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Sodium Butyrate-Mediated Induction of the Glycoprotein Hormone α-Subunit Gene: Requirement for Continued Protein Synthesis, Identification of a Butyrate-Responsive Element, and Inhibition of Promoter Activation by 2-Deoxyglucose

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Abstract Production of the glycoprotein hormone α-subunit (GPHα) was enhanced by sodium butyrate (Btr) in HeLa cells. Induction of the HeLa α-subunit gene by Btr was inhibited by the simultaneous addition of cycloheximide (CHX), indicating a requirement for continued protein synthesis. Transient expression assays using plasmids containing the GPHα gene promoter fused to the chloramphenicol acetyltransferase (CAT) reporter gene demonstrated that the GPHα promoter is inducible by Btr in HeLa cells, and this induction could be prevented by 2-deoxyglucose (dGlc). CAT production driven by the SV-40 early promoter, the cytochrome P-450-IA1 promoter, and the Rous sarcoma virus long terminal repeat was also enhanced by Btr, but the augmented synthesis was not inhibited by the addition of dGlc, demonstrating that the effect is restricted only to some promoters. CAT synthesis could be induced by Btr when the GPHα promoter extended upstream to position −169 (relative to the transcription start site at +1) but not when the promoter terminated at −150, classifying the DNA between these termini as a Btr-responsive element (BRE). This region overlaps the composite trophoblast-specific enhancer. Inactivation of enhancer subdomains by site-directed mutagenesis confirmed the deletion analysis and ranked their response to Btr as CRE < TSE < URE < αACT. Electrophoretic mobility shift analysis failed to detect any significant difference among several enhancer binding proteins in nuclear extracts from untreated and Btr-treated cells. Together, these results suggest that Btr-mediated induction of the α-subunit gene in HeLa cells is manifest either through the synthesis of a new transcription factor(s), which is inhibited by CHX but required for increased transcription from the GPHα gene promoter, or through the activity of existing factors that may require glycosylation or phosphorylation by a modification system that is inducible by Btr and inhibited by dGlc and CHX. These results further suggest that the factor is not an enhancer-binding protein or that Btr increases its transactivation potential without altering its DNA-binding activity. J. Cell. Biochem. 74:242–263, 1999.

Key words: chorionic gonadotropin; butyrate; deoxyglucose; gene regulation; butyrate-responsive element

Abbreviations used: αACT, GPHα activation element; ABP, αACT binding protein; BRE, butyrate-responsive element; Btr, butyrate; CG, chorionic gonadotropin; CHX, cycloheximide; CAT, chloramphenicol acetyltransferase; CRE, cAMP-responsive element; dGlc, 2-deoxyglucose; GPH, glycoprotein hormone; HEPES, N-2-hydroxyethylpiperazine-N′-2′-ethanesulfonic acid; kbp, kilobase pair(s) PCR, polymerase chain reaction; SCFA, short-chain fatty acid; SSC, standard saline citrate; TSE, trophoblast-specific element; TSEB, TSE-binding protein; URE, upstream regulatory element; UREB, URE-binding protein.

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Human chorionic gonadotropin (hCG) is a glycoprotein hormone composed of two dissimilar subunits α and β. The heterodimer (αβ) and free α-subunit are secreted as eutopic proteins by the syncytiotrophoblast cells of the developing placenta. Free α-subunit is also produced in an ectopic manner by a variety of tumors and tumor-derived cell lines of nonplacental origin [Blackman et al., 1978; Rosen and Weintraub, 1974; Ghosh and Cox, 1976; Ruddon et al., 1979]. The synthesis of hCG and free α-subunit can be increased in vitro by a variety of chemical inducers [Ghosh et al., 1977; Tamura and Cox, 1988; Cox and Park, 1989; Ruddon et al., 1980]. Millimolar concentrations of sodium butyrate (NaBtr) enhance production of the glycoprotein hormone α-subunit (GPHα) in nontrophoblastic tumor cell lines such as ChaGo (bronchogenic carcinoma) and HeLa (cervical carcinoma), but not by trophoblastic tumor cell lines like JEG-3 and JAr (choriocarcinomas) [Chou et al., 1977; Hussa et al., 1978; Ruddon et al., 1980]. However, this distinction is qualified by more recent observations showing that Btr acts synergistically with cAMP to enhance GPHα production in JEG-3 cells, although it has no effect alone [Campain et al., 1994].

The mechanism(s) by which Btr elevates gene expression is unknown, but it is of interest because of the potential use of Btr in chemotherapeutic regimens as a differentiating agent [Perrine et al., 1988, 1989; Ginder et al., 1984]. Several reports suggest that changes in chromatin structure that result from the inhibition of histone deacetylase may be a significant factor [Candido et al., 1978; Sealy and Chalkley, 1978]. In this regard, transient transfection assays have indicated that Btr causes an increase in transcription of the GPHα gene [Darnell, 1984]. Other studies have demonstrated that a substantial lag is evident between the time of Btr addition and the increase in steady-state levels of GPHα mRNA [Cox et al., 1987]. The lag is greater than the time required to observe an increase in acetylated histone forms [Sealy and Chalkley, 1978]. These observations suggest the possibility that the Btr-mediated induction of the GPHα gene is indirect.

The enhanced synthesis of HeLa α-subunit in response to NaBtr is influenced by the culture media formulation [Cox and McClure, 1983] and by the concentration of glucose in a given medium [McClure and Cox, 1984]. Hexoses that support induction of the HeLa α-subunit in medium containing pyruvate as an energy source are identical to those that stimulate the glycosylation of lipid-linked oligosaccharides and enhance the synthesis of various glycoproteins (i.e., glucose > mannose > galactose > fructose) [Stark and Heath, 1979; Turco, 1980]. It is also of interest that in Chang liver cells, Btr supports α-subunit glycosylation in glucose-free medium [Morrow et al., 1983]. Previous results from this laboratory demonstrate that induction of GPHα by NaBtr in HeLa cells can be prevented by the simultaneous addition of 2-deoxyglucose (dGlc) [Cox, 1981]. The effect of dGlc on α-subunit synthesis is manifest in two ways, one involving the post-translational modification of the polypeptide and one involving the steady-state levels of GPHα mRNA [Cox et al., 1987]. The HeLa α-subunit is aberrantly glycosylated in the presence of dGlc as evidenced by an increased sensitivity of its oligosaccharide side chains to endoglycosidase H [Cox et al., 1987]. Deoxyglucose prevents the increase in GPHα mRNA produced by NaBtr when both compounds are simultaneously added to HeLa cultures [Cox et al., 1987]. The effect of dGlc on α-subunit glycosylation is understandable, in view of the documented effects of the sugar on protein glycosylation in general [Eagon and Heath, 1977; Datema and Schwartz, 1978, 1979]. The inhibition of mRNA production is less clear. The effect could be transcriptional, perhaps by depletion of cellular UTP, or post-transcriptional, perhaps by altering mRNA turnover.

The experiments presented were carried out to better define the mechanism by which Btr induces the GPHα gene. The results suggest that the short chain fatty acid (SCFA) acts through one or more pathways to activate transcription of the gene through a promoter motif localized to the trophoblast-specific enhancer [Jameson et al., 1988]. The inhibition by cycloheximide and dGlc of promoter activation by NaBtr suggests that the SCFA may act indirectly by increasing the production or post-translational modification (e.g., glycosylation, phosphorylation) of a new, limiting, or inactive transcription factor. Of the promoters tested that were inducible by NaBtr in HeLa cells, only that of the GPHα gene was significantly reversed by dGlc. This, and the failure of dGlc to inhibit the effect of Btr on GPHα induction in JEG-3 cells, indicate that the effects were both promoter and cell specific. They further suggest

that effects of dGlc on general features of metabolism, such as ATP production, are probably not involved, as these would be expected to influence transcription from multiple promoters in various cell types. The levels of three enhancer binding proteins involved in regulating GPHα promoter activity were similar in nuclear extracts from untreated and Btr-treated HeLa cells, suggesting that their transactivation potential may be altered in the absence of any obvious change in DNA binding activity.

MATERIALS AND METHODS

Materials

Sigma Chemical Company (St. Louis, MO) was the source of dGlc, G418, and cycloheximide (CHX). Sodium butyrate was purchased from Metheson, Coleman, and Bell. Amersham was the supplier of [^{14}C]chloramphenicol, and New England Nuclear was the supplier of [α-^{32}P]dCTP and GeneScreen hybridization membrane. Culture medium and calf serum were supplied by Grand Island Biological Company, and fetal bovine serum (FBS) was obtained from Hyclone. Restriction endonucleases were purchased from Promega (Madison, WI) or Life Technologies.

Cell Culture

HeLa cell line CCL 2.2 was obtained from the American Type Culture Collection. HeLa A19 was cloned from 5-azacytidine-treated HeLa 2.2 parent cultures and will be described in more detail elsewhere (D.E. Cosgrove and G.S. Cox, unpublished communication). All cells were maintained as monolayer cultures in Eagle's minimum essential medium supplemented with 6 mM L-glutamine, 5% calf serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. JEG-3 cells were obtained from the American Type Culture Collection (HTB 36) and maintained as monolayers in RPMI-1640 medium with the supplements listed above plus 5% FBS.

Dot-Blot Hybridization

A modification of the procedure by White and Bancroft [1982] was used for RNA dot hybridization. Details of lysate preparation and RNA purification were as reported by Cox et al. [1987] and Cosgrove et al. [1989]. Membranes were spotted with RNA samples, baked at 80°C for 2 h, and prehybridized overnight in a mixture containing 50% deionized formamide, 1 M NaCl, 10% dextran sulfate, 0.2% polyvinylpyrollidone, 0.2% bovine serum albumin (BSA), 0.2% Ficoll, and 100 μg/ml of denatured and sonicated salmon sperm DNA. Recombinant hCG-α cDNA [Fiddes and Goodman, 1979] was labeled by nick translation to high specific activity (7.5×10^8 cpm/μg DNA) using [α-^{32}P]dCTP and the procedure described by Rigby et al. [1977]. Molecular hybridization was performed for 24 h at 42°C in the mixture above containing 50,000 cpm of labeled cDNA per cm^2 of membrane. Membranes were then washed twice in 2× standard saline citrate (SSC) at room temperature for 5 min, twice in 2× SSC plus 1% sodium dodecyl sulfate (SDS) at 65°C for 30 min, and twice in 0.1× SSC at room temperature for 30 min (1× SSC contains 15 mM sodium citrate, pH7, and 150 mM NaCl). Filters were exposed to XAR film at −70°C, and signals were quantified by optical scanning densitometry.

Plasmids

The plasmids pSV_0CAT and pSV_2CAT were provided by Dr. Bruce Howard [Gorman et al., 1982a]. pSV_2CAT contains the bacterial chloramphenicol acetyltransferase (CAT) gene placed under control of the SV-40 early region promoter. pSV_0CAT is a promoterless derivative of pSV_2CAT and was used as a null control for CAT expression and to construct the plasmid pα1.6CAT. The latter was carried out by established methods [Maniatis et al., 1982], first linearizing pSV_0CAT with the restriction endonuclease *Hin*dIII and then ligating it to a 1.6-kbp *Eco*RI/*Bam*HI fragment (containing promoter-proximal upstream sequences of the human GPHα gene) after filling in the *Bam*HI and *Eco*RI ends with Klenow polymerase and attaching *Hin*dIII linkers. Correct orientation of the insert was verified by restriction enzyme analysis. The plasmids pRSVCAT [Gorman et al., 1982b] and pRNH12c [Hines et al., 1988] were kindly provided by Dr. Ronald Hines. These plasmids contain the Rous sarcoma virus LTR and the promoter for human cytochrome P-450-IA1 fused to CAT, respectively. The $pBLCAT_3$ vector was kindly provided by Dr. G. Schutz. The plasmid pSV_2neo [Southern and Berg, 1982], kindly provided by Dr. William Chaney, contains the gene for an aminoglycoside phosphotransferase under control of the SV-40 promoter and enhancers, rendering cells that integrate the plasmid into their genome in an active state resistant to the neomycin analog

G418 sulfate. Where indicated, the pCMVlacZ vector, which was generously donated by Dr. Solon Rhode, was coprecipitated with the pαCAT expression plasmids at a DNA ratio of 1 : 2 to monitor transfection efficiency.

