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Transcriptional Activation of the Adenine Phosphoribosyltransferase Promoter by an Upstream Butyrate-Induced Moloney Murine Sarcoma Virus Enhancer-Promoter Element

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It has been documented that the activity of a specific promoter can be occluded by the presence of another promoter element upstream. We present evidence for a phenomenon contradictory to that predicted by the promoter occlusion theory. Transcription from the hamster *aprt* (adenine phosphoribosyltransferase) promoter was augmented instead of repressed in transfected mouse L cells when an upstream Moloney murine sarcoma virus enhancer-promoter element was induced with butyrate. Without an adjacent Moloney murine sarcoma virus element, butyrate could not activate the *aprt* promoter.

A fundamental problem in biology is to understand the mechanisms by which genes are expressed. *cis*-Acting DNA sequences required for gene regulation can be operationally separated into promoters and enhancers. Promoters are essential for the initiation of transcription and are usually located immediately upstream from the RNA start site. Enhancers can stimulate the rate of transcription from promoters in a relatively distance- and orientation-independent manner. Both promoters and enhancers are composed of arrays of sequence motifs that bind transcription factors which may determine distinct levels of transcription from promoters (7, 16, 20).

Promoter activities of eucaryotic genes may also be modulated by their sequence context. Deletion of the promoter in the 5′ long terminal repeat (LTR) of the avian leukosis retrovirus activates its 3′ LTR promoter (4). Similar transcriptional interference occurs between duplicated alpha-globin gene constructions (19). The phenomenon of promoter occlusion is likely to be caused by an inhibition of transcriptional initiation from a downstream promoter as readthrough transcripts destabilize initiation complexes within the downstream promoter region. Such transcriptional interference may have a profound influence on the activity of cellular promoters arranged in tandem or on the expression of cellular genes which are located downstream from an integrated retrovirus.

We have conducted experiments to study the interaction between a retrovirus promoter and an adjacent housekeeping promoter. We report here that transcriptional initiation from a downstream *aprt* (adenine phosphoribosyltransferase) promoter was augmented, rather than repressed, by an upstream Moloney murine sarcoma virus (MSV) enhancer-promoter when the MSV enhancer was induced with butyrate (9), a chemical known to produce a variety of effects on cells including increased transcription of certain genes (12). In agreement with the findings of Emerman and Temin (8), who showed an epigenetic gene suppression phenomenon by infecting cells with retroviral vectors containing adjacent cistrons, we have shown by transfecting cells with plasmid constructs containing adjacent promoters that promoter occlusion may not be a general principle.

MATERIALS AND METHODS

Plasmids. Plasmids were prepared by banding in two cycles of CsCl density gradients after alkaline lysis (15). Figure 1 shows the architecture of the plasmids constructed for this study. The plasmid pHaprt-7 was constructed by cloning the 3.9-kilobase (kb) *Bam*HI fragment of pHaprt-1 (14) into the *Bam*HI site of pUC19. pHaprt-9 was constructed by deleting the 1.2-kb *Xba*I fragment (from the internal *Xba*I site to the polylinker *Xba*I site) from pHaprt-7. pHaprt-4 was constructed by cloning the 3.6-kb *Tha*I-*Hin*dIII fragment of pHaprt-1 into the *Sma*I-*Hin*dIII site of pUC19, thus deleting the 5′ noncoding region upstream of the *Tha*I site (Fig. 2D). pM-LTR4 was constructed by cloning the *Eco*RI-*Sma*I fragment of pm1sp (25), which contains the Moloney murine sarcoma virus (MSV) enhancer-promoter, into the *Eco*RI-*Sma*I site of pUC19. pM-LTR5 was constructed by cleaving pM-LTR4 with *Xba*I (thus deleting the fragment extending from the MSV *Xba*I site to the polylinker *Xba*I site), followed by the attachment of *Bam*HI linkers after *Xba*I ends were filled in. pMVHA4-4 was constructed by cloning the 3.6-kb *Tha*I-*Hin*dIII fragment containing the promoterless *aprt* gene into the *Sma*I-*Hin*dIII site of pM-LTR4. pMVHA9-20 was constructed by cloning the 2.5-kb *Xba*I fragment of pMVHA4-4 into the *Xba*I site of pUC19. pMVHA4-7 and pMVHA4-7R were constructed by cloning the 3.9-kb *Bam*HI fragment containing the full-length *aprt* gene into the BamHI site of pM-LTR4. pMVHA5-7 and pMVHA5-7R were constructed by cloning the same fragment containing the full-length *aprt* gene into the *Bam*HI site of pM-LTR5. pHATZ8-18U was constructed by cloning a *Hin*dIII-*Pvu*II fragment, which contains the *Bam*HI-*Pvu*II fragment of the *aprt* gene (Fig. 2D), into the *Hin*dIII-*Sma*I site of pTZ18U.

