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Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate

Cornelia M.Gorman+ and Bruce H.Howard
Laboratory of Molecular Biology, National Cancer Institute, Division of Cancer Biology and Diagnosis, National Institutes of Health, Bethesda, MD 20205, and

Raymond Reeves
Program in Biochemistry and Biophysics, Washington State University, Pullman, WA 99164, USA

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ABSTRACT

We have studied the effects of sodium butyrate on DNA-mediated gene transfer in an effort to investigate interrelationships between chromatin structure and expression of recombinant plasmids. Our results demonstrate that butyrate affects the early stages of gene activity following DNA uptake at least two levels. First, the number of cells able to express foreign DNA increases from 10% to up to 40%. Second, there is an increase in enhancer-dependent transcription, approximately 30 fold in HeLa cells, involving the SV40 early promoter. Stable transformation efficiencies increase to 4% and 10% in HeLa S3 and monkey kidney CV-1 cells, respectively. Finally, expression of integrated recombinant plasmid genes is reinducible by a second treatment five weeks after initial exposure to this agent.

INTRODUCTION

DNA-mediated gene transfer has become an important technique for the study of mammalian gene regulation. Despite its widespread use, however, the steps that precede expression of exogenously introduced DNA remain poorly understood. Evidence from a number of laboratories indicates that prior to expression foreign DNA molecules are transported to the nucleus and incorporated into chromatin structures. Because relatively little is known, in particular, concerning the relationship between chromatin formation and subsequent gene function, we have investigated effects of an agent known to alter chromatin structure (1-10), sodium butyrate, on the expression of exogenous DNA.

Since cellular DNA and viral replication can be inhibited by butyrate (8,11) we chose nonreplicating recombinant plasmids (12) for our studies. The plasmids used allowed us to monitor: transient expression by chloramphenicol acetyltransferase (CAT) activity (13), transfection efficiency (the percentage of cells expressing the exogenous DNA) using fluorescent antibody to CAT (14), and stable transformation using selection for either the bacterial *E. coli* xanthine-guanine phosphoribosyltransferase (gpt) gene (15, 16) or the Tn5 aminoglycoside phosphotransferase (neo)

gene (17).

Our results show that butyrate treatment immediately following the uptake of plasmid DNA influences both transient expression and stable transformation. The magnitude of the butyrate-induced increase in CAT activity in primate cells seen as early as 12 hrs after pSV2cat transfection is partially dependent on the presence of SV40 enhancer sequences in this plasmid. A second transient effect of butyrate is an increase in the percentage of cells expressing plasmid DNA.

Early effects on plasmid expression frequently correlate with stable transformation efficiencies. We have found that butyrate causes a 5-10 fold increase in the number of colonies obtained following selection for the E. coli gpt marker in CV-1 cells or the neo marker in Hela and NIH/3T3 cells. In CV-1 cells stable transformation frequencies increase to as high as 10% and in Hela S3 cells to as high as 4%.

Finally, butyrate treatment immediately following DNA-mediated transfection can affect subsequent expression of integrated plasmid DNA containing a hybrid SV40 early promoter-CAT transcriptional unit. Whereas CAT activity in stable clones derived from cells that have never been exposed to butyrate is unaffected by butyrate treatment, CAT activity in stable clones derived from cells initially exposed to butyrate is frequently stimulated 4 to 7 fold by subsequent treatment.

MATERIALS AND METHODS

Plasmid preparation. Care is taken in preparing the plasmid DNA for transfection. It is important to use DNA free of chromosomal DNA and in an unnicked state. Therefore we routinely use plasmid DNA prepared by double banding in CsCl (18). The effect that the preparation of plasmid DNA has on transfection efficiency is described elsewhere (19).

Cell culture and transfection. Calcium phosphate (20) is used for transfection as described (13, 14). We have optimized these procedures to yield maximum transient expression as well as stable transformation (19). Standard selection conditions for the dominant selection using the E. coli gpt gene (16) were used in CV-1 cells and mouse NIH/3T3 cells. These experiments used either pSV2cat-SVgpt (Fig. 1), pSV2gpt or pRSVgpt (19). For dominant selection based on expression of the Tn5 neomycin resistance gene, G418 (Schering) was used at a concentration of 800 μg/ml (36% active) for NIH/3T3 cells or 1 mg/ml (42% active) for HeLa cells.

Either pSV2neo (17) or pRSVneo (19) was used for selection in NIH/3T3 and HeLa S3 cells.

