

Dual Effects of 2-Deoxyglucose on Synthesis of the Glycoprotein Hormone Common α -Subunit in Butyrate-Treated HeLa Cells

G. STANLEY COX,* DANIEL S. McCLURE, AND DOMINIC E. COSGROVE

Department of Biochemistry, University of Nebraska Medical Center, Omaha, Nebraska 68105

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Sodium butyrate (Btr) (3 mM) causes a 10-fold increase in production of the glycoprotein hormone α -subunit in HeLa cells. The following report demonstrates that this response could be inhibited about 95% by 5 mM 2-deoxy-D-glucose (dGlc), whereas α -subunit production in uninduced cells was affected little or not at all. Addition of D-mannose restored the Btr induction of HeLa- α in cultures that had been treated with dGlc. When the α -subunits secreted by cells cultured in Btr plus dGlc or in Btr alone were compared by gel filtration (Sephadex G-75) and lectin affinity (concanavalin A and ricin) chromatography, differences were noted that probably reflect changes in their carbohydrate moieties. Immunoprecipitation of [³⁵S]methionine-labeled HeLa- α and incubation with endoglycosidase H indicated that the subunit secreted from cells in the presence of dGlc contained oligosaccharide side chains that were not processed to the complex type. Cells that were simultaneously treated with Btr plus dGlc showed no increase in α -subunit production over cells receiving Btr only; in contrast, cells that were preincubated with Btr for either 16 or 36 h before dGlc was added exhibited high levels of subunit synthesis. Measurement of α -mRNA levels at various times after Btr and dGlc were added to cultures indicated that Btr brought about a dramatic increase in α -specific mRNA about 24 h after being added to cultures. This increase could be prevented by dGlc when added simultaneously with Btr but not when added after a 24-h preincubation. Although dGlc prevented the induction of α -subunit and α -mRNA in response to Btr, it had no effect on histone hyperacetylation, suggesting that if this chromatin modification is necessary for the induction process, it is not in itself sufficient. Together, the data demonstrate that dGlc inhibits the accumulation of α -subunit mRNA normally produced in response to Btr and that the subunit produced contains altered oligosaccharide constituents.

Production of the glycoprotein hormone α -subunit by HeLa cells (derived from a cervical carcinoma) is one of numerous examples of ectopic protein production by tumors, i.e., the synthesis of proteins not normally expressed by the cell type from which the tumor was derived (1, 3, 21, 24, 29, 31). These proteins can be used clinically to diagnose neoplastic conditions and to monitor the progress of surgery, radiation, and chemotherapy. However, the molecular basis for ectopic gene expression is unknown, and the mechanism by which such genes are regulated is important for understanding the metabolism of the neoplastic cell.

The α -subunit is produced at variable rates by different HeLa lines (5, 17, 22, 23) and is inducible by a number of agents such as hydroxyurea, bromodeoxyuridine, aphidicolin, and Btr (9, 18, 19, 34), although the mechanism of induction has not been determined. In a previous report from this laboratory (6), it was observed that the Btr-mediated induction of the α -subunit in HeLa cells could be prevented completely by dGlc. Because this sugar derivative was known to inhibit protein glycosylation (12-14), the results suggest that Btr also might be affecting glycoprotein production. Support for the latter possibility comes from recent reports by Morrow et al. (27) and McClure and Cox (26), describing enhanced protein glycosylation and α -subunit synthesis by Btr in Chang liver cells and HeLa cells when cultured in glucose-free medium, conditions known to restrict the transfer of oligosaccharides from dolichol intermediates to nascent peptides (38, 40).

These phenomena have been examined in more detail, since understanding the mechanism of action of Btr and dGlc may further our understanding of α -subunit gene expression.

The results described below suggest that dGlc has at least two effects on α -subunit production in Btr-treated HeLa cells, one at the level of posttranslational or cotranslational subunit modification and one at the level of α -mRNA synthesis or degradation.

MATERIALS AND METHODS

Abbreviations: α -mRNA, α -subunit mRNA; Btr, sodium butyrate; Bt₂cAMP, N⁶,O^{2'}-dibutyryl-3',5'-cyclic AMP; ConA, concanavalin A; dGlc, 2-deoxyglucose; endo H, endo- β -N-acetylglucosaminidase H; Glc, glucose; hCG, human chorionic gonadotropin; MEM, minimum essential medium; RIA, radioimmunoassay; SDS, sodium dodecyl sulfate; TAT, aminotransferase.

Materials. Sigma Chemical Co. was the source of dGlc, α -methyl-D-glucoside, 3-O-methyl-D-glucoside, 6-deoxy-D-glucose, D-galactose, 2-deoxy-D-galactose, 6-deoxy-D-galactose, 2-deoxy-D-ribose, D-arabinose, D-ribose, D-xylose, D-fructose, D-mannose, N-acetyl-D-glucosamine, polyvinylpyrrolidone, and *Ricinus communis* agarose. Sephadex G-75 superfine, ConA-Sepharose, and Ficoll were purchased from Pharmacia, Inc., and all reagents for SDS-polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories. GIBCO Laboratories supplied powdered MEM (with Earle salts), powdered RPMI 1640 medium, calf serum, and antibiotics. Bovine lactoperoxidase and bovine serum albumin were obtained from Worthington Diagnostics, and endo H was purchased from Boehringer Mannheim Biochemicals. Amersham Corp. was the source of [α -³²P]dCTP (800 Ci/mmol), and New England Nuclear Corp. supplied Na¹²⁵I (17 Ci/mg), [1-¹⁴C]butyric acid (14 mCi/mmol), and GeneScreen hybridization membrane. Btr was purchased from Matheson, Coleman, and Bell. Purified

* Corresponding author.

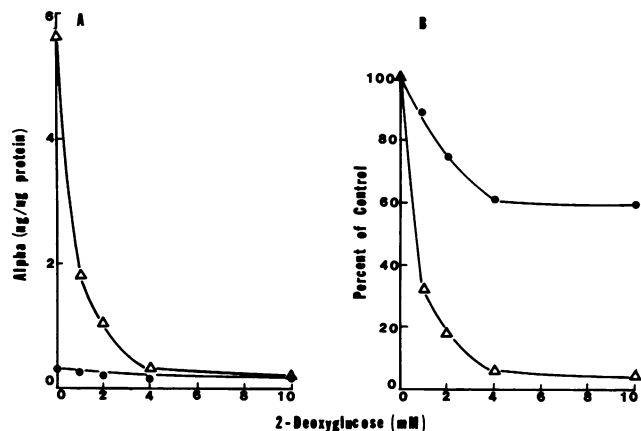


FIG. 1. Effect of dGlc on the Btr-mediated induction of HeLa- α . Cultures at near confluence were supplemented with fresh RPMI 1640 medium with (Δ) or without (\bullet) 3 mM Btr and the concentrations of dGlc as indicated in the figure. Media and cells were harvested after 72 h and assayed as described in Materials and Methods. Panel B expresses the data in panel A as a percentage of the sample receiving no dGlc.

hCG- α (CR-123) was a gift from the National Institute of Child Health and Human Development (Center for Population Research). Purified calf thymus histones and pCG α (pBR322 with hCG- α cDNA inserted in the *Hind*III site) were generous gifts from Roger Chalkley and John Fiddes, respectively.

