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Differential Expression of the Human Gonadotropin α Gene in Ectopic and Eutopic Cells

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We have analyzed the regulation of the α gonadotropin gene in eutopic placental cells and ectopic tumor cells by constructing a series of plasmid vectors containing α genomic 5′ flanking DNA placed upstream of the gene encoding the bacterial enzyme chloramphenicol acetyltransferase (CAT). These plasmid DNAs were transfected into a eutopic (JAr) and an ectopic (HeLa) cell line. Both cell types expressed the CAT gene from plasmid constructs containing as much as 1,500 base pairs (bp) and as little as 140 bp of α 5′ flanking DNA; JAr cells were considerably more efficient than HeLa cells. Ectopic and eutopic cells differed qualitatively in their expression from these α-CAT constructs when cells were treated with cAMP or butyrate. Butyrate induced α expression in HeLa cells but not in JAr cells, while cAMP induced expression in JAr cells. These results are consistent with and extend previous observations suggesting that there are cell-specific differences in the regulation of α gene expression in ectopic and eutopic cells. However, by using deletion constructs of the α-CAT gene, we found that the basal expression and cell-specific induction of the α gene in ectopic and eutopic cells were dependent on the same 140 bp of α 5′ flanking DNA. These 140 bp were sequenced and found to contain a 9-bp stretch of DNA homologous with the consensus viral enhancer sequence. Such features of α expression common to both ectopic and eutopic cells may be involved in the coordinate expression of the α gene and the tumorigenic phenotype observed in each cell type.

The α gonadotropin gene is expressed during both the normal development of the human placenta (eutopic production) and as a concomitant of tumor formation. Two classes of α-expressing tumors can be distinguished: eutopic tumors, derived from the placental trophoblast, and ectopic tumors, derived from a variety of nontrophoblastic cells. The relationship between the regulation of expression of the single-copy (2) α gene in eutopic cells and that in ectopic cells is unclear. The eutopic cells that first express α mRNA during development of the placenta (19) have a uniquely aggressive growth pattern for a normal tissue, resulting in invasion and metastasis of placental cells into the maternal host during gestation (32). Experiments in which somatic cell fusions are made with HeLa cells, an ectopic α-producing tumor, and normal human fibroblasts result in stable cell lines that are no longer tumorigenic in vivo and no longer express α protein (38). Rare segregants of these fusions that lose a specific pair of chromosomes regain the capacity to cause tumors in vivo and also regain the capacity to express α protein (38). Thus, a relationship between α expression and a tumorigenic phenotype is evident in both eutopic and ectopic cells.

Despite these observations, there is evidence that differences might exist in the control of α gene expression in eutopic and ectopic cells. The induction of α mRNA expression during the differentiation of the human placenta (19) is correlated with the exit of those cells from the cell cycle (30, 31, 34), while α transcription in the ectopic tumor line HeLa is independent of the cell cycle (10). In ectopic and eutopic cell lines, α expression appears to respond differently to cyclic AMP (cAMP) and sodium butyrate. For example, cAMP induces α protein synthesis in eutopic cell lines (7, 21–23), but has little or no effect on α protein levels in ectopic cells (21; unpublished data). Conversely, butyrate stimulates α transcription in HeLa cells but has no apparent effect on α protein levels in eutopic cells (8, 10, 23, 36).

To analyze further the control of α gene expression and its relationship to tumorigenicity, we have prepared plasmid expression vectors containing 5′ flanking sequences of the human α gene and introduced them into ectopic (HeLa) and eutopic (JAr) cell lines. Transcription from such plasmids was demonstrated in both cell types; while apparently more efficient in eutopic cells than in ectopic cells, transcription was dependent on the same 140 base pairs (bp) of α 5′ flanking genomic DNA. In each cell type, inducers of α expression, namely butyrate and cAMP, had distinct effects. Our results demonstrate both specific distinctions and parallels in the control of α expression in eutopic placental cells and ectopic tumors and are discussed with respect to the relationship between α gene expression and the tumorigenic phenotype.

MATERIALS AND METHODS

Suppliers. [^{35}S]methionine (>600 Ci/mmol) and [^{14}C]chloramphenicol (46.9 mCi/mmol) were purchased from New England Nuclear Corp. [α-^{32}P]dNTP and [γ-^{32}P]ATP (>3,000 Ci/mmol) were purchased from Amersham Corp. Restriction endonucleases were from New England BioLabs or Bethesda Research Laboratories. T4 polynucleotide kinase and exonuclease III were from New England BioLabs, T4 DNA ligase and the Klenow fragment of *Escherichia coli* DNA polymerase I were from New England Nuclear Corp., and S1 nuclease was from Miles Laboratories, Inc. Purified chloramphenicol acetyltransferase (CAT) was from P-L Biochemicals. Synthetic dodecamer *Hin*dIII linkers were purchased from Collaborative Research, Inc. CsCl was purchased from Varlacoid Chemical Co. The sodium salt of butyric acid was obtained from J. T. Baker Chemical Co., and

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cAMP analogs were obtained from Sigma Chemical Co. Bovine calf serum was from KC Biologicals.

Cell culture and transfection. Plasmids pSV2 and pSV0 and HeLa cells were gifts from L. Laimins, National Institutes of Health, and JAr cells were a gift of R. Ruddon, University of Michigan, Ann Arbor. Cells were passaged in monolayer in Dulbecco modified Eagle medium (DMEM) supplemented with 10% bovine calf serum and glutamine.

Plasmid DNAs for HeLa cell transfection were banded twice on CsCl gradients and assayed for supercoils (form I DNA) on agarose gels. For transfecting into JAr cells, plasmids were banded once on CsCl gradients, linearized by restriction endonuclease digestion, treated sequentially with RNase A (10.0 μg/ml for 60 min at 37°C) and proteinase K (50.0 μg/ml for 20 min at 37°C), and thoroughly extracted with phenol-chloroform-isoamyl alcohol. Plasmid DNA was transfected (10.0 μg per 10-cm dish); no carrier DNA was added.

Cells were plated (10^6 cells per 10-cm dish) 20 h before transfection and fed with fresh medium 4 h before transfection. Plasmid DNAs were transfected by a $CaPO_4$ precipitation method described previously (17) as modified by Wigler et al. (41). Ethanol-precipitated DNA was redissolved in 0.25 M $CaCl_2$, added dropwise to 2× HBS (280 mM NaCl, 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 1.5 mM Na_2PO_4 [pH 7.12]) while vortexing, and allowed to stand for 20 to 30 min. The precipitate was gently pipetted with polystyrene pipettes, and 1.0 ml was added dropwise per 10-cm dish. After 16 to 20 h, the DNA precipitate was removed, and cells were washed twice with DMEM and fed with fresh medium. For induction experiments, reagents were added 36 h later. In all experiments, cells were harvested for assay of CAT activity and protein determination 48 h after removal of the DNA precipitate.

CAT and protein assays. Cell extracts were prepared and assayed for CAT activity as described by Gorman et al. (16). To ensure that assays were in the linear range and to reduce variability, one-third of each reaction mixture was removed at various times for analysis. After separation of chloramphenicol (CM) from its acetylated forms by ascending thin-layer chromatography and visualization by autoradiography, the amount of acetylated CM produced was determined by cutting out the appropriate regions and quantitating them in a scintillation counter. Units of CAT activity were calculated from standard curves made from reactions with known amounts of purified CAT and labeled CM of known specific activity. Protein concentrations were determined by the method of Bradford (3), with bovine serum albumin as the standard.

