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Two cytotoxic cell proteinase genes are differentially sensitive to sodium butyrate

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ABSTRACT

The 5′-flanking regions of two cytotoxic cell protease genes, CCP1 and 2, are sufficient to confer cytotoxic T lymphocyte-specific expression when fused to a reporter gene. The two regulatory regions are, however, differentially sensitive to treatment of the recipient cell, MTL 2.8.2, with sodium butyrate. With CCP1 a six-fold increase in cat expression was observed, whereas CCP2 was insensitive to the butyrate treatment. One major butyrate-sensitive regions was defined in the CCP1 5′-flanking sequence between −243 to −112 and another less effective one between-682 to −427. These fragments of DNA were also able to confer responsiveness to butyrate when ligated to a heterologous fos promoter. These sequences within the 5′ flank of CCP1 share homology with other elements that have been defined as butyrate-responsive. We believe that our results argue against a pleiotropic affect of butyrate such as histone acetylation. More likely sodium butyrate is mediating a specific stimulation of transcription through modification of the activities of selected transcriptional regulatory proteins that in turn affect their interactions with proteins bound to the promoter.

INTRODUCTION

Cytolytic lymphocytes play an important role in defense against viral and neoplastic diseases. It now appears that there is more than one way in which these cells can lyse their targets (1,2). One mechanism involving the directed exocytosis of potential effector molecules from cytoplasmic granules has received considerable attention recently (3,4). These subcellular organelles contain a family of serine esterases (5,6,7), a pore-forming protein called perforin or cytolysin (8,9), proteoglycans (10,11,12), a lipase (13) and other less well characterized molecules (4). The contents of the granules are released upon interaction with the target and are believed to be active participants in the annihilation of this cell (reviewed in Young (3), Tschopp and Nabholz, (4)).

During T cell stimulation, the genes that encode the granule-proteins are transcriptionally activated. Thus, in order to understand how T cell activation is controlled one must identify the elements that regulate the expression of this family of genes. Although the detailed events that control the expression of the individual genes are not known, two of the serine protease genes, C11 and B10 (encoding cytotoxic cell proteases (CCP) 1 and 2, also known as granzymes B and C) were recently shown to be differently regulated (14). Both C11 and B10 contain distinct regulatory elements responsible for their cell-specific distribution and temporal expression. Early observations, made following transfection of cytotoxic T lymphocytes (CTLs) in the presence of sodium butyrate, indicated that C11 and B10 had a different sensitivity to this inducing agent (15). While both 5′-flanking regions were equally effective in driving *cat* transcription in the absence of butyrate, C11 clearly showed a stronger ability to do so in the presence of the agent.

Sodium butyrate has been the subject of many previous studies particularly as an inducer of differentiation. It has been shown to affect gene expression at a number of different levels including chromatin structure (16,17,18,19,20), transcription (21,22,23,24,25), and mRNA half-life (26). However, there are only a few reports (27,28,29,30) on the presence of DNA sequences that control butyrate-sensitivity of proximal genes. Here we describe the effect of sodium butyrate on C11- and B10-directed *cat* gene expression. The reason behind the differential butyrate-responsiveness of the two serine protease genes appears to be defined by distinct genetic elements within the 5′-flanking regions of both B10 and C11. Furthermore, these butyrate-sensitive sequences can confer butyrate-responsiveness to heterologous viral and cellular promoters such as tk and c-fos that are not normally responsive to this agent. The difference in butyrate sensitivities of the two genes argues against any pleiotropic effect of sodium butyrate. Similar gene-specific butyrate stimulation has recently been reported for a chicken β-globin gene (31).

MATERIALS AND METHODS

Cell lines and tissue culture

The cytotoxic T cell line MTL 2.8.2 was generated from CBA/Balb/c mice as described previously (32). It is an IL2 dependent cell line that can proliferate in the absence of antigen.