Cell culture. HeLa S3 cells were maintained in spinner culture at densities of 3×10^5 to 8×10^5 cells per ml in MEM with spinner salts or RPMI 1640 medium supplemented with 6% calf serum, 0.06% L-glutamine, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml. To initiate an experiment, spinner cells were plated in 25- or 75-cm² T flasks (Corning Glass Works) at a density of about 2×10^4 to 3×10^4 cells per cm² and supplemented with MEM (5.5 mM Glc) or RPMI 1640 medium (11 mM Glc) containing the additions as noted above. When the cultures neared confluence, the medium was replaced with fresh medium containing Btr, dGlc, and other agents as indicated in the table and figure legends.

Column chromatography. Conditioned media or partially purified HeLa- α were chromatographed on Sephadex G-75 superfine and immobilized lectins (ConA-Sepharose and ricin-agarose) as described previously (7, 8, 26).

Quantitation of α -subunit and protein. The levels of α -subunit were determined in a specific RIA as described previously (6), and protein concentration was determined by the method of Lowry et al. (25) with bovine serum albumin as standard. Levels of subunit accumulation were somewhat variable from one experiment to another, but duplicate samples in the same experiment generally agreed within $\pm 17\%$.

Isolation and electrophoresis of histones. Cells were grown in 75-cm² flasks with RPMI 1640 medium to near confluence. Fresh medium was added and supplemented with 3 mM Btr or 5 mM dGlc. After 72 h, the cell sheet was collected and the cells were washed twice by centrifugation in 10 mM Tris hydrochloride (pH 7.4) buffer containing 100 mM KCl and 10 mM MgCl₂ (TKM). The cells were suspended and allowed to swell for 20 min on ice in 10 mM Tris hydrochloride (pH 7.4) containing 10 mM KCl and 0.15 mM magnesium acetate (LTKM). Cells were broken by several strokes of a Dounce homogenizer, and nuclei were removed by centrifugation at

1,200 \times g for 3 min. The nuclei were washed twice with LTKM buffer and suspended in 3 ml of 10 mM Tris hydrochloride (pH 7.4)–0.5 mM NaHSO₃–6 mM Btr per 75-cm² flask. The nuclei were lysed by homogenization and centrifuged at 17,000 \times g for 20 min. The resulting pellet was suspended in 0.5 ml of 0.4 N H₂SO₄, incubated for 10 min on ice, and centrifuged at 12,000 \times g for 10 min. Four volumes of 95% ethanol were added to each supernatant, and the mixtures were kept at -20°C overnight. The precipitates were collected by centrifugation at 12,000 \times g for 10 min and suspended in 0.9 N acetic acid.

Electrophoresis of histones was essentially as described by Panyim and Chalkley (30). Gels containing 15% acrylamide in 6.25 M urea at pH 3.2 were polymerized in cylindrical tubes (0.6 by 18 cm); running buffer was 0.9 N acetic acid. Gels were preelectrophoresed at 2 mA per gel for 9 h with pyronin Y as a mobility marker. Histone samples were run at 2 mA until tracking dye reached the end of the gel. Gels were stained in Coomassie blue, destained, and scanned at 600 nm with a Gilford 250S spectrophotometer, gel scanner, and recorder.

Isolation of HeLa RNA. Cells were grown to 80 to 90% confluence in 25-cm² flasks and then exposed to effectors as indicated in the table and figure legends. The cell sheet was scraped into the overlying medium and collected by centrifugation at 1,000 \times g for 10 min. Pelleted cells were washed three times with 5 ml of ice-cold 50 mM Tris hydrochloride (pH 7.4) containing 0.15 M NaCl. Cytoplasmic extracts were prepared by a modification of the White and Bancroft (43) procedure. The final cell pellets were suspended in 180 μ l of ice-cold buffer containing 10 mM Tris hydrochloride (pH 7.0) and 1 mM EDTA and then lysed with two 20- μ l additions of

TABLE 1. Effect of various sugars on the induction of HeLa- α by Btr^a

| Addition | α -Subunit induction | |
|----------------------------|-----------------------------|-----|
| | ng/ μ g of protein | % |
| Expt 1 | | |
| None | 0.45 | |
| Btr | 4.50 | 100 |
| Btr plus | | |
| dGlc | 0.60 | 13 |
| 6-Deoxyglucose | 3.69 | 82 |
| 2-Deoxygalactose | 1.18 | 26 |
| 6-Deoxygalactose | 3.55 | 79 |
| 2-Deoxyribose | 3.85 | 86 |
| Arabinose | 2.80 | 62 |
| Ribose | 3.31 | 74 |
| Xylose | 4.40 | 98 |
| Fructose | 4.60 | 102 |
| Galactose | 4.60 | 102 |
| Mannose | 6.12 | 136 |
| Expt 2 | | |
| None | 0.22 | |
| Butyrate | 4.87 | 100 |
| Butyrate plus | | |
| dGlc | 0.26 | 5 |
| N-Acetyl glucosamine | 0.18 | 4 |
| 3-O-Methyl glucoside | 4.02 | 83 |
| α -Methyl glucoside | 1.85 | 38 |

^a Cells in 75-cm² flasks were cultured for 72 h in MEM supplemented with the compounds listed in the table. At that time, the medium was used to determine α -subunit (RIA), and the cell sheet was used to measure cell protein. Btr was present at 3 mM, while all of the sugars and derivatives were added at 5 mM.

5% Nonidet P-40 with a 5-min mixing on ice between the additions. Nuclei were removed by centrifugation at 4°C. For dot-blot analysis, a sample (200 μ l) of the supernatant was transferred to a sterile microtube (1.5 ml) containing 120 μ l of 20 \times SSC buffer (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7]) and 80 μ l of 37% (vol/vol) formaldehyde. The mixture was heated at 60°C for 15 min and then spotted directly onto nitrocellulose or stored at -70°C. For Northern blot analysis, the postnuclear supernatant was made 0.1% in SDS and 10 mM in EDTA, and RNA was extracted four times with phenol-chloroform-isoamyl alcohol (49:49:2) that had been equilibrated with 0.5 M Tris hydrochloride (pH 9). Ammonium acetate (0.5 M) and ethanol (70%) were added to the final aqueous phase, which was kept at -20°C overnight. The RNA was collected by centrifugation, redissolved in a small volume of sterile water, and analyzed as described below.