Plasmid construction. Plasmid pα6/10 was digested with *Eco*RI and *Bam*HI, and the ends were filled in with Klenow enzyme. The 1.5-kilobase-pair (kbp) fragment containing the 5′ flanking region of the α gene was isolated from a polyacrylamide gel by electroelution and purified on a DE52 column (37). The fragment was then ligated to phosphorylated *Hin*dIII linkers at a ratio of linker to fragment ends of 75:1. After digestion with an excess of *Hin*dIII, free linkers were removed on a polyacrylamide gel, and the fragment was isolated and purified as described above. After ligation into the *Hin*dIII site of pBR322, recombinants were isolated as Amp^r Tet^s colonies in *E. coli* HB101. The 1.5-kbp *Hin*dIII fragment containing α sequences was isolated and purified from this vector (pBRα) and inserted into the *Hin*dIII site of plasmid pSV0 to generate pαCAT.

To generate deletions in the α sequences of pαCAT, pαCAT was digested with *Bgl*II, and 2.5-μg portions were digested with 40 U of exonuclease III at 30°C for 1.0 to 3.0 min. Single-stranded DNA was digested with 60 U of S1 nuclease per 1.7 pmol of 5′ ends. After digestion with *Nde*I and filling in ends with Klenow, plasmids were self-ligated at a concentration of approximately 5 nM to allow efficient intramolecular ligation. The amount of α 5′ flanking DNA in various deletion mutants was determined by restriction analysis. The structures of clones pΔ140, pΔ60, and pΔ45 were confirmed by chemical sequencing (29). Each plasmid was digested with *Hin*dIII, end labeled with Klenow and [α-^{32}P]dCTP, digested with *Hin*dIII, and isolated on a polyacrylamide gel for sequencing. pΔ140 was also labeled on the complementary strand by labeling at the *Hin*dIII end to confirm its sequence.

RESULTS

Constructions. The human α gonadotropin gene has previously been isolated from a human library as a single 16-kbp insert (2) and subcloned as three *Eco*RI fragments into pBR322 (M. Boothby, R. Darnell, and I. Boime, unpublished data). One subclone (pα6/10) contains the first and second exons encoding the α gene, separated by 8.5 kbp, as well as 1.5 kbp of sequence 5′ to the first exon (Fig. 1). Evidence that transcription actually initiates at this first exon was provided by Fiddes and Goodman (11), whose sequencing data and mapping experiments located a probable cap site for the α gene 38 bp upstream from the *Bam*HI site within the first exon (Fig. 1).

An expression vector containing the simian virus 40 (SV40) early promoter upstream of the CAT gene, pSV2-CAT (pSV2), constructed by Gorman et al. (16), was used as a positive control in transfection experiments and as the parental plasmid for the constructs (Fig. 1). A promoterless construct derived from pSV2, pSV0-CAT (pSV0 in Fig. 1), was used as a negative control in transfection experiments. In the deletion of the SV40 promoter fragment from pSV2, pSV0 was constructed to contain a single *Hin*dIII site immediately 5′ to the CAT gene (Fig. 1), providing a convenient site for the insertion of transcriptional promoter sequences. Figure 1 illustrates the strategy taken to construct the α expression vector pαCAT. A 1.5-kbp fragment containing 5′ α genomic flanking sequences was ligated to *Hin*dIII linkers and inserted into the *Hin*dIII site of pSV0. The fragment of α genomic DNA chosen begins 38 bp downstream of the cap site, and extends 1.5 kbp 5′-ward from the cap site. The resulting plasmid, pαCAT, is shown in Fig. 1.

Transfections with pαCAT in JAr cells. To determine the in vivo levels expressed by the 1.5 kbp of α 5′ flanking DNA, we first transfected a eutopic cell line (JAr, derived from a choriocarcinoma) with pαCAT and compared the level of expression with those of pSV2 and pSV0. DNA was transfected by $CaPO_4$ precipitation as described previously (17, 41), and cell extracts were assayed for CAT activity 48 h later (Fig. 2). Extracts of cells transfected with either pSV0 (Fig. 2, lanes 1 through 3), pSV2 (lanes 4 through 6), or pαCAT (lanes 7 through 9) were incubated with ^{14}C-labeled CM, and samples were removed at various times. Since the CAT gene product converts CM into mono- and diacetylated derivatives (16), detection of acetylated forms of CM resolved on silica gel thin-layer plates is a sensitive assay for expression of the CAT gene in transfected cells.

Extracts from JAr cells transfected with pSV2 have high levels of CAT activity (Fig. 2, lanes 4 through 6). Extracts from these cells converted 20% of the labeled CM to acetylated forms within 30 min. Comparison of these results