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These cells were cultured in RPMI 1640 medium (Gibco Laboratories, Life Technologies, Inc., Grand Island, NY) supplemented with 10% (v/v) fetal calf serum (HyClone Laboratories, Inc., Logan, Utah), 20 mM HEPES, pH 7.5, antibiotics and 100 μM of β-mercaptoethanol. This is referred to as RHFM medium. MTL 2.8.2 cells were maintained in RHFM containing 30 units/ml of recombinant human IL2.

Plasmid construction

The parental vectors pGEM1cat and pGEM2cat referred to as pGEMcat are derived from the promoterless vectors pGEM1 and pGEM2 (Promega Corporation). They allow insertion of 5′-flanking fragments at various positions within the multiple cloning site (MCS) region. pFCΔ56 also referred to as pFoscat in this paper was provided by W. Leonard (NIH) and contains the mouse c-fos essential promoter sequences, defined by Gilman *et al.* (33) (namely the TATA box without the GC boxes and the cAMP consensus sequences), linked to the cat gene and downstream SV40-derived sequences from pSV0cat in pUC13. pTKcat was provided by R. Miksicek (Heidelberg) and includes the herpes virus thymidine kinase promoter region −109 to +51 (34,35) ligated upstream of the cat cartridge and polyadenylation site of pSV2cat in pUC8. This promoter region contains the TATA box, the CCAAT box and two SP1 sites (GC boxes).

Parental plasmids containing the 5′-upstream region of both B10 and C11 (36), respectively 1648 bp (−1617 to +31) and 1078 bp (−961 to +117) were excised from genomic clones with EcoR1-Acc1 or EcoR1-Tth111 1 and subcloned in pUC13. The various fragments shown in this paper were prepared from the parental B10 and C11 plasmids by restriction enzyme cleavage. Table 1 presents the various constructs used in the current study with the corresponding portion of B10 or C11 5′-flanking region contained within each vector. A map of B10 and C11 5′-upstream regions is also presented in Figure 1. Plasmid stocks were propagated in *E. coli* strain DH5α and isolated using the alkaline lysis procedure (37).

Transfections and cat assays

MTL 2.8.2 cells were transfected following a DEAE dextran protocol adapted and optimized for cytotoxic T cell lines (15). Sodium butyrate was added to a final concentration of 7.5 mM for 13 hours immediately following transfection. Cells were harvested and chloramphenicol acetyl transferase (cat) activity assays were performed as previously described (38,15). The concentration in the lysates was determined by the Bio-Rad protein assay. An equal amount of protein (usually 100 μg) from each sample was incubated with 0.014 μCi of 45 mCi/mmol, 0.1 mCi/ml (^{14}C)chloramphenicol (Dupont/NEN products), 4 mM acetyl coenzyme A (Pharmacia) and 0.5 M Tris-HCl pH 7.8 in a final volume of 100−150 μl at 37°C for 4 hours. Reaction products were visualized by autoradiography at room temperature and the relative increases in *cat* activity were determined by liquid scintillation quantification of the acetylated and unacetylated (^{14}C)chloramphenicol after thin layer chromatography. Each series of *cat* assays were performed a minimum of three times and within each set of experiments the parent vectors (pGEMcat, pFCΔ56 and pTKcat) were done in triplicate.

Table 1. Summary of the C11 and B10 5′-end-containing plasmids[a]

Constructs	Restricted fragments utilised from the 5′-flanking region	Genomic position of the 5′-fragments
B10: p1648cat	EcoRI-AccI	−1617 to +31
p1080cat	PstI-AccI	−1049 to +31
C11: p896cat	TaqI-AvaII	−828 to +68
p495cat	HaeIII-AvaII	−427 to +68
p311cat	AluI-AvaII	−243 to +68
p180cat	HinfI-AvaII	−112 to +68
p439cat	AluI-AluI	−682 to −243
p437cat	HaeIII-HaeIII	−864 to −427

[a] Full length C11 and B10 5′-upstream regions were cleaved with the specific restriction enzymes indicated and the various fragments cloned into three distinct *cat* plasmids: pGEMcat, pFCΔ56 and pTKcat after polishing the ends with Klenow and/or T4 DNA polymerase and the addition of HindIII linkers as detailed elsewhere (14). The position of the fragments denoted is in reference to the transcriptional start site +1.