Dot-blot hybridization. The dot-blot hybridization method used was a modification of that described by Kafatos et al. (20). Cytoplasmic RNA samples were quantified spectrophotometrically (assuming 1 A_{260} = 40 μ g of RNA per ml) prior to the addition of formaldehyde as described above. Appropriate volumes to yield 3, 10, and 30 μ g of RNA were diluted with SSC in a 96-well microtiter dish to give a final volume of 200 μ l and 15 \times SSC. A sample of 150 μ l was applied to nitrocellulose (BA85; Schleicher & Schuell, Inc.) or GeneScreen by using a manifold apparatus from Schleicher & Schuell. Each well was washed twice under gentle vacuum with 200 μ l of sterile 15 \times SSC, and then the filters were air dried and baked at 80°C in vacuo for 1 to 2 h.

Recombinant hCG- α cDNA (15) was labeled by nick translation to high specific activity ($>2 \times 10^8$ cpm/ μ g) by using [α - 32 P]dCTP and a modification of the procedure described by Rigby et al. (33). Membranes were prehybridized overnight at 42°C in a solution (100 μ l/cm 2) containing 50 mM Tris hydrochloride (pH 7.5), 1 M NaCl, 1% SDS, 0.1% sodium PP $_i$, 50% deionized formamide, 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinylpyrrolidone,

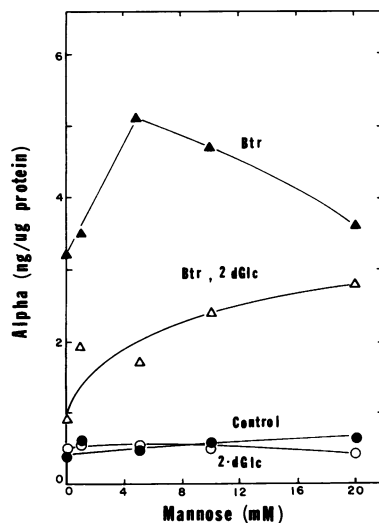


FIG. 2. Ability of mannose to restore induction in dGlc-treated cells. Fresh RPMI 1640 medium was added to near-confluent cultures and supplemented with 3 mM Btr (\blacktriangle), 3 mM Btr plus 5 mM dGlc (\triangle), 5 mM dGlc (\circ), or no additions (\bullet). Mannose was added to individual flasks at the concentrations indicated in the figure. Media and cells were harvested after 72 h and assayed as described in Materials and Methods.

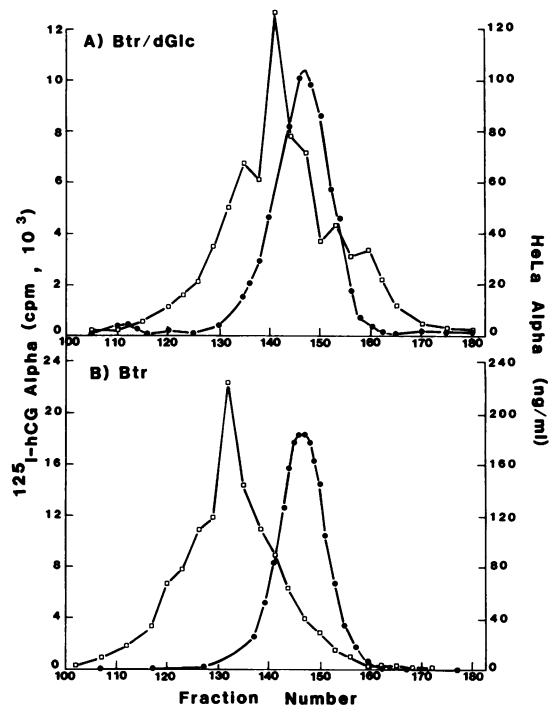


FIG. 3. Gel filtration chromatography of HeLa- α . Media (80 ml) from cells cultured in the presence of Btr plus dGlc (A) or Btr alone (B) were lyophilized to dryness and suspended in 0.1 M NH_4HCO_3 . Samples containing 5,725 ng (panel A) or 6,340 ng (panel B) of HeLa- α were mixed with 2×10^5 to 3×10^5 cpm of ^{125}I -labeled hCG- α and chromatographed on Sephadex G-75 (2.5 by 140 cm). The column was developed with 0.1 M NH_4HCO_3 , and fractions of 2.5 ml were collected and assayed for HeLa- α (RIA; \square) or hCG- α (^{125}I ; \bullet).

10% dextran sulfate, and 100 μ g of denatured and sonicated salmon sperm DNA per ml. Molecular hybridization was carried out for 24 to 48 h at 42°C in the above mixture (50 μ l/cm 2) containing 50,000 cpm of [32 P]cDNA per cm 2 . Following hybridization, filters were washed twice in 2 \times SSC at room temperature for 5 min, twice in 2 \times SSC-1% SDS at 65°C for 30 min, and twice in 0.1 \times SSC at room temperature for 30 min. Filters were exposed for 3 to 5 days to Kodak XAR film at -70°C. Hybridization was quantified by scanning the autoradiographs with a Bromma 2202 Ultrosan laser densitometer (LKB Instruments, Inc.) and Gelscan program.

Northern blot analysis. The procedure used was basically that as described by Thomas (39). The RNA samples (20 μ g) were heated for 5 min at 65°C in a solution containing 20 mM sodium phosphate (pH 7.4), 6% (vol/vol) formaldehyde, and 50% (vol/vol) formamide and then subjected to electrophoresis through 1% (wt/vol) agarose gels. The electrode buffer (20 mM sodium phosphate [pH 7.4] containing 6% formaldehyde) was recirculated between electrode reservoirs by using a peristaltic pump. Following electrophoresis, the RNA was transferred to nitrocellulose or GeneScreen in 20 \times SSC. Prehybridization (16 h) and hybridization (48 h) were carried out at 42°C in sealed plastic bags containing 50% formamide, 5 \times SSC, 1% SDS, 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 50 μ g of denatured salmon sperm DNA per ml. The ^{32}P -labeled α -cDNA probe (1×10^8 to 3×10^8 cpm/ μ g) was prepared as described above. Following hybridization, filters were washed twice