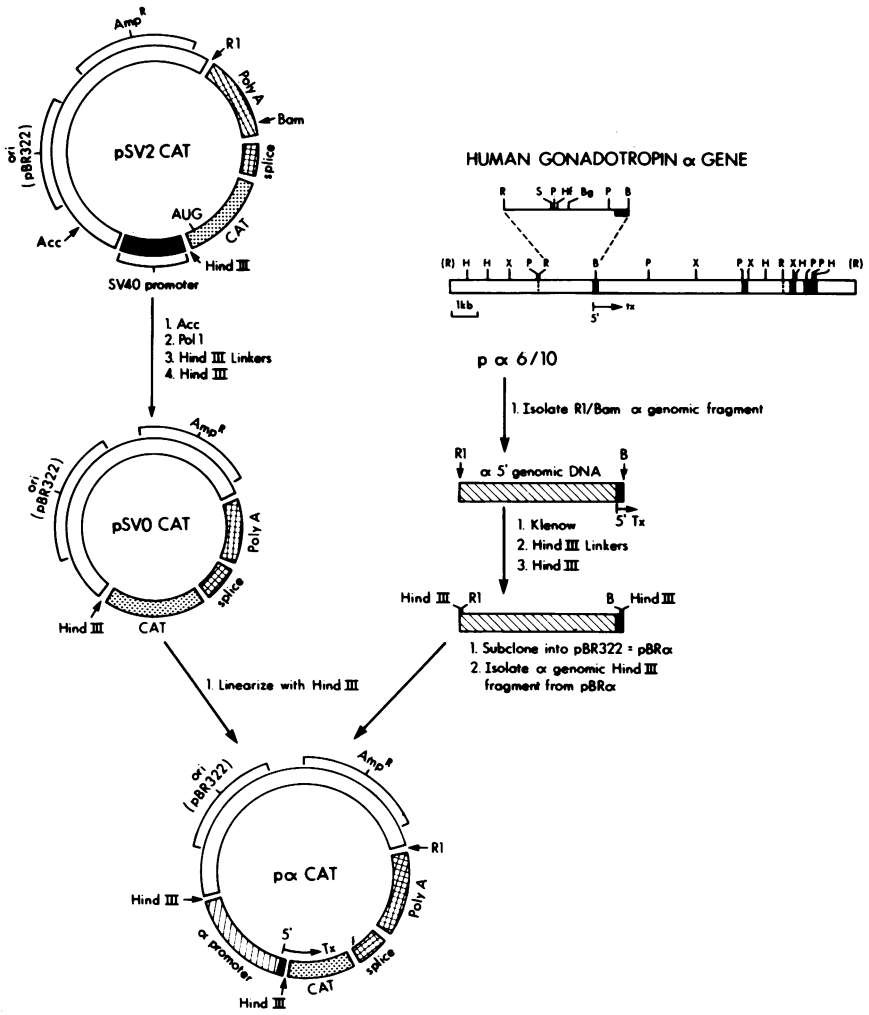


FIG. 1. Construction of pαCAT. The left side of the figure shows the parental plasmids used in constructing pαCAT, pSV2 CAT and pSV0 CAT (15, 16). The functional elements of pSV2 CAT are indicated (counterclockwise, not to scale): the Amp^r gene and origin of replication from pBR322; a 330-bp segment containing the SV40 early promoter region, including both 72-bp repeat sequences (enhancers); the CAT gene from the pBR322-Tn9 plasmid of *E. coli*; the SV40 small t intron, including splice recognition sequences; and a fragment of the SV40 early region containing a polyadenylation site. pSV0 CAT was derived from pSV2 CAT by deleting the SV40 early promoter region and inserting an artificial *Hin*dIII site at the resulting junction of pBR322 and CAT sequences. The right side of the figure illustrates the preparation of α genomic DNA in the construction of pαCAT. The restriction sites in and flanking the human α gene (2, 11; unpublished data), indicated by the open box, are abbreviated as follows: R, *Eco*RI; H, *Hin*dIII; X, *Xba*I; P, *Pst*I; S, *Sma*I; Hf, *Hin*fI; Bg, *Bgl*II; B, *Bam*HI. The 10-kbp *Eco*RI fragment containing the two 5′ exons of the α gene was subcloned into the *Eco*RI site of pBR322 to create pα6/10. From pα6/10, a 1.5-kbp *Eco*RI-*Bam*HI fragment of 5′ flanking α DNA was isolated for the construction of pαCAT. Synthetic *Hin*dIII linkers were added to the *Eco*RI-*Bam*HI fragment, which was inserted into the *Hin*dIII site of pBR322 to generate pBRα. After amplification, this same *Hin*dIII fragment was isolated from pBRα and subcloned into the *Hin*dIII site of pSV0 CAT to generate pαCAT. Transcription (Tx) from α sequences into the CAT gene in pαCAT is indicated by the arrow.

with previous reports indicates that in JAr cells, pSV2 is expressed 0.3-, 10-, and 20-fold as efficiently as in CHO, HeLa, and NIH-3T3 cells, respectively (normalized to number of cells per assay [15]). That this activity was specifically a function of the transcriptional sequences present in pSV2 is indicated by the lack of CAT activity in cells transfected with pSV0 (Fig. 2, lanes 1 through 3). Thus, JAr cells provide a competent host cell for transfection experiments. Cells transfected with form I (supercoils) expressed much less CAT than did the linearized form. This contrasts to that seen for HeLa cells (see below).

pαCAT expressed CAT activity at readily detectable levels in JAr cells (Fig. 2, lanes 7 through 9). The percentage of CAT activity detected from pαCAT relative to pSV2, averaged from three experiments (Table 1), was 38%; this ratio was highly consistent between experiments. Thus, transcription from the α promoter was relatively efficient compared with transcription from the viral promoter of pSV2 (Table 1). The CAT signal from these plasmids varied between 10- and 100-fold over the background CAT activity detected in extracts from pSV0-transfected cells (Table 1). The data in Table 1 also demonstrate that the absolute value of CAT activity in lysates from cells transfected with either pαCAT or pSV2, when normalized to protein content per assay, varied only twofold between experiments. Thus, 1.5 kbp of α 5′ flanking DNA are capable of directing transcription of the transfected CAT gene in JAr cells.

Induction of α expression in JAr cells. Radioimmunoassay

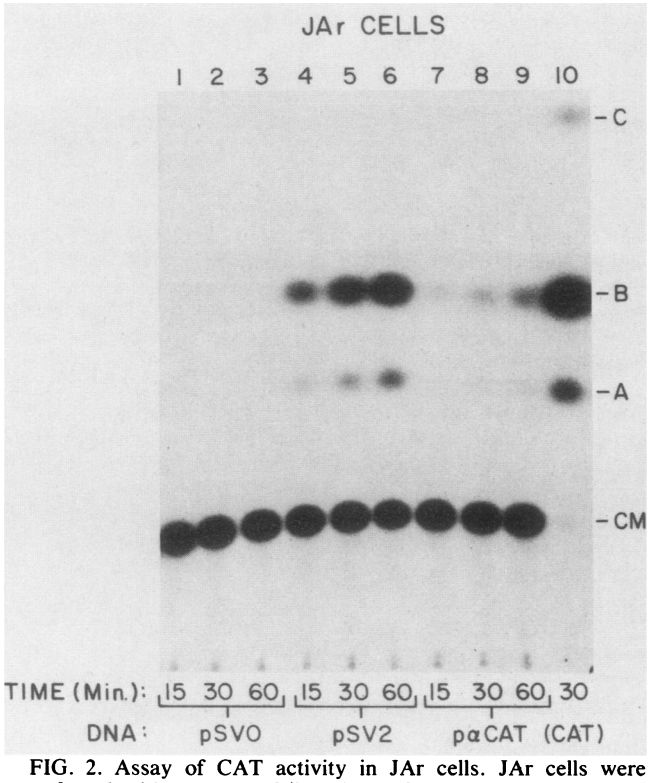


FIG. 2. Assay of CAT activity in JAr cells. JAr cells were transfected with 10.0 μg of linearized pSV0 (lanes 1 through 3), pSV2 (lanes 4 through 6) or pαCAT (lanes 7 through 9) DNA. Cell extracts were assayed 48 h later for CAT activity in aliquots removed at 15, 30, and 60 min as indicated. After extraction with ethyl acetate and concentration, samples were spotted on thin-layer silica gels to resolve substrate (CM) from its acetylated products: 1-acetyl chloramphenicol (A), 3-acetyl chloramphenicol (B), and 1,3-diacetyl chloramphenicol (C). Lane 10 shows the products obtained when chloramphenicol was incubated with purified CAT (2 U from P-L Biochemicals). The autoradiograph is shown; the spots in this gel were counted, and results are presented in experiment 1 of Table 1.

analyses have shown that the levels of α and β subunits of human chorionic gonadotropin are increased after treatment of JAr cells with cAMP but not with butyrate. To confirm these observations, JAr cells were treated with either 1 mM 8-Br-cAMP or 5 mM butyrate for 24 h and protein synthesis was assayed by pulse-labeling cells for 60 min with [^{35}S]methionine. Figure 3 shows the results of immunoprecipitating the α and β subunits of human chorionic gonadotropin from extracts of these cells; it is clear that while cAMP induced both α and β protein synthesis, butyrate was without effect on stimulating synthesis of either subunit. These results also suggest that the induction of α subunit previously reported with dibutyryl cAMP is a result of the action of the cAMP, and not of butyrate, a metabolite of dibutyryl cAMP.

