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MEMORY OF BUTYRATE INDUCTION BY THE MOLONEY MURINE SARCOMA VIRUS ENHANCER-PROMOTER ELEMENT

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SUMMARY: We reported previously that a housekeeping gene could be converted to a butyrate-inducible gene by replacing its cognate promoter with the Moloney murine sarcoma virus enhancer-promoter element. In this study we report that the activated transcriptional state could be propagated from mother to daughter cells after the withdrawal of the inducer.

Eukaryotic transcriptional signals include enhancers and promoters that determine distinct levels and patterns of transcription from specific genes. Emerging evidence indicates that the activity of enhancer-promoter elements are controlled by multiple *trans*-acting transcriptional factors that recognize and interact with such *cis*-acting DNA elements, and by other factors that interact with these DNA-binding proteins. The chromatin structure of specific domains that encompass the gene also determines the degree of transcription (1,2,3,4). Some enhancer-promoter elements are inducible by environmental stimuli, presumably through a cascade of events that translate an extracellular signal to the activation of transcription of specific genes. After withdrawing the inducer from the environment.

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transcription may either decline to its basal level or persist at the augmented level in the absence of the inducer. In one example, the dexamethasone-induced transcription of the mammary tumor virus enhancer-promoter was switched off after hormone withdrawal although the altered chromatin structure persisted (5,6). An insight into the propagation of gene regulation patterns is of fundamental importance to understanding cell commitment, differentiation, and gene regulation.

We have demonstrated previously (7,8) that the adenine phosphoribosyltransferase (*aprt*) gene could be converted from a housekeeping gene to an inducible gene when its cognate *aprt* promoter was replaced by the Moloney murine sarcoma virus (*MSV*) enhancer-promoter element. We demonstrate here that the butyrate-activated transcriptional state was imprinted in daughter cells for many generations after withdrawing butyrate from the culture medium.

MATERIALS AND METHODS

Plasmids-The plasmid pMVHA4-4 was constructed by fusing the *MSV* enhancer-promoter element to a promoterless hamster *aprt* gene (8). The plasmids pMVHA9-20 and pHaprt-9 were both pUC-19 based constructs containing the complete coding sequence of the hamster *aprt* gene driven by different promoters (8). The plasmids 18S rRNA and 28S rRNA were pGEM vectors containing the rat 18S and 28S ribosomal RNA genes, respectively (9).

Cell cultures-LAT cells (mouse L cells deficient in both adenine phosphoribosyltransferase and thymidine kinase) were grown in Dulbecco modified Eagle's medium (DMEM) containing 10% serum (equal amounts of fetal bovine serum and calf serum). LAT cell lines containing stable integrants of the recombinant *MSV-aprt* transgene were constructed by transfecting LAT cells with pMVHA4-4 as described (8), followed by the selection of APRT-positive cells from monoclones in AA medium (DMEM containing 0.05 mM azaserine, 0.1 mM adenine, and 10% dialyzed serum). For butyrate induction, sodium butyrate (*n*-butyric acid neutralized to pH 7.0 with NaOH) was added to a final concentration of 5 mM to the medium. Cells were incubated in butyrate-containing medium for 2 days, and washed three times with PBS before being returned to butyrate-free medium.

Nuclear run-on transcription assay-Cells were harvested at indicated time points by trypsinization. A fraction of cells was lysed for the determination of APRT enzyme activities as previously described (8). Remaining cells of the same population were lysed in a Dounce homogenizer at a concentration of 10^7 cells per ml in 10 mM Tris, pH 7.5/10 mM NaCl/3 mM $MgCl_2$/0.5% NP-40. Nuclei were

resuspended in 100 µl of 50 mM Tris, pH 8.0/40% glycerol/5 mM $MgCl_2$/0.1 mM EDTA, followed by mixing with an equal volume of 10 mM Tris, pH 8.0/5 mM $MgCl_2$/300 mM KCl/6 mM DTT/2 units per µl of RNasin/1.5 mM each of ATP, CTP, GTP/250 µCi [α-^{32}P]UTP. After the labeling reaction had proceeded for 40 minutes at 30° C, cold UTP was added to a final concentration of 0.1 mM, and the reaction proceeded for another 10 minutes. Total nuclear RNA was isolated by guanidinium thiocyanate-phenol extractions as described (10). ^{32}P-labeled RNA probe (10^6 cpm) was hybridized to denatured plasmid DNAs immobilized on nytran membranes in 6X SSPE/0.5% SDS/50 µg per ml denatured salmon sperm DNA at 68° C by standard protocols (11). The blot was washed once in 6X SSPE/0.5% SDS at room temperature, once in 6X SSPE/0.1% SDS at room temperature, twice in 1X SSPE/0.5% SDS at 37°C, and twice in 0.1X SSPE/0.5% SDS at 68°C. The dried blot was exposed to X-ray film at -80° C with an intensifying screen. Signals were quantitated by densitometry.