RESULTS

Effect of sodium butyrate on transfected C11 and B10-driven cat gene expression in MTL2.8.2 cells

MTL2.8.2 cells were transfected with either pC11 896cat or pB10 1080cat, treated with sodium butyrate for 13 hours at a concentration of 7.5 mM and left to recover for various lengths of time (0, 6, 24 or 48 hours) in butyrate-free medium. Cells were then harvested for *cat* determination. As shown in Figure 2 (panel A), the C11-driven *cat* gene activity in MTL2.8.2 cells treated with butyrate for 13 hours was higher (2 fold) than that in untreated cells. Following discontinuation of the drug treatment, the level of *cat* activity increased 3 fold (6 fold above the untreated sample) and remained high up to 48 hours in the recovery medium. B10 (panel B), on the other hand, showed only a slight modulation (1.5 fold increase) in the *cat* activity levels following the addition of butyrate that we do not believe is significant.

The 5′-flanking regions of C11 can confer butyrate-responsiveness to heterologous promoters

To determine if butyrate-sensitivity of the 5′-upstream region was dependent on sequences found within the promoters, the large 5′-fragment of C11 was fused to heterologous sequences i.e. the c-fos promoter in pFoscat and the viral herpes tk promoter in pTkcat vectors. Following transfection into MTL2.8.2 cells and butyrate treatment (7.5 mM for 13 hours), cells were

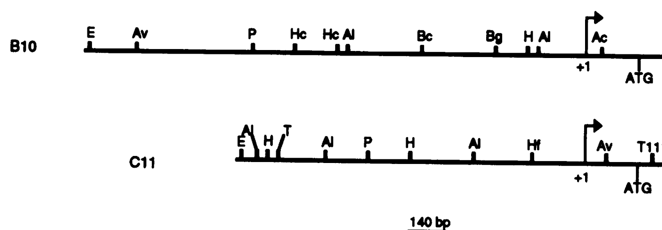


Figure 1. The B10 and C11 5′-flanking regions showing the restriction endonuclease sites used to generate the various constructs presented. E: EcoRI, Av: AvaII, P: Pst1, Al: AluI, Hc: HincII, Bg: BglII, H: HaeIII, Ac: Acc1, T: Taq1, Hf: HinfI, T111:Tth111 1. The transcriptional initiation site is represented by +1. The translational start site ATG is also shown.

supplemented with fresh medium depleted of inducing agent and incubated for a further 35 hours before harvest. The data presented in Table 2 demonstrates that although the parental vectors (pFoscat and pTkcat) were not significantly sensitive to butyrate, the fusion of the C11 5′-flanking regions conferred upon them butyrate-responsiveness. In order to control for any possible artefacts caused by having two promoters firing in the same orientation, the C11 fragments were inserted in both orientations and gave essentially the same results. The effect on the Tk promoter was quite small but was particularly notable with the c-fos basal promoter where an 11 fold induction in *cat* activity was measured. Thus, it appears that the addition of approximately 1 kilobase sequence from C11 is sufficient to increase stimulation by butyrate in a number of different promoter contexts.

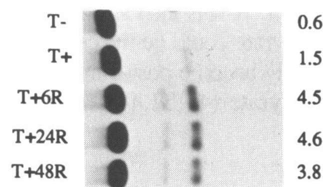
Mapping butyrate-responsive sequences within the C11 5′-flanking region

We next examined the 5′-flanking region of the CCP1 protease gene to define the genetic elements that could be responsible for the butyrate sensitivity observed. A series of deletion fragments spanning the 5′-end of the C11 gene were generated using restriction cleavage sites. These fragments were fused to the *cat* reporter gene in pGEMcat and transfected into MTL2.8.2 cells. Following butyrate treatment (7.5 mM for 13 hours), cells were supplemented with fresh medium free of inducer and incubated for a further 35 hours before harvest. Figure 3 represents a detailed dissection of the 5′-end of C11 in which at least one butyrate-inducible region was identified. The deletion of the sequences between −828 and −243 lead to a small decrease (22%) in the fold stimulation by butyrate. However, removal of the sequences downstream from −243 (i.e. −243 to −112) resulted in a 3.6 fold (72%) loss in butyrate-inducibility. This denoted a proximal site responsive to sodium butyrate induction.