Cell cultures. LAT cells (mouse L cells deficient in both APRT and thymidine kinase) were grown in Dulbecco modified Eagle medium containing 10% serum (equal amounts of fetal bovine serum and calf 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. Long-term clonal transfectants were produced by

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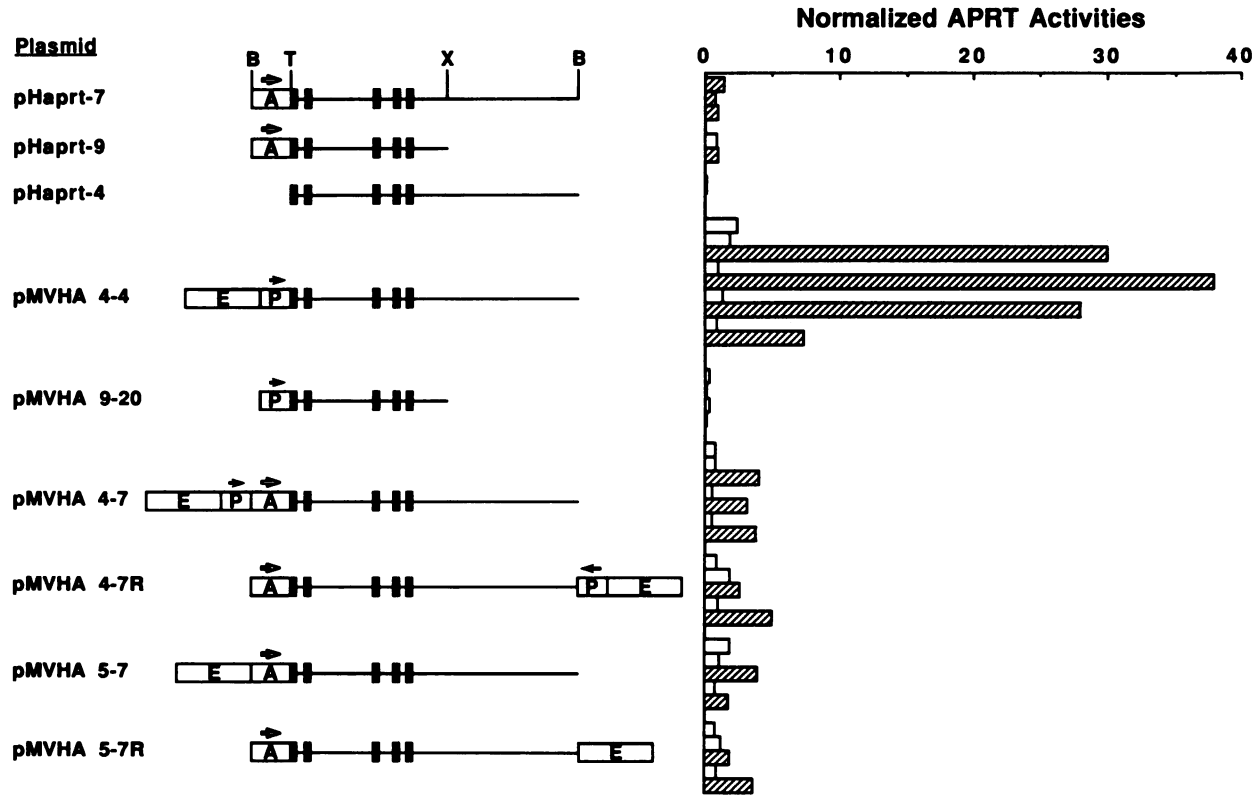