Butyrate treatment. Butyric acid was neutralized in a fume hood with sodium hydroxide to give sodium butyrate as a stock solution of 500 mM. Cells were exposed to butyrate following glycerol shock (21) by adding this agent directly to the fresh medium. Different concentrations of butyrate were used depending on the cell type: CV-1 monkey kidney cells, 10 mM; NIH/3T3, 7 mM; HeLa S3, 5 mM; CHO, 2 mM. Treatment was for 12-14 hrs. Both the treated and control cells were then washed and either harvested for enzyme assay (13) or refed. AT 36 hrs cells were stained with fluorescent antibody for CAT (14) or split into selection (16, 17).

Transient expression. For enzyme assay of CAT activity 10 cm plates were seeded with 5 x 10^5 CV-1 cells and transfected with various CAT containing plasmids as described (13). CAT assays using C^{14} chloramphenicol (CM) are detailed elsewhere (13). For a time course of CAT expression cells were harvested at 12, 24, 36, 48 and 60 hrs after the addition of butyrate. For the data in Fig. 2, 10 µl of C^{14} CM with 10 µl cellular extract were used in the reactions which were run for 30 min. To compare CV-1 and L cells (Table 2), 5 µl of extract were used in a reaction with 5 µl C^{14} CM run for 15 min. CHO and HeLa S3 reactions contained 5 µl of C^{14} CM with 10 µl of cellular extract and were run for 15 min. For the comparison of pSV2cat and pSV1cat, assays contained 10 µl extract and were run for 25 min (CV-1 cells) or 50 µl extract run for 40 min (HeLa S3 cells). Note that butyrate is removed at the 12 hr time point. To determine the transfection efficiency during this transient period we stained cells with rhodamine labelled CAT antibody (14). To determine the percentage of cells expressing the pSV2cat-SVgpt plasmid, multiple random fields were located using a phase microscope. The number of cells per field were counted and compared with the number of fluorescent cells in the same field.

Analysis of stable transformants. Cells were split into the appropriate selective medium (16, 17). At two weeks plates were stained and the number of colonies present were counted. To further analyze the stable transformants, colonies of CV-1 cells transfected with pSV2cat-SVgpt were isolated from treated and untreated plates. Colonies were transferred into cloning medium containing xanthine, hypoxanthine and thymidine and expanded to three 150 mm flasks; two flasks of each clone were used for high molecular weight DNA preparations. Cells were lysed using sodium doedecyl sulfate

and proteinase K for one hour at 37° C. Following heat treatment for 10 min at 55° C cells were again incubated at 37° C for one hour. The lysates were extracted with phenol and chloroform-isoamyl alcohol followed by an extraction with chloroform-isoamyl alcohol. Following the addition of sodium acetate, high molecular weight DNA was spooled and dialyzed overnight against 50 mM Tris pH 7.9, 50 mM NaCl, 10 mM EDTA. To remove RNA each sample was treated with RNAse followed by a chloroform-isoamyl alcohol extraction. DNA was again spooled followed by dialysis.

DNA-DNA dot blots were performed for an estimate of integrated copy number. DNA concentrations were determined with the DABA reaction. 3 and 7 μg of DNA from each clone were spotted onto nitrocellulose. The DNA was treated with an equal volume of 0.1 N sodium hydroxide followed by one third volume of 0.5 M sodium phosphate (dibasic). The salt concentration of each sample was adjusted prior to spotting so that the final concentration of saline sodium citrate (SSC) was 6x. DNA was added to the nitrocellulose using a vacuum manifold. Standards were CV-1 monkey DNA with pSV2cat-SVgpt DNA added to mimic 0, 1, 10, 50, 100 copies per genome. Hybridization with nick-translated DNA probe (22) included 10% dextran sulfate-50% formamide to enhance the signal (23). $3x10^6$ cpm/ml were hybridized at 42°C for 16 hrs. Following several washes with 0.1xSSC to remove unhybridized counts the nitrocellulose was dried and exposed to X-Ray film with an intensifying screen for 3 days at -70°C.

To determine CAT activity in these stable transformants duplicate plates were seeded ($1x10^6$ cells/ 150 mm dish). After 24 hrs. one plate was treated with 10 mm butyrate. Treatment was for 24 hrs at which time treated and untreated cells were harvested and lysed in 200 μl of buffer for CAT assay, which was run at 37° C for two hrs.

RESULTS

Butyrate treatment: Effects on Morphology and Cell Growth. Following the addition of butyrate to tissue culture cells, changes in growth and morphology were observed as expected (24-26). Cells were visibly flattened, perhaps slightly swollen. These effects were greatest at the higher concentrations of butyrate used with CV-1 cells. At 36 hrs after the addition of butyrate (22 hrs after the butyrate had been removed) the cell densities in the treated flasks were 10% to 50% less, depending on the cell type, than that of the untreated flasks, presumably due to the change in growth pattern induced by butyrate.