MTL2.8.2 cells were also transfected with the pFCΔ56 series of constructs and following treatment with butyrate for 13 hours, were left in drug-free medium for 35 hours before *cat* determination. The results presented in figure 4 indicate that sequences between −243 and +68 contain a major butyrate responsive element. Furthermore deletion of the region between −243 to −112, defined as butyrate-sensitive in the C11 natural context (Figure 3), resulted in a 7.5 fold (87%) decrease in fold stimulation.

The region between −828 to −427 also appears to contain sequences that respond to butyrate since its deletion resulted in

A. C11



B. B10

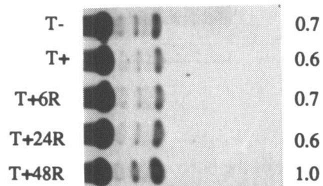


Figure 2. Effect of sodium butyrate on C11- and B10-directed *cat* activity. MTL2.8.2 cells were transfected with either pC11 896cat (panel A) or pB10 1080cat (panel B) and treated with sodium butyrate for 13 hours at a concentration of 7.5 mM. Following this step, the cells were either collected immediately or allowed to recover for 6, 24, or 48 hours at 37°C/5% CO_2 atmosphere in butyrate-free medium. Cells were then harvested for *cat* determination (see Materials and Methods). The absolute percentage of chloramphenicol acetylation is given on the right. T(−) refers to untreated transfected MTL2.8.2 cells, T(+) to butyrate-treated transfected cells and R to recovery time. Exposure time was 24 hrs at room temperature for C11 and 15 days at room temperature for B10.

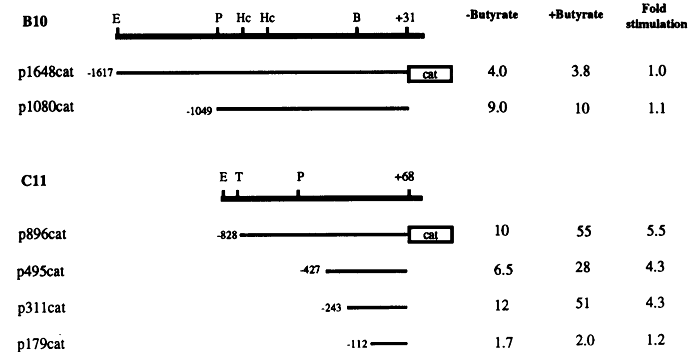


Figure 3. Effect of sodium butyrate on C11-cat fusion constructs in MTL2.8.2 cytotoxic T cells. Fragments of the 5′-flanking sequences of C11 were ligated upstream of the cat gene in pGEMcat vectors as described under Materials and Methods and were transfected into MTL2.8.2 cells. Cells were subjected to butyrate at a concentration of 7.5 mM for 13 hours. Following a media change, cells were allowed to recover for 35 hours before harvest and *cat* determination. The fold stimulation was determined by assaying both untreated and treated cells. Numbers presented are *cat* activities relative to the parent promoterless pGEMcat plasmid. Absolute values of chloramphenicol acetylation normalized to 1.0 were for untreated cells 0.27 and for butyrate-treated cells 0.30. Each value represents the mean of at least three experiments. Abbreviations for the restriction enzyme cleavage sites are E: EcoRI, P: Pstl, T: Taql.