Construction of Deletion and Point Mutations in the GPHα Promoter

A collection of 5′-truncated promoter mutants was constructed according to standard procedures using DNA restriction fragments or PCR products ligated into the polylinker upstream of the CAT gene in $pBLCAT_3$ [Luckow and Schutz, 1987]. It is noted that the order in the $pBLCAT_3$ polylinker of restriction sites used for above constructions is 5′-*Hin*dIII/*Pst*I/*Xba*I/*Bam*HI/*Bgl*II/*Xho*I-3′.

pα(−1101/+3)CAT. A 1.7-kbp fragment extending from −1637 to +48 was liberated from a GPHα genomic clone [Fiddes and Goodman, 1981] by digestion with *Eco*RI and *Bam*HI. A portion of the isolated fragment was digested with *Sma*I, resulting in a blunt end at −1114. The $pBLCAT_3$ vector was linearized with *Xba*I, and recessed ends were filled in with Klenow fragment of *Escherichia coli* DNA polymerase I (hereafter abbreviated Klenow polymerase). The vector was digested with *Bam*HI and ligated to the *Sma*I/*Bam*HI fragment with T4 DNA ligase. This pα(−1114/+48)CAT vector was digested with *Pst*I, and the 1.1-kbp fragment was isolated and subcloned into the *Pst*I polylinker site of $pBLCAT_3$.

pα(−846/+3)CAT. A 0.89-kbp fragment extending from −846 to +48 was liberated from the GPHα genomic clone by digestion with *BglII* and *Bam*HI. The 3′-recessed ends were filled in with the Klenow polymerase, and the blunt-ended fragment was subcloned into the filled-in and dephosphorylated *Xba*I site of $pBLCAT_3$ to produce pα(−846/+48)CAT. This vector was digested with *Pst*I, and the liberated fragment was subcloned into the *Pst*I site of $pBLCAT_3$.

pα(−295/+3)CAT. A 398-bp fragment (extending from −295 to +103) was amplified by the polymerase chain reaction (PCR) [Mullis et al., 1986]. A 10-kbp *Eco*RI fragment of the GPHα genomic clone, extending from about 2 kbp upstream of exon I to the middle of intron B was used as the template. The upstream and downstream primers were 5′-CTAAGCTTGTGTCAGCTTTCAGGATGTT-3′ and 5′-CATATTACCTTTCTCTGGG-3′, respectively. The PCR product was purified by gel electrophoresis and ligated into the polylinker of TA 3000 (Invitrogen). One clone, designated hereafter as TA 398, has the fragment oriented such that it is flanked by the *Xma*III and *Not*I polylinker sites on the 5′ and 3′ ends as it normally resides in the GPHα promoter. The TA 398 vector was digested with *Hin*dIII plus *Bam*HI, and the 0.35-kbp fragment was force cloned into $pBLCAT_3$ that had been similarly digested with *Hin*dIII plus *Bam*HI. This pα(−295/+48)CAT vector was digested with *Hin*dIII plus *Pst*I, and the 0.3-kbp fragment was purified and subcloned into the *Hin*dIII/*Pst*I restriction sites of $pBLCAT_3$.

pα(−180/+3)CAT. The pα(−295/+48)CAT vector was digested with *Hin*dIII plus *Xho*I and used as the template in a PCR, primed in the forward direction with oligonucleotide U-180 (5′-gcgaagcttCAAAAATGACCT-3′) and in the reverse direction with oligonucleotide CAPA (5′-GTTCTCAGTAACTGCAGTTAATGAAGTC-3′). The U-180 primer extends from −181 to −170 and provides a *Hin*dIII site in a 5′ leader (lowercase), and the CAPA primer contains the native *Pst*I site centered at +1 with 11 bp of flanking DNA on each side. The resulting 0.2-kbp fragment (−180 to +15) was digested with *Hin*dIII and *Pst*I and subcloned into the corresponding sites of $pBLCAT_3$.

pα(−169/+3)CAT. The TA 398 vector was digested with *Bsu36*I plus *Bam*HI, and the released fragment (−169/+48) was gel purified, filled in using Klenow fragment, and subcloned into the blunted *Xba*I site of $pBLCAT_3$. The resultant pα(−169/+48)CAT vector was digested with *Pst*I, and the 0.17-kbp fragment was purified and subcloned into the *Pst*I site of $pBLCAT_3$.

pα(−150/+3)CAT. The promoter fragment for this vector was generated by PCR using pα(−295/+48)CAT as template DNA, CAPA oligonucleotide as the reverse primer, and U-CRE (5′-GCGAAGCTTCAAATTGACGTCATTG-3′) as the forward primer. The latter oligonucleotide contains the distal CRE and surrounding sequence. The purified fragment was trimmed by digestion with *Hin*dIII and *Pst*I and ligated into the polylinker of $pBLCAT_3$.

pα(−100/+3)CAT. The TA 398 vector was digested with *Eco*RI and *Hin*dIII to generate a 0.4-kbp fragment. This was isolated by gel electrophoresis, then digested with *Rsa*I to liberate four fragments of 225, 136, 70, and 14 bp. The 225-bp *Rsa*I/*Eco*RI fragment (containing GPHα

promoter sequence from −100 to +103 and 22 bp of polylinker) was digested with *Bam*HI and purified by polyacrylamide gel electrophoresis and phenol extraction. It was directionally subcloned into $pBLCAT_3$ that had been digested with *Xba*I, filled in with Klenow polymerase, and then digested with *Bam*HI. This pα(−100/+48)CAT vector was digested with *Pst*I, and the released fragment was subcloned into the *Pst*I site of $pBLCAT_3$. All constructions were verified by a combination of restriction site analysis, PCR (using primers that yield product in an orientation-dependent manner), and dideoxy sequencing. The deletion vectors are designated by the 5′/3′ termini of the GPHα promoter.

Point mutations were introduced into the GPHα promoter by PCR. Separate upstream primers contained the desired base changes and were used in conjunction with a common downstream primer anchored in the first exon (5′-CATATTACCTTTCTCTGGG-3′). The template consisted of the 20-kbp GPHα genomic clone in pBR322. Upstream primers corresponded to the GPHα genomic clone from −181 to −147 and contained a 5′ extension (lower case letters) that provided a *Hin*dIII recognition site. The wild-type sequence and positions mutated (indicated in parentheses to the right of the base) are indicated: 5′-gcaagcttCAAAAATGAC(−172)CTA(−169)AGGGTTGAAACAAGATAA(−151)GATCAAATTG-3′. Mutations, in pairs, were C to T at −172, A to G at −169, and A to T at −151. These sites were chosen to effect changes in DNA binding and enhancer function at subdomains TSE, URE, and α-ACT, respectively. The resulting vectors were named for the elements that retained their wild-type sequence. For example, T (TSE) contains mutations at −169 and −151 to inactivate both URE and α-ACT; U (URE) contains mutations at −172 and −151 to inactivate both TSE and α-ACT; and A (α-ACT) contains mutations at −172 and −169 to inactivate both TSE and URE. In gel mobility shift experiments (see Results), it was shown that these substitutions effectively eliminated protein binding to the corresponding oligonucleotides. The mutagenic oligonucleotides in combination with the exon I primer generated a 292-bp PCR product that was purified by electrophoresis through 1% agarose. After electroelution, the fragment was digested with *Hin*dIII (at a site provided in the forward primer) and *Pst*I (at a site centered at the GPHα gene cap site). These were subcloned into the polylinker upstream of the CAT reporter gene in $pBLCAT_3$ [Luckow and Schutz, 1987]. All clones were selected by hybridization of colony blots [Grundstein and Hogness, 1975] with the wild-type oligonucleotide and confirmed by dideoxy sequencing using a primer complimentary to the CAT gene. These mutants provided a collection of CAT expression vectors in which activity of the composite enhancer was derived from only one of the three *cis* elements.

DNA-Mediated Transfection and Reporter Gene Assay

All plasmids used in transfection experiments were prepared by CsCl gradient centrifugation or by chromatography on QIAGEN columns; their supercoiled conformation was verified by agarose gel electrophoresis. The day before transfection, cells were plated into 100-mm dishes at 5×10^5 cells per dish. DNA-calcium phosphate coprecipitation was performed as described previously [Graham and van der Eb, 1973], using 10 μg of each CAT reporter plasmid followed by a glycerol shock [Parker and Stark, 1979]. For transient expression assays, the cells were replenished after 24 h with 10 ml of fresh medium supplemented with effectors as indicated in the tables and figure legends and assayed for CAT activity 24 h later. HeLa cells were selected for stable integration and expression of CAT by coprecipitation of pα1.6CAT and pSV_2neo, followed by selection with G418 at 400 μg/ml. After 2 weeks, concentrations of G418 were reduced to 200 μg/ml, at which time mock-transfected cultures contained only cell debris and no viable cells. Pooled clones were treated with the indicated effectors 24 h after cells were plated; they were harvested after another 24 h and assayed for CAT activity. Chloramphenicol acetyltransferase was measured in transient and stable transfectants as outlined previously [Gorman et al., 1982a, 1982b]. Extracts were prepared from washed cells by freeze-thaw and sonication, heating at 65°C for 5 min, and incubation with [^{14}C]chloramphenicol and acetyl CoA for 3 h. Reaction mixtures were then extracted with 1 ml of ethyl acetate, dried, and chromatographed on silica gel thin-layer chromatography (TLC) plates in chloroform/methanol (95 : 5). After autoradiography, spots corresponding to the acetylated chloramphenicol derivatives were

scraped from the plate and quantified by liquid scintillation spectrometry. Protein concentration in the extracts was determined using the Bradford dye binding assay [Bradford, 1976] with BSA as the standard. Activity is expressed as μmoles of [^{14}C]chloramphenicol converted to acetylated forms or as percent of chloramphenicol converted to acetylated derivatives per mg of extract protein. Activity of β-galactosidase was determined in a colorimetric assay using extracts without heat pretreatment [Hall et al., 1983]. Parameters of protein concentration and incubation time were chosen to maintain linearity of the reaction.