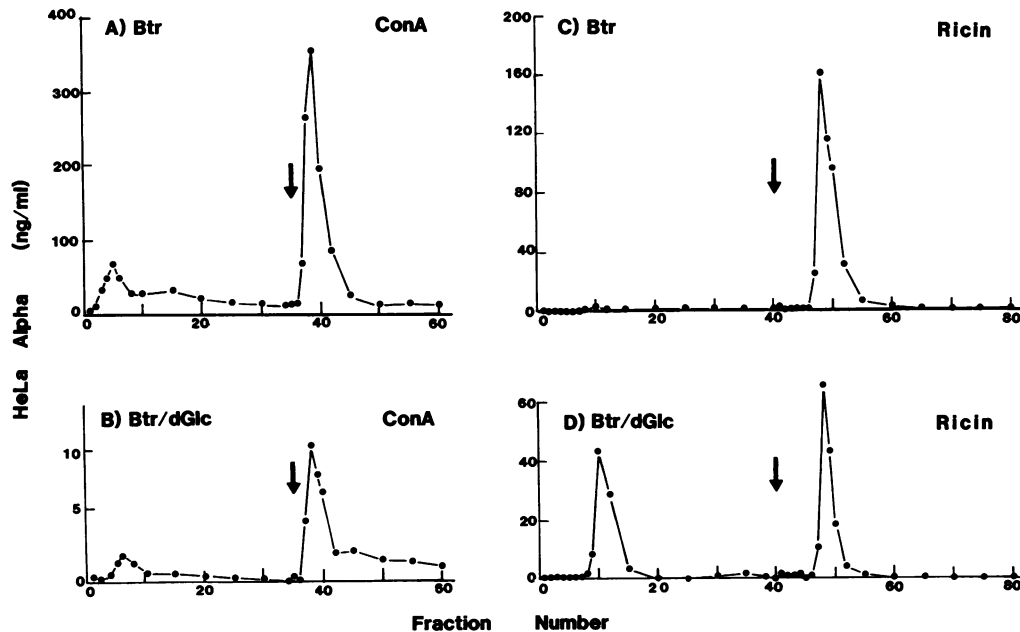


FIG. 4. Lectin chromatography of HeLa- α . Concentrated culture media were dialyzed to remove glucose and then chromatographed as described previously (6, 8) on ConA-Sepharose directly or on ricin-agarose following mild acid hydrolysis (60 mM H_2SO_4 at 80°C for 90 min) to remove terminal sialic acid. Conditioned media were collected from cultures containing 3 mM Btr (panels A and C) or 3 mM Btr plus 5 mM dGlc (panels B and D). Arrows denote elution of adsorbed proteins with phosphate-saline buffer containing 0.2 M α -methylglucoside for ConA (panels A and B) or 0.2 M galactose for *Ricin communis* agglutinin (panels C and D). Selected fractions were assayed for α -subunit by RIA.

for 10 min at room temperature in $2\times$ SSC-0.1% SDS-0.1% sodium PP_i , twice for 15 min at 55°C in the same solution, and twice for 15 min at 55°C in $0.1\times$ SSC-0.1% SDS-0.1% sodium PP_i . The filters were air dried and exposed to Kodak XAR film at -70°C for about 7 days.

RESULTS

Inhibition by dGlc of the Btr-mediated induction of α -subunit. dGlc significantly inhibited the production of HeLa- α in response to Btr but only marginally reduced subunit levels in uninduced cells (Fig. 1). The analog reduced subunit production in induced cultures down to approximately the level observed for untreated cells. Accumulation of immunoreactive material was unaffected or depressed slightly (10 to 40%) in control cultures but was consistently inhibited 80 to 95% in Btr-stimulated cells. The inhibition by dGlc was reversible for exposure times up to at least 36 h, since washing the cell sheet and refeeding with fresh medium without dGlc restored the capacity of Btr to stimulate α -subunit production (data not presented). It was also noted that the continuous presence of Btr was required for sustained induction. dGlc was unable to reverse some of the other effects of Btr on cell morphology, cell growth, and DNA synthesis (D. S. McClure and G. S. Cox, unpublished data).

The effect of various sugars and derivatives on the Btr-mediated induction of HeLa- α was examined in efforts to define the aspects of structure necessary to suppress induction. The data summarized in Table 1 indicate that dGlc and 2-deoxygalactose were most effective in preventing α -subunit accumulation, whereas their 6-deoxy counterparts were relatively ineffective. The pentoses examined had little or no effect, except perhaps for arabinose; the unmodified hexoses supported induction to levels greater than 100%.

Since 3-*O*-methyl glucoside is not phosphorylated, lack of inhibition by this compound (Table 1, experiment 2) and the 6-deoxy derivatives (experiment 1) suggest that metabolites rather than the free sugars are the active inhibitors. Even though *N*-acetyl glucosamine was a potent inhibitor, it was not studied further because it was much more cytotoxic than the other agents and inhibited basal subunit production.

To determine whether dGlc might be affecting subunit secretion rather than synthesis, we determined the intracellular levels of HeLa- α in Btr-induced cultures supplemented with increasing concentrations of dGlc. The percentage of intracellular subunit increased relative to the combined total of intracellular plus extracellular subunit in a culture, but the increase was not sufficient to account for the depletion of extracellular material (data not presented). Hence, an inhibition of protein secretion per se does not adequately explain the effect of dGlc on HeLa- α production, since there was not a substantial accumulation of intracellular, incompletely processed product.

The uptake and metabolism of [^{14}C]butyrate in cells cultured in RPMI 1640 medium was examined in the absence and presence of 5 mM dGlc to assess any possible changes which could account for the effect of the analog. When Btr was present at isotopic concentrations, 5.0% of the label was released as $^{14}CO_2$, 88.0% was extracted with $CHCl_3-CH_3OH$, and 7.1% was incorporated into material insoluble in $CHCl_3-CH_3OH$. dGlc altered these values to 7.5, 83.9, and 8.6%, respectively. When cells were pretreated for 36 h with 3 mM Btr prior to the addition of label ([^{14}C]butyrate in the absence of unlabeled Btr), the proportion of ^{14}C converted to $^{14}CO_2$ increased to 15.2%, while label in the $CHCl_3-CH_3OH$ extract and residue decreased to 78.6 and 6.2%, respectively. The addition of dGlc during the preincubation changed the distribution such that 19.3, 65.8, and 14.9% of the label was recovered in CO_2 , lipid, and nonlipid material, respectively. It seems unlikely, although possible, that these

small changes in Btr metabolism are responsible for its failure to induce HeLa- α in the presence of dGlc.

It is known that dGlc is a potent inhibitor of protein glycosylation (12–14). One of several means by which dGlc could depress glycoprotein synthesis is by sequestering nucleotides required for sugar nucleotide formation, particularly as GDP-dGlc (12, 13). Since mannose also participates in glycosylation reactions as the GDP derivative (GDP-mannose), it is noteworthy that mannose could restore elevated α -subunit production to cultures containing Btr and dGlc (Fig. 2). Increasing the Glc concentrations also alleviated the inhibition by dGlc, but galactose and fructose had little or no effect (data not presented).