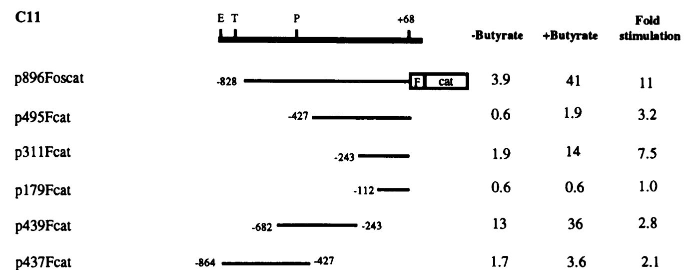


Figure 4. Butyrate-responsiveness of the c-fos promoter conferred by C11 5′-upstream sequences. Fragments of the 5′-flanking regions of C11 gene were ligated upstream of the minimal fos promoter in pFCΔ56 and transfected into MTL2.8.2 cells. Following butyrate exposure for 13 hours at a concentration of 7.5 mM, cells were resuspended in fresh medium and incubated for a further 35 hours before harvest and *cat* determination. The *cat* activities relative to the parental pFCΔ56 plasmid are shown. The absolute values for pFCΔ56-driven *cat* activities were 0.88% for untreated cells and 1.2% for butyrate-treated cells. The fold induction was determined by comparing untreated and treated samples. All values given represent the average of at least three experiments. For abbreviations see Figure 4.

a 71% loss in fold stimulation. In the natural promoter context, the removal of this region showed a minor effect in sodium butyrate inducibility. Further deletion of sequences upstream from −243 (−427 to −243) resulted in an increase (2.4 fold or 57%) in butyrate-inducibility. Constructs p439Fcat and p437Fcat both contain sequences between −682 to −427 and all showed a stimulation of *cat* activity (2 to 3 fold) after butyrate treatment. This series of deletion constructs delineated a minor butyrate-inducible area in C11 (−682 to −427) indicating the presence of butyrate-sensitive sequences between these boundaries. Even though the effect is quite small, previous studies have also implicated this region in transcriptional control (14,36).

DISCUSSION

Many reports have indicated that sodium butyrate can modulate gene activity in a variety of cell lines. It has also been shown that this agent can have different consequences on the induction of specific RNAs in the same cell (30,18,22,23). We demonstrated, in an earlier study, that sodium butyrate treatment induces C11-driven *cat* gene expression five fold over that of B10-driven *cat* gene expression in the cytotoxic T cell line MTL2.8.2 (15). In this report, we investigated the reason behind this differential sensitivity to butyrate.

The length of the sodium butyrate treatment and the concentration of inducer chosen for our experiments were based on a previous optimization study for transient expression in MTL2.8.2 cells (15). Many other groups have used concentrations ranging from 1 to 10 mM for 30 minutes to 7 days and measured changes in mRNA level from 5 to 80 fold depending on the experimental conditions (26,22,29,30,21). Undoubtedly, shorter or longer exposures will have different consequences on the level of modulation of gene expression by butyrate. In the current report, the transfected *cat* gene expression driven by the C11 5′-flanking region was induced two fold following exposure to butyrate (7.5 mM) for 13 hours and was further increased to four fold after 6 hours in butyrate-free medium (Figure 2, panel A). The *cat* activity remained high up to 48 hours in this recovery buffer. For the B10-directed *cat* gene expression, the addition or discontinuation of butyrate had no significant effect as the levels of *cat* activity measured were identical to those of untreated cells (panel B).

The experiments described above also suggested the possibility that specific sequences or areas within the 5′-upstream region of C11 could be particularly sensitive to butyrate. Table 2 showed that it is indeed the case. When the 5′-flanking regions of C11 were fused to heterologous promoters such as the cellular fos and viral tk, both were able to confer butyrate responsiveness to the normally insensitive promoters. C11 showed sensitivity to the inducer in its natural context (see Figure 2, panel A) and it was therefore not surprising to see that it could confer sensitivity to other genes. The magnitude of the effect was however different for each promoter : endogenous 6 fold, fos 11 fold and tk 2 fold. This result underscores the conclusions of a number of investigators that effect of regulatory sequences depends upon the promoter context (40−42).

