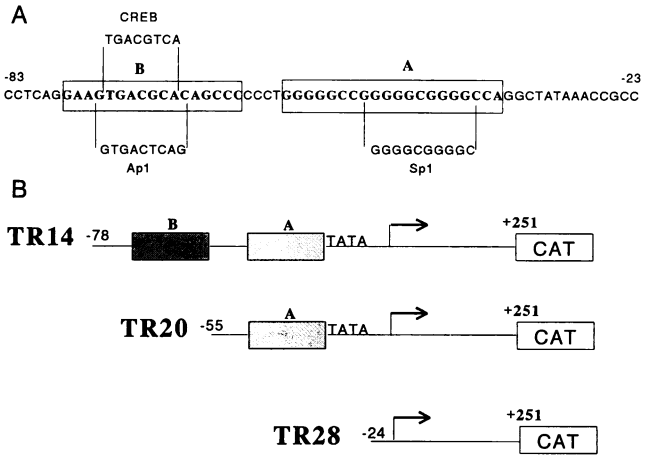

FIG. 1. (A) Sequence of the regulatory region of the TR gene promoter. Boxes indicate elements that are protected from DNase I digestion in footprinting experiments using HeLa nuclear extracts (3, 26, 28). Also indicated are sites that are similar to consensus recognition sequences of several known transcription factors. (B) Schematic drawing of the promoter constructs carried by the stable transformants used in this report. Boxes labelled A and B correspond to elements A and B shown in panel A.

DNA synthesis. The kinetics of induction of DNA synthesis by serum in the stable transformants was determined by pulse-labelling with [^{3}H]thymidine. The cells were grown to confluence in 35-mm-diameter culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 5% calf serum and allowed to enter quiescence. They were then stimulated by adding serum to 20%. At the times indicated, the cells were washed three times in DMEM containing 0.1% bovine serum albumin. They were then incubated for 2 h in the same medium containing 1 μCi of [^{3}H]thymidine (90 Ci/mmol). At the end of this incubation, the cells were washed three times in Dulbecco's phosphate-buffered saline and then incubated overnight at 4°C in 5% trichloroacetic acid (TCA). They were washed four times in cold 5% TCA and then solubilized in 0.5 ml of 1 N NaOH. The sample was neutralized with HCl and counted in a scintillation counter.

For some experiments, induction of DNA synthesis under defined conditions was determined by a method similar to that of Dicker and Rozengurt (8). The cells were grown to confluence in 24-well microtiter dishes in DMEM containing 10% calf serum and allowed to enter quiescence. The medium was then changed to a 1:1 mixture of Waymouth's medium and DMEM that contained no serum. One day later, the cells were stimulated with various reagents for 24 h in the presence of [^{3}H]thymidine (1 μCi, 90 Ci/mmol). The cells were rinsed in serum-free medium and incubated overnight at 4°C in 10% TCA in serum-free medium. The medium was removed, and the cells were incubated overnight at 4°C in 5% TCA. The cells were solubilized in 0.2 ml of 1 N NaOH, and the level of [^{3}H]thymidine incorporated into DNA was determined in a scintillation counter.

CAT assays. The stable transformants were grown to confluence in DMEM containing 10% calf serum and allowed to enter quiescence. For serum stimulation experiments, serum was added to a concentration of 20%. Experiments using serum-free medium were carried out in the defined system described by Dicker and Rozengurt (8). After reaching confluence, the cells were incubated for 24 to 48 h in medium consisting of 50% DMEM and 50% Waymouth MB752 medium. Serum or other reagents were added directly to the medium, and incubation was continued for the time indicated in each figure. CAT activity was measured as described by Gorman (12), by using ^{14}C-labelled chloramphenicol. To quantitate the level of enzyme activity, the radioactive spots were excised from the thin-layer chromatography plates and counted in a scintillation counter.

DNase I footprinting. To prepare the probe for footprinting analyses, 20 μg of *Sal*I-digested pTRSaf was 5′ end labelled by using the Klenow fragment in the presence of 250 μM dATP, dGTP, and TTP and 50 μCi of [α-^{32}P]dCTP. The reaction was incubated at room temperature for 1 h and chased with cold dCTP (250 μM final concentration) for 10 min. The labelled DNA was ethanol precipitated and digested with *Eco*RV. The appropriate 5′-end-labelled DNA fragment was isolated by electrophoresis on a 2% agarose gel in 45 mM Tris–45 mM borate–1 mM EDTA. This probe contains TR sequences from positions −114 to +11.

Footprinting reaction mixtures contained the end-labelled probe (0.5 to 1 ng)–30 μg of HeLa nuclear protein (9)–100 μg of poly(dI-dC) per ml in buffer consisting of 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.9), 20% glycerol, 0.1 M KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Reaction mixtures contained unlabelled oligonucleotide competitors as described in the figure legends. After incubation on ice for 20 min, DNase I was added at a final concentration of 0.1 to 4.2 μg/ml and digestion was carried out at room temperature for 1 min. The reaction was immediately stopped by addition of 1 reaction volume of stop buffer (0.6 M ammonium acetate, 0.1% sodium dodecyl sulfate, 0.1 mM EDTA). The DNA was ethanol precipitated, dissolved in 98% formamide loading buffer, heated to 95°C for 10 min, and separated on an 8% polyacrylamide sequencing gel. After electrophoresis, the gel was fixed in 10% acetic acid–10% methanol for 20 min, transferred to Whatman 3MM filter paper, dried under vacuum, and exposed to Kodak film overnight at −70°C.