Effects of dGlc on the HeLa α -subunit. Physicochemical characterization of the tumor protein produced under various culture conditions suggests that an altered subunit accumulates in the presence of dGlc. Conditioned medium from cells incubated in the presence of 3 mM Btr or 3 mM Btr plus 5 mM dGlc was concentrated and chromatographed on Sephadex G-75 superfine. Immunoreactive material from cultures treated with Btr and dGlc (Fig. 3A) emerged in a greater elution volume than did material from cells cultured with Btr only (Fig. 3B), indicating a lower apparent molecular weight.

It has previously been reported (7) that after mild acid hydrolysis (60 mM H_2SO_4 at 80°C for 90 min), HeLa- α coeluted with ^{125}I -labeled urinary hCG- α during gel filtration chromatography. In the absence of hydrolysis, the tumor protein eluted ahead of the normal α -subunit. Because sialic acid is removed from oligosaccharide side chains under these conditions of hydrolysis, the results were taken to suggest that the HeLa subunit is more highly sialylated than its normal counterpart and that the additional mass, altered conformation, or increased negative charge modifies its elution profile. On the basis of this interpretation, it seems likely that the subunit secreted in the presence of dGlc has altered carbohydrate side chains.

This conclusion was tested more directly by chromatographing the HeLa protein on immobilized lectins. About 80% of the tumor protein secreted by cells in the presence of Btr or Btr plus dGlc was specifically retained by ConA-Sepharose and could be eluted with 0.2 M α -methylglucoside (Fig. 4A and B). Similarly, over 95% of the α -subunit synthesized in response to Btr was adsorbed to ricin-agarose and specifically eluted with 0.2 M galactose (Fig. 4C). In contrast, as much as 50% of the immunoreactive α -subunit in medium from cells incubated with both Btr and dGlc was not retained by the ricin matrix. On the basis of the specificity of ConA (mannose or Glc) and ricin (galactose), these results suggest that core glycosylation occurs in the presence of dGlc, but terminal processing leading to the complex-type oligosaccharide side chains may be altered.

Evidence to support this conclusion is illustrated by data presented in Fig. 5. Cells in culture media containing Btr or dGlc or both were incubated with [^{35}S]methionine. Radiolabeled material was immunoprecipitated from culture media with anti- α serum, incubated with endo H, and analyzed by electrophoresis on SDS-polyacrylamide gels. HeLa- α from control groups migrated at M_r 24,000 (lane 1), whereas authentic urinary hCG- α migrated slightly faster, at M_r 22,000 (arrow). In cultures exposed to 5 mM dGlc, three species of HeLa- α were evident, having M_r 23,000, M_r 20,500, and M_r 17,000, respectively (lane 3). Similar patterns were evident for subunits recovered from cultures also containing 3 mM Btr (lanes 5 and 7). When α -subunit from dGlc-treated cells (plus or minus Btr) was incubated with

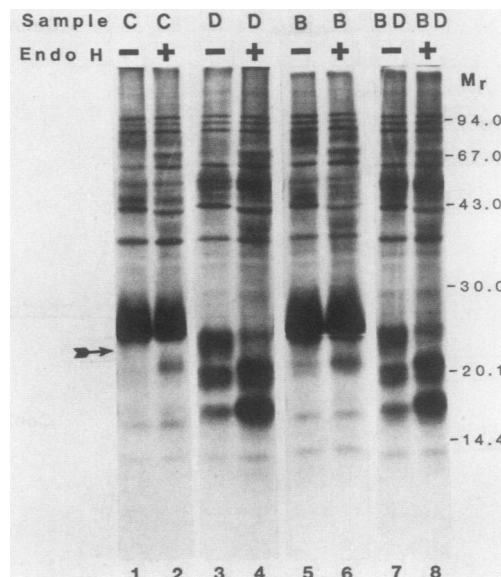


FIG. 5. Effect of endo H on the α -subunit secreted by HeLa cells in the presence of dGlc. Cells were labeled for 24 h with [^{35}S]methionine (100 μ Ci/ml) in medium containing 5 mM dGlc (D), 3 mM Btr (B), 3 mM Btr plus 5 mM dGlc (BD), or no additions (C). Secreted proteins were concentrated by precipitation with $(NH_4)_2SO_4$ and incubated with anti- α serum. Immune complexes were precipitated with *Staphylococcus aureus* cells, washed three times in buffer containing 1% Triton X-100 and 2 M urea, and then washed once in 50 mM Tris hydrochloride (pH 6.8). Immunoprecipitated proteins were released by boiling the washed pellets in 40 μ l of 10 mM Tris hydrochloride (pH 6.8) buffer containing 1% SDS and 1% 2-mercaptoethanol. Duplicate portions (18 μ l) were made 0.12 M in sodium citrate (pH 5.8) and incubated under N_2 at 37°C for 8 h with either nothing (-) or 0.01 U of endo H (+). In addition, each tube was supplemented with 1 mM phenylmethylsulfonyl fluoride and 10 μ g of aprotinin per ml. Samples (approximately 125,000 cpm) were then electrophoresed at 25 mA through a 12.5% polyacrylamide slab gel containing SDS. The gels were soaked in En 3 Hance (New England Nuclear), dried, and subjected to fluorography. Molecular size markers and urinary hCG- α (CR-123) were labeled with [3H]formaldehyde by reductive methylation (32) and run on parallel lanes. The molecular sizes indicated are in kilodaltons.

endo H, the prominent band at M_r 23,000 was greatly diminished, with a concomitant increase in material migrating with M_r 20,500 and M_r 17,000 (lanes 4 and 8). Endo H had only a small effect on the subunit synthesized in the absence of dGlc (lanes 2 and 6). These results are interpreted to indicate that dGlc leads to the formation of underglycosylated forms of α -subunit that are effective substrates for endo H, suggesting the absence of complex-type chains and the presence of a high-mannose core on these molecules.

Effects of dGlc on α -mRNA. Induction of α -subunit in HeLa cultures generally occurs after an initial lag of 12 to 24 h following Btr addition (19, 34). Subunit levels continue to increase for at least 72 h in the presence of the inducer but decline when the fatty acid is removed from the culture (data not presented), indicating a requirement for its continued presence. Thus, it was of interest to add dGlc to cultures at various times following the addition of Btr to determine whether induction of HeLa- α was sensitive to the sugar derivative at all times during the incubation period. The results presented in Fig. 6 demonstrate that dGlc (5 mM) added to cultures simultaneously with Btr inhibited subunit