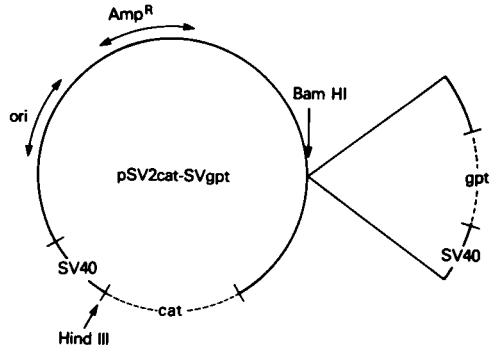

Fig. 1. Diagram of pSV2cat-SVgpt plasmid. Origin and ampicillin resistance gene of pBR322 are indicated. The SV40 early promoter is joined to the CAT gene at the Hind III site. The SVgpt transcriptional unit is inserted into the unique Bam site.

Transient Expression Following Butyrate Treatment. To understand how butyrate might be influencing transient expression, we first determined the number of cells expressing high enough levels of CAT to be positive when fluorescently stained for CAT activity. Transfection efficiencies using the plasmid pSV2cat-SVgpt (Fig. 1; 13) in CV-1 cells were monitored by fluorescent staining with rhodamine-labeled anti-CAT (14). Fig. 2 shows representative fields of cells from butyrate treated and untreated plates. The number of cells counted per plate and the percentage of cells which are fluorescent are shown in Table 1. In untreated plates we scored 10% of the CV-1 cells transiently expressing the CAT enzyme. By comparison, when butyrate treated plates were scored, we consistently observed an increase in the percentage of fluorescent cells (Fig. 2; Table 1), such that the percentage of flourescent cells ranged from 25%-40%. Since there were fewer cells present after butyrate treatment, the absolute number of fluorescent cells were 2 fold higher in the butyrate treated plates. This two fold increase was seen with pSV2cat-SVgpt and pSV2cat, as well as with pRSVcat, which uses the Rous sarcoma virus (RSV) long terminal repeat (LTR) as a promoter (14).

Fig. 3 shows time courses of CAT activity levels in treated and untreated plates transfected with pRSVcat and pSV2cat. The time course of pRSVcat expression shows a two fold increase in CAT activity in response to butyrate treatment. This is consistent with the increase in the number of cells expressing CAT. The enzyme levels in the treated plates increase