The sequences of the oligonucleotides used in this study are as follows: TR(−79/−37), AGGAAGTGACGCACAGCCCC CCTGGGGGCCGGGGGCGGGGCCA; Sp1, TGCACGGG GCGGGGCCTGCA; Ap1, AGCTTGATGAGTCAGCCGG ATC; CRE, CCCGGGTGACGTCACGGGGA; Egr-1, AG CGTCGCGGGGGCGCACGT; PEA3, TCGAGCAGGAA GTTCGA. All oligonucleotides were used in double-stranded form, and the other strand for each oligonucleotide was the exact complement.

RESULTS

Deletion analysis of mitogen-responsive elements in the TR gene promoter. Figure 1A shows a diagram of the region of the TR promoter analyzed in this study. The boxes labelled A and B represent regions of the promoter that were previously shown to be protected by factors from HeLa nuclear extracts in DNase I footprinting experiments (3, 26, 28). Both sites have sequences that are similar to the consensus recognition sites of known transcription factors. Region A is a GC-rich element that contains within it a site identical to the consensus sequence for Sp1 (16). Region B has a site that is similar to the consensus sequences for both Ap1 and CREB but is not a strong match for either. Previously we have shown that microinjection of oligonucleotides that span both sites A and B was able to block serum induction of DNA synthesis but that oligonucleotides specifying either site alone were ineffective (27).

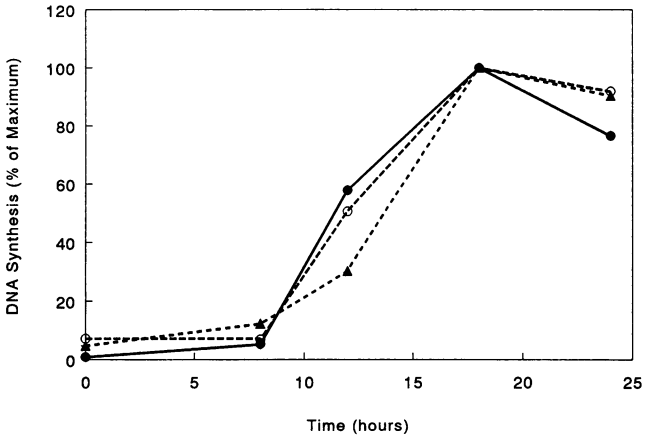

FIG. 2. Kinetics of induction of DNA synthesis in response to serum in Swiss 3T3 cells stably transformed with TR promoter constructs TR14 (▲), TR20 (○), and TR28 (●). Confluent cultures were stimulated with serum for the time indicated and then pulse-labelled with [^{3}H]thymidine for 2 h before determination of TCA-insoluble counts incorporated into DNA. Each point is the average of duplicate samples.

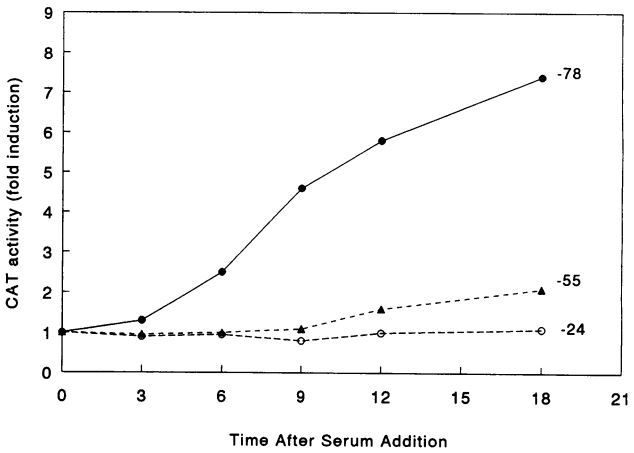

FIG. 3. Serum induction of CAT activity in Swiss 3T3 cells stably transformed with TR promoter constructs. The deletion endpoint of the construct carried by each transformant is indicated on the right. The length of time (in hours) that each culture was stimulated with serum is indicated at the bottom.

Thus, to analyze these elements in more detail, we constructed a series of deletion mutations that span the region of interest. Figure 1B shows a diagram of the informative deletion mutations utilized in this study. Construct TR14 extends to position −78 and therefore retains both protein-binding sites A and B. Deletion mutation construct TR20 extends to position −55 and retains region A but has lost region B. Deletion mutation TR28 extends to position −24 and has lost both regions A and B and the TATA box. These promoter constructs were linked to the bacterial CAT gene and cotransfected into Swiss 3T3 cells along with pSV2Neo (33). Stable transformants were selected by using G418, and cell lines were established by pooling all of the resistant colonies from each construct to minimize any effects that the sites of integration might have on utilization of the TR promoter. The average numbers of copies integrated in each cell line were estimated to be 2.3, 1.3, and 4.1, respectively, for constructs TR14, TR20, and TR28. Thus, all of the stable cell lines carried similar numbers of the integrated CAT constructs.