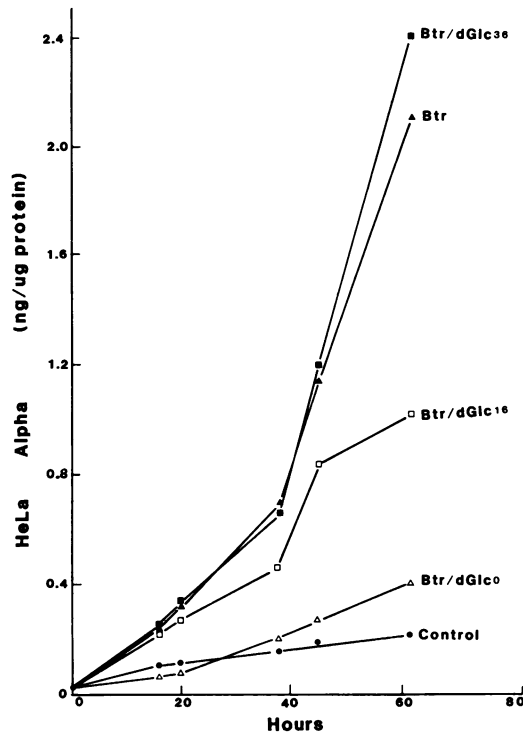


FIG. 6. Effect of delayed dGlc addition on Btr induction of HeLa- α . Cells at moderately high density were given fresh medium containing no additions (●), 3 mM Btr (▲, □, ■), or 3 mM Btr plus 5 mM dGlc (Btr/dGlc⁰; △). dGlc was added to one of the Btr flasks after 16 h (Btr/dGlc¹⁶; □) and to another Btr flask after 36 h (Btr/dGlc³⁶; ■). The third Btr flask received no further addition (▲). Media samples were withdrawn at the times indicated and assayed for α -subunit. Cell protein was determined when the cells were harvested at 62 h.

induction down to levels approximating those of uninduced cultures (as noted above; Fig. 1 and 2). However, when the sugar derivative was added 16 h after cells were preincubated with 3 mM Btr (Fig. 6; Btr/dGlc¹⁶), α -subunit levels were significantly elevated relative to those of control cultures and cultures receiving Btr and dGlc at zero time (Btr/dGlc⁰); addition of the inhibitor 36 h after Btr addition (Btr/dGlc³⁶) had no effect on subunit accumulation. These results suggest that a process necessary for the Btr-mediated induction of HeLa- α and sensitive to the action of dGlc or its metabolites occurs during the initial lag period. Elevated subunit synthesis can continue unabated if the sugar is added after the preincubation.

Previous studies have demonstrated that the induction of α -subunit in HeLa cells by Btr can be prevented by cycloheximide and actinomycin D (19, 34), indicating that synthesis of both RNA and protein is required. This, as well as the fact that induction becomes relatively less sensitive to dGlc between 16 and 36 h after exposure to Btr (Fig. 6), suggests that the short-chain fatty acid may stimulate the production of α -mRNA during the initial incubation, and this could subsequently be translated in the presence of the sugar analog.

The experiment described in Fig. 7 was carried out to test this possibility. The solid bars demonstrate again that dGlc added simultaneously with Btr reduced significantly the accumulation of α -subunit during a 72-h incubation (compare Fig. 7B and C), whereas little or no reduction was evident

when cells were exposed to Btr for 24 h prior to dGlc addition (compare Fig. 7B and D). The steady-state levels of α -mRNA were also determined at various times after Btr and dGlc addition (hatched bars). It can be seen (compare Fig. 7A and B) that at 24 h, Btr caused an increase in α -mRNA levels, which then declined over the next 48 h. A similar result was observed when dGlc was added at 24 h, although the subsequent decrease in α -mRNA at 48 and 72 h was more rapid (compare Fig. 7B and D). In contrast, little or no increase in α -mRNA was observed when dGlc was added simultaneously with Btr (Fig. 7C). These data indicate that dGlc prevents accumulation of α -mRNA when cells are cultured with both reagents simultaneously but has less effect on transcript levels when cells are exposed to Btr for 24 h prior to dGlc addition.

Northern transfer analysis (39) was also used to examine α -mRNA isolated from cells exposed to the various effectors. A prominent hybridization signal corresponding to an RNA of about 850 nucleotides (relative to 28S and 18S rRNA) was detected in RNA from control cells (Fig. 8, lane 1). A more intense signal was observed in RNA from Btr-treated cells (lane 2), which was significantly diminished when cells were simultaneously exposed to Btr and dGlc (lane 4). These results confirm the dot-blot analysis and demonstrate that hybridization was to a unique molecular species. Basal levels of α -mRNA were also reduced by dGlc in this experiment (lane 3).

A well-recognized response of a variety of cells to Btr is an increase in their content of acetylated histones, especially histones 3 and 4 (H3 and H4) (2, 4, 10, 35). This is thought to occur by an inhibition of histone deacetylase activity by the four-carbon fatty acid (35). It has been suggested that hyperacetylated histones may be correlated with transcriptionally active genes, since both are organized into a chromatin structure that is more readily accessible to DNase than is bulk chromatin (36, 37, 41). Since the elevation of steady-state levels of α -mRNA in Btr-induced cells appears to result from an increase in transcription (11), it was of interest to examine histones in cells treated with Btr and dGlc. To this end, cells were cultured with 3 mM Btr, 3 mM Btr plus 5 mM dGlc, or no additions. After 48 h, histones were extracted from isolated nuclei and analyzed by electrophoresis in acid-urea polyacrylamide gels. There was a decrease in material migrating as H3 and H4 and a concomitant increase in more slowly migrating histone forms in samples prepared from Btr-treated cells compared with those isolated from untreated cells (Fig. 9). The decreased mobility results from increased acetylation of these histone subfractions. It is noted that dGlc had no effect on the hyperacetylation of histones in the Btr-treated cells. Thus, the results presented in Fig. 7 to 9 suggest the conclusion that dGlc interferes with a process in the production of α -mRNA in response to Btr subsequent to or irrespective of histone modification.

DISCUSSION

The results described above indicate that dGlc affects the production of the glycoprotein hormone α -subunit in HeLa cells by at least two mechanisms, one at the level of posttranslational (or cotranslational) modification and one at the level of mRNA synthesis (or degradation). The first is manifested in the accumulation of a subunit exhibiting altered elution profiles during Sephadex G-75 and ricin-agarose chromatography and increased sensitivity to endo H, most probably the consequence of modified or incomplete oligosaccharide side chains. The second is an inhibition