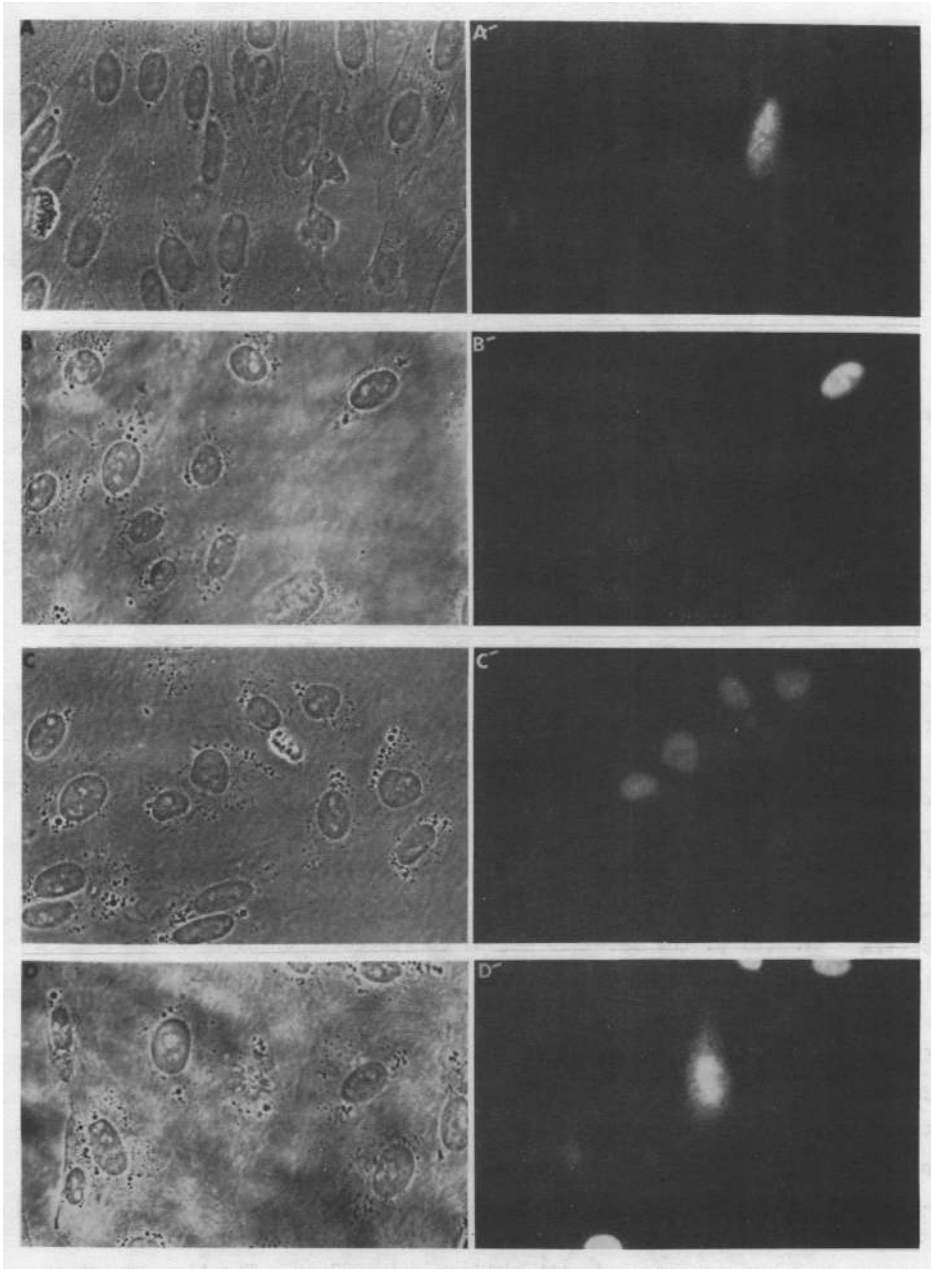

Fig. 2. Immunofluorescence of CAT positive cells 36 hrs after transfection. The number of fluorescent cells in the untreated plates (A, A', B, B') are compared with butyrate treated plates (C, C', D, D'). Cells are shown in phase contrast (A, B, C, D) and after fluorescent staining (A', B', C', D').

Table 1. Butyrate effect on transfection of CV-1 cells with pSV2cat-SVgpt. The number of cells counted/35 mm dish is shown along with the number of fluorescent cells/35 mm dish. Data are shown for butyrate treated and untreated plates.

	# of cells counted 35 mm dish	# of fluorescent cells 35 mm dish
+ BUTYRATE	202	72
	120	49
	177	44
	157	49
	134	25
	average % of positive cells	32
- BUTYRATE	272	30
	273	34
	170	18
	118	14
	134	15
	average % of positive cells	11

until 36 hrs (Fig. 3) and then decline slightly by 60 hrs (data not shown). In contrast to the results obtained with pRSVcat we observed that in plates transfected with pSV2cat and treated with butyrate the level of CAT activity is 10–12 fold higher than in untreated plates transfected with this plasmid.

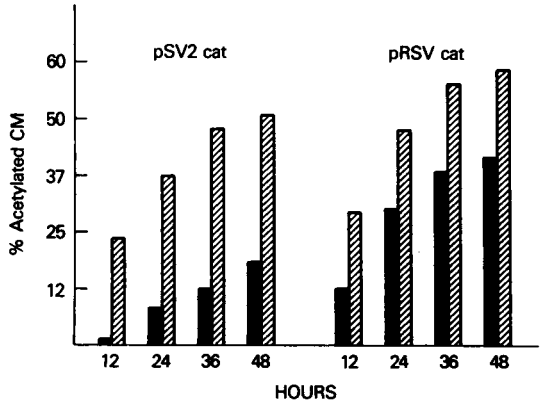

Fig. 3. Graph comparing transient expression levels of CAT in untreated cells (solid bars) with expression in butyrate treated cells (hatched bars). The expression of CAT from the pSV2cat and pRSVcat plasmids is compared at 12 hr intervals following the addition of butyrate. Note butyrate is removed at the 12 hr time point.