Each cell line was also analyzed for serum induction of DNA synthesis. The cells were grown to confluence and allowed to become quiescent. They were then stimulated by increasing the serum concentration to 20%. At the time points indicated, they were pulse-labelled with [^{3}H]thymidine to estimate the level of DNA synthesis (Fig. 2). All three stable cell lines initiated DNA synthesis between 8 and 12 h and reached a maximum level around 18 h post serum stimulation. Thus, all of the cell lines responded to serum in nearly identical manners and retained the temporal pattern of the parental 3T3 cell line (28).

The abilities of the promoter constructs to respond to serum were tested by growing the stable lines to confluence, allowing them to enter quiescence, and then increasing the serum concentration to 20% as described above. At various time points, the cells were harvested and the level of CAT activity was assayed. These results are shown in Fig. 3. In the cell line carrying TR14, which extends to position −78 and retains both protein-binding sites A and B, there was a marked increase in CAT activity following serum stimulation. There was a slight increase between 0 and 3 h, with the major increase occurring after 6 h of stimulation. We observed up to 12.5-fold stimulation by serum relative to the basal level in quiescent cells, with an average of ~6-fold induction (see Fig. 4 also).

In the cell line carrying construct TR20, which extends to position −55 and has lost element B, CAT activity was also induced by serum stimulation but to a much smaller extent than in the cell line carrying construct TR14 (−78 construct). We observed a slight increase between 6 and 9 h poststimulation and an approximately twofold increase by 18 h. No increase in CAT activity in response to serum stimulation was observed in the cell line carrying the TR28 (−24) construct. This mutant had a basal level of CAT activity nearly equivalent to that of the stable transformants described above, even though it had lost both regions A and B and the TATA box. Since this construct extends 251 bp downstream of the major transcriptional start site (Fig. 1), it is possible that these downstream sequences contribute to the basal activity observed. However, constructs carrying both elements A and B but extending only to position +11 retained the ability to respond to serum (data not shown). Also, it is possible that neighboring elements at the sites of integration contribute to the basal activity of the −24 construct. However, the lack of mitogen responsiveness in this mutant demonstrated that the increase in activity observed with the −78 and −55 constructs was not due a serum-induced increase in either overall transcription or translation. Likewise, the results observed cannot be due to increased stability of either CAT mRNA or the CAT enzyme following serum stimulation. From these results, we can conclude that the region encompassing element B (−78 to −55) is necessary for the response to serum and that full responsiveness involves elements downstream of −55.

Responsiveness of the TR promoter to purified growth factors. We further analyzed the ability of these promoter elements to respond to purified growth factors under defined conditions. Insulin, epidermal growth factor (EGF), and bombesin have all been shown to be mitogenic to 3T3 fibroblasts. When added alone, however, they do not elicit a potent mitogenic response as measured by induction of DNA synthesis (31). But when added in combination they act

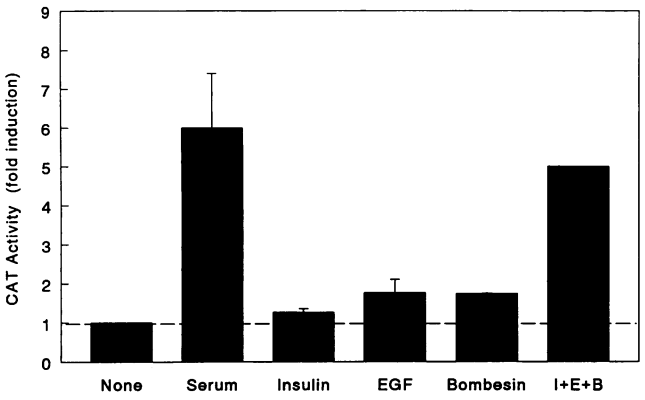

FIG. 4. Induction of CAT activity by serum and defined growth factors. The stable transformant carrying the −78 deletion construct, which retains full serum responsiveness, was used for these experiments. Insulin (I) was used at a concentration of 1 μg/ml, EGF (E) was used at 100 ng/ml, and bombesin (B) was used at 400 ng/ml. These represent levels that stimulate maximal induction of DNA synthesis in these cells. Induction was carried out under serum-free conditions as described in Materials and Methods. The level of activity is shown relative to that of control, uninduced cells.

synergistically to stimulate cell proliferation. This is thought to be due to the abilities of these various factors to activate different signalling pathways that cooperate to stimulate events that are obligatory to transition to the S phase (31).

The following experiments were carried out on cells in defined serum-free medium as described in Materials and Methods. Figure 4 shows that when insulin alone was added to TR14 (−78) stably transfected cells, there was only a slight enhancement of CAT activity. Both EGF and bombesin, when added individually, induced an approximately twofold increase in CAT activity compared with the level in unstimulated quiescent cells. When any two of these factors were added together, slight increases in CAT activity were observed relative to the individual factors (data not shown). However, when the cells were stimulated with all three factors (insulin, EGF, and bombesin) induction of CAT activity was nearly equal to that seen with serum.