The effect of cAMP on CAT expression was examined in transfected JAr cells treated with 1 mM dibutyryl cAMP. In pαCAT-transfected JAr cells, CAT activity was induced 2- to 2.5-fold (Table 1). Induction was specific for α sequences; CAT expression in pSV2-transfected cells was unaffected by treatment with cAMP (Table 1). These results indicate that the 1.5-kbp section of DNA flanking the α gene contains sequences that regulate transcription in response to cAMP. Further, this regulation of α expression is consistent with the induction of the endogenous α subunit in response to cAMP in JAr cells (Fig. 3) and predicts that this response is also mediated by an induction of transcription of the α gene. Consistent with these results were analyses of Northern blots of the steady-state levels of α mRNA derived from JAr cells treated with cAMP; α mRNA was induced two- to fivefold (R. Darnell, M. Boothby, and I. Boime, unpublished observations).

JAr cells were transfected with pαCAT and incubated with 5 mM butyrate for the final 12 h before cell extracts were assayed for CAT activity (Table 1). Butyrate had no significant effect on CAT activity in JAr cells. However, the same α 5′ flanking sequences are competent to respond to butyrate when transfected into HeLa cells, as demonstrated below. These results suggest that butyrate does not affect α transcription in JAr cells.

Transfections with pαCAT in HeLa cells. In initial experiments with HeLa cells as host cells for transfections, it was demonstrated that there was little or no CAT activity in extracts of cells transfected with linearized pSV2 (data not shown). This result was repeated with several independent preparations of linearized plasmid, each of which had been expressed in JAr cells, as well as in three independent strains

TABLE 1. JAr cell CAT assays

Expt, assay (DNA + Inducer)	CAT Activity (μmol/g per min)[a]	Induction (fold)[b]
Expt 1		
Bckd (pSV0CAT)	0.11 ± 0.05	
pSV2CAT	3.67 ± 0.31	
pSV2CAT + cAMP	3.75 ± 0.19	1.02
pαCAT	1.19 ± 0.13	
pαCAT + cAMP	2.96 ± 0.27	2.50
Expt 2		
Bckd (pSV0CAT)	0.05 ± 0.01	
pSV2CAT	1.49 ± 0.10	
pSV2CAT + cAMP	1.31 ± 0.03	0.88
pSV2CAT + butyrate	1.53 ± 0.03	1.03
pαCAT	1.05 ± 0.09[c]	
pαCAT + cAMP	2.13 ± 0.16	2.03
pαCAT + butyrate	1.28 ± 0.08	1.22
Expt 3		
Bckd (pSV0CAT)	0.01 ± 0.003	
pSV2CAT	1.77 ± 0.35	
pSV2CAT + cAMP	2.09 ± 0.38	1.18
pSV2CAT + butyrate	2.49 ± 0.70	1.41
pαCAT	0.54 ± 0.05	
pαCAT + cAMP	1.04 ± 0.09	1.93
pαCAT + butyrate	0.57 ± 0.07	1.06

[a] Each number represents the average of 3 CAT assays from a single transfection extract ± standard error of the mean, as detailed in Materials and Methods; units are normalized to grams of total protein and incubation time per assay, ± the standard error of the mean.

[b] Ratio of CAT activity of induced cell extract to that of uninduced cell extract.

[c] Average of two assays: activities were 1.07 and 1.01.

of HeLa cells (data not shown). However, when form I rather than linearized pSV2 was used for transfection into HeLa cells, CAT activity was readily detectable (Table 2). These results indicate that HeLa and JAr cells differ significantly in either the process by which they take up foreign DNA or the process by which they express it.

We quantitated the results from several experiments in which form I plasmid pSV2 was transfected into HeLa cells (Table 2). Transfection efficiencies in these experiments varied by 3.5-fold, from 0.80 to 2.80 U of CAT activity (Table 2). This variability was comparable to that seen in JAr cells, in which transfection efficiency varied by 2.5-fold (Table 1). It has been suggested that variables such as differences in seeding density may lead to significant changes in transfection efficiencies between experiments (15). Thus, averaging results between several experiments generates large errors. Nonetheless, our data suggest that the level of CAT activity was not significantly different in either HeLa cells or JAr cells transfected with pSV2 (1.9 versus 2.3 CAT U, respectively; average values from Tables 1 and 2). Thus, in the conditions used in these transfection experiments (assuming that pSV2 transcription is not differentially regulated in JAr and HeLa cells), HeLa cells were approximately as efficient as JAr cells at expressing transfected plasmid DNA.

In extracts of HeLa cells transfected with pαCAT we could only detect a very low level of CAT activity (Table 2). The strongest signal from pαCAT was seen in experiment 1 of Table 2 (also in an additional experiment not shown), where the activity of pαCAT was 4.0-fold over background levels; in later experiments, this signal was closer to the background signal seen from pSV0. In some experiments, a significant amount of CAT activity could be detected under prolonged assay conditions in extracts from HeLa cells transfected with pSV0 (for an example, see Fig. 7). This was unexpected, since previous reports have demonstrated that no detectable signal could be detected from extracts from cells transfected with promoter-defective CAT constructs, even after a 12-h CAT assay (39). The reason that pSV0-transfected HeLa cells variably expressed detectable levels of CAT activity is unclear.

TABLE 2. HeLa cell CAT assays

Expt, assay (DNA + Inducer)	10^{-1} CAT activity (μmol/g min)	Induction (fold)[b]
Expt 1		
Bckd (pSV0CAT)	0.18 ± 0.09	
pSV2CAT	20.89 ± 1.49	
pSV2CAT + butyrate	15.20 ± 0.58	0.73
pSV2CAT + cAMP	18.24 ± 1.40	0.87
pαCAT	0.85 ± 0.26	
pαCAT + butyrate	2.50 ± 0.17[c]	2.94
pαCAT + cAMP	1.13 ± 0.27	1.33
Expt 2		
Bckd (pSV0CAT)	0.40 ± 0.04	
pSV2CAT	27.55 ± 1.74	
pSV2CAT + butyrate	60.16 ± 10.75	2.18
pαCAT	0.48 ± 0.11	
pαCAT + butyrate	1.97 ± 0.24	4.1[d]
Expt 3		
Bckd (pSV0CAT)	0.030 ± 0.001	
pSV2CAT	7.88 ± 1.42	
pSV2CAT + butyrate	8.91 ± 1.24	1.13
pαCAT	0.04 ± 0.01	
pαCAT + butyrate	0.49 ± 0.01	12.3[d]

[a] Data and CAT activity units are as described in footnote *a* of Table 1 except that CAT units are to be multiplied by 10: each number is the average of three points ± the standard error of the mean.
[b] Ratio of CAT activity of induced cell extracts.
[c] Average of two assays: activities were 2.50 and 2.50.
[d] Quantitative fold induction may be unreliable due to low signal-to-background ratio (see the text).

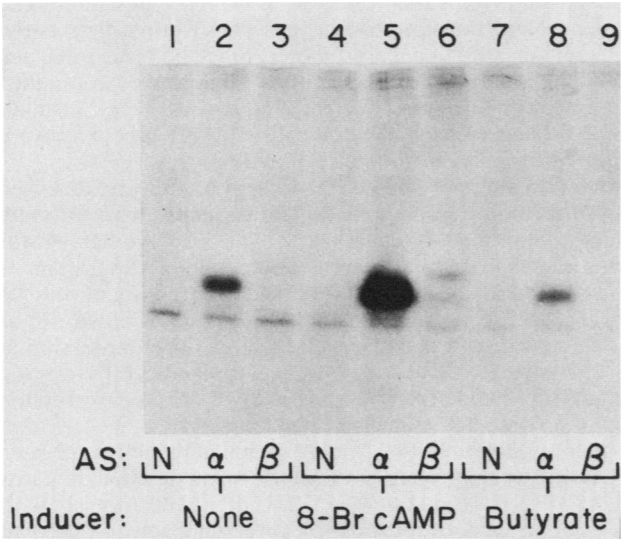


FIG. 3. Differential effects of cAMP and butyrate on α subunit protein synthesis in JAr cells. JAr cells were incubated for 24 h in the presence of either 1 mM 8-Br-cAMP (lanes 4 through 6) or 5 mM butyrate (lanes 7 through 9). Cell extracts were preadsorbed with normal rabbit serum and immunoprecipitated with anti-human chorionic gonadotropin α antiserum (α; lanes 2, 5, and 8), anti-human chorionic gonadotropin β antiserum (β; lanes 3, 6, and 9) or normal rabbit serum (N; lanes 1, 4, and 7).