RESULTS

The butyrate-induced transcriptional state was propagated to daughter cells independent of the inducer

Figures 1 and 2 show that the butyrate-activated transcriptional state of the *MSV-aprt* transgene persisted for many generations

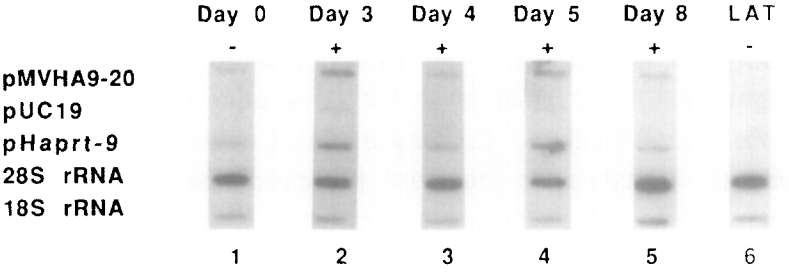

Figure 1. Nuclear run-on transcription assay of the *MSV-aprt* transgene. Cells derived from a stable clone produced by transfecting LAT cells with pMVHA4-4 were incubated in medium containing 5 mM butyrate for 2 days (Days 0-2), washed with PBS, and incubated in butyrate-free medium for another 6 days (Days 2-8). Nuclei were harvested for *in vitro* transcription reactions and ^{32}P-labeled mRNAs were hybridized to immobilized plasmid DNAs as described in text either before butyrate treatment on Day 0 (lane 1) or 1-6 days after butyrate withdrawal from the culture medium (lanes 2-5). Nontransfected parental LAT cells were included as a negative control (lane 6). The - sign indicates that cells were not treated by butyrate. The + sign indicates that cells were treated with butyrate prior to harvest.

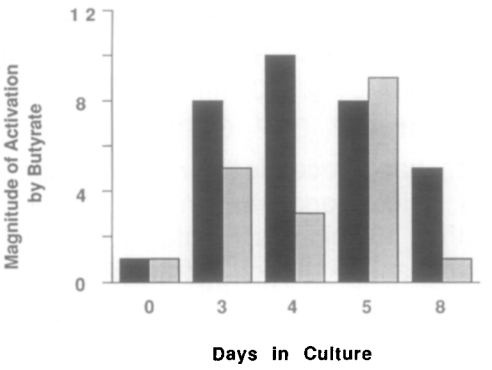

Figure 2. Magnitude of activation of the *MSV-aprt* transgene by butyrate treatment. The amount of ^{32}P-labeled mRNA hybridized to specific denatured plasmid DNA in Figure 1 was quantitated by densitometry. The amount of *aprt* signal on a per cell basis was first obtained by normalizing the signal to that of the ribosomal RNA extracted from the same population of cells at indicated time points. The magnitude of butyrate-induced transcription was then obtained by normalizing the fraction containing the induced signal to that containing the non-induced on Day 0. The magnitude of butyrate-induced APRT enzyme acitvities was obtained by normalizing enzyme activities in induced cells to the level of non-induced when equal amounts of proteins were assayed under identical conditions. The mean value from duplicate plates of cells was used for normalization. Multiple experiments have shown that the enzyme level was stable without butyrate treatment (7,8). Solid bar: the magnitude of activation of APRT enzyme levels by butyrate treatment. Stippled bar: the magnitude of activation of transcriptional rates of the *MSV-aprt* transgene by butyrate treatment.

after butyrate withdrawal. The rate fluctuated between 3- to 9-fold higher than the basal level 1-3 days after removing butyrate from the culture medium. On Day 8 (i.e., 6 days after butyrate withdrawal), transcription declined to approximately the same rate as that on Day 0 (i.e., before butyrate treatment). Transcriptional rates of the 18S and 28S ribosomal RNA genes were stable when run in parallel as reference genes. In parental LAT cells, the *aprt* signals were completely absent, even after prolonged exposure of the film, a clear indication that the *aprt* signals detected in transfectants were produced by the transgene which was absent in nontransfected parental cells. There were also trace amounts of pUC19 signals detected when the *aprt* signals were high, which may be due to some readthrough transcripts extended into flanking pUC19 vector sequences when the *MSV* promoter was actively transcribing. Hybridization of the labeled-mRNA to a separate blot yielded a nearly identical pattern (data not shown).