These results suggest that multiple signalling pathways interact to generate the full response of the TR promoter. Therefore, we examined the effects of various reagents that are known to activate specific intracellular signalling pathways on TR promoter usage. We found that some of these reagents acted synergistically to activate expression from the TR promoter constructs to extremely high levels.

Synergistic activation of the TR promoter by vanadate, TPA, and cAMP. Vanadate has been shown to be a specific inhibitor of tyrosine phosphatases (24, 30, 34). Treatment of normal cells with vanadate leads to elevated levels of phosphotyrosine-containing proteins (19, 37) and the appearance of a transformed phenotype (18). With the −78 construct (TR14), which retains both elements A and B (Fig. 1), addition of 60 μM sodium orthovanadate alone, in the absence of any serum, led to enhanced CAT expression (Fig. 5A). Induction by vanadate was dose dependent, as shown in Fig. 6. Concentrations of vanadate above 60 μM applied over long periods were toxic to these cells and resulted in lower levels of CAT activity.

These cells were also treated with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). TPA is known to activate protein kinase C directly, thereby replacing diacylglycerol in phospholipase-mediated signalling pathways. When added alone at concentrations of 20 to 200 ng/ml to serum-free cultures of the stable transformant carrying the −78 TR promoter construct, TPA stimulated an approximately twofold increase in CAT activity (Fig. 5 and 6). However, when TPA was added together with vanadate, the two reagents acted synergistically to stimulate a nearly 40-fold increase in expression (Fig. 5A).

Expression was further enhanced when the cAMP analog dibutyryl cAMP (db-cAMP) was added together with vanadate and TPA (Fig. 5A). Under these conditions, we observed an up to 85-fold increase in CAT activity, but this is probably an underestimate, since the chloramphenicol substrate became depleted owing to the high level of the CAT enzyme in the assay. When added by itself, db-cAMP had no effect on CAT expression at any concentration that we tried (Fig. 6). It appeared to enhance the effect of TPA slightly but to inhibit activation by vanadate (Fig. 5A).

We carried out similar assays by using the stable transformant TR20, which carries the −55 TR promoter construct (Fig. 5B) and therefore has lost element B (Fig. 1). This construct was still responsive to vanadate, although at a somewhat reduced level. It did not respond at all to TPA alone. Thus, the twofold activation observed with the −78 construct appears to require element B. Surprisingly, TPA still acts synergistically with vanadate to induce a high level of CAT expression. Thus, TPA may act through two separate mechanisms to activate the TR promoter.

As expected, db-cAMP had no effect by itself on the −55 construct (Fig. 5B). However, it was no longer able to act synergistically with TPA and vanadate to induce elevated CAT activity. Thus, this effect of db-cAMP also requires sequences between positions −55 and −78 (element B).

A stable cell line (TR28) carrying the −24 TR promoter construct was also analyzed. As described above, this construct lacked both elements A and B but retained a basal level of expression that was only slightly diminished relative to that of the −78 and −55 constructs. This construct did not respond to serum (Fig. 2), and Fig. 5C shows that it also did not respond to vanadate, TPA, db-cAMP, or any combination of these reagents. Thus, the induction and synergistic activation described above appear to require the same elements that are required for serum activation.

Superactivation of the TR promoter by vanadate, TPA, and db-cAMP is a delayed response. Induction of both the endogenous TR gene (28) and the TR promoter-CAT constructs (Fig. 2) by serum is a delayed response occurring several hours after stimulation. Thus, it is important to determine whether the kinetics of induction by reagents that mimic specific intracellular signalling pathways is similarly delayed. Cells carrying the −78 TR promoter construct were grown to confluence and allowed to enter quiescence. They were then stimulated either by serum or by vanadate plus TPA and db-cAMP under serum-free conditions. At the time points indicated, the cells were harvested and assayed for CAT activity (Fig. 7). The synergistic effects of vanadate, TPA, and db-cAMP led to superactivation relative to the induction seen with serum. However, in both cases the kinetics of induction were nearly identical, with the major increase in CAT activity occurring later than 6 h after stimulation. This suggests that together these reagents act on the same processes that are stimulated by serum to lead to TR promoter activation.

We also analyzed the mitogenic properties of these reagents by analyzing their abilities to induce cells to enter the S phase. Quiescent cells (TR14) in serum-free medium were