When HeLa cells were transfected with pαCAT, the observed CAT activity was about 2.2-fold above background levels (cells transfected with pSV0; Table 2). In contrast, CAT activity in pαCAT-transfected JAr cells averaged 20-fold over background activity (Table 1). Similarly, the level of expression from pαCAT relative to pSV2 was much greater in JAr cells than in HeLa cells (Tables 1 and 2). Since HeLa cells appear to be approximately as competent as JAr cells in transcribing pSV2 yet significantly less competent in expressing transfected pαCAT DNA, we conclude that the expression of pαCAT is at least an order of magnitude higher in JAr cells than in HeLa cells. This is consistent with basal levels of expression of the α gene in the two cell types. Northern analyses revealed much more α mRNA in JAr than in HeLa cells; in the latter case, the level of endogenous α mRNA was barely detectable in several experiments.

Induction of α expression in HeLa cells. CAT activity was increased significantly when HeLa cells transfected with pαCAT were incubated in the presence of butyrate for 12 h (Table 2). The extent of induction was difficult to quantitate

accurately due to the low basal expression of α; there was an average of a sixfold induction with a large variation (Table 2). When pSV2-transfected HeLa cells were incubated in the presence of butyrate, no significant change in the level of pSV2 expression was observed (Table 2). In one experiment, (experiment 2 in Table 2) pSV2-mediated CAT expression did appear to be induced 2.2-fold after butyrate treatment, but this result was not reproducible; in addition, this activity was significantly less than the induction seen in pαCAT-transfected cells. Thus, the induction of pαCAT expression after butyrate treatment is specific to the α 5′ flanking genomic sequences. These results are consistent with previous data demonstrating that butyrate stimulates transcription of the endogenous α gene 10-fold in HeLa cells (10). The effect of butyrate on α expression appears to be specific to HeLa cells, since JAr cells transfected with pαCAT and treated with butyrate showed no induction of CAT expression (Table 1). In a single experiment, cAMP (8-Br-cAMP rather than dibutyryl cAMP was used, since the latter is metabolized intracellularly to butyrate and cAMP) had no effect on pSV2 expression and a minimal effect (1.3-fold induction) on pαCAT expression in HeLa cells (experiment 1 in Table 2).

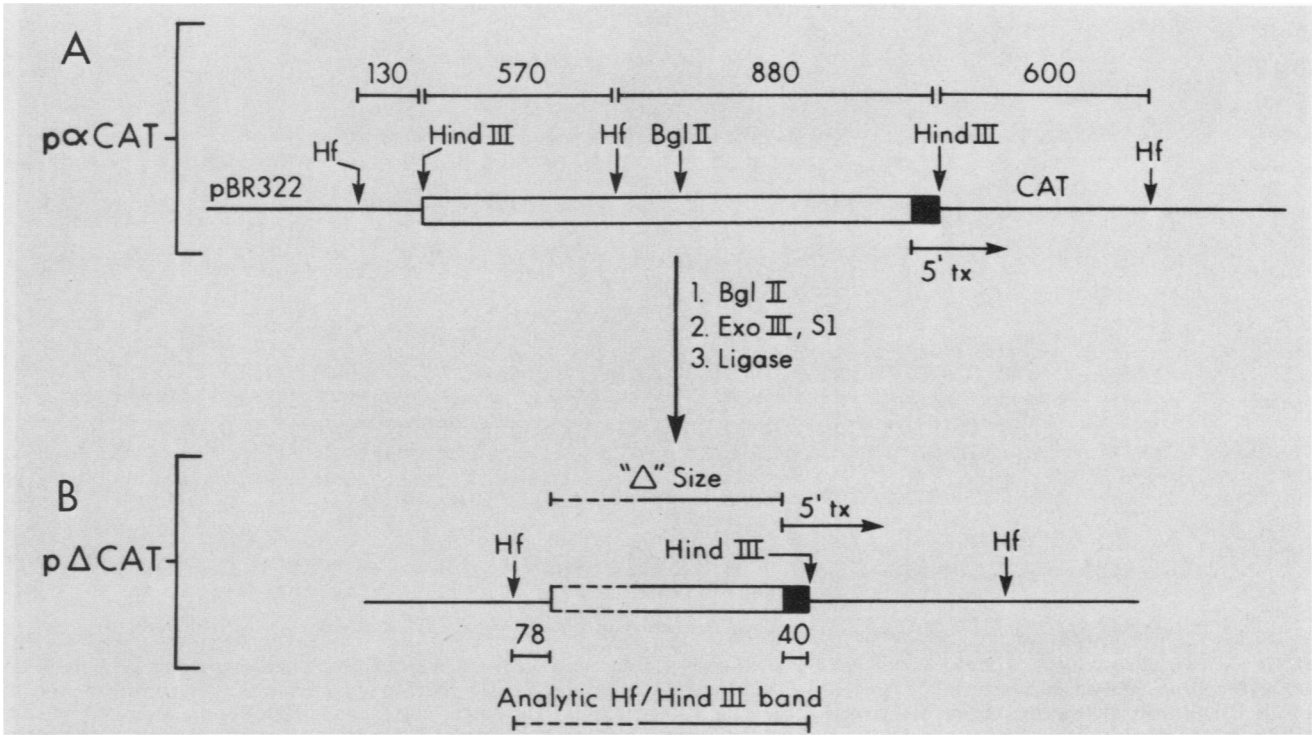
pαCAT deletion mutants. To determine the location of the functional regions in the 5′ flanking sequence of the α gene that are involved in pαCAT expression, a series of deletions of the 5′ end of the α gene were produced (Fig. 4). The deletions were constructed so that each would have decreasing amounts of 5′ flanking α DNA in an otherwise identical plasmid background. pαCAT was linearized with *Bgl*II, which cuts pαCAT in the middle of the 1.5 kbp of 5′ flanking α DNA. Portions were then digested with exonuclease III for various time periods to give deletions with endpoints throughout the 800 bp of α DNA downstream from the *Bgl*II site. After digestion of single-stranded DNA with S1 nuclease, plasmids were digested with a second restriction endonuclease, *Nde*I. This secondary digestion ensured that the ends of each deletion endpoint would be ligated to an identical point in the pBR322 sequences of pαCAT. After ligation, transformants were screened by sizing the fragments produced in *Hin*dIII-*Hin*fI double digests (Fig. 4). A full range of deletion mutants that contained from 680 (pΔ680) to 20 (pΔ20) nucleotides of α 5′ flanking sequences was obtained.