Preparation of Nuclear Extracts

HeLa cells at about 75% confluence were incubated for 24 h in the absence or presence of 3 mM NaBtr. They were scraped into the medium, collected by centrifugation, and washed in ice-cold buffer containing 50 mM potassium phosphate (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5 mM EGTA. The final pellets were dispersed into TE buffer (10 mM Tris-Cl, pH 7.4, 1 mM EDTA) and immedediately diluted with an equal volume of 2× nuclear wash buffer (20 mM HEPES, pH 8.0, 10 mM NaCl, 30% sucrose, 2 mM EDTA, 1% Triton X-100, 2 mM dithiothreitol, 10 mM $MgCl_2$, and 2 mM phenylmethylsulfonyl fluoride). This was incubated at 0°C for 10 min, then underlaid with 30% sucrose, and centrifuged at 3,000*g* for 30 min at 5°C to collect nuclei. The supernatant fluid was removed, and the pellet was resuspended in a solution containing 10 mM HEPES (pH 8.0), 500 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5 mM spermidine. After the suspension was incubated on ice for 60 min, the nuclei were cleared by centrifugation at 10,000*g* for 10 min. The supernatant fraction was dialyzed extensively against the buffer system described by Manley et al. [1980]. For the preparation of extracts from Btr-treated cells, all solutions were also supplemented with 3 mM NaBtr.

Electrophoretic Mobility Shift Analysis

DNA-binding proteins were assayed by incubating ^{32}P-labeled oligonucleotides (10,000 cpm, approximately 0.5 ng) with 10 μg of unfractionated nuclear extract protein and 2 μg of poly (dI-dC)-poly (dI-dC) in 25 μl of a solution containing 12% glycerol, 12 mM HEPES (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 4 mM Tris-Cl (pH 7.9), 0.6 mM EDTA, and 0.6 mM dithiothreitol. This mixture was incubated at 22°C for 20 min before loading onto a 5% polyacrylamide gel, and DNA-protein complexes were separated from unbound probe by electrophoesis at 180–200 V for 90–120 min in buffer containing 90 mM Tris base, 64.6 mM boric acid, and 2.5 mM EDTA at pH 8.3. Sequences of the sense strands for the oligonucleotides used for binding and competition were as follows: TSE, 5′-GCAAATGACCTAAGGGTTGAAACG-3′; αACT (GATA), 5′-GAAACAAGATAAGATCAAAT-3′; CRE, 5′-CGGCAAATTGACGTCATGGTAAG-CCC-3′; BRE, 5′-TAAGGGTTGAAACAAGATA-AG-3′; HET, 5′-GACTTCATAACTGCAGTTAC-TGAGAAC-3′. The T (TSE), U (URE), and A (αACT) probes were double-stranded oligonucleotides corresponding to the mutagenic primers described above. In addition, a null (N) oligonucleotide contained base changes at all three positions (−172, −169, −151), and a wild-type (W) oligonucleotide had no mutations. Complimentary oligonucleotides (10 μg each) were annealed in 40 μl of a solution containing 20 mM Tris-Cl (pH 7.4), 2 mM $MgCl_2$, and 50 mM NaCl by placing the microcentrifuge tube in 1 liter of water at 95°C and allowing the mixture to cool until the bath reached ambient temperature. DNA (50–100 ng) was labeled with T4 polynucleotide kinase and [α-^{32}P]ATP.

RESULTS

Protein Synthesis Is Required For Induction of GPHα mRNA by NaBtr in HeLa Cells

The steady-state levels of GPHα mRNA showed a linear increase from 12 to 48 h after HeLa 2.2 cultures were treated with 3 mM NaBtr (Fig. 1A). During the course of several experiments, the maximum increase of 5- to 15-fold occurred between 24 and 48 h after the addition of Btr; mRNA levels declined between 48 and 72 h of Btr treatment. In untreated cultures, the steady-state levels of GPHα mRNA remained at a relatively constant level over this same time period. Extrapolation of the data shows that induction of mRNA occurred after a lag of about 6 h, and an increase in secreted α-subunit was detected in Btr-treated cells after a lag of about 14 h. Accumulation of subunit was linear from 24 to 72 h, reaching levels about 6-fold higher than uninduced cells at 72 h. It is intriguing that a 13.5-fold increase in GPHα mRNA produced only a 6-fold increase in

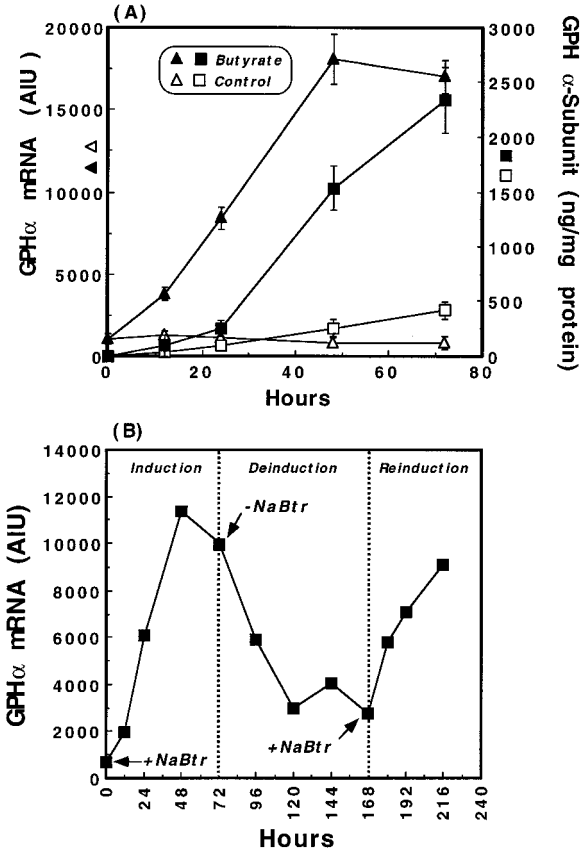

Fig. 1. Kinetics of GPHα gene induction by NaBtr in HeLa cells. **A:** Replicate cultures were left untreated (open symbols) or supplemented with 3 mM NaBtr (closed symbols). At the times indicated, media were aspirated for determination of secreted α-subunit by RIA (□, ■). Cell sheets were harvested, lysed and assayed for GPHα mRNA by dot blot hybridization (△, ▲). **B:** Kinetics of induction, deinduction, and reinduction of the GPHα gene by NaBtr in HeLa cells. Replicate cultures in T-25 flasks were supplemented with 3 mM NaBtr at time-zero. Duplicate flasks were harvested at 12, 24, 48, and 72 h after NaBtr addition and processed for dot-blot hybridization (Induction). Medium from the remaining cultures was aspirated, and cell sheets were washed twice, then covered with 5 ml of fresh medium not containing NaBtr. Duplicate cultures were processed 24, 48, 72, and 96 h after NaBtr had been washed out (Deinduction). At that time, the remaining cultures were replenished with fresh medium again supplemented with 3 mM NaBtr. Duplicate cultures were harvested and processed 12, 24, and 48 h after the second addition of NaBtr (Reinduction). Cytoplasmic lysates were diluted and spotted on nylon membranes to give 1, 3, and 10 μg of RNA. These were hybridized to ^{32}P-labeled GPHα cDNA and quantified as described previously [Cosgrove et al., 1989]. Values are reported as arbitrary integrator units (AIU) and are the average of duplicate cultures at two different RNA concentrations.

secreted α-subunit, suggesting that post-transcriptional effects may contribute to the induction process. In this regard, an earlier report from this laboratory showed that NaBtr decreases the apparent half-life of GPHα mRNA by approximately 2-fold [Cosgrove and Cox, 1990]. The effect of NaBtr on expression of the GPHα gene is reversible, as illustrated in Fig. 1B. When HeLa cells were treated with a single dose of 3 mM NaBtr, the levels of GPHα mRNA increased steadily for about 48 h and then began to decline. When the cells were washed and replenished with medium not containing NaBtr after 72 h of induction, the rate of mRNA decline was enhanced, and mRNA levels decreased over the next 96 h, at which time cultures were again supplemented with 3 mM NaBtr. At that time, GPHα mRNA levels increased without an apparent lag for the next 48 h. The overall rate and extent of mRNA accumulation were less in the reinduction phase as compared with the initial induction phase.

In order to determine whether continued protein synthesis is required for induction of GPHα mRNA by NaBtr, it should be possible to measure steady-state levels of mRNA in HeLa cells treated with NaBtr in the absence and presence of translation inhibitors such as cycloheximide (CHX). However, this rationale is complicated by the previous discovery that the GPHα gene is inducible in HeLa 2.2 cells by CHX and puromycin [Cox et al., 1990]. Consequently, a HeLa clone (A19) in which the production of GPHα mRNA is significantly less responsive to CHX but is as responsive to NaBtr as the parental cells was used to carry out this analysis. The characteristics of these cells are summarized in Table I.

The A19 cells were pretreated with NaBtr for periods of 0, 6, 12, 24, and 36 h before the addition of CHX. All cells were harvested at 48 h, and RNA dot hybridization was performed. As noted in Figure 2, a 32-fold increase in GPHα mRNA levels occurred after culturing

TABLE I. Effect of Cycloheximide and Sodium Butyrate on the Induction of α-Subunit mRNA in HeLa Cell Lines[a]

HeLa Cells	α-Subunit mRNA (AIU)		
	Basal	CHX	Butyrate
CCL 2.2	1,530 (1.0)	15,350 (10.0)	24,000 (15.7)
Clone A19	4,380 (1.0)	10,524 (2.4)	90,770 (20.7)

[a]The cell lines indicated were grown to near-confluence, then treated for 24 h with 10 μg/ml CHX or for 48 h with 3 mM NaBtr. A control set of cultures received only fresh medium (basal). Cytoplasmic lysates were prepared and analyzed by dot-blot hybridization to ^{32}P-labeled GPHα cDNA. Levels of mRNA were quantified by scanning densitometry of the autoradiogram. The mRNA levels are expressed as arbitrary integrator units (AIU); numbers in parentheses were normalized to control (uninduced) levels.

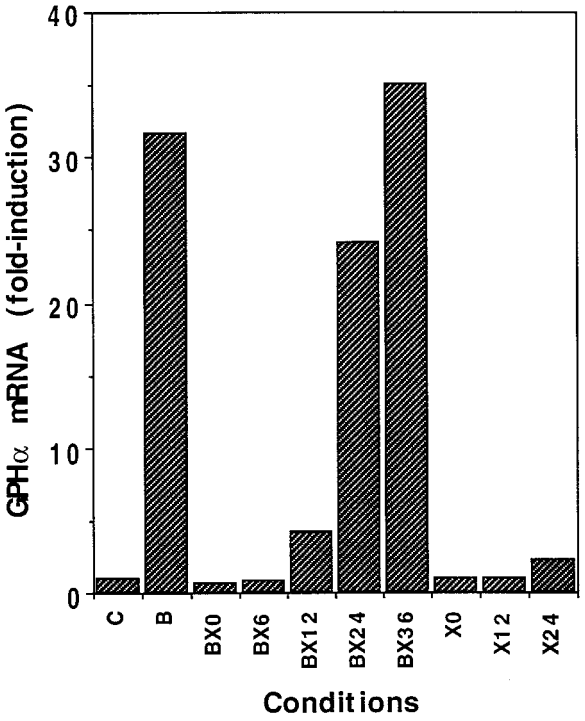

Fig. 2. Effects of protein synthesis inhibition on α-subunit induction by NaBtr. Treatment of HeLa A19 cultures with various combinations of 3 mM NaBtr and/or 10 μg/ml CHX was as follows: (C) no additions, harvested at 48 h; (B) NaBtr added at zero time, harvested at 48 h; (BX0) NaBtr and CHX added at zero time, harvested at 48 h; (BX6, BX12, BX24, BX36) NaBtr added at zero time, followed by addition of CHX at either 6, 12, 24, or 36 h, harvested at 48 h; (X0, X12, X24) CHX added at zero time or at 12 or 24 h, harvested at 48 h. Total RNA was isolated by phenol extraction before analysis by dot hybridization. The autoradiogram was quantified by optical scanning densitometry, and the data are presented as arbitrary integrator units after normalizing to the uninduced sample (C).

the A19 cells in 3 mM NaBtr for 48 h (B). Induction of GPHα mRNA by NaBtr was completely inhibited by CHX when both compounds were added simultaneously at zero time (BX0). These data also confirm that NaBtr induction of the GPHα gene occurs after a lag of 12–24 h. That is, when cells were pretreated with NaBtr for 24 or 36 h before the addition of CHX (BX24 and BX36), the levels of GPHα mRNA were similar to those in cells receiving no CHX (B); but cells treated with NaBtr for only 6 h (BX6) or 12 h (BX12) before the addition of CHX showed little or no induction of the GPHα gene. Addition of CHX alone produced only marginal changes in the levels of GPHα mRNA after periods of 48 h (X0), 36 h (X12), and 24 h (X24). These samples are the parallel controls for comparison with BX0, BX12, and BX24, respectively. The time lag and the requirement for protein synthesis are consistent with a model in which the synthesis or modification of a new or limiting protein(s) is required for induction of the GPHα gene by Btr in HeLa cells.