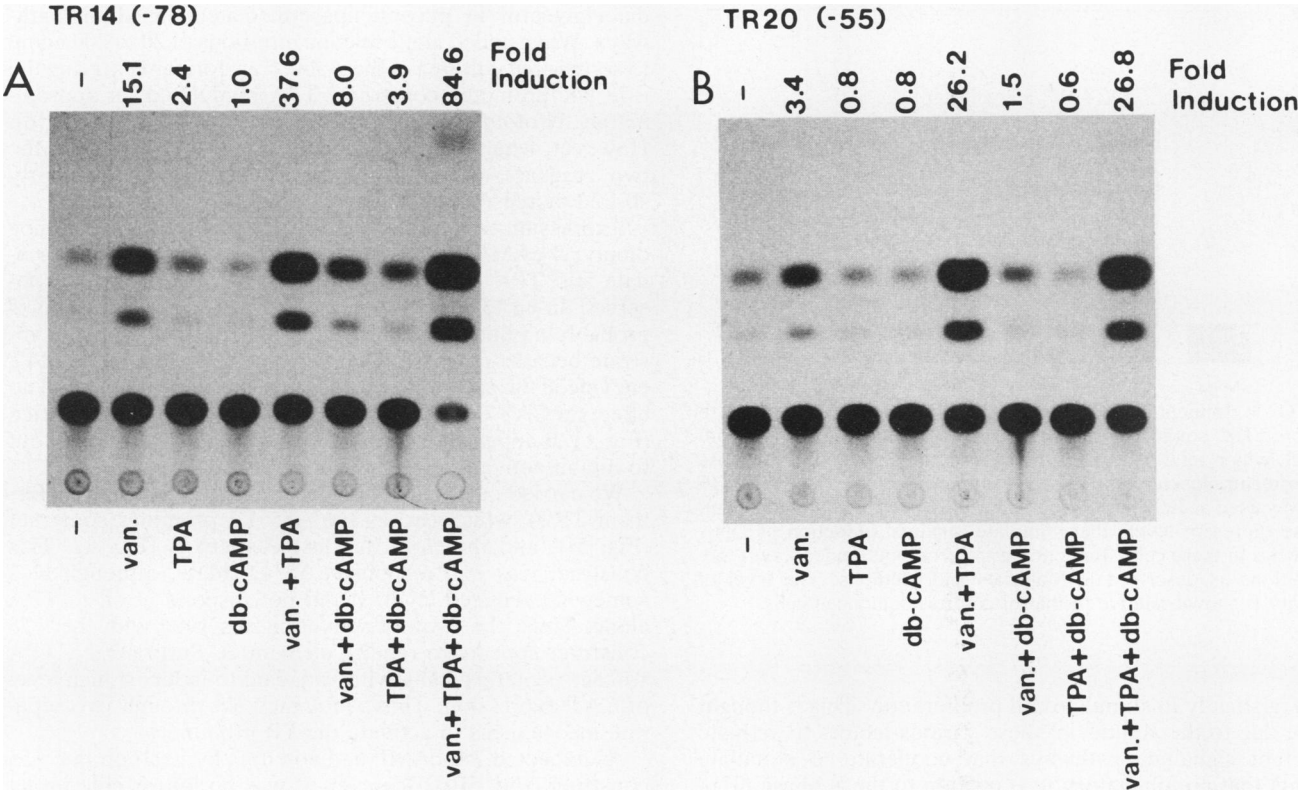

FIG. 5. Stimulation of Swiss 3T3 stable transformants with vanadate (van.), TPA, and db-cAMP. (A) The TR14 (−78)-transformed cell line was stimulated under serum-free conditions with 60 μM sodium orthovanadate, 100 ng of TPA per ml, 400 μM db-cAMP, or the combinations of these reagents indicated. Fold induction relative to the uninduced control shown in the first lane is indicated at the top. In panel B the TR20 (−55)-transformed cell line was used, and in panel C the TR28 (−24)-transformed cell line was used under exactly the same conditions described for panel A.

treated with serum, vanadate, TPA, or db-cAMP in the presence of [^{3}H]thymidine over a 24-h period and then assayed for incorporation of the label into TCA-insoluble material (Fig. 8). As expected, serum stimulated DNA synthesis in these cells. Both vanadate and TPA have previously been shown to be mitogenic, and both were able to stimulate significant increases in DNA synthesis under these experimental conditions. In Swiss 3T3 cells, cAMP analogs have been shown to enhance the mitogenic activities of numerous mitogens (31) but to be insufficient to induce

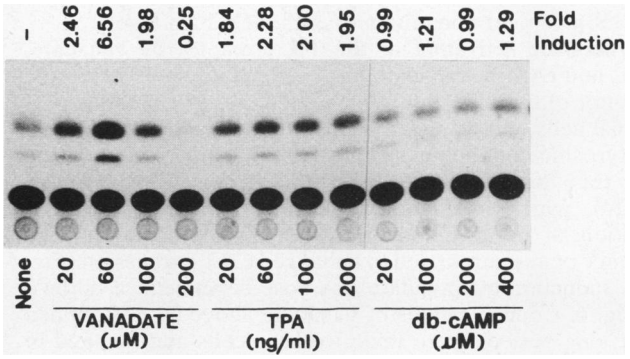

FIG. 6. Responses of the TR14 (−78)-transfected cell line to various doses of sodium orthovanadate, TPA, and db-cAMP. Reagent concentrations are shown at the bottom, and levels of induction relative to the unstimulated control are indicated at the top.