FIG. 1. Effect of MSV transcription signals on expression of an adjacent *aprt* gene. Transient APRT activities produced by each plasmid were normalized to those produced by pHaprt-7 in the same transfection experiment. Each bar represents the value obtained from a single dish of cells. LAT cells were incubated in normal medium (□) and medium containing 5 mM butyrate (▨) for 2 days after transfection. Boxes: A, 5′ noncoding region of the *aprt* gene containing the *aprt* promoter (open arrows show the direction of transcription of the *aprt* promoter); P, MSV *Xba*I-*Sma*I fragment containing promoter elements (5, 10) (solid arrows show the direction of transcription of the MSV promoter); E, *Eco*RI-*Xba*I fragment containing the MSV enhancer repeats (5, 10, 13, 25); solid, *aprt* exons. The remaining unlabeled sequences are introns or 3′ noncoding regions of the *aprt* gene. B, *Bam*HI; X, *Xba*I; T, *Tha*I.

transfecting LAT cells with the plasmid without any carrier DNA (24). APRT-positive cells were selected in AA medium (Dulbecco modified Eagle medium containing 0.05 mM azaserine, 0.1 mM adenine, and 10% dialyzed serum). Transient transfectants were produced by DEAE-dextran as previously described (22), except that the concentration of DEAE-dextran (molecular weight = 5×10^5; Pharmacia) was reduced to 250 μg/ml. A suboptimal concentration of DNA (e.g., 0.5 μg/6-cm dish) was transfected into cells for transient expression, and cells were harvested 2 days after transfection.

APRT enzyme assays. Lyophilized cells were suspended in buffer A (50 mM Tris [pH 7.5], 30 mM KCl, 10 mM $MgCl_2$). The same amount of protein from different cell extracts, under linear assay conditions, was added to an equal volume of the APRT reaction buffer (100 mM Tris [pH 7.5], 2 mg of bovine serum albumin per ml, 16 mM $MgCl_2$, 1.5 mM 5-phosphorylribose 1-pyrophosphate, 25 μM adenine, 50 μCi of [^{3}H]adenine per ml) at 37°C. The enzyme activity was terminated by the addition of the stop buffer (50 mM sodium acetate, 2 mM sodium phosphate [monobasic], pH 5.0). Nucleotides were precipitated by $LaCl_3$ and collected on Whatman glass fiber GF/C filters. Enzyme activities were quantitated by scintillation counting.

RNase mapping. The scheme for RNase mapping is outlined in Fig. 2A. The 5′ region of the hamster *aprt* gene, including the promoter and part of exon 1, was transcribed into a 413-nucleotide (nt) antisense RNA by T7 RNA polymerase in vitro. ^{32}P-labeled RNA probe (10^5 cpm) was hybridized to 80 μg of total cellular RNA isolated from cells by guanidinium thiocyanate-phenol extractions as previously described (2). Single-stranded regions were digested by RNase A plus T_1 (17). RNase-resistant fragments were fractionated on an 8 M urea–12% polyacrylamide sequencing gel. The gel was exposed to X-ray film at room temperature without an intensifying screen.

RESULTS

Effect of 5′ or 3′ deletions on *aprt* expression. To define the transcriptional unit of the *aprt* gene, deletions were made from both ends of the cloned fragment. The fragment in pHaprt-7 contains 392 base pairs (bp) of 5′ noncoding sequence (Fig. 1 and 2A and D) and 1.6 kb of 3′ noncoding sequence. pHaprt-7 synthesized as much APRT as the longer construct pHaprt-1 in transient expression assays (data not shown). pHaprt-9 was made by deleting a 1.2-kb 3′ sequence of the cloned fragment in pHaprt-7. Such a 3′ deletion did not hamper the activity of the *aprt* gene (Fig. 1). The promoterless *aprt* gene (i.e., pHaprt-4) contained only five nucleotides of 5′ nontranslated sequence. This 5′ deletion greatly reduced APRT activity relative to that obtained with pHaprt-7 in transfected LAT cells, although activity was not totally abolished (Fig. 1). It is thus clear that the 392