Table 2. Transient expression levels of CAT activity shown as % chloramphenicol (CM) acetylated. Levels of CAT in CV-1, L cells, CHO and HeLa S3 are compared using the plasmids $pSRM_2cat$, pRSVcat and pSV2cat. Values shown are the average of three experiments. Cells were harvested at 12 hrs immediately following butyrate treatment. Conditions for assays in each cell type are given in Experimental Procedures.

cell type	plasmid	% acetylated CM -butyrate	% acetylated CM +butyrate	relative increase
L cells	$pSRM_2cat$	16	48	3x
	pRSVcat	6	38	6x
	pSV2cat	9	36	4x
CV-1	$pSRM_2cat$	0.8	3	4x
	pRSVcat	22	39	2x
	pSV2cat	2	21	10x
CHO	$pSRM_2cat$	8	16	2x
	pRSVcat	4	12	3x
	pSV2cat	17	46	3x
HeLa S3	$pSRM_2cat$	0.3	4	13x
	pRSVcat	8	98	12x
	pSV2cat	1.5	66	44x

The use of butyrate raises the levels of CAT activity from pSV2cat (SV40 early promoter) to levels similar to those seen with pRSVcat (Rous LTR) (Fig. 3). The largest difference in CAT activity is observed at the 12 hr time point. In part this could reflect the fact that butyrate treatment is reversible. However, a direct effect of butyrate on transcription cannot be excluded.

To investigate whether the relatively large increase in CAT activity observed with pSV2cat in CV-1 cells following butyrate treatment might be associated with specific plasmid-host cell interaction(s), we tested pSV2cat, pRSVcat and a third plasmid $pSRM_2cat$ in other cell types (Table 2). $pSRM_2cat$ is identical to pSV2cat except that the 72 bp repeated enhancer sequence of SV40 (28, 29) has been replaced by the Moloney sarcoma virus enhancer sequence (30, 31). In CHO and L cells all three plasmids showed similar increases in levels of CAT activity (2-6 fold) following butyrate treatment (Table 2). In CV-1 cells transfected with $pSRM_2cat$ or pRSVcat DNA, CAT activity increased 2-3 fold following the addition of butyrate; as before monkey kidney cells transfected with pSV2cat showed a 10-12 fold increase in CAT levels (Table 2). It is noteworthy that butyrate

Table 3. For comparison of the effect of butyrate on the SV40 enhancer, the plasmid pSV2cat was used for the enhancer "+" DNA and pSV1cat for the enhancer "-" DNA. The data are presented as % of chloramphenicol acetylated by cellular extracts following transfection with these plasmids. The butyrate effect shows the increase in enzyme levels obtained with enhancer "+" and enhancer "-" DNA following butyrate treatment. The enhancer effect shows the difference in enzyme levels between the pSV2cat and pSV1cat in the presence and absence of butyrate.

CV-1 Cells	butyrate		butyrate effect
DNA	-	+	
enhancer			
-	0.8	2.5	3X
+	6	68	12X
enhancer effect	7X	23X	
Hela Cells			
	butyrate		butyrate effect
DNA	-	+	
enhancer			
-	0.2	0.5	2.5X
+	2	61	30X
enhancer effect	10X	120X	

treatment did not override, but rather increased, the host specificity demonstrated for the SV40 virus enhancer in CV-1 cells (31, 32). We also tested these plasmids in human cells, which are "semi-permissive" for SV40. Butyrate had a larger overall effect on the expression of all three plasmids in Hela cells, increasing the levels of CAT over 10 fold with both pRSVcat and $pSRM_2cat$. However, again there appeared to be a specific host effect, since pSV2cat expression in these cells was increased 40 fold (Table 2). These data suggest that butyrate has an added effect on the SV40 early region promoter particularly in permissive (CV-1) and "semi-permissive" (Hela) cells.

To test whether this effect is dependent on the SV40 enhancer region we transfected cells with either pSV2cat, containing this enhancer sequence, or with pSV1cat, which is lacking the enhancer region but has readily detectable levels of transcription initiating from the core early region promoter (13). Table 3 shows the increased effect of butyrate on plasmids containing the SV40 enhancer expressed in CV-1 and Hela cells. In the

Table 4. Effect of butyrate treatment on stable transformation of CV-1 cells. $5x10^5$ cells were transfected with 5 μg form I plasmid DNA (pSV2cat-SVgpt), treated with 10 mM butyrate for 12 hrs or left untreated, and replated into mycophenolic acid-xanthine-HAT selective medium at 36 hrs.

	transfection efficiencies/100 mm dish $5x10^4$ cells plated	
EXPERIMENT	+ butyrate	- butyrate
1	$1x10^{-1}$	$9x10^{-3}$
2	$1x10^{-1}$	$9x10^{-3}$
	transfection efficiencies/100 mm dish $1x10^4$ cells plated	
1	$1.5x10^{-1}$	$8x10^{-3}$
2	$1x10^{-1}$	$7x10^{-3}$