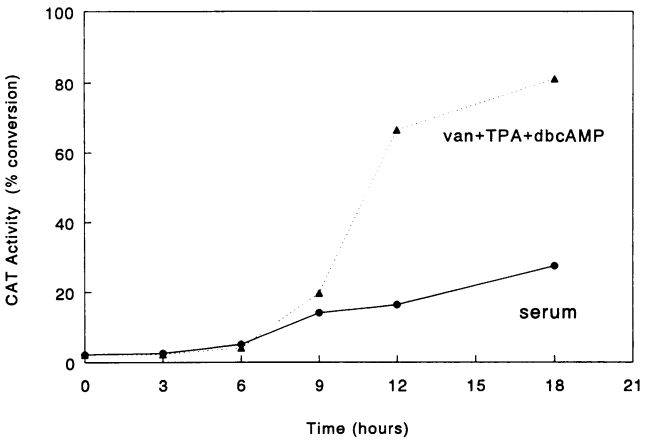

FIG. 7. Time course of induction of CAT activity in the TR14 (−78)-transformed cell line by either serum (●) or the combination of 60 μM sodium orthovanadate (van), 100-ng/ml TPA, and 400 μM db-cAMP (▲).

DNA synthesis when added alone. We also observed no induction of DNA synthesis by db-cAMP in our experiments. Interestingly, we were unable to demonstrate any significant additive or synergistic effects of the various combinations of vanadate, TPA, and db-cAMP on entry into the S phase (data not shown).

DNase I footprinting of the mitogen-responsive region of the TR promoter. The data presented above show that at least two elements are involved in the response of the promoter to serum and in superactivation of the promoter by vanadate, TPA, and db-cAMP. Previous footprinting experiments (3, 26, 28) have demonstrated that there are two binding sites for nuclear factors in this region (Fig. 1). Within the footprinted region are sequences that are similar to the consensus binding sites for a number of different transcription factors. Therefore, we carried out a more detailed analysis of this region by DNase I footprinting assays in the presence of unlabelled oligonucleotides that specify these consensus sequences (Fig. 9). In the absence of any unlabelled competitor, elements A and B were clearly protected from digestion. Protein-DNA interactions were not detected at any other sites in this region; however, two DNase-hypersensitive regions appeared to be induced downstream of

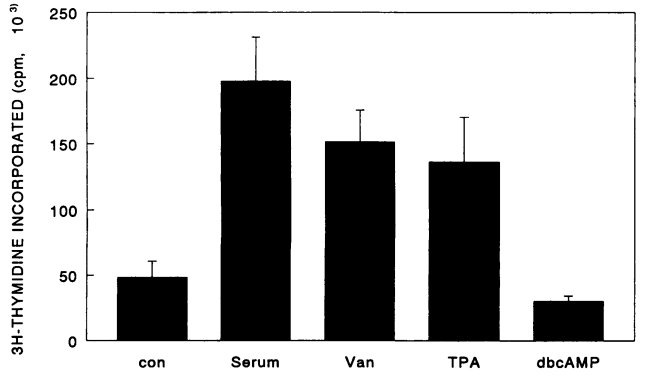

FIG. 8. Stimulation of DNA synthesis in Swiss 3T3 cells. Quiescent cultures (con) in serum-free medium were stimulated for 24 h in the presence of [³H]thymidine by 20% serum, 60 μM sodium orthovanadate (Van), 100-ng/ml TPA, or 400 μM db-cAMP.

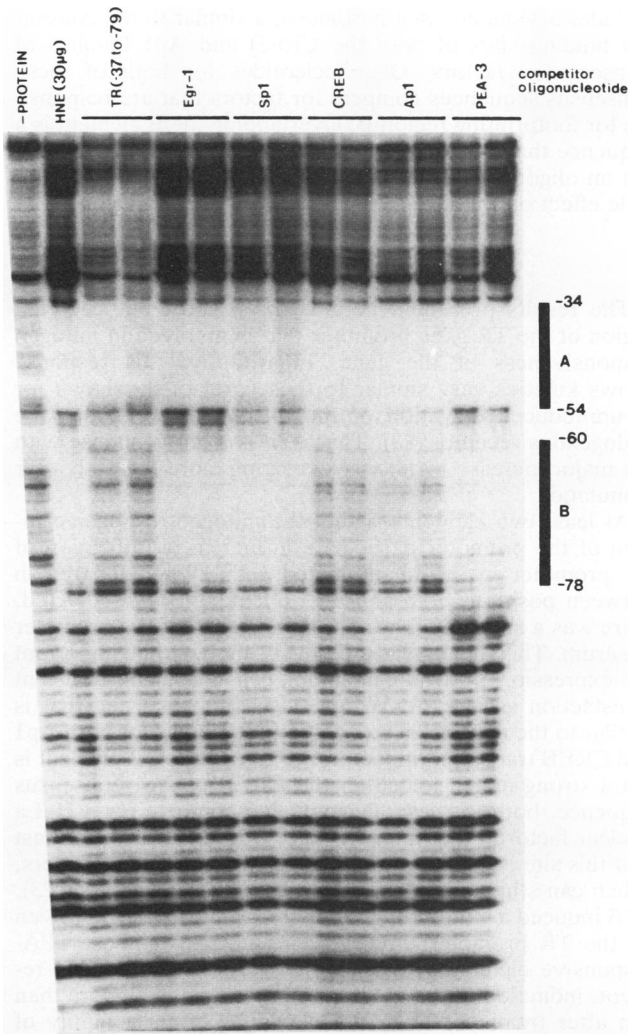

FIG. 9. DNase I footprint analysis in the presence of competitor oligonucleotides that encode binding sites for known transcription factors. The reactions were carried out with 30 μg of nuclear protein. The competitor oligonucleotide used is indicated at the top of each lane. Two concentrations of each competitor were used. The left lane of each pair utilized a 325-fold excess of the unlabelled competitor relative to the labelled probe, and the right lane utilized a 650-fold excess. HNE, HeLa nuclear extract.