GPHα Gene Expression in HeLa Cells Treated With NaBtr and dGlc

When HeLa cells were simultaneously treated with 3 mM NaBtr and 5 mM dGlc in medium containing 5 mM Glc, only a modest increase in secreted α-subunit could be detected (Fig. 3A). However, when cells were preincubated with NaBtr for 24 h before dGlc addition, the hexose was unable to prevent the induction of GPHα. The accumulation of subunit in the pretreated cultures was comparable to that of cultures treated with 3 mM NaBtr in the absence of dGlc. Figure 3B shows that the effects of dGlc on Btr induction of GPH α-subunit were accomplished by parallel changes in the steady-state levels of GPHα mRNA. That is, dGlc added simultaneously with NaBtr prevented the increases in α-subunit mRNA that were produced in cultures receiving either NaBtr alone or NaBtr initially followed after 24 h with dGlc. In experiments not presented, it was found that dGlc caused a greater increase in the percentage of α-subunit that remained intracellular in Btr-treated cultures as compared with uninduced cultures. However, when absolute values of secreted and intracellular subunit were examined, calculations indicated that the amount of subunit sequestered in cells treated with NaBtr and dGlc was not sufficient to account for the dramatic reversal in levels of secreted α-subunit when these cultures were compared with those treated with NaBtr only. Thus, taken together, these results support the conclusion that the changes in subunit production were largely the result of changes in the levels of GPHα mRNA.

Inhibition by dGlc of the Btr-Mediated Induction of the GPHα Promoter Is Tissue Specific

Previous studies have suggested that the α-subunit gene is regulated differently in cell lines derived from trophoblastic and nontrophoblastic tumors [Chou et al., 1977; Hussa et al., 1978; Ruddon et al., 1980]. For example, cAMP induces the gene 10- to 15-fold in JEG-3 choriocarcinoma cells but elevates GPHα mRNA levels only 1- to 2-fold in HeLa cells. By contrast, the gene is induced 10- to 15-fold by NaBtr in HeLa cells, but the SCFA does not increase, and

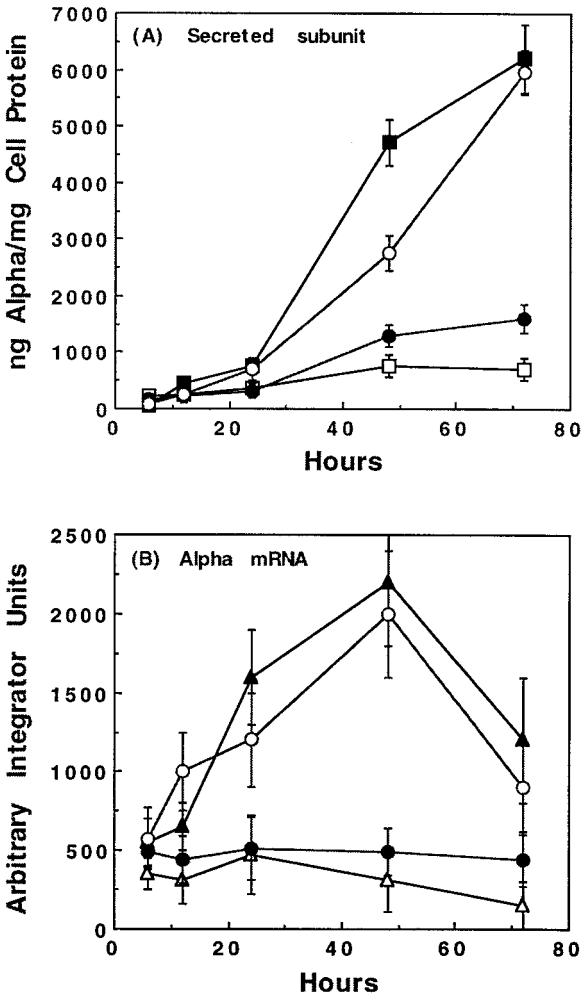

Fig. 3. Effect of dGlc on induction of the GPHα gene by NaBtr. **A:** Series of HeLa cultures in 25-cm^2 flasks received no additions (□), 3 mM NaBtr (■), 3 mM NaBtr plus 5 mM dGlc at zero time (●), 3 mM NaBtr plus 5 mM dGlc after 24 h (○). At the times indicated on the abscissa, media were aspirated and assayed for α-subunit by RIA and normalized to the amount of cell protein. **B:** HeLa cells were cultured under the same conditions as described in A. They were harvested at the times indicated and used for preparing lysates as described by White and Bancroft [1982]. Hybridization to ^{32}P-labeled GPHα cDNA and densitometry of autoradiograms were carried out as described by Cosgrove et al. [1981]. Replicate 25-cm^2 flasks received no additions (△), 3 mM NaBtr (▲), 3 mM NaBtr and 5 mM dGlc at zero time (●), and 3 mM NaBtr at zero time followed by 5 mM dGlc at 24 h (○).

in some cases decreases, mRNA levels in JEG-3 cells. However, in the presence of cAMP, NaBtr acts synergistically to increase steady-state levels of GPHα mRNA from 25- to 50-fold in JEG-3, indicating that the dichotomy of cAMP and Btr responses in trophoblastic (JEG-3) and nontrophoblastic (HeLa) tumor cell lines is not as distinct as previously thought [Campain et al., 1994]. Thus, it was of interest to compare the effects of dGlc on GPHα mRNA levels in cells that produce the α-subunit as either an ectopic (HeLa) or eutopic (JEG) product. The hybridization results (not presented) showed that 1 mM 8-Br-cAMP increased the steady-state level of GPHα mRNA about 11-fold in JEG-3 cells, whereas 3 mM NaBtr produced only a 2-fold increase. These agents acted synergistically to elevate transcript levels about 32-fold. Deoxyglucose (10 mM, relative to 11.5 mM Glc in RPMI-1640 medium) had little or no effect on basal levels or on the levels generated in response to cAMP, NaBtr, or cAMP plus NaBtr. Values in the presence of dGlc were 90–160% of those in its absence. These results suggest that Btr is acting through distinct pathways to induce the gene in HeLa and JEG-3 cells and that only one pathway is sensitive to dGlc.

Effect of dGlc on Induction of the GPHα Gene by NaBtr Is Mediated Through Its Promoter

As shown in a previous communication [Cox et al., 1987] and confirmed above, Btr induction of the GPHα gene in HeLa cells can be negated by dGlc. Under these conditions (i.e., equimolar Glc and 2-dGlc), the hyperacetylation of histones by Btr is unaffected [Cox et al., 1987], suggesting that this chromatin modification is not sufficient for induction. Consequently, it was of interest to determine whether NaBtr could enhance transcription from the GPHα promoter in a DNA-mediated transient expression assay and whether this effect could be negated by dGlc. The levels of CAT activity in HeLa cells transfected with pα1.6CAT were increased about 4-fold by NaBtr, and this was reduced to basal (uninduced) levels by the simultaneous addition of dGlc (Fig. 4 and Table II). The pSV$_2$CAT plasmid, which contains the SV-40 early region promoter, exhibited high activity in HeLa cells that could be further induced about 5-fold by NaBtr. However, induction of the viral promoter by NaBtr was inhibited only 27% or less by the addition of 5 mM dGlc. To further examine the promoter dependence for reversal of Btr induction by dGlc, transient expression assays were performed with plasmids in which the CAT gene was driven by either the human cytochrome P-450-IA1 gene promoter or the Rous sarcoma virus LTR. The P-450-IA1-CAT and RSV-CAT constructs were induced 6.4- and 4.2-fold, respectively, by Btr treatment but, as in the case with pSV$_2$CAT,

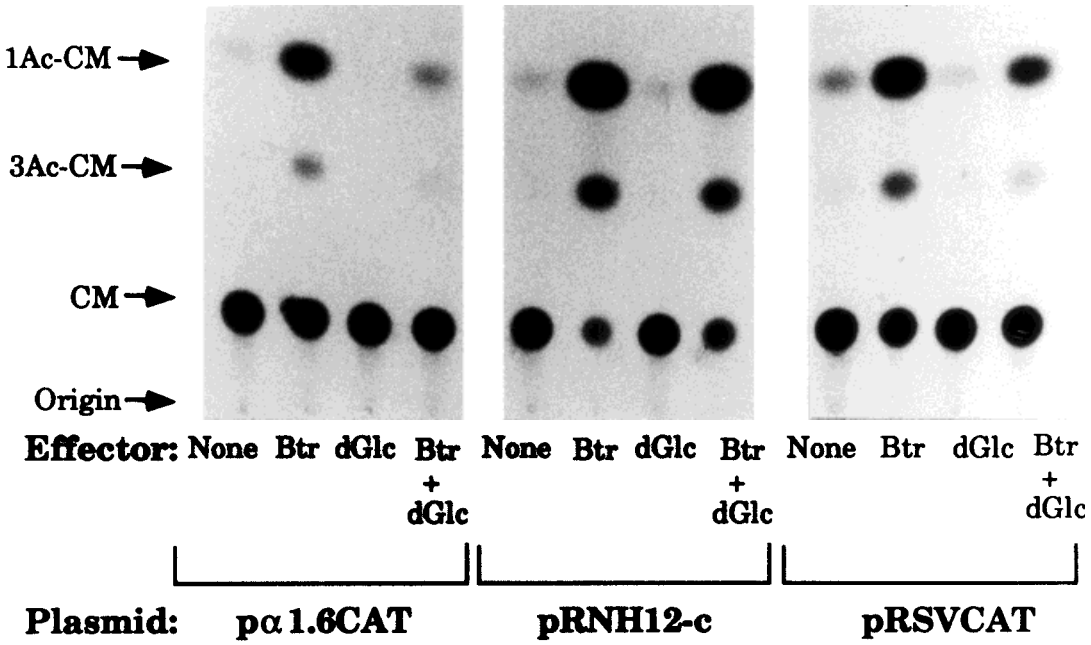

Fig. 4. Effect of NaBtr and dGlc on the promoters of cytochrome P450-IA1, GPHα, and the Rous sarcoma virus LTR. HeLa 2.2 cells were transfected with pα1.6CAT, pRNH12c, and pRSVCAT. At 24 h after the glycerol shock, cells were treated with 3 mM NaBtr in the absence and presence of 5 mM dGlc. After an additional 24 h, the cells were assayed for CAT activity, a direct measure of the ability of the promoters to be influenced by these effectors. Transfection with pSV_0CAT provided a measure of background CAT production in these experiments, as the pα1.6CAT vector was derived from this promoterless plasmid. [^{14}C]Chloramphenicol (CM) and its acetylated forms (1Ac-CM, 3Ac-CM) are indicated.

induction of transferase activity by NaBtr was not significantly inhibited by dGlc. The results of a single experiment are shown in Figure 4, and the averages of several determinations are quantified and summarized in Table II.