Several of these deletion mutants (pΔ45, pΔ60, and pΔ140) were sequenced to confirm their fidelity and endpoints. In addition, sequencing of pΔ140 provided new information on the 5′ sequences flanking the α gene, and both strands were sequenced to obtain reliable data (Fig. 5). Fiddes and Goodman have previously sequenced the first 89 bp upstream of the cap site (11), and our results were identical, except that at −82 we found a T instead of a C. Whether this reflects a polymorphism in the 5′ flanking sequence of the α gene is uncertain. We noted several features of the α 5′ flanking sequence and have shaded these areas in Fig. 5. Two structural regions common to eucaryotic promoters are shaded, the TATA box at −29 to −23 and a sequence that corresponds to the consensus sequence for a CAAT box at −94 to −90. A third shaded region, a 9-bp sequence between −48 and −40, matches almost exactly the consensus enhancer sequence of a variety of viral promoters (the consensus enhancer sequence is GTGGAAAG [27, 40] and the corresponding α sequence is GTGGAAAC). In addition, we compared the α sequence to the sequence for bovine α gene (14). A high degree of homology (83%) was observed in the region between −142 and −23. This suggests that elements in the immediate 5′ flanking sequence are associated with controlling the expression of gonadotropin α-type genes.

Transfection with pαCAT deletion mutants. To determine those regions of α 5′ flanking DNA involved in expression of the α gene, several of the deletion mutants were transfected into JAr and HeLa cells. In JAr cells, duplicate transfections were performed, and cAMP was added to one of each set of duplicates 12 h before harvesting the cells for CAT assays. The results of CAT assays from this experiment (which corresponds to experiment 3 in Table 1) are shown in Fig. 6. Each line represents the CAT activity in samples of cell extracts from individual JAr cell transfections, determined at several times to ensure that the assay was in the linear range. Figures 6A and B recapitulate several points noted in Table 1: first, neither cAMP nor butyrate had a significant effect on pSV2-mediated CAT expression (Fig. 6A); and second, induction with butyrate had no effect on pαCAT-mediated CAT expression in JAr cells (Fig. 6B), while induction with cAMP increased pαCAT expression approximately twofold (Fig. 6B). Deletion mutants pΔ245, pΔ190, pΔ140 (and pΔ100, data not shown) all expressed basal levels of CAT activity that are comparable to those produced by pαCAT in JAr cells, and in each case, this level of expression doubled after treatment with cAMP (Fig. 6C through E). pΔ140 appeared to express only 50% of the level of CAT produced from pαCAT or the larger deletion mutants; it is not yet certain whether this result is significant. However, when JAr cells were transfected with deletion mutants pΔ60 or pΔ45, only background levels of CAT activity were produced in either the presence or absence of cAMP (Fig. 6F and G).

These results establish several points. First, CAT expression from α vectors transfected into JAr cells appeared to depend specifically on the presence of sequences between −60 and −140. Second, the induction of α expression with cAMP in JAr cells, while only twofold, was reproducible and dependent on the presence of some sequences within the pΔ140 mutant. Finally, the absence of α expression in cells transfected with either pΔ60 or pΔ45 provides additional evidence that transcription in these CAT constructs is dependent on a functional α promoter. Since both the pΔ60 and pΔ45 mutants retained a TATA box, and pΔ60 also retained the enhancer-like sequences at −40 to −48 (Fig. 5), additional sequences such as perhaps the CAAT box at −90 are required for efficient promoter function.

Deletion mutants pΔ245, pΔ140, and pΔ45 were also used to transfect duplicate plates of HeLa cells, with butyrate added to one plate of each pair 12 hours before harvest for CAT assays (Fig. 7). Interpretation of this experiment is complicated by the low levels of basal expression of pαCAT in HeLa cells (see above). Figures 7A and B illustrate, as shown in Table 2, that the high level of CAT expression in pSV2-transfected HeLa cells was not significantly affected by butyrate, while the background level of CAT expression in pαCAT-transfected cells was increased dramatically after 12 h of induction with butyrate. Similar results were seen when HeLa cells were transfected with deletion mutants pΔ245 (Fig. 7C), pΔ140 (Fig. 7D), or pΔ100 (not shown). pΔ45 (or pΔ60; data not shown) expressed levels of CAT that were one-half the background signal seen with pSV0 in the presence or absence of butyrate, and one-third of the basal signal seen with pαCAT. Thus, in HeLa cells transfected with either pαCAT or pΔCAT deletion mutants containing at least 140 bp of α 5′ flanking DNA, butyrate appeared to induce transcription levels that were very low but were still significantly above the background level. However, the background level of CAT activity from pΔ45, a mutant not



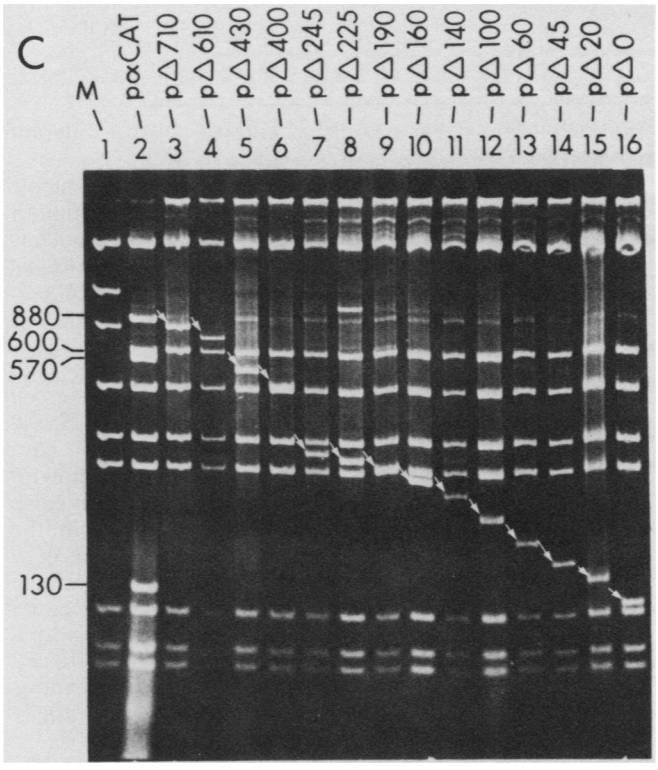


FIG. 4. Construction and characterization of 5′ α deletion mutants (pΔCAT). Deletion mutants in α 5′ flanking sequences were constructed as detailed in Materials and Methods. Briefly, pαCAT contains a single *Bgl*II site within the 5′ flanking sequences of the α gene 750 bp upstream of the CAP site. After pαCAT was digested with *Bgl*II, plasmids were treated with exonuclease III for various times to create a series of deletion mutants. They were characterized by analyzing restriction fragment sizes after *Hin*fI-*Hin*dIII double digestion. Comparison of panels A and B indicates that in such a digest, the 600-bp fragment from pαCAT should remain in the pΔCAT mutants, and the 880-, 570-, and 130-bp fragments from pαCAT should become a single band of varying size dependent on the deletion size (illustrated as the analytic *Hin*fI-*Hin*dIII band in panel B). These bands are indicated by the downward arrows in panel C. Each deletion mutant was named according to the length of α 5′ flanking DNA that it contained. Each band from the *Hin*fI-*Hin*dIII digest contains DNA in addition to α 5′ flanking DNA (as indicated in panel B), 79 bp of pBR322 DNA and 40 bp of α 5′ transcribed DNA. Thus, 118 bp was subtracted from the band sizes determined from the analytical gel to determine the actual length of α 5′ flanking DNA and, thus, the name of each mutant. Band sizes were determined with the aid of DNA markers as in lane 1 (a *Hin*fI digest of equimolar amounts of pSV0 CAT and pSV2 CAT).

expected to be competent to promote transcription (see above), was unaffected by butyrate. These results suggest that at least the first 140 bp of α 5′ flanking sequences are necessary to promote basal transcription in HeLa cells. Further, the action of butyrate to induce pαCAT expression in HeLa cells is also dependent on the presence of this stretch of 5′ flanking DNA.