position −34. Footprinting experiments were also carried out in the presence of the oligonucleotides specifying binding sites for the transcription factors that potentially recognize the TR promoter elements. As expected, the oligonucleotide spanning the TR promoter from positions −34 to −79 blocked binding within both elements B (−60 to −78) and A (−34 to −54), although titration of factors that bind to element A appeared to be incomplete at the unlabelled-competitor levels used. In addition, note that disruption of protein binding by this oligonucleotide also blocked formation of DNase-hypersensitive sites in the downstream region. An oligonucleotide specifying an Sp1-binding site appeared to block the footprint in region A partially but had no effect on binding in region B. An oligonucleotide specifying a binding site for Egr-1, another transcription factor with a GC-rich binding site, had no effect at all on the observed pattern of footprinting. As noted above, element B

includes a sequence that is somewhat similar to the consensus binding sites of both the CREB and Ap1 families of transcription factors. Oligonucleotides for both of these consensus sequences compete for factors that are responsible for footprinting region B. Overlapping the B element is a sequence that is identical to the recognition site for PEA-3, but an oligonucleotide specifying this binding site had very little effect on the DNase protection pattern in region B.

DISCUSSION

The results presented here define a short but complex region of the TR gene promoter that is involved in mitogen responsiveness of the gene. Importantly, this response shows kinetics very similar to those previously shown for serum-induced elevation of the mRNA that encodes the endogenous receptor (28). That is, it is a late response with the major increase in activity occurring more than 6 h after stimulation.

At least two elements within the mitogen-responsive region of the promoter participate in increased utilization of the promoter in serum-stimulated cells. When the region between positions −78 and −55 (element B) was deleted, there was a substantial loss in the response of the promoter to serum. This region was previously shown to be important for expression in a human tumor cell line using transient transfection assays (6). Within this region is a site that is similar to the consensus recognition sequences for both Ap1 and CREB transcription factors. Although the TR element is not a strong match for either the CREB or Ap1 consensus sequence, both of these elements can compete for a HeLa nuclear factor(s) that interacts here (Fig. 9). It is of interest that this site is also required for response to phorbol esters, which can stimulate transcription by activating Ap1 (1, 23). TPA induced a modest twofold increase in expression driven by the TR promoter; however, in contrast to most TPA-responsive elements that respond very rapidly to this reagent, induction of the TR promoter was delayed more than 6 h after treatment (data not shown). Ap1 is a family of transcription factors that are dimers and heterodimers of the *jun* and *fos* oncogene products. Most members of the *jun* and *fos* gene families are immediate-early genes and are activated in less than 1 h following mitogen stimulation. The c-*fos* gene product is expressed transiently and returns to basal levels within 2 to 3 h (10, 36). Interestingly, several *fos*-related antigens display an extended duration of expression following serum stimulation (10). Thus, it is possible that one of these factors could participate in regulating the TR promoter through element B. In this regard, it is worthwhile to note that oligonucleotides that specify either the consensus Ap1-binding site or the CREB site can compete for binding to element B (Fig. 9). Thus, it is probable that a factor in this class is involved in the serum-induced increase in TR promoter activity.

A second region of the TR promoter, downstream of element B, also participates in the response to mitogens. Although the deletion mutations used in this study cannot precisely define the position of this element, it most likely involves the highly GC-rich region (element A) immediately downstream of element B. This is supported by previous microinjection experiments which indicate that elements A and B cooperate with each other during mitogenic induction (27). In addition, we found that serum stimulation led to induction of a factor that bound with specificity to element A (data not shown).

Element A contains a consensus sequence for transcription factor Sp1. Footprinting experiments (Fig. 9) demonstrated that by competition, an oligonucleotide specifying an Sp1 consensus sequence partially eliminated factors that bind to element A. In other experiments (26), we have found that element A can bind multiple nuclear factors, including Sp1 or Sp1-like factors. It should be pointed out that factors other than Sp1 have been shown to bind to GC boxes, including factors LSF (15), Ap2 (29), ETF (17), and p53 (2). We have also detected nuclear factors that compete with Sp1-like factors for binding to element A but bind independently of the Sp1 consensus sequence (26). It is interesting that an EGF-responsive element in the gastrin gene promoter has been mapped to a GC-rich element somewhat similar to the motif found in the TR promoter (25). Although the gastrin element has an Sp1 consensus binding site, it does not appear to bind Sp1. Also, a GC-rich element in the mouse thymidine kinase promoter has been shown to bind Sp1, as well as a factor called Yi (4). Yi-binding activity is induced by mitogen stimulation and has been proposed to play a role in the increased expression of thymidine kinase in the late G_1 phase. We do not know the relationship, if any, of these factors to the proteins that interact with element A in the TR promoter. Further characterization of these factors is necessary to determine their role in the mitogen responsiveness of the TR promoter.