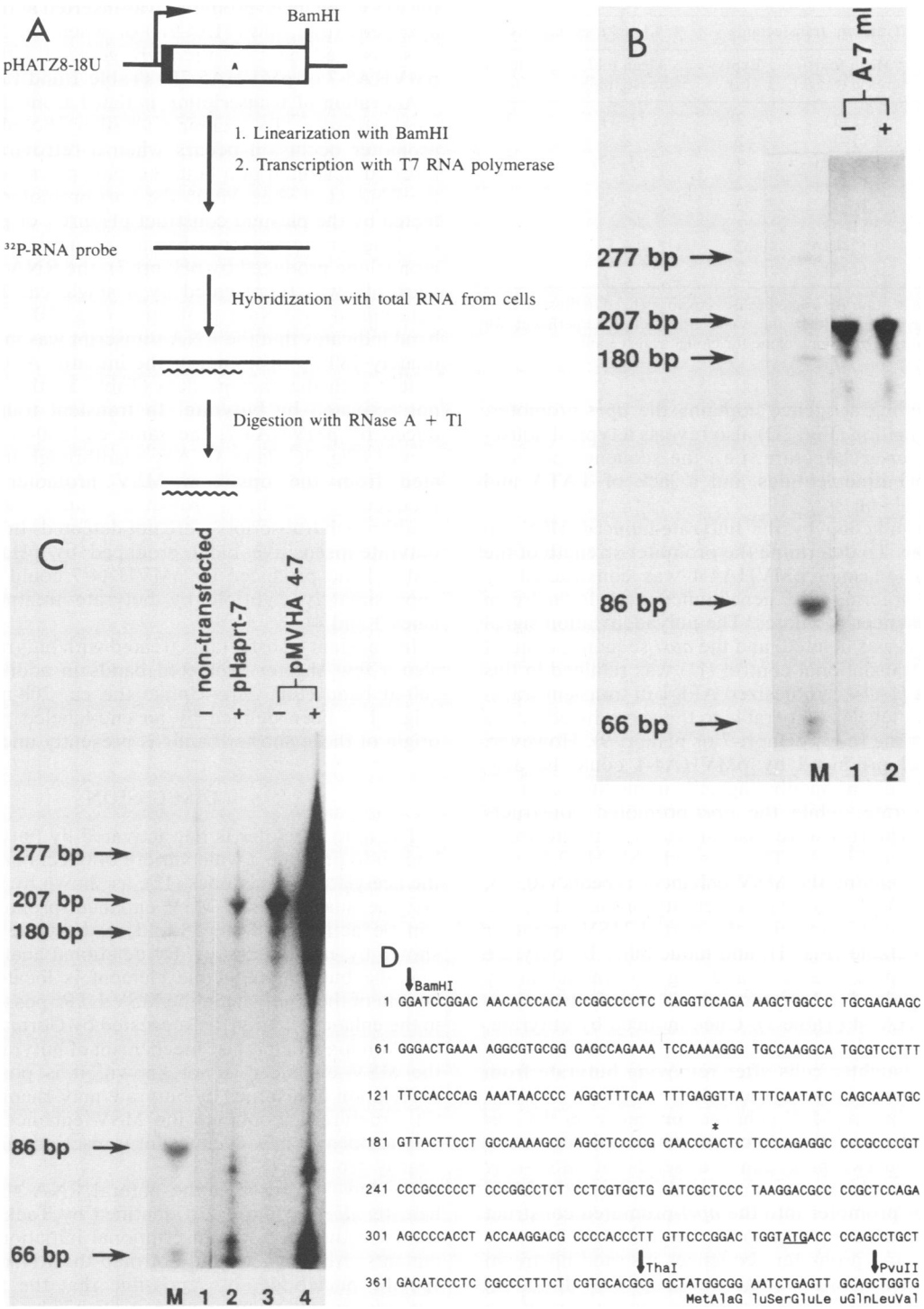

FIG. 2. RNase protection assays. (A) Scheme for RNase mapping, with the direction of transcription (arrow) of the T7 promoter in the plasmid derived from pTZ18U. Boxes: A, see legend to Fig. 1; solid, exonic sequence upstream of the *Pvu*II site in exon 1 of the *aprt* gene. (B) RNA from the clonal transfectant A-7m1 (lanes 1 and 2). (C) RNA from nontransfected LAT cells (lane 1), RNA from transient transfectants produced by pHaprt-7 (lane 2), and pMVHA4-7 (lanes 3 and 4). RNA size markers produced by transcribing DNA fragments of defined lengths with T7 RNA polymerase are shown in panels B and C, lanes M. +, Cells incubated in 5 mM butyrate for 2 days before harvest; −, cells incubated in normal medium at all times. (D) Nucleotide sequence of the 5′ region of the hamster *aprt* gene (18). *, One of the proposed RNA start sites. The ATG codon in the 5′ noncoding region is underlined. The partial amino acid sequence of exon 1 is included.