absence of an enhancer (pSV1cat) the stimulation of CAT activity following butyrate treatment is 2-3 fold, perhaps reflecting the increase in the number of cells expressing the plasmid DNA. In the presence of the SV40 enhancer sequence the butyrate effect is 12 and 30 fold, respectively, for CV-1 and Hela cells. In these experiments with conventional non-butyrate conditions, the increase in enzyme levels due to the enhancer is approximately 7-10 fold in both cell types. In CV-1 cells butyrate appears to facilitate the function of the SV40 enhancer sequence yielding an increase in expression of 25 fold. A parallel result is seen in Hela cells where the increase is more than 100 fold in the presence of both the SV40 enhancer and butyrate treatment. These data indicate that the butyrate effect is partially enhancer dependent and that the magnitude of the increase in CAT activity involves plasmid-host cell interactions. We wish to emphasize that these results deal with the function of enhancer sequences as an integral part of the SV40 early region promoter. The effect of butyrate on these sequences when the enhancer is placed in artificial positions may be more complex (unpublished results).

Effects of Butyrate on Stable Transformation Frequency. In Table 4 we show that butyrate treatment increases stable transformation of CV-1 cells transfected with pSV2cat-SVgpt. The same results have been achieved with pSV2gpt. This increase is approximately 10 fold and results in transfection efficiencies of approximately 10%. A discussion of the high stable transformation efficiencies obtainable in CV-1 and other primate cells is presented elsewhere (19). In some stable transformation experiments we used the vector pRSVgpt (19), which uses the RSV LTR to direct the bacterial gpt gene. Interestingly, in CV-1 experiments where this vector was used,

Table 5. Effect of butyrate on stable transformation in NIH/3T3 and Hela cells. 5 µg form I plasmid DNA was used to transfect $5x10^5$ cells. Cells were treated with butyrate for 12 hrs or left untreated and replated in selective media at 36 hrs. To determine the above transfection efficiencies NIH/3T3 cells were plated at $5x10^4$/10 cm plate and Hela S3 cells were plated at $1x10^4$/10 cm plate.

cell type	DNA	selection	+butyrate	-butyrate
NIH/3T3	pSV2gpt	MX-HAT	$1x10^{-3}$	$2x10^{-4}$
	pSV2neo	G418	$3x10^{-3}$	$5x10^{-4}$
Hela S3	pRSVneo	G418	$4x10^{-2}$	$5x10^{-3}$

we saw only a small increase in the number of colonies in the treated plates over untreated plates (data not shown). This small increase suggests that with the Rous LTR we may have reached gpt expression levels optimum for establishment of stable colonies of CV-1 cells without the aid of butyrate. Table 5 shows the increases in transformation efficiency when NIH/3T3 and Hela cells are exposed to butyrate using either the gpt or neo marker. With butyrate treatment, 4% of the Hela S3 cells transfected form stable colonies. Tables 4 and 5 illustrate the fact that the butyrate effect on stable transformation may vary with cell type. This is not surprising since butyrate responsiveness also varies with cell type (27).

CV-1 colonies selected for gpt expression (16) were picked from treated and untreated plates and amplified in selective media. It was observed that butyrate treated colonies were easier to subclone and grew more rapidly than untreated colonies. The number of copies of integrated plasmid was determined by DNA-DNA dot blots for each of 11 clones. In these clones the integrated plasmid copy number of pSV2cat-SVgpt averaged 10. Within the limits of this analysis, we could not detect a significant difference in the number of integrated plasmids in the treated and untreated clones.

CAT Activity in Stable Transformants. To determine whether butyrate treatment affects subsequent expression of integrated plasmid copies, the following experiment was performed. Duplicate flasks of CV-1 cells were transfected with pSV2cat-SVgpt and were either treated for 12 hrs with butyrate or left untreated. Following selection for gpt expression, clones were picked and grown for 5 weeks in the absence of butyrate. Four clones which had never been exposed to butyrate and 7 clones which had received the initial butyrate treatment 5 weeks earlier were analyzed. Two plates of each clone were prepared for CAT assay. One plate was harvested 48 hrs

Table 6. CAT activity in pSV2cat-SVgpt clones selected with MX-HAT. Percentages of chloramphenicol acetylated (AC-CM) by extracts from untreated or treated butyrate clones are shown in the center column. Levels of CAT activity following a secondary butyrate treatment of the gpt selected clones are given in the third column.