Identification of a Btr-Responsive Element in the GPHα Gene

The above results suggest that 1.6 kbp of GPHα 5′-flanking DNA is sufficient to provide a transcriptional response to NaBtr that is sensitive to dGlc. Thus, it was of interest to identify the Btr-responsive element (BRE) inferred from the transfection experiments. A collection of reporter plasmids was constructed by inserting DNA fragments of variable length into a polylinker located upstream of the CAT gene in $pBLCAT_3$ [Luckow and Schutz, 1994]. These GPHα promoters and the relative positions of binding sites for a number of transcription factors are depicted in Figure 5. The CAT levels were increased about 4-fold in HeLa cells transfected with each of the pαCAT vectors until the promoter was truncated to −180. The vectors terminating at −169, −150, and −100 generated CAT activity in Btr-treated cells that was 360%, 160%, and 73%, respectively, of the activity produced in untreated cells. Thus, these results suggest that a *cis*-regulatory element necessary for the response to NaBtr is located between nucleotides −169 and −150. The fact that a fraction of the Btr response was lost when DNA between −180 and −169 and between −150 and −100 was deleted suggests that sequence information flanking the −169/−150 motif may also contribute to full activity of the element.

The above results implicate a region of the GPHα gene 5′-flanking DNA that approximately corresponds to an element previously characterized as a trophoblast-specific enhancer [Jameson et al., 1989]. This is a composite enhancer containing binding sites for at least three separate *trans*-acting factors [Jameson et al., 1989; Pittman et al., 1994]. The most proximal, α activation element (αACT), abuts the distal cAMP-responsive element (CRE) and probably binds one or more members of the GATA factor family [Steger et al., 1991, 1994]. The most distal element has been called distal-TSE or URE-2 [Jameson et al., 1988], referred to here as trophoblast-specific element (TSE).

TABLE II. Effect of dGlc on Btr-Mediated Induction of CAT Activity Driven by a Variety of Promoters[a]

Cells	Plasmid	Effector	CAT activity
HeLa 2.2	pα1.6CAT	None	0.12 ± 0.02 (1.0)
		dGlc	0.14 ± 0.03 (1.1)
		NaBtr	0.51 ± 0.07 (4.1)
		NaBtr + dGlc	0.11 ± 0.02 (0.9)
	pSV_2CAT	None	0.52 ± 0.09 (1.0)
		NaBtr	2.59 ± 0.38 (5.0)
		NaBtr + dGlc	1.89 ± 0.38 (3.6)
	pRNH12c	None	0.10 ± 0.01 (1.0)
		dGlc	0.14 ± 0.02 (1.4)
		NaBtr	0.70 ± 0.11 (6.4)
		NaBtr + dGlc	0.80 ± 0.16 (7.6)
	pRSVCAT	None	0.06 ± 0.01 (1.0)
		dGlc	0.04 ± 0.01 (0.6)
		NaBtr	0.24 ± 0.04 (4.2)
		NaBtr + dGlc	0.22 ± 0.04 (3.8)
HeLa ST1.6	pα1.6CAT (integrated)	None	0.07 ± 0.01 (1.0)
		dGlc	0.07 ± 0.01 (1.0)
		NaBtr	0.23 ± 0.04 (3.5)
		NaBtr + dGlc	0.07 ± 0.01 (1.1)

[a]HeLa 2.2 cells were transfected with the plasmids indicated. Cultures were replenished 24 h later with fresh medium and the effectors 3 mM NaBtr and 5 mM dGlc. After another 24 h, cells were harvested and assayed for CAT activity. HeLa ST1.6 cells were cultured 24 h in the presence of effectors and assayed for CAT. The amount of chloramphenicol converted to acetylated forms was determined by scraping the acetylated chloramphenicol spots from thin-layer chromatography (TLC) plates and counting by liquid scintillation spectrometry. Protein concentration in cell extracts was determined by the Bradford [1976] assay using bovine serum albumin as a standard. CAT activity and standard error are expressed as μmoles of [^{14}C]chloramphenicol converted to acetylated forms per mg of extract protein; values in parentheses are normalized to the samples receiving no effectors.

Located between, and probably overlapping, the TSE and αACT motifs is an element referred to previously as the proximal-TSE or URE-1 [Jameson et al., 1988; Pittman et al., 1994], referred to here as URE (upstream regulatory element). Thus, it was of interest to determine more precisely which of these elements (TSE, URE, αACT) mediate the induction by NaBtr.

Point mutations were introduced into the GPHα enhancer so that two of the three subdomains were inactivated, leaving expression of a CAT reporter gene dependent on the third element in conjunction with the downstream tandem CRE and basal promoter elements (e.g., CAAT, TATA). To verify that the selected base changes were sufficient to disrupt the enhancer domains, the ability of mutated oligonucleotides to interfere with binding of the appropriate enhancer-binding proteins (i.e., TSEB, UREB, ABP or GATA factor) with their cognate *cis* elements (i.e., TSE, URE, αACT or GATA element) was determined. The extrapolation assumed here is that failure of a mutated oligonucleotide to support protein binding will be reflected by a concomitant inactivation of the corresponding domain such that it does not contribute to enhancer activity in transient expression assays.

As shown in Figure 6A, a single DNA-protein complex with low mobility was produced when ^{32}P-labeled TSE oligonucleotide was incubated with HeLa nuclear extracts (lane 1), which was effectively eliminated by excess unlabeled W (lanes 2 and 3), TSE (lanes 4 and 5), and T (lanes 6 and 7), but not by U (lanes 8 and 9), A (lanes 10 and 11), and N (lanes 12 and 13) oligonucleotides. Similarly, Figure 6B shows that a single, rapidly migrating complex was produced with a ^{32}P-labeled U probe (lane 1), and this could be disrupted by a 200-fold excess of wild-type (W) and U oligonucleotides (lanes 2 and 3) but not by excess TSE (lane 4), αACT (lane 5), and TSE plus αACT (lane 6) oligonucleotides. When ^{32}P-labeled αACT was the probe (Fig. 6C), a single complex was produced that migrated slightly faster than the TSEB complex but significantly slower than the UREB complex. The ABP complex was disrupted by

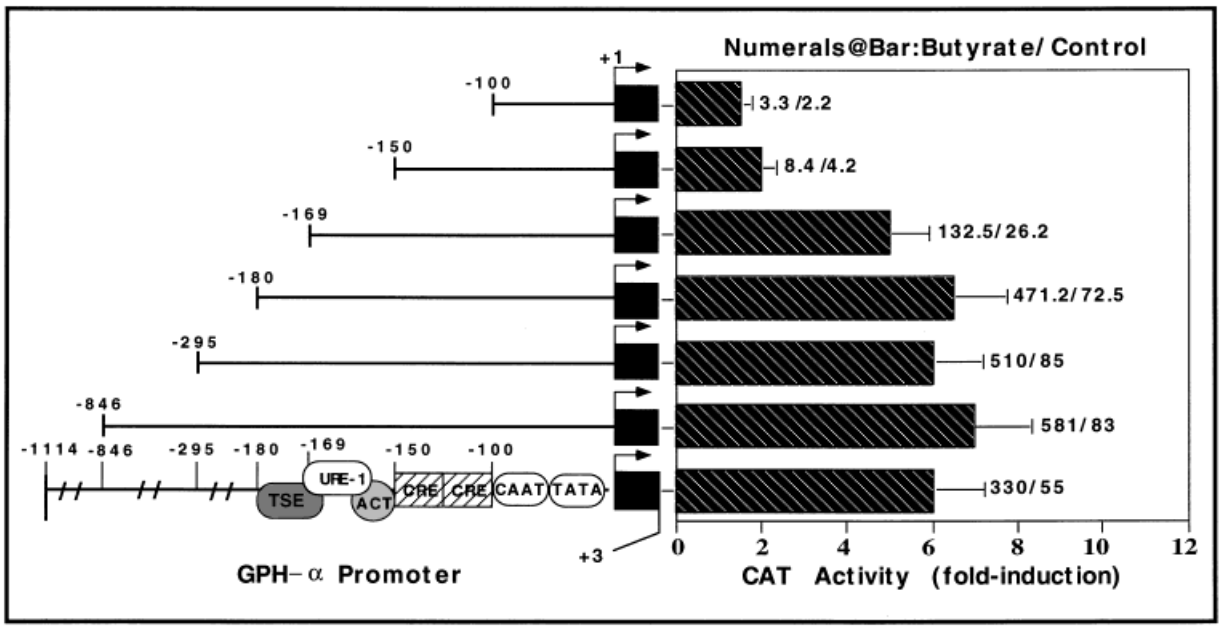

Fig. 5. Identification of a Btr-responsive element in the GPHα gene 5′-flanking DNA. The pαCAT expression vectors (10 μg) were coprecipitated with pCMVlacZ (5 μg) and transfected into HeLa cells using the calcium phosphate method [Graham and van der Eb, 1973]. At 24 h after the glycerol shock, one set of transfected cultures was treated with 3 mM NaBtr, while the other received no additions. After another 24 h, all cells were harvested and assayed for CAT and β-galactosidase. The ratio of CAT:βgal activities was determined for each vector in Btr-treated and untreated cells. The numerical values for the normalized CAT activity are provided at the end of each bar, which depict the fold-induction (Btr/Control). The relative position of *cis*-acting elements in the GPHα promoter are schematically presented at the left (not drawn to scale). The numbers indicate the 5′-termini of the truncated promoter mutants. Bent arrow, transcription start site; black box, exon I. The geometric symbols indicate (from right to left) the TATA box, CAAT box, cAMP-response elements (CRE), α activation element (αACT), upstream regulatory element (URE), and trophoblast-specific element (TSE).

supplementing binding reactions with excess W (lanes 2 and 3), αACT (lanes 4 and 5), and A (lanes 10 and 11) oligonucleotides. The null (N) oligonucleotide had no effect on complex formation, and the T and U oligonucleotides showed no effect on complex formation at 50 ng but caused a slight reduction at 200 ng (an approximately 400-fold excess). When the wild-type (W) oligonucleotide was used as a probe (results not presented), two complexes were produced with HeLa nuclear extracts that migrated coincident with TSEB and ABP, and these could be specifically eliminated with TSE and αACT oligonucleotides, respectively. Complexes migrating slower than TSEB could not be detected, suggesting that ternary complexes of DNA and TSEB plus ABP were not formed or were not stable under the in vitro conditions of assay. The UREB complex could be detected only when TSE and αACT were both mutated, suggesting that TSEB and ABP were strong competitors for UREB binding if all three enhancer subdomains were active. Together, these results demonstrate that the point mutations at −172, −169, and −151 effectively disrupted interactions between the enhancer elements and their cognate binding proteins.