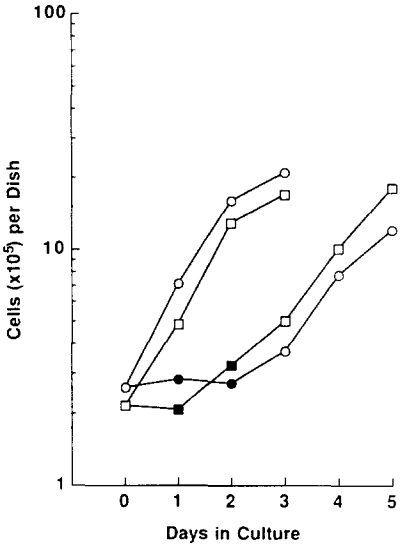

Figure 3. Butyrate-arrested cellular replication. Cells were seeded at a density of 2 X 10^5 cells per 6-cm plate. One group of cells were incubated in medium containing 5 mM butyrate for 2 days (Days 0-2), washed with PBS, and incubated in butyrate-free medium until harvest. Cells were counted daily before and after butyrate treatment. Cells of a control group without butyrate exposure were counted in parallel. Open circle: transfected cells containing the *MSV-aprt* transgene harvested in the absence of butyrate. Solid circle: transfected cells containing the transgene harvested in the presence of butyrate. Open square: nontransfected LAT cells harvested in the absence of butyrate. Solid square: nontransfected LAT cells harvested in the presence of butyrate. Each data point represents the mean value from duplicate plates.

Figure 2 shows that the APRT enzyme level was also augmented by butyrate treatment as previously shown (8), and the induced activity persisted after butyrate withdrawal.

Butyrate-arrested cellular replication was reversed immediately after butyrate withdrawal

To test whether the post-butyrate effect was due to a broad cellular effect, rather than a specific stimulation of the *MSV* enhancer-promoter element, we measured the rate of cellular replication before and after butyrate treatment. Figure 3 shows that cell growth was arrested by butyrate, and returned to normal almost immediately after butyrate withdrawal. The observation was consistent with previous findings that butyrate could arrest DNA synthesis and cell division (12, 13). There was apparently no "memory" for butyrate effect upon growth arrest in future generations after depleting butyrate from the culture medium.

DISCUSSION

By measuring the transcriptional rate of a recombinant *MSV-aprt* transgene in transfected LAT cells at different time points after butyrate treatment, we provide evidence that the active transcriptional state transiently imprinted upon the gene by butyrate could be extended into future generations in the absence of the inducer. Butyrate induction of transcription was specifically mediated via the *cis*-acting *MSV* enhancer-promoter element since substituting this element with a housekeeping promoter resulted in no response to butyrate (8). Butyrate produces a wide variety of effects on cells (14) including the modification of high-mobility-group proteins, arrest of DNA replication and cell growth, and the acetylation of histones via the inhibition of histone deacetylase. Although much has been learned about the action of butyrate upon cells, the molecular mechanisms that mediate "butyrate memory" remain to be elucidated. It is unlikely that "butyrate memory" is mediated by butyrate molecules remained intracellularly after butyrate withdrawal from the culture medium because there was no "memory" of the butyrate effect upon cellular replication in daughter cells when butyrate was no longer present in the culture medium, suggesting that there were no intracellular butyrate molecules after butyrate withdrawal, at least not sufficient to arrest cell divisions. Futhermore, the *MSV-aprt* transgene could be activated by butyrate only after the gene had entered the cell (8). Attempts to activate the transgene by treating cells with butyrate prior to transfection have all failed (data not shown).

The propagation of the pattern of gene expression from mother to daughter cells has been attributed to propagation of active transcription complexes to progenies during multiple rounds of cell division (15), and/or modified DNA or chromatin structures may gain the capacity to template their own structure independent of the initial inductive event (16). It is also possible that induced or modified transcription factors are inheritable. Although we provide no evidence as to how such a "memory" for transcriptional induction has been achieved, the studies present a model system which may be instrumental in providing new insights into gene regulatory mechanisms.

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