TABLE 1. Induction of APRT activities by butyrate

Plasmid	Normalized APRT activity in[a]:			
	Transient transfectants		Clonal transfectants	
	Mean fold increase (range)	Expts (*n*)	Mean fold increase (range)	Clones (*n*)
pHaprt-7	1 (1–1)	3	1 (1–1)	3
pHaprt-9	1 (1–1)	2		
pMVHA4-4	21 (8–38)	4	7 (3–12)	3
pMVHA9-20	1 (1–1)	2		
pMVHA4-7	6 (6–7)	3		
pMVHA4-7R	3 (1–5)	3		
pMVHA5-7	3 (2–4)	2		
pMVHA5-7R	3 (2–4)	2		

[a] APRT activities in butyrate-treated cells were normalized to those in their nontreated counterparts of either the same transfection experiment for transient transfectants or the same clone for clonal transfectants.

bp of 5′ noncoding sequence contains the *aprt* promoter. Sequence information (Fig. 2D) also reveals a typical housekeeping promoter architecture, i.e., the sequence contains clusters of CpG dinucleotides and a lack of TATA and CCAAT boxes (1, 6).

APRT overproduction by the butyrate-induced MSV enhancer-promoter. To determine the promoter strength of the MSV LTR, the plasmid pMVHA4-4 was constructed by placing the promoterless *aprt* gene immediately downstream of the MSV enhancer-promoter. The polyadenylation signal of the MSV LTR was deleted, and the *aprt* sequence context for eucaryotic translational control (11) was retained in this construct. pMVHA4-4 synthesized APRT in transient transfection assays at levels comparable to those produced by the wild-type *aprt* gene (e.g., pHaprt-7 or pHaprt-9). However, the APRT level produced by pMVHA4-4 could be augmented 8- to 38-fold by incubating cells in medium containing 5 mM butyrate, while the *aprt*-promoted constructs pHaprt-7 and pHaprt-9 were insensitive to butyrate treatment (Table 1 and Fig. 1). The plasmid pMVHA9-20 was constructed by deleting the MSV enhancer repeats (10, 13, 25) from pMVHA4-4. The APRT activity produced by the *Xba*I-*Sma*I fragment containing the partial MSV promoter was barely detectable (Fig. 1), and inducibility by butyrate was also interrupted (Table 1 and Fig. 1). Induction by butyrate was retained in long-term clonal transfectants produced by pMVHA4-4 (Table 1). Once induced by butyrate, the high APRT level in several clones persisted for a few generations in daughter cells after removing butyrate from the culture medium (data not shown).

Effect of an adjacent MSV enhancer-promoter on the *aprt* promoter. To examine the effects of retroviral regulatory signals on a housekeeping gene, a series of MSV-*aprt* recombinant plasmids was constructed that placed the MSV enhancer and/or promoter into the *aprt*-promoted construct pHaprt-7 in various ways. The construct pMVHA4-7 has the MSV enhancer and promoter sequences inserted upstream of the *aprt* promoter in the same transcriptional direction; pMVHA4-7R has these MSV sequences downstream of the structural *aprt* gene in reverse orientation. pMVHA5-7 has only the MSV enhancer sequence added 5′ to the *aprt* promoter, and pMVHA5-7R has the MSV enhancer 3′ to the gene.

Introduction of the *aprt* promoter between the MSV promoter and the *aprt* coding sequences, as in the plasmid pMVHA4-7, reduced the magnitude of butyrate induction from an average of 21- to 6-fold (Table 1 and Fig. 1). Negligible increases of APRT activities relative to those produced by the wild-type *aprt* gene were observed when the MSV enhancer-promoter was inserted at the 3′ end of the *aprt* gene (e.g., pMVHA4-7R) or when the MSV enhancer was inserted next to the *aprt* gene at either end (e.g., pMVHA5-7 or pMVHA5-7R) (Table 1 and Fig. 1).