Promoters were constructed by PCR mutagenesis to contain these same point mutations, which were used to drive CAT expression in transient transfection experiments. The expression vectors are named for the enhancer elements not mutated using the abbreviations T (for TSE), U (for URE), A (for αACT), and C (for tandem CREs). In several experiments, a vector with an intact enhancer (pαTUAC-CAT) produced high levels of CAT that were increased from 4- to 8-fold in response to NaBtr. By contrast, the marginal (1.5- to 2.5-fold) induction of CAT by NaBtr in cells transfected with pαC-CAT shows that mutation of all three domains in the composite enhancer significantly restricted the GPHα promoter response to the SCFA (Fig. 7). The levels of CAT activity in cells transfected with vector pαTC-CAT, pαUC-CAT, and pαAC-CAT were increased 6-fold, 9-fold, and 17-fold, respectively (Fig. 7). Because the response of theses enhancers is limited to the

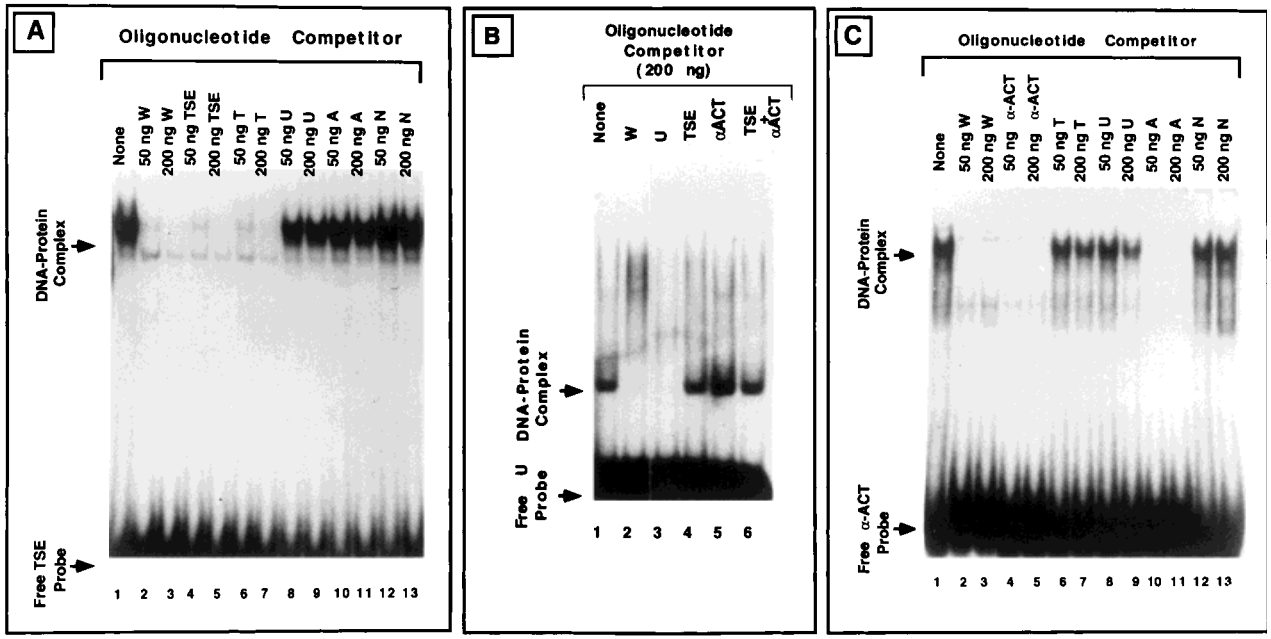

Fig. 6. Effect of point mutations on the binding of HeLa nuclear proteins to GPHα enhancer subdomains. Oligonucleotides extending from −181 to −141 were synthesized to contain the following base changes: T, A(−169)G plus A(−151)T; U, C(−172)T plus A(−151)T; A, C(−172)T plus A(−169)G; N, C(−172)T plus A(−169)G plus A(−151)T. A wild-type (W) oligonucleotide was left unchanged. DNA binding was carried out as described under Materials and Methods with 10 μg of HeLa nuclear extract protein and 10,000 cpm (approx. 0.5 ng) of the probe designated. **A:** TSE. **B:** U. **C:** αACT. Unlabeled oligonucleotide competitors were added at a 100- or 400-fold excess as indicated above the gel lanes. B: Competing oligonucleotides were present at 200 ng levels. Extracts were preincubated with competitors for 10 min before probe addition. Incubations were at 22°C (A, C) or 4°C (B).

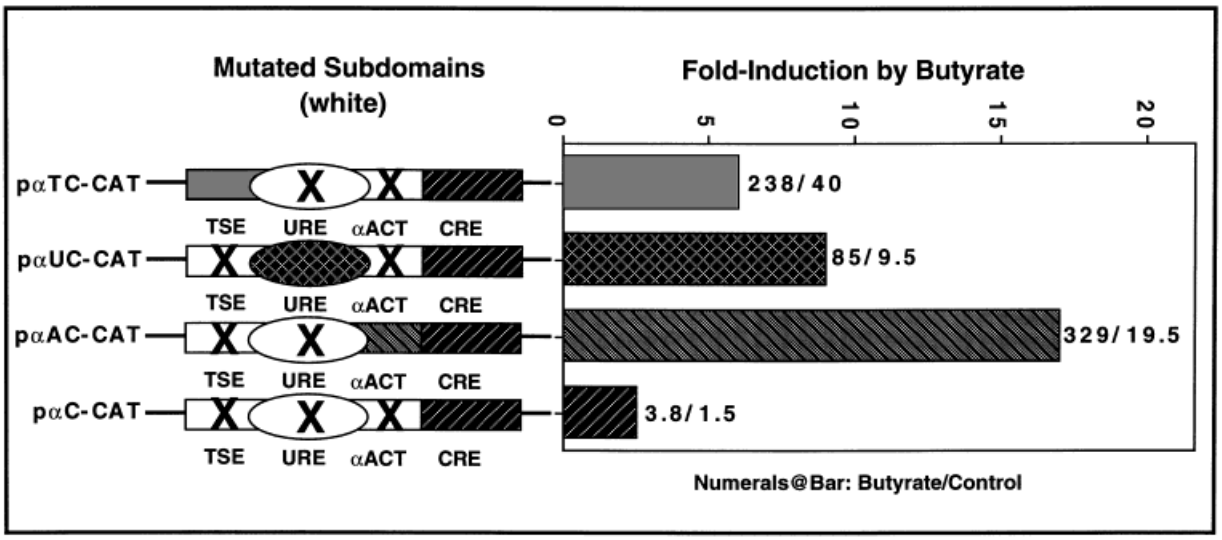

Fig. 7. Response of enhancer subdomain(s) to NaBtr. HeLa cultures were transfected in quadruplicate with calcium phosphate precipitates containing 10 μg of plasmid DNA as indicated. These include pαTC-CAT, pαUC-CAT, pαAC-CAT, pαC-CAT, and pαTUAC-CAT. After 24 h, one-half of the cultures received no additions, and one-half received 3 mM NaBtr. Cells were harvested after another 24 h and assayed for CAT as described under Materials and Methods. The enhancer cartoons show mutated subdomains in white (with an X) and wild-type elements in various patterns. Bars in the graph are patterned to match the unmutated element and represent the Btr-induced CAT activity relative to the uninduced activity produced from the corresponding vector. These values are given as a numerical ratio (Btr/Control) at the bar termini. Values for the wild-type enhancer (pαTUAC-CAT) were 428/54 (7.9-fold).

one remaining element not inactivated by mutagenesis (in concert with the tandem CREs), these results support those obtained initially with the deletion mutants that implicate the region from −169 to −150, which contains both the URE and αACT elements. The site-directed mutants provided a means to further refine localization of the BRE; they demonstrate that the greatest response to the SCFA was provided by the αACT element and progressively lower responses by the URE and TSE subdomains.

Induction of the GPHα Promoter by NaBtr in Cells Stably Transfected With pα1.6CAT Is Inhibited by dGlc

It has been demonstrated previously that Btr can enhance CAT synthesis when a variety of plasmids are transfected into cells for assay by transient expression [Gorman et al., 1983; Goldstein et al., 1989]. This general effect is independent of whether the corresponding cellular gene is induced by the SCFA and may reflect uncharacterized effects on plasmid uptake, stability, or histone deposition [Gorman et al., 1983]. Consequently, an attempt was made to control for this nonspecific type of induction by constructing stable transfectants carrying the α-subunit promoter-CAT chimera. HeLa 2.2 cells were co-transfected with pα1.6CAT and pSV_2neo, and stable transfectants were selected on the neomycin analog G418 as described under Materials and Methods. HeLa ST1.6–1 and HeLa ST1.6–2 refer to G418-resistant cultures selected from HeLa 2.2 cells transfected with pα1.6CAT plus pSV_2neo at DNA ratios of 5:10 and 15:10, respectively. The results presented in Figure 8 and Table II demonstrate that NaBtr induced CAT activity from the stably integrated GPHα promoter, and this induction was inhibited by dGlc down to uninduced levels. Deoxyglucose had no effect on basal CAT activity. The results presented in Figure 8 also show that tunicamycin (TM), which inhibits asparagine-linked glycosylation, had little or no effect on basal or induced CAT production. It is concluded that the integrated and unintegrated CAT constructs respond similarly to NaBtr and dGlc.

Enhancer Binding Proteins in Nuclear Extracts from Cells Cultured in the Absence and Presence of NaBtr

Electrophoretic mobility shift analysis was used to investigate whether NaBtr produced a change in the activity of one or more DNA-binding proteins that interact with a cluster of *cis*-regulatory elements in the 5′-flanking DNA

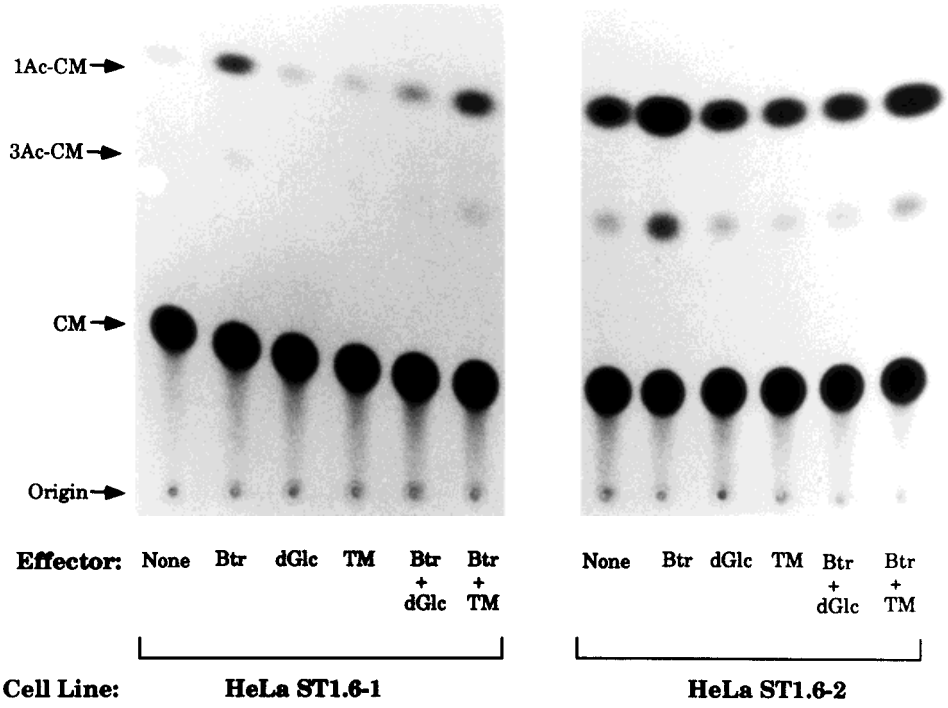

Fig. 8. Effect of NaBtr and dGlc on CAT activity in HeLa 2.2 cultures stably transfected with pα1.6 CAT. Stable transfectants were selected as described under Materials and Methods. HeLa ST1.6-1 and HeLa ST1.6-2 cells were plated into 60-mm dishes at 5×10^5 cells/dish in medium without G418, and after 24 h were treated with 3 mM NaBtr, 5 mM dGlc, and 2 μM tunicamycin (TM) as indicated. Cell extracts were prepared 24 h later, and 25 μg of cell lysate protein was assayed for CAT activity by thin-layer chromatography (TLC), followed by autoradiography.