Initial transfection conditions	Clone	% AC-CM	% AC-CM with subsequent butyrate treatment
-Butyrate			
	2	1	1
	3	2	2
	4	1	1
	7	1.5	1.5
+Butyrate			
	2	4	24
	3	2	2
	4	8	36
	5	3.5	21
	7	3	18
	9	4	30
	10	1.5	1.5

after plating. The other plate was incubated for 24 hrs and then treated with butyrate for 24 hrs prior to harvesting. Low levels of CAT activity were present in the clones which were initially untreated, and these levels of CAT activity did not change with subsequent butyrate treatment (Table 6). The levels of CAT activity in the clones initially treated with butyrate were generally higher than in the untreated clones, though there was some variation. The butyrate clones were responsive to a secondary butyrate treatment, showing an increase of 4-7 fold in CAT activity in 5 to 7 clones examined (Table 6). These data suggest that, in addition to the effect butyrate has on transient expression, this agent may have a long lasting effect on gene activity. This effect on the expression of integrated plasmids could result from changes in the chromatin structure of either the cellular locus of integration or of the plasmid itself.

DISCUSSION

We have studied how an agent known to influence gene expression by modifying cellular chromatin may affect the expression of exogenous genes. To do so we monitored the effects of sodium butyrate on DNA-mediated gene transfer using recombinant plasmids which allow for the study of transient

as well as stable expression levels of CAT, the number of cells expressing exogenous DNA, and stable transformation efficiency. The plasmid pSV2cat-SVgpt (Fig. 1) was particularly useful in this regard.

Transient Expression: Increase in Percentage of Cells Expressing Plasmid DNA. We have seen a 3-4 fold increase in the percentage of CV-1 cells expressing the CAT plasmids following butyrate treatment (Fig. 2; Table 1). For two reasons we think it unlikely that butyrate is affecting the initial step(s) of DNA uptake from the medium. First, butyrate treatment follows the removal of the DNA-calcium phosphate co-precipitate as well as two washes and glycerol shock (21). Second, Loyter et al. (33, 34) have demonstrated that virtually all the cells of a monolayer population can take up detectable amounts of the co-precipitate; thus the initial uptake is probably not a limiting factor in transfection-competent cell types such as those used in these experiments. Butyrate can change the cell cycle (27) and this change may be affecting the capacity of the cells to transport the DNA to the nucleus, thereby increasing the number of cells in a population which can express exogenous DNA. Alternatively butyrate may be increasing the number of cells in the population which are highly transcriptionally active (4-7).

There are several mechanisms by which butyrate may facilitate the packaging of plasmid DNA into chromatin, allowing for more efficient transcription, and thus increase the number of cells expressing detectable levels of CAT. Mertz (35) and Harland et al. (36) have shown that one important variable for proper transcription is the conformation of the plasmid DNA. Butyrate may be affecting the plasmid conformation by influencing various levels of chromatin structure within the plasmid. Butyrate has been shown to uncouple histone synthesis from DNA replication, potentially increasing cellular histone levels (37). Additionally butyrate could facilitate formation of active chromatin in either of two ways. First, newly modified histones could be assembled more rapidly into chromatin (38). Secondly, histones in newly forming chromatin could be preferentially modified (39) resulting in a chromatin structure which is more accessible for transcription.

Not only is the formation of nucleosomal complexes important for proper transcription (35), but often other protein-DNA interactions are necessary for efficient RNA synthesis (40). Perry and Chalkley (41) suggest that butyrate induced modifications allow for conformational changes in chromatin which increase the accessibility of regulatory proteins, examples of which

could be RNA polymerases or other proteins that may bind to the enhancer regions (31). There is precedent for highly active regions of viral as well as cellular chromatin to be associated with both modified histones and non-histone chromosomal proteins (10, 42-44).

Transient Expression: Enhancer Dependent Effects. We discuss evidence above that indeed butyrate may be affecting the transcriptional levels of plasmid DNA by acting on chromosomal proteins. In our experimental system the effect butyrate has on gene activity is partially enhancer dependent, and, in particular, the magnitude of the increase seen with butyrate treatment relates to specific plasmid-host cell interactions. The greatest butyrate effects are seen with the intact SV40 early promoter in monkey (permissive) and human ("semi-permissive") cells. Large increases are also observed with pRSVcat and $pSRM_2cat$ plasmids in Hela cells, which are very responsive to butyrate (8, 25-27). The latter increases may reflect weaker enhancer function in the retroviral components of pRSVcat and $pSRM_2cat$.

The effects of butyrate on SV40 enhancer function are particularly interesting because work done with SV40 minichromosomes has shown an extended SV40 origin region, including the 72 bp repeated enhancer region (28, 29) to have a nucleosomal phasing pattern that differs from the remainder of the viral genome (45, 46). This region, and the analogous region in polyoma, is also hypersensitive to DNase I (47-49), although only a proportion of the viral population exists in this sensitive state at one time (45, 49). Host range and specificity of some polyoma mutants have been correlated with changes in the levels of histone hyperacetylation in these regions (42, 44, 49). These results suggest that changes in the chromatin structure within this area, parallel to those induced by butyrate, facilitate viral expression.