As presented in Figures 3 and 4, distinct butyrate-sensitive regions were identified for the C11 gene. Sequences between −828 and −427 and between −243 and −112 were defined as responsive in the natural C11 promoter context. Although the level of responsiveness was low for the area delineated by nucleotides −828 to −427, its butyrate sensitivity was confirmed and further narrowed to nucleotides −682 to −427 when fused to the c-fos basal promoter where a 3.5 fold increase in *cat* activity was measured (compare Figures 3 and 4). Sequences between −243 and −112 had the most impact on butyrate stimulation in both promoter contexts with a 7.5 fold increase upon fusion to c-fos. These results are consistent with those of Gorman and Howard (27) which observed a 2 to 10 fold enhancement in SV40-directed *cat* activity in cells that had been treated for 12 hours with 10 mM sodium butyrate. They speculated that one of the possible mechanisms by which butyrate might facilitate the transient expression of exogenously supplied foreign genes was to promote their assembly into 'active' or 'open' chromatin. It was also suggested that possible targets of the action of butyrate could be the cellular DNA-binding proteins. These presumably become post-translationally modified following exposure to butyrate (43,22,44). A direct consequence of this

Table 2. Differential butyrate-sensitivity of the 5′-flanking regions of C11 and B10 when placed in front of different promoters[a]

Constructs	−Butyrate		+Butyrate		Fold Stimulation	
pGEMcat	1.0[b]	(0.3)[c]	1.0	(0.3)	1.0	(1.1)
pB10 1080cat	9.0	(2.4)	10.0	(3.1)	1.2	(1.3)
pC11 896cat	10.0	(2.7)	55.0	(17.0)	5.5	(6.3)
pFoscat	1.0	(0.9)	1.0	(1.2)	1.0	(1.4)
pC11F 896cat	3.9	(3.4)	41.0	(49.0)	11.0	(12.0)
pTKcat	1.0	(5.3)	1.0	(7.4)	1.0	(1.4)
pC11Tk 896cat	1.2	(6.5)	2.1	(16.0)	1.8	(2.4)

[a] MTL2.8.2 cytotoxic T cells were transfected with 15 μg of plasmid DNA following a DEAE dextran procedure as described elsewhere (15). One series was treated with sodium butyrate for 13 hours at a concentration of 7.5 mM then fed fresh medium without drug for another 35 hours before harvest and *cat* determination.

[b] Values given are relative to the parental vector set at 1.0 and represent the mean of three or more experiments which agreed within 10%. Within each series of experiments, the parental vectors were done in triplicate.

[c] Numbers in parentheses represent absolute values of *cat* activity (% chloramphenicol acetylation).

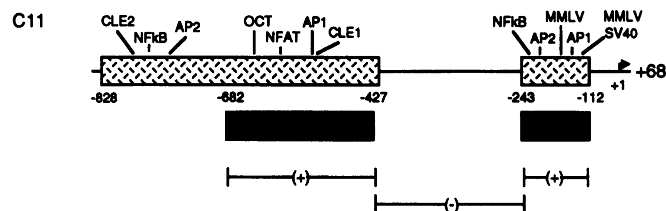


Figure 5. Summary of the butyrate-responsive elements mapped within the C11 5′-upstream regions and their relation to the cis-acting regulatory elements defined by Frégeau and Bleackley (14). Butyrate-sensitive sequences are represented by boxes; those identified in the natural serine protease C11 5′-context are dotted and those with the heterologous promoter c-fos are filled. Negative (−) and positive (+) symbols delineate the regulatory areas of C11 that down-regulate and up-regulate transcription. Sequences within the butyrate-sensitive regions which are homologous to regulatory motifs identified in numerous genes are also shown. CLE1 or CLE2, conserved lymphokine element 1 or 2; NFκB, nuclear factor of kappa light chain in B cells; AP1 or AP2, activating protein 1 or 2; OCT, octamer motif; NFAT, purine box of the binding site of nuclear factor of activated T cells; MMLV, Moloney murine leukemia virus enhancer core, SV40, simian virus 40 enhancer core. The numbering is in reference to the transcription start site +1.

action would be to alter the network of interactions between transcriptional factors that bind to regulatory regions and change the fate of gene expression. It is more likely that constructs containing sequences from −682 to −427 or −243 to −112 are sensitive to butyrate because these sequences are target sites for specific factors which are particularly affected by the modifications induced by the agent. Indeed, the C11 butyrate-sensitive sequences map to DNAse1 hypersensitive regions previously defined (36). Such regions usually correspond to chromatin domains that are transcriptionally active. This suggests that the genetic elements that are contained within the butyrate-responsive areas may attract specific factors which are involved in the activation of the C11 gene.