of the GPHα promoter. These include the TSE, αACT, and tandem CRE motifs located between −180 and −110 relative to the transcription start site (+1) and the putative BRE encompassing nucleotides −170 to −150. Proteins that interact with each of these elements to generate complexes migrating slower than the unbound probe were detected in nuclear extracts prepared from HeLa cells cultured in the absence or presence of 3 mM NaBtr. As seen in Figure 9, there was little or no difference in TSE-, αACT-, and CRE-binding activities in extracts from untreated and Btr-treated cells. In each case, specificity of binding was demonstrated by competition with a 200-fold excess of homologous (TSE, αACT, CRE, BRE) and heterologous (Het) oligonucleotides. Reproducible changes in URE binding activity between extracts prepared from untreated and Btr-treated cells could not be demonstrated (data not presented). A nuclear protein that interacts with the putative BRE (Fig. 10) has not been identified previously, although the element partially overlaps the TSE on the 5′ end (TAAGGG) and the αACT motif on the 3′ end (AGATAA). The results presented in Figure 9 (CGA-BRE) show that a specific DNA-protein complex (marked with an arrow) was produced when the ^{32}P-labeled BRE probe was incubated with nuclear extracts from untreated and Btr-treated HeLa cells. The complex migrated faster than the TSE-TSEB and αACT-ABP complexes, indicating that it was neither of these. There was a slower complex (designated with a filled box) that comigrated with an αACT-ABP complex when these were analyzed on the same gel (data not presented). Thus, these results suggest that in addition to complex formation by the BRE-binding protein, sufficient sequence is available at the 3′ end of the oligonucleotide for weak interaction with a GATA factor under the binding conditions employed. Deoxyglucose had no effect on DNA-binding activity of the above factors in the absence or presence of NaBtr (data not presented).

DISCUSSION

It has been established that a wide spectrum of genes are expressed at significantly higher levels in cells cultured in the presence of SCFA like propionate and Btr [Kruh, 1982]. It has also been documented for a few genes that their transcript levels are reduced in response to Btr

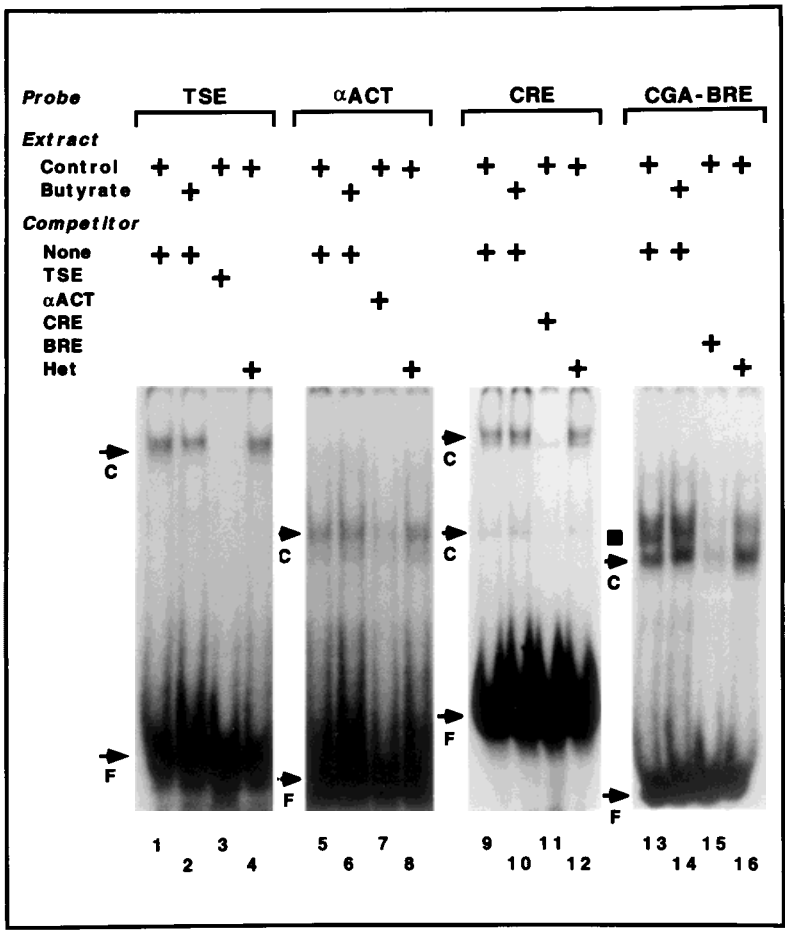

Fig. 9. Electrophoretic mobility shift analysis of enhancer binding proteins in nuclear extracts from untreated and Btr-treated HeLa cells. Nuclear extracts were prepared from approximately confluent HeLa cultures that had been left untreated or exposed to 3 mM NaBtr for 24 h. Binding of ^{32}P-labeled oligonucleotides to 10 μg of protein was carried out in reaction mixtures described previously [Campain et al., 1994]. The DNA-protein complexes (C) were separated from free probe (F) by electrophoresis on native polyacrylamide gels. Incubation and electrophoresis were carried out at 22°C for TSE, αACT, and CRE and at 4°C for CGA-BRE. Oligonucleotides contained motifs for enhancer binding proteins (TSE, **lanes 1–4**; αACT, **lanes 5–8**; CRE, **lanes 9–12**) and the putative Btr-responsive element (BRE, **lanes 13–16**).

[McKnight et al., 1980; Ormandy et al., 1992]. The effect of CHX has been examined in some systems to determine whether induction (or repression) by Btr is dependent on continued protein synthesis. In this regard, genes can generally be grouped into one of two categories. The first includes genes that are induced by Btr with rapid kinetics (e.g., 2–4 h), and the increase in mRNA is not attenuated by simultaneous treatment with CHX or other inhibitors of protein synthesis. The second group includes genes that are induced by SCFA only after a significant lag (e.g., 6–12 h), and cells treated simultaneously with CHX and Btr show little or no increase in mRNA. These properties are frequently used to classify genes as immediate or delayed response genes with respect to a particular stimulus. Our results place the GPHα gene in the second category when induced by NaBtr in HeLa cells (i.e., prominent lag, inhibited by CHX), but in the first category when assayed in JEG-3 cells (i.e., nominal lag, not inhibited by CHX). The latter classification may be oversimplified, however, as NaBtr is ineffective in JEG-3 cells unless CHX or cAMP is also present [Campain et al., 1994].

The data summarized in Figure 2 demonstrate that protein synthesis is required for induction of HeLa α-subunit mRNA by NaBtr. The choice of HeLa clone A19 for demonstrating this effect was critical since it had been shown previously that the α-subunit gene could be induced by CHX itself in HeLa 2.2 cells [Cox et al., 1990]. Because expression of the α-subunit gene is only nominally increased by CHX in clone A19, the effect of inhibiting protein synthesis on α-subunit mRNA production should be limited to those processes required for Btr induction. The window of time during which CHX can prevent the induction of GPHα mRNA by NaBtr is comparable to the lag time required to observe an increase in α-subunit mRNA in response to the SCFA (Fig. 1). Although other interpretations are possible, a significant lag is consistent with the synthesis of a new protein(s) required for Btr induction of HeLa α-sub-

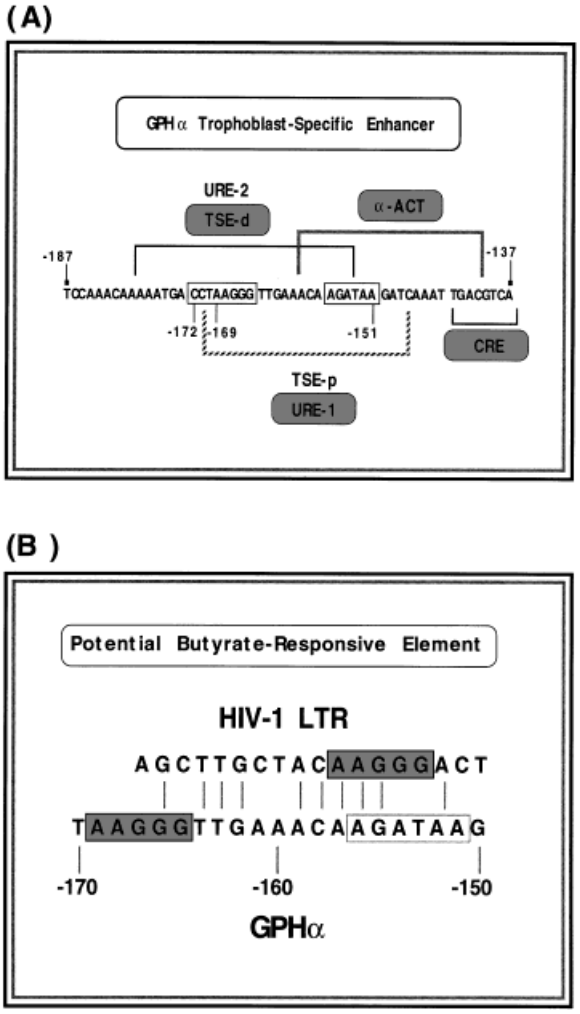

Fig. 10. Structure of the composite trophoblast-specific enhancer and comparison of the GPHα and HIV-1 Btr-responsive elements. **A:** Relationship of the adjacent and overlapping domains (brackets) of the composite enhancer. Adjacent to the distal CRE (at right) is the αACT element, which contains a GATA motif (boxed). The distal TSE (TSE-d or URE-2) joins αACT with a few base pair (bp) overlap. A consensus sequence for TSE binding proteins (CCTNNGGG) is boxed. The URE-1 (or proximal TSE) domain occupies a central position in the enhancer and exhibits significant overlap with the TSE-d and αACT elements. Bases at positions −151, −169, and −172 were mutated (in pairs) to inactivate the αACT, URE, and TSE domains, respectively. **B:** The GPHα promoter between −150 and −170, which is required for maximal induction by Btr (Fig. 5), is compared to a motif in the HIV-1 LTR that was previously shown to mediate viral induction by Btr [Bohan et al., 1989]. The alignment was made to maximize sequence identity. Open box sequence corresponds to a consensus GATA motif (A/TGATAA/G). Shaded nucleotides highlight identical pentamers occurring in both elements.

unit. Once the putative factor accumulates, elevated production of α-subunit mRNA can continue in the presence of CHX.