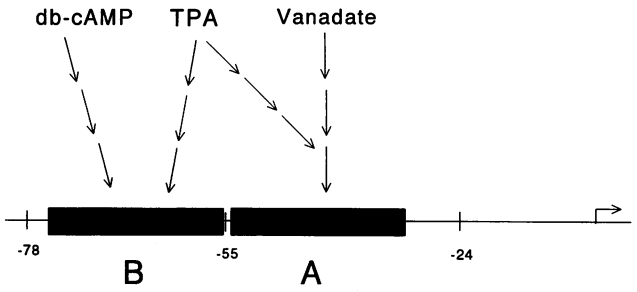

FIG. 10. Model depicting the regions of the TR promoter through which vanadate, TPA, and db-cAMP appear to act to induce expression.

Figure 10 shows a model that summarizes the results of the experiments involving vanadate, TPA, and db-cAMP. Activation of the TR promoter by vanadate requires the sequence downstream of position −55, which includes GC-rich element A. The tumor promoter TPA requires element B to stimulate a twofold increase in expression when added alone but nevertheless can act synergistically with vanadate when the B element is deleted. Thus, TPA appears to act on two separate pathways. db-cAMP had no effect on expression when added alone but had a synergistic effect when added together with vanadate and TPA. This effect of db-cAMP requires the presence of the B element.

Treatment of cultured cells with vanadate has been shown to stimulate DNA synthesis (32) and cell proliferation (18) and to transform normal fibroblasts phenotypically (18). In vitro, vanadate has been shown to inhibit tyrosine phosphatases selectively (24, 30, 34), and in living cells, vanadate leads to enhanced tyrosine phosphorylation of numerous proteins (19, 37). It is thought that stimulation of cell growth and induction of the transformed phenotype by vanadate are due primarily to the elevation of tyrosine phosphorylation brought about by inhibition of dephosphorylation (18, 19). Since the same region of the TR promoter that is responsive to mitogen stimulation is required for induction by vanadate,

it is likely that this effect is mediated by a pathway involving tyrosine phosphorylation. Because there are numerous tyrosine kinases that phosphorylate multiple targets and cause an array of pleiotropic effects, it is not possible to determine from these experiments a specific mechanism or pathway that may be responsible for vanadate-induced expression of the TR promoter constructs. It should also be mentioned that vanadate has been reported to have other effects on cellular activities that may or may not be the result of enhanced tyrosine phosphorylation. For example, Vargas et al. (35) have reported that vanadate specifically inhibits the turnover of short-lived proteins. Thus, it is possible that vanadate allows accumulation of a factor that is involved in activation of the TR promoter but that is normally rapidly degraded.

The tumor promoter TPA apparently affects expression from the TR promoter by two different mechanisms. It is able to induce expression weakly through the B element, which contains a sequence similar to a TPA-responsive element (1, 23) that is recognized by the Ap1 family of transcription factors. Thus, it is likely that one of the members of this family of factors is involved in the response of the B element to TPA. It is interesting that the induction of TR promoter activity by TPA is also a delayed response; therefore, it is unlikely to involve an Ap1 complex containing c-*fos*.

The second, and more striking, effect of TPA is its ability to act synergistically with vanadate in the induction of the TR promoter constructs. Surprisingly, this does not require the B element of the TR promoter. Thus, TPA appears to modulate a component of the signalling pathway through which vanadate functions. TPA treatment of cells is known to lead to increased tyrosine phosphorylation of specific cellular proteins (11, 20). Thus, it is possible that TPA treatment leads to activation of a specific tyrosine kinase that acts on a set of target proteins that cannot be dephosphorylated in the presence of vanadate. This, in turn, may lead either directly or indirectly to increased expression through the TR regulatory region.

The synergistic effect of adding db-cAMP together with vanadate and TPA is most likely the result of a cAMP-dependent pathway. This is supported by the fact that it requires the B element, which contains a site very similar to the consensus cAMP-responsive element (Fig. 1). It is intriguing that this site did not confer responsiveness to db-cAMP when the reagent was added alone. Thus, this element is not typical of the cAMP-responsive elements found in most other genes.

It is interesting that the kinetics of induction by the combination of vanadate, TPA, and db-cAMP are nearly identical to those seen with serum. This suggests that activation of the TR promoter by these reagents requires the same events that are activated by growth factors and that both early and late events are necessary. The superactivation by these reagents relative to the activation observed with serum may result from the continuous stimulation of signalling events that are normally only transiently activated by growth factors.

In summary, we found that vanadate, TPA, and db-cAMP act synergistically to superactivate expression of human TR gene promoter constructs in quiescent cells. This response is a delayed event and appears to involve the same signalling pathways initiated by serum factors to stimulate TR expression. The extraordinary level of induction by these reagents suggests that they will be useful in determining the molecular mechanisms that control late events that are critical for progression into the S phase.

ACKNOWLEDGMENTS

We thank Robin Miskimins for numerous encouragements and critical evaluation of the manuscript.

This work was supported by Public Health Service grant GM43976 from the National Institute of General Medical Sciences.

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