DISCUSSION

Differential regulation of α expression in ectopic and eutopic cells. We have observed two significant differences in the expression of α 5′ flanking DNA transfected into ectopic and eutopic cells. First, a quantitative difference in the basal expression of α sequences was demonstrated. By using

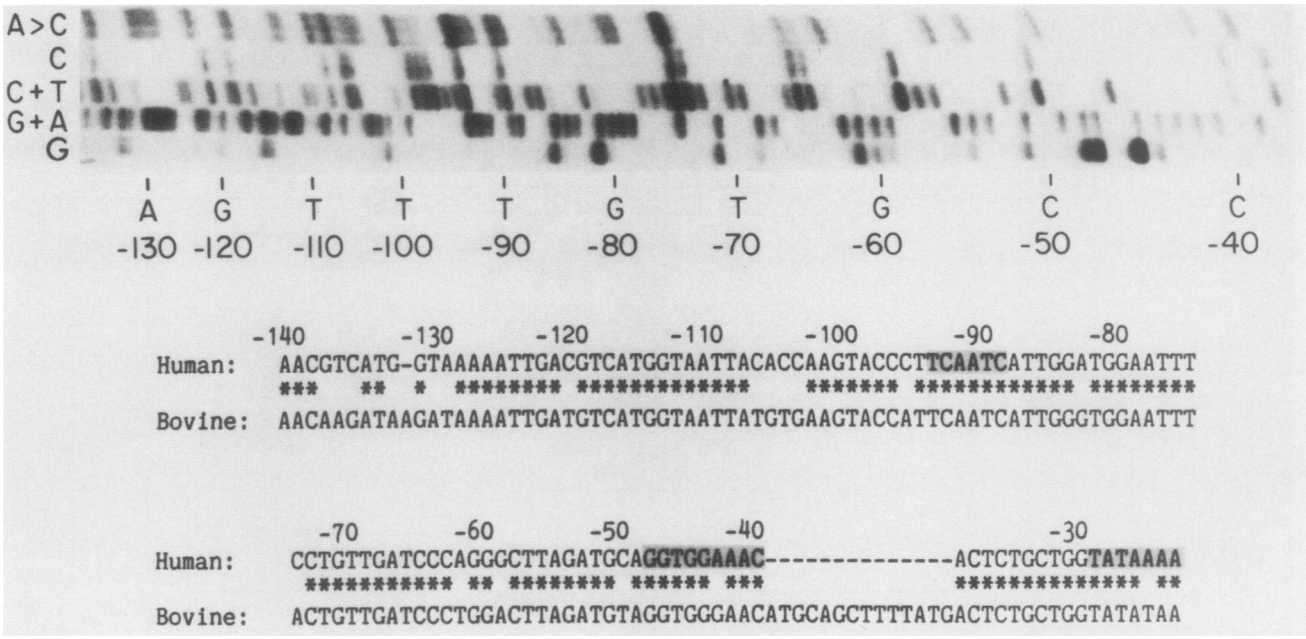


FIG. 5. 5′ Flanking sequences of the human and bovine α genes. In the top panel is an autoradiograph of a sequencing gel obtained by chemical sequencing of pΔ140 labeled at the *Hin*dIII site (see Materials and Methods). The position of the sequence in bp upstream from the cap site is indicated. Below, the human and bovine 5′ flanking α sequences are compared; nucleotide homologies are indicated by stars; numbers refer to bp upstream of the human cap site; shaded regions are (upstream to downstream) the CAAT box, an enhancer like sequence, and the TATA box.

expression from pSV2 as an internal standard for transfection efficiency in each cell type (39) the same genomic α sequences were expressed at least 10-fold more efficiently in JAr (eutopic) than in HeLa (ectopic) cells. Second, a qualitative difference was observed in the expression of pαCAT in each cell type. In HeLa cells, α transcription was induced by butyrate, whereas in JAr cells, α transcription was unaffected by butyrate but was induced by cAMP.

These results are consistent with previous observations on the expression of the endogenous α subunit. Basal levels of α protein synthesis have been shown to be consistently higher in eutopic cells than in ectopic cells (35, 36). Further, cell-specific responses of the endogenous α gene to cAMP and butyrate parallel to those obtained with transfected α sequences have been reported at the protein level in eutopic cells (Fig. 3) (7, 8, 21–23, 36) and ectopic cells (7, 10, 12, 23) and at the transcriptional level in ectopic cells (10). Thus, regulation of the transfected pαCAT constructs appears to be a valid reflection of regulation of the endogenous α gene.

Our results predict differential transcriptional control of the endogenous α gene in ectopic and eutopic cells. Previous work has demonstrated that the CAT activity transiently present in cells after plasmid transfection is an accurate reflection of mRNA production from that plasmid (16, 26, 39). Similarly, the CAT activity we have measured after pαCAT transfection is likely to represent transcription dependent on 5′ flanking α DNA. Thus, altered expression of pαCAT is consistent with differential transcriptional controls of the endogenous α gene in ectopic and eutopic cells. Further, since pαCAT expression was measured under conditions where the plasmid expression vector was extrachromosomal (20), our results suggest that *trans*-acting factors are involved. Finally, since differential expression of the α gene was observed from identical constructs transfected into either ectopic or eutopic cells, a different factor(s) may mediate α expression in each cell type.

Common features of α expression in ectopic and eutopic cells. We have observed that the expression or induction of transfected α sequences, in either ectopic or eutopic cells, is dependent on a single common fragment of α 5′ flanking genomic DNA. While plasmids containing 45 to 60 bp of α 5′ flanking DNA (pΔ45 and pΔ60) did not express CAT in either eutopic (Fig. 6) or ectopic (Fig. 7; data not shown) cells, constructs containing 140 bp (pΔ140) were expressed and induced by either butyrate or cAMP in the appropriate cell type (Fig. 6 and 7). Results identical to those with pΔ140 have been obtained in preliminary experiments with constructs containing only 100 bp (pΔ100; data not shown). Further, in neither cell type were we able to distinguish sequences necessary for basal α from those necessary for the induction of α expression. Thus, a single fragment of α DNA, 100 to 140 bp in length, is sufficient to allow both the basal and cell-specific induction of α expression in either ectopic or eutopic cells. Similar results have been reported when the CAT system was used to assay adenovirus E2 promoter function (24); sequences required for basal expression and E1A-mediated induction of E2 were essentially inseparable.