Inhibition by dGlc of the induction by NaBtr is restricted to certain promoters. Results presented above (Table II, Fig. 4) show that 5 mM dGlc can inhibit the induction by NaBtr of a CAT reporter gene dependent for expression on 1.6-kbp of GPHα gene 5′-flanking DNA. Deoxyglucose has little or no effect on basal CAT activity in cells transfected with this plasmid. Transient expression of the CAT reporter gene linked to a variety of promoters is elevated by Btr, frequently to an extent greater than that produced by the GPHα construct. Of the four promoters tested, only that of the GPHα gene was stimulated by the SCFA in a manner that could be significantly inhibited by dGlc. Elevation of CAT activity in transfected cultures treated with NaBtr may be due to the opening or loosening of chromatin structure that results from histone hyperacetylation. Deoxyglucose would not be expected to prevent enhanced expression from transfected plasmids that were activated by Btr in this manner. Moreover, previous studies from this laboratory have demonstrated that Btr increases histone acetylation in HeLa cells treated concurrently with dGlc [Cox et al.,1987], indicating that histone modification is not sufficient to induce α-subunit production. In order to avoid the general effect that Btr has on transient transfection assays [Gorman et al., 1983; Goldstein et al., 1989], HeLa 2.2 cells were cotransfected with pα1.6CAT and pSV_2neo, and stable clones (selected with G418) were analyzed for CAT activity after treating cells with NaBtr in the absence and presence of dGlc (Fig. 8). The fact that dGlc inhibits Btr induction from the integrated GPHα promoter minimizes any possible effects these compounds might have on plasmid uptake or stability in the transient expression assays.

The results with a nested set of pαCAT deletion vectors (Fig. 5) and site-directed mutations in the composite enhancer (Fig. 7) suggest that a Btr-responsive element lies between nucleotide −169 and −150 in the GPHα promoter, and in particular, may be centered over the αACT domain of the composite enhancer. This conclusion is based on two observations. First, expression from pα(−169)CAT is highly inducible by Btr, whereas expression from pα(−150)CAT is only marginally elevated by the SCFA. Second, when promoter activity was dependent on a

single enhancer domain in concert with the CRE, the greatest response to Btr was provided by αACT followed in order by URE and TSE. Thus, a *trans*-acting factor(s) that mediates Btr action most likely interacts with the 20 bp of DNA bounded by the 5′ termini of the deletion vectors or interacts with proteins that bind either the αACT or URE subdomain or both. Moreover, participation by CREB/ATF factors cannot be entirely excluded, as expression of pα(−150)CAT and pαC-CAT are stimulated to a small degree by NaBtr. It is noted from the analyses summarized in Figure 7 that some of the individually mutated enhancer elements are more highly inducible than the wild-type promoter. The explanation for this is unknown, but it is probably a reflection of the fact that the enhancer subdomains provide significantly different contributions to the basal promoter activity (TSE > αACT > URE). Moreover, in experiments not presented, it has been observed that expression of pαTUC-CAT is less than that from pαTC-CAT, suggesting a competition by TSEB and UREB for overlapping binding sites and a concomitant decrease in CAT production. Hence, it is suggested that a variety of interactions among the subdomains may similarly affect the induction by NaBtr.

Specific DNA-protein complexes were identified for TSE, αACT, and CRE probes when these were incubated with extracts from untreated and Btr-treated HeLa cells, as judged by successful competition with a 200-fold excess of homologous oligonucleotide and no competition by a similar excess of an unrelated oligonucleotide (Fig. 9). However, little or no difference in abundance or migration of the individual complexes was evident in samples containing extracts from Btr-treated cells. Similarly, the potential BRE identified in Figures 5 and 7 is specifically bound by a factor in HeLa nuclear extracts, but consistent differences in binding activity or complex mobility were not evident in response to NaBtr. These results suggest either that Btr may affect transactivation by one or more of these factors without influencing their DNA-binding activity, or that another factor, undetectable by this method because of low abundance or competition for binding to an overlapping site by a more abundant protein, may be the Btr-responsive factor. They further suggest that dependence on protein synthesis (Fig. 2) is not on production of these DNA-binding proteins themselves, but perhaps on synthesis of a protein(s) that interacts with the αACT, BRE, or TSE binding proteins or provides activation by post-translational modification.

It has long been suggested that the molecular mechanism for gene activation by Btr was based on the inhibition of histone deacetylase by the SCFA [Boffa et al., 1978; Sealy and Chalkley, 1978a]. This results in a significant increase in the level of acetylated histones, which are known to be enriched in transcriptionally active chromatin, and which should provide a less tightly packed nucleosome array [Sealy and Chalkley, 1978b]. However, more recent reports suggest that the increased DNase sensitivity of genes does not automatically lead to their activation [Birren et al., 1987], and DNA motifs have been identified in the promoter of inducible genes that may serve as Btr-responsive elements (BRE). For example, it has been shown that activation of the γ-globin gene in murine erythroleukemia cells by NaBtr is dependent on a DNA region located between −725 and −569 of the γ-gene promoter [Glauber et al., 1991]. The human γ-globin gene is induced by NaBtr in K562 cells by a mechanism thought to involve the competition of CDP (CCAAT displacement protein) and CBP (CCAAT-box binding protein), perhaps through a loss of CDP binding activity [McDonaugh and Niehaus, 1991]. Yeivin et al. [1992] have shown that the adenine phosphoribosyltransferase (APRT) gene from Chinese hamster ovary (CHO) cells could be induced 10- to 40-fold when the gene was linked to the Moloney murine sarcoma virus (MoMSV) enhancer in association with either the APRT or MoMSV promoter. No effect was observed for NaBtr on these promoters without the enhancer, and the greater response was noted with the MoMSV promoter (43-fold) compared with the APRT promoter (11-fold). Transcription from the human *mdr*-1 gene is reversibly enhanced by NaBtr in cultured colon carcinoma cells [Morrow et al., 1994]. This induction is at lease partially dependent on a Y-box motif (CTGATTGGCT) located between −82 and −73 of the *mdr*-1 promoter. Two regions have been mapped in the HIV-1 long terminal repeat (LTR) that contribute to viral activation by NaBtr in a wide range of cell types, both lymphoid and nonlymphoid [Golub and Volsky, 1991; Bohan et al., 1989]. These regions include the TATA box and Sp1 motifs located from −17 to −65 and a more distal

motif (AGCTTG) located from −117 to −103. The C11 serine protease gene is activated by NaBtr in cytotoxic T cells. Two Btr-sensitive regions were identified in the C11 promoter; these exhibit extensive similarity to sequences in the HIV-1 LTR that respond to NaBtr. Butyrate can also suppress gene activity, and Johnston et al. [1992] have shown that NaBtr inhibits myogenesis by interfering with transcriptional activation brought about by MyoD and myogenin. The latter belong to a structurally related family of basic helix-loop-helix (bHLH) proteins that interact with a core motif related to CANNTG. The HLH region of MyoD was reported to be essential for Btr inhibition.

The fact that several distinct elements have been identified as being Btr-responsive is intriguing and suggests several possible mechanisms of action:

1. Post-translational modification (e.g., phosphorylation, acetylation, and glycosylation) of *trans*-regulatory proteins that interact with these *cis* elements may be a common theme by which each of the factors is stimulated with respect to DNA binding or transactivation.
2. An accessory protein, which is shared by multiple factors and whose quantity or activity is modified by Btr, interacts with these other regulatory factors to stimulate transcription but is not itself a DNA binding protein.
3. Protein factors that bind to the Btr-responsive elements must interact with acetylated or phosphorylated histones, which are elevated in Btr-treated cells, to elicit their maximal response.
4. The action of Btr is manifest by changes in chromatin structure as the result of histone modification, but specific rather than pleiotropic effects are suggested because the distinctive *cis* elements that are identified depend on their relative position to critical nucleosomes that must be displaced to activate the promoter. That is, multiple factors are identified because their cognate binding sites are packaged in the critical nucleosome.

Which of these possible mechanisms may be operative for induction of the GPHα gene is unknown. The 20 bp of GPHα 5′-flanking DNA required for induction of the CAT reporter gene in transient transfection assays has striking sequence similarity to a Btr-responsive motif in the HIV-1 LTR [Bohan et al., 1989]. These are compared in Figure 10 and are aligned to maximize the identity. In addition to the 10/15 matching base pairs (vertical lines), both the HIV-1 and GPHα fragments contain the pentamer AAGGG (shaded), although they are positioned at opposite ends of the respective elements. This argues in favor of involvement by the same, or at least similar, DNA binding proteins, although it must be noted that the GPHα gene and HIV-1 LTR are active in epithelial cells and T lymphocytes, respectively. The GPHα BRE, but not the HIV-1 LTR, also contains a GATA factor consensus sequence (WGATAR, open box), suggesting the potential involvement of one or more members of this protein family. This latter possibility is further supported by the results summarized in Figure 7.

Of the enhancer subdomains, the αACT or GATA element clearly provides the greatest response to NaBtr, with smaller, although still significant, responses contributed by the URE and TSE domains. Promoters with only the CRE also show enhanced transcription in the presence of NaBtr, but the magnitude of induction is significally less than that achieved with the upstream enhancer.

The delay in GPHα gene induction by NaBtr suggests that its effect in HeLa cells is indirect. This is supported by the data presented in Figure 2 showing that the increase in GPHα mRNA in response to NaBtr is inhibited by CHX. A similar window for Btr action was noted when induction by the SCFA was inhibited by the simultaneous addition of dGlc but not by the delayed addition of dGlc after pretreatment with NaBtr for 24 h. Cycloheximide and dGlc do not appear to inhibit the same process because dGlc does not lead to GPHα induction in other HeLa cell clones that are inducible by CHX [Cox et al., 1990 and unpublished observations]. Taken together, these results suggest that the *trans*-regulatory protein that activates the BRE, either by binding directly to the element or by interacting with other factors that do, must be synthesized as a primary response to Btr or be modified by an enzyme that is upstream of the factor in a Btr signaling cascade.

The mechanism by which dGlc eliminates any effect of Btr on GPHα gene expression is not yet defined. High exogenous concentrations

of dGlc could significantly reduce intracellular concentrations of ATP (as a result of its phosphorylation by hexokinase to generate deoxyglucose-6-P, which is not metabolized further) or UTP and GTP (resulting from the formation of UDP-dGlc and GDP-dGlc). However, trivial explanations such as metabolic disruption or inhibition of overall RNA synthesis can be ruled out by the fact that elevated CAT synthesis can be sustained in the presence of NaBtr and dGlc in HeLa cells transfected with plasmids employing promoters other than that of GPHα (e.g., P-450-IA1) (Fig. 4), and the hexose has little or no effect on induction of the GPHα gene by NaBtr in cAMP-treated JEG-3 cells (unpublished results).

Although several possibilities can be suggested in the case of GPHα gene regulation, induction by Btr is thought to be mediated by the synthesis or post-translational modification of a new or limiting transcription factor that interacts with either the GPHα gene promoter sequences or another transcription factor to enhance expression of the gene in HeLa cells. The most likely candidates for post-translation modification are glycosylation and phosphorylation. This interpretation is supported by the protein synthesis requirement for Btr induction of GPHα mRNA, by the lag time preceding induction, and by the specific inhibition of transcription by dGlc when CAT production is dependent on the GPHα promoter. Earlier reports that demonstrate a glucose requirement for induction of α-subunit by NaBtr [McClure and Cox, 1984] are also consistent with this interpretation, as glucose greatly stimulates protein glycosylation [Turco, 1980; Staneloni et al., 1980]. The existence of such a regulatory protein is not unprecedented. Several transcription factors, such as AP-1, AP-2, TFIIIA, and Sp1, are known to be glycosylated [Jackson and Tjian, 1988]. Based on lectin binding studies, these are thought to contain O-linked N-acetylglucosamine, which in some cases appears to be necessary for factor activity [Jackson and Tjian, 1988]. In future experiments, it will be of interest to examine the glycosylation status of GPHα enhancer binding proteins in cells exposed to NaBtr.

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