Our data support the idea that butyrate may be influencing the transcriptional efficiency of plasmid DNA by hyperacetylation, leading to an "opening" of chromatin in a critical region such as that spanning an enhancer and adjacent promoter. This effect seems especially important in papovaviruses. Our strongest evidence is the large increase in expression seen with the SV40 enhancer in permissive and "semi-permissive" cells. An analogous result has been seen with the BK virus enhancer sequence in CV-1 and Hela cells (C. Gorman, N. Rosenthal, G. Khoury and B. Howard, unpublished results).

Stable Transformation. Though the data for transient and stable expression are presented separately, they are not independent events. We have shown

that transient levels of expression of dominant selectable markers correlate well with the final success of establishing stable transformants (14, 19). This correlation suggests that the increased levels of plasmid gene product stimulated by butyrate during the transient period could be directly increasing the number of cells able to survive the initial stage of selection. Alternatively, butyrate could have an added effect by establishing a more active chromatin structure in the enhancer region of the plasmid, which in turn allows for continued expression of the selectable gene. One suggested mechanism for how enhancer sequences can influence the number of stable transformants is by changing chromatin structure at the site of integration, providing an open region for transcription initiation (50). Butyrate could be increasing the efficiency with which these enhancer sequences, upon integration, create new actively transcribing regions of chromatin.

It is also possible that butyrate may directly change the transcriptional activity of the cellular DNA at the locus of integration. Most data suggest that exogenous DNA is randomly integrated into the cellular chromatin (51), and the low efficiency of stable clones compared with the number of cells which take up DNA suggests one limitation may be the failure to integrate within an active region of the chromosome (52, 53). Butyrate has been shown to cause stable changes in chromatin associated with increased transcription (7, 54). In CHO cells which were thymidine kinase deficient (tk-), butyrate treatment induced expression of the thymidine kinase gene leading to a high percentage of tk+ revertants capable of growing in selective medium (7). Potentially this reversion could be due to a change in chromatin structure leading to an increase in transcription of the tk gene. Thus while we did not detect more copies of the integrated pSV2cat-SVgpt plasmid, in the butyrate treated cells integration may occur more often in active regions of chromatin, whose structure may then be maintained to allow for continued transcription of the selectable marker. In effect fewer cells may "lose" the selectable gene by integration into inactive domains of the host chomosome.

CAT Expression in Stable Clones. Five of seven clones isolated from butyrate treated cells showed an increase in the levels of CAT activity when reexposed to butyrate. The retention of responsiveness to butyrate suggests a stable change in the chromatin structure in these clones or, alternatively, a preference for integration of these plasmids within butyrate responsive regions of the host chromosome. Results from certain hormone activated systems may be relevant to the first suggestion, that the plasmid

chromatin has been modified in such a way that it can remain responsive to butyrate after integration. In some steroid responsive systems a primary event is the rapid acetylation of histones (55). Although this acetylation ms transient, the increase in transcription remains, as though a stable conformational change induced by the modification of histones spreads over a region of the chromosome (55). Perry and Chalkley (41) have shown that the effect of butyrate on histone acetylation is similar in that it affects contiguous nucleosomes in an entire region of chromatin. With our butyrate treatment we may be mimicking hormone activity by eliciting a reinducible conformational change in chromatin. Additional experiments from Yamamoto and co-workers suggest that with mammary tumor virus (MTV) both the specific promoter sequence and the locus of integration are important for hormone responsiveness. In one study (56) two clones, each of which contained a single integrated virus copy, were found to have very different expression patterns: neither clone produced MTV specific RNA in the absence of hormone; however, one clone was found to be inducible upon the addition of hormone while the other clone seemed to be permanently inactive. From DNase I sensitivity studies Feinstein et al. (56) concluded that position of the MTV clones within the chromosome determined whether these genes could remain responsive to hormones. In a later study Ucker et al. (57) further analyzed two inducible clones. In this comparison these workers concluded the initial responsiveness of a gene, as well as its integrated position within the chromosome, modulate expression. By analogy we suggest it is possible that, following butyrate treatment, a relaxation of some regions of chromosome occurs and that plasmids, preferentially integrated into these regions, acquire responsiveness to butyrate. The effect butyrate has on mammalian chromosome is not uniform; there are regions which are responsive to butyrate while others are not. The variation in responsiveness in CAT levels seen in the stable clones could be illustrative of this phenomenon.

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+Present Address: Eukaryotic Molecular Biology Group, Cancer Research Campaign, Biochemistry, Imperial College of Science and Technology, London SW7 2AZ, UK

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