An intriguing feature of this 100- to 140-bp fragment of α DNA is that it contains a 9-bp DNA sequence homologous with the consensus viral enhancer sequence (27, 34, 40). Tissue-specific expression of a variety of genes, including immunoglobulins (1, 13, 33), chymotrypsin, and insulin (39), are thought to be dependent on the presence of an enhancer-like sequence. Similarly, induction of the metallothionein gene by glucocorticoids and cadmium (25), mouse mammary tumor virus transcription by glucocorticoids (5, 28), and adenovirus E2 expression by E1A (24) are thought to be

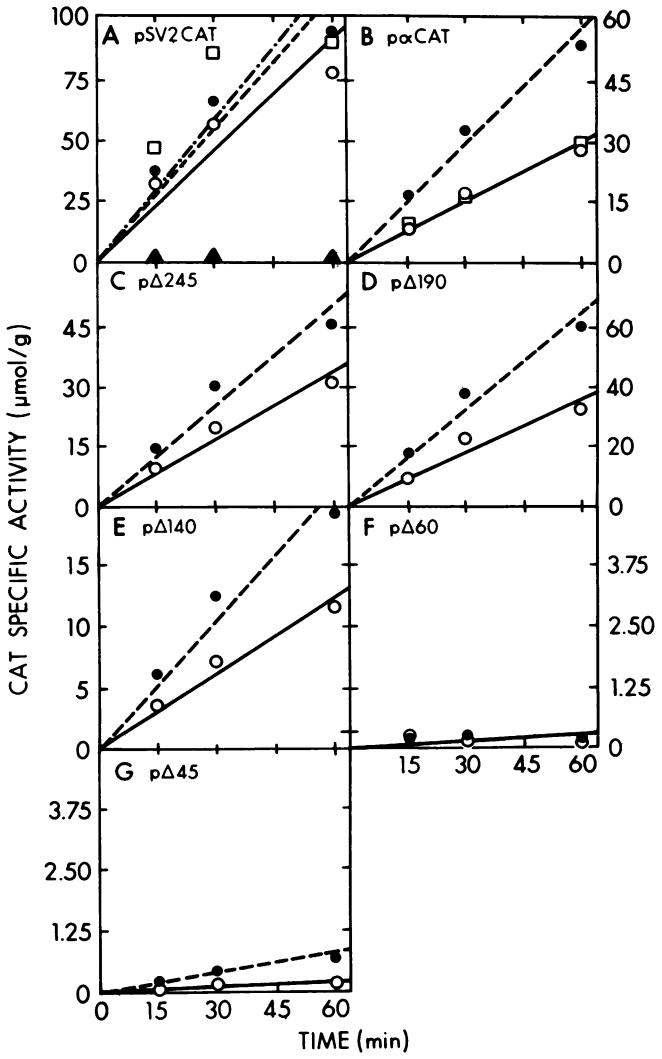


FIG. 6. CAT enzyme activity from cAMP induced and uninduced pΔCAT vectors in transfected JAr cells. Levels of CAT enzyme produced per microgram of protein in JAr cells transfected with pSV2 (A), pSV0 (A, ▲), pαCAT (B), pΔ245 (C), pΔ190 (D), pΔ140 (E), pΔ60 (F), and pΔ45 (G) are plotted as a function of assay time. Uncorrected counts per minute from thin-layer chromatographs were converted to units of molarity by using standard curves from reactions with purified CAT of known specific activity and normalized to protein content per assay. Cells treated with inducers were incubated with 1 mM dbcAMP or 5 mM butyrate for 12 h before extracts for CAT assays were prepared (see Materials and Methods). The results of pSV0, pSV2, and pαCAT transfections are quantitated in experiment 3 of Table 1. Symbols: ○, plasmid control; ●, plasmid plus 8-Br-cAMP; □, plasmid plus butyrate.

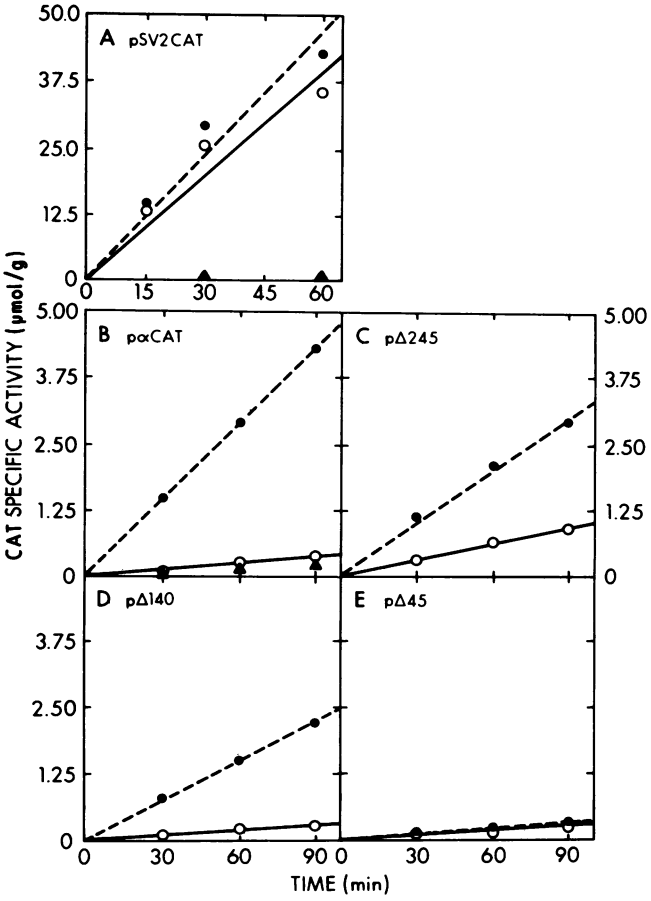


FIG. 7. CAT enzyme activity from butyrate induced and uninduced pΔCAT vectors in transfected HeLa cells. Levels of CAT enzyme produced in HeLa cells transfected with pSV2 (A), pSV0 (A and B ▲) pαCAT (B), pΔ245 (C), pΔ140 (D), and pΔ45 (E) per microgram of protein per assay are plotted as a function of time. Butyrate-treated cells were incubated in 5 mM butyrate for 12 h before extracts were prepared for CAT assays. The results of pSV0, pSV2, and pαCAT transfections are quantitated in experiment 3 of Table 2.

activated by sequences that contain either enhancer-like features or proven enhancers. An α enhancer-like sequence may similarly be involved in the cell-specific expression or induction of the α gene in ectopic and eutopic cells.

Some aspects of α gene regulation may be mediated by DNA sequences other than those we have identified. While previously identified DNA sequences involved in the transcriptional control of mammalian genes have been found within an area 200 to 300 bp upstream from the cap site, they have generally been located further upstream than those shown here (5, 18, 25, 28). In the bovine α gene (14), an 18-bp sequence between −165 and −182 is homologous to the progesterone receptor-binding site (9). In addition, several genes have been shown to contain control elements or potential control elements within their transcribed regions, including the immunoglobulin enhancer (1, 13, 33), and the β globin gene (6, 42). A family of RNA species preferentially expressed in tumor cells has been postulated to be coordinately regulated by a conserved sequence that is present within the mature RNA species (4).

Our results suggest that the α gene is differentially regulated in ectopic and eutopic cells. Basal expression of the α gene in both cell types, as well as its cell-specific induction, is dependent on the same 140 bp of 5′ flanking α genomic DNA. Thus the coordinate regulation of the α gene and its possible linkage to the tumorigenic phenotype in eutopic and ectopic cells may be mediated by related soluble transcription factors acting on a common sequence.

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