Activation of transcription initiated from the downstream *aprt* promoter. To examine whether the phenomenon of promoter occlusion occurs when a retroviral promoter is inserted upstream of a housekeeping promoter, we mapped the RNA start sites within the *aprt* promoter in cells transfected by the plasmid construct pHaprt-7 or pMVHA4-7. In the long-term clonal transfectant A-7m1 (an APRT-positive monoclone produced by pHaprt-7), the RNA transcriptional start site was represented by a single ca. 200-nt band as determined by RNase protection (Fig. 2B). This ca. 200-nt band indicates that the RNA transcript was initiated approximately 180 bp upstream of the initiator ATG codon. Consistent with the enzyme data (Table 1), this transcript was not activated by butyrate. In transient transfectants produced by pMVHA4-7, the same ca. 200-nt band was observed (Fig. 2C, lane 3). Any readthrough transcripts initiated from the upstream MSV promoter should have produced a 413-nt protected band; however, such readthrough transcripts were not detected. In contrast to the butyrate-insensitive band produced by pHaprt-7, the ca. 200-nt band produced by pMVHA4-7 could be augmented approximately eightfold by butyrate treatment (Fig. 2C, lanes 3 and 4).

In transient transfectants treated with butyrate, there were also a few shorter protected bands in addition to the ca. 200-nt band (Fig. 2C). Unlike the ca. 200-nt band whose origin has been defined by an end-labeled probe (23), the origin of these shorter bands is presently unclear.

DISCUSSION

The *aprt* promoter is not activated by butyrate, an agent known to produce a wide variety of effects on cells, notably the acetylation of histones (12), as shown by both RNA and enzyme analysis. The MSV enhancer-promoter, however, can be activated 8- to 38-fold by butyrate treatment, as shown by enzyme assays. By deletional analysis, we know that the butyrate-inducible element is located further upstream of the *Xba*I site in the MSV LTR, possibly embedded in the enhancer region, as suggested by Gorman and Howard (9). Although the precise mechanism of butyrate induction of the MSV enhancer is not known, it is possible that the acetylation of histones by butyrate may change the chromatin structure surrounding the MSV enhancer in a specific way to open up new avenues for transcription factors to bind nearby promoters.

We have shown that the natural RNA start site of the hamster *aprt* promoter as identified by Tackney et al. (23) was also the site for transcriptional initiation in our transfectants. Although we did not map the RNA start site to a specific nucleotide, it is possible that the transcript was initiated from the consensus cap site 177 bp upstream of the initiator ATG codon (Fig. 2D), i.e., an A flanked by a string of pyrimidines on the noncoding strand (3) in that region. With the construct pMVHA4-7, in which the MSV enhancer-promoter element was placed in tandem with the *aprt* promoter in the same transcriptional direction, we expected to see transcription initiating from the upstream MSV promoter and overriding the downstream *aprt* promoter as a result of promoter occlusion (4, 19), especially when the

MSV enhancer was induced by butyrate. To our surprise, RNase protection assays indicated that transcription was initiated only from the downstream *aprt* promoter, and the ca. 200-nt protected product could be increased by butyrate treatment when there was an upstream MSV enhancer-promoter. It is unclear how an adjacent MSV enhancer-promoter element could convert a butyrate-insensitive *aprt* promoter to a butyrate-responsive one. Another question that remains unanswered is why the RNA start site was selected at a downstream weak promoter which lacks a TATA box instead of at the upstream MSV promoter. Although the mechanisms may be far from being understood, this finding challenges the generality of promoter occlusion.

It is common to find multiple RNA start sites for a housekeeping promoter which lacks an apparent TATA box (21). It is not understood why there was only a single RNA start site for the hamster *aprt* promoter both in CHO cells (23) and in the clonal transfectant A-7m1 that we produced, while multiple RNA start sites emerged when transient transfectants were treated with butyrate. Our results provide evidence that the physical properties of the transfected DNA template in transient transfectants may be different from those in stable clones.

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