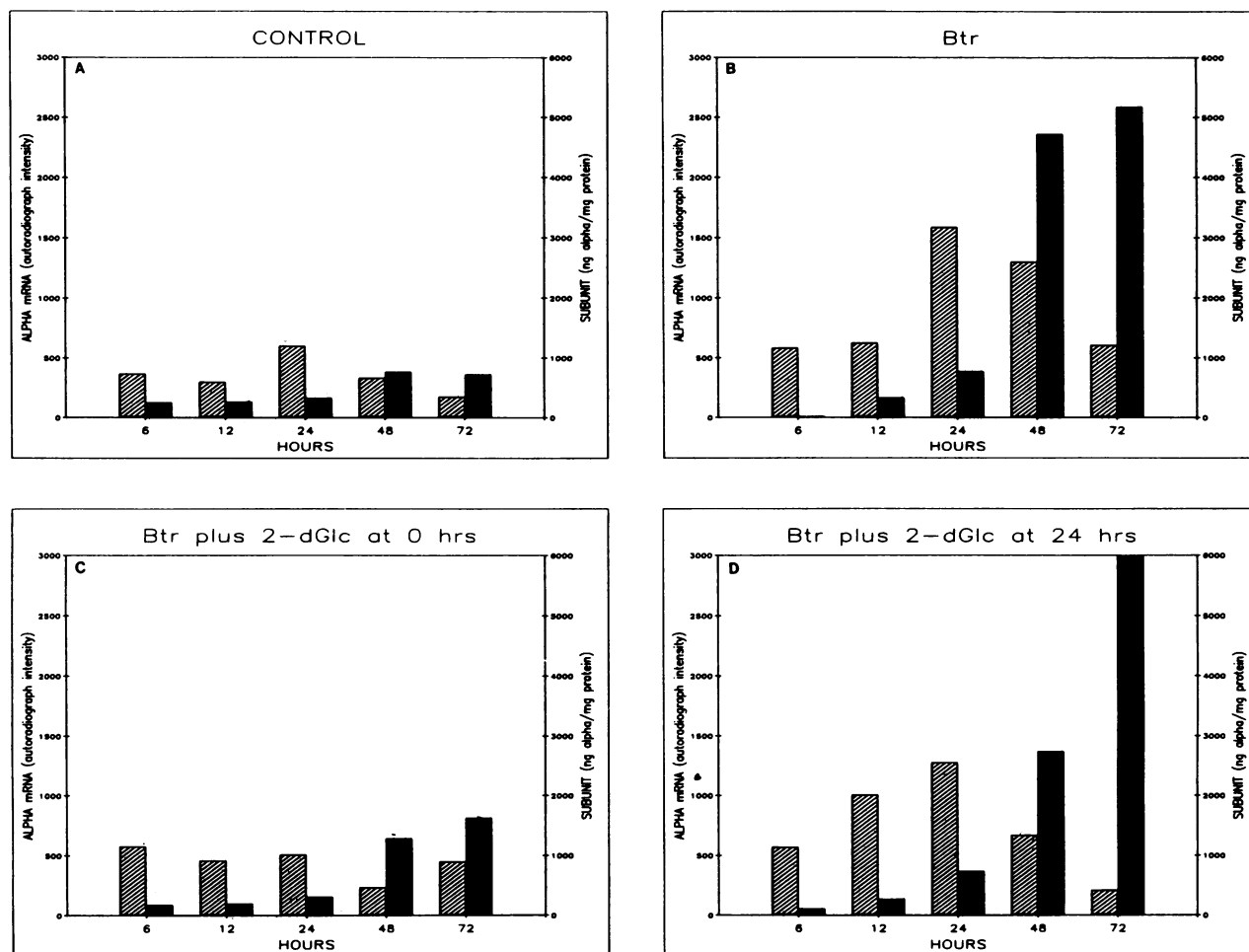


FIG. 7. Effect of Btr and dGlc on α -subunit and α -mRNA accumulation. A series of cultures in 25-cm² flasks received no additions (A), 3 mM Btr (B), 3 mM Btr plus 5 mM dGlc at zero time (C), or 3 mM Btr at zero time plus 5 mM dGlc after 24 h (D), as described in the legend to Fig. 6. At the times indicated in the figure, media and cells were harvested and assayed for α -subunit by RIA (solid bars) or for α -mRNA by dot-blot hybridization (hatched bars), as described in Materials and Methods. The data presented represent the average of two independent experiments.

by the sugar of the increase in α -mRNA accumulation normally produced in response to Btr. It is not known whether the latter effect is on transcriptional activity or mRNA stability.

At present, the consequences of subunit modification in dGlc-treated cells are not known. The secreted protein apparently contains at least the core oligosaccharide since it binds to ConA-Sepharose (mannose specific), but these may not be processed since much of the protein is not bound by ricin-agarose (galactose specific) and is susceptible to endo H action. This might be expected in view of the results obtained by Datema and Schwarz (12, 13) showing that GDP-dGlc inhibits the formation of lipid-linked oligosaccharides in virus-infected chicken embryo fibroblasts. However, in contrast to their suggestion that the shortened oligosaccharides are not transferred to protein *in vitro*, the present results seem to indicate that the α -subunit secreted by HeLa cells in the presence of equimolar Glc and dGlc is glycosylated but that the oligosaccharide side chains may indeed be abnormal. This glycosylation occurs despite a 78 to 89% inhibition of [³H]glucosamine incorporation into total secreted HeLa proteins under the same conditions (6). It has previously been observed that induction of HeLa- α by Btr is

dependent on the presence of Glc in the culture medium and that Btr can stimulate protein glycosylation under conditions of Glc depletion (26, 27). Moreover, the same hexoses which restore induction in Glc-free medium (26) are exactly those which reverse the inhibition caused by dGlc (mannose > galactose > fructose; data not presented). Since these are also the same sugars which promote the formation of glycosylated oligosaccharide-dolichol intermediates (40), it is tempting to speculate that at least one action of Btr is mediated through protein glycosylation.

It is possible that subunit synthesis is directly coupled to glycosyl transfer or that degradation of aberrant subunits is enhanced. In regard to this latter possibility, it is noteworthy that significant accumulation of a nonglycosylated subunit (M_r 12,000) was not detected in dGlc-treated cultures (Fig. 4B and 5). This is in contrast to the secretion of an apparently nonglycosylated immunoglobulin light chain by a mouse myeloma tumor cell suspension cultured in the presence of dGlc (14). The difference in these results and those presented above may be reconciled by the difference in medium supplements in the two studies; present experiments were carried out with media containing both Glc and dGlc, while the previous studies were performed with media

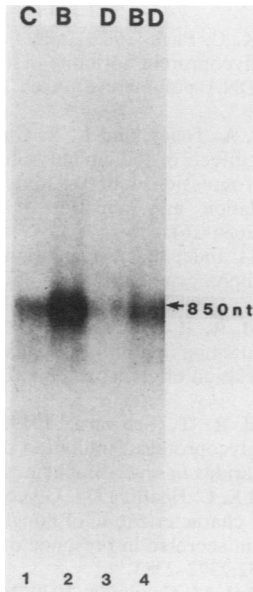


FIG. 8. Northern blot analysis of α -mRNA. Total cellular RNA was prepared as described in Materials and Methods from HeLa cultures 48 h after they had received no additions (lane 1), 3 mM Btr (lane 2), 5 mM dGlc (lane 3), or 3 mM Btr plus 5 mM dGlc (lane 4). Samples containing 20 μ g of RNA were electrophoresed through formaldehyde-containing agarose gels, transferred to a GeneScreen membrane, and hybridized to 32 P-labeled α -cDNA (1.3×10^8 cpm/ μ g) as described in Materials and Methods.

containing no Glc. Furthermore, preliminary experiments suggest that α -subunit levels can be increased (although not to normal levels) when cells are preincubated with a mixture of protease inhibitors before Btr and dGlc are added (G. S. Cox, unpublished observations). In this regard, Weintraub et al. (42) observed that 50 to 65% of the nonglycosylated thyroid-stimulating hormone subunits produced in dispersed mouse thyrotropic tumor cells in the presence of tunicamycin were degraded intracellularly before secretion. This degradation appeared to be specific for thyroid-stimulating hormone subunits compared with total 35 S-labeled proteins.

The data presented in Fig. 6 and 7 demonstrate that exposure of HeLa cells to Btr for approximately 24 h (between 16 and 36 h) prior to dGlc addition is sufficient to support α -subunit synthesis at rates comparable to those of Btr-induced cells not receiving dGlc. The data suggest that elevated subunit synthesis can continue for at least 48 h in the presence of the sugar analog once mRNA levels increase in response to the carboxylic acid (about 24 h). The continued presence of Btr is required to maintain elevated α -subunit production (McClure and Cox, unpublished observations), and dGlc does not inhibit Btr uptake at concentrations that prevent subunit induction (6).

The increase in histone acetylation in response to Btr may be necessary for the pronounced increase in α -mRNA observed at 24 h but is clearly not in itself sufficient, since dGlc added simultaneously with Btr prevents the increase in α -specific mRNA without affecting histone hyperacetylation (Fig. 7 to 9). Thus, it is suggested that dGlc interferes with the induction process at a step between chromatin modification (if this is a necessary event) and the appearance of specific transcripts. At least three possibilities can be considered, and these are currently under investigation. They are (i) decreased availability of ribonucleoside triphosphates, (ii) decreased activity of the transcription complex

(RNA polymerase II and accessory factors), and (iii) turnover of α -mRNA (or hnRNA).

It is interesting to compare the present results with those of Firestone and Heath (16), who examined the effect of dGlc on the cAMP-mediated induction of alkaline phosphatase (a membrane-bound glycoprotein) in mouse L cells. They noted that the net production of alkaline phosphatase protein was suppressed when protein glycosylation was inhibited; but in contrast to the effect of dGlc on HeLa- α (Fig. 6), complete inhibition was observed when the addition of dGlc took place 20 h after the addition of Bt₂cAMP as well as when the addition of the glycosylation inhibitor took place in conjunction with that of the cyclic nucleotide. A lower-molecular-weight, nonglycosylated form of alkaline phosphatase was not detected in L-cell cultures treated with dGlc, and the authors suggested that the nonglycosylated form of alkaline phosphatase was intrinsically more sensitive to endogenous rough endoplasmic reticulum-associated

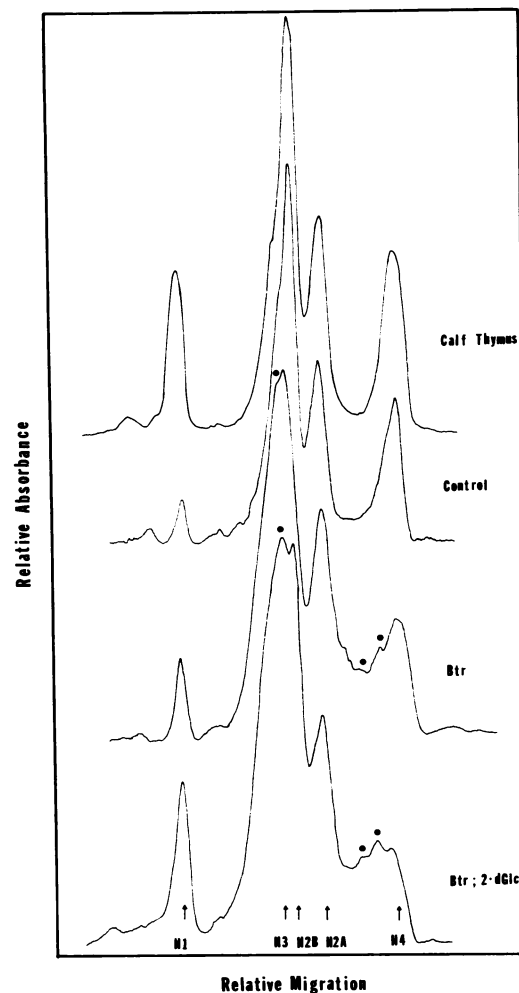


FIG. 9. Effect of Btr and dGlc on histone acetylation. Histones were isolated from cells cultured for 48 h with 3 mM Btr, 3 mM Btr plus 5 mM dGlc, or no additions, and analyzed by electrophoresis on acid-urea polyacrylamide gels as described in Materials and Methods. Electrophoresis was toward the cathode (right), and purified calf thymus histones were run as markers (arrows). Spectrophotometric scans of the stained gels are presented. Hyperacetylated forms of histones 3 and 4, which migrate on the anodic side of the parental species, are noted (solid circles).

proteases than was the native glycosylated protein. However, the small amount of alkaline phosphatase activity detected in cells exposed to Bt₂cAMP and dGlc quantitatively bound a ricin-derivatized Sepharose column, suggesting that the observed activity was due to a low-level functioning of the metabolic pathways involved with protein glycosylation. Similar conclusions may be applicable for the results obtained in the present study. *In vitro* translation of L-cell mRNA isolated from cultures containing both Bt₂cAMP and dGlc indicated that functional alkaline phosphatase mRNA was produced in quantities similar to those produced in cells treated only with Bt₂cAMP. This is in contrast to the results presented above, which indicate that dGlc prevents the increase in α -mRNA normally produced in response to Btr. Thus, different proteins (alkaline phosphatase and hCG- α) or different cell types (mouse L cells and human carcinoma cells) or both may respond quite differently to this sugar analog (or one of its metabolites); and the additional changes in cellular metabolism elicited by Btr and Bt₂cAMP further expand the number of interactions which may bring out a change in cell phenotype.

It does not seem unusual for low-molecular-weight metabolites to have multiple effects on the same (or different) cell products. For example, Noguchi et al. (28) have suggested a dual role for Bt₂cAMP on the synthesis of TAT in rat liver. The cyclic nucleotide induces TAT in two distinct ways; one is pretranslational and involves a transient and rapid increase in TAT mRNA activity, while a second appears to involve a delayed but sustained increase in translation of basal levels of TAT mRNA.

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