Similar observations were obtained from groups who studied the effect of sodium butyrate on expression of transfected DNA molecules. Tang and Taylor (29) demonstrated that a nonresponsive promoter such as adenine phosphoribosyltransferase (APRT) could be induced by butyrate by placing an inducible Moloney sarcoma virus (MSV) enhancer-promoter upstream from it. It was suggested 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. Another possibility was the activation of trans-acting factors following modifications induced by butyrate; factors which would bind to the MSV enhancer and participate in the formation of the transcriptional complexes to activate APRT. In the same vain, Dorner *et al.* (30) demonstrated that treatment of CHO cells with butyrate increased the expression of erythropoietin, Factor VIII and von Willebrand factor from stably integrated and amplified genes

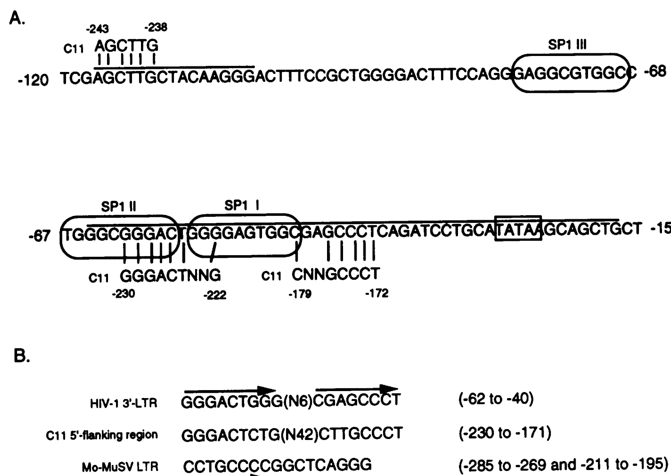


Figure 6. Homologies between HIV-1 3′-LTR butyrate-responsive sequences and those of C11 5′-flanking regions. Panel A: the nucleotide sequence of HIV-1 3′ LTR (isolate BH10) comprising both butyrate-sensitive regions mapped by Bohan *et al.* (28). HIV-1 butyrate-responsive areas are indicated by a line above the sequences (−117 to −103 and −65 to −17). Sequences from C11 butyrate-responsive regions that show homology to HIV-1 3′ LTR elements are given above and underneath. The numbering refers to the position of the sequences within the 5′-flanking regions of C11. N refers to pyrimidine or purine. The TATA box and SP1 binding sites I, II and III identified within the HIV-1 3′ LTR are also shown. Panel B: the 17 bp homologous sequence found between HIV-1 3′ LTR, C11 5′-end butyrate-sensitive areas and Moloney murine sarcoma virus LTR enhancer. The respective position of this 17 oligomer within each context is given.

which utilize the adenovirus major late promoter in combination with the SV40 enhancer for transcription. They indicated that the combination of promoter and enhancer is important in determining the responsiveness of integrated transcription units to butyrate. The same conclusion had been reached by Gorman and Howard, who showed that the SV40 promoter was very sensitive to butyrate but that the deletion of the enhancer reduced this induction by a factor of 6 to 10 (27).

The results represented in Figures 3 and 4 are summarized in Figure 5. Cis-acting genetic elements within C11 5′-flank appear to include the positive regulatory regions −682 to −427 and −243 to −112 and the negative regulatory region −427 to −243 (14). The proximal C11 target site for sodium butyrate induction was located between −243 to −112 relative to the transcriptional start site (+1) which corresponds to the area comprising the regulatory elements reported to exert a positive effect on C11 expression. Because the boundaries of the C11 promoter have not been defined yet, we do not know if any of the binding sites for RNA polymerase II basal transcription are also part of this proximal site for sodium butyrate induction. Fragment −243 to −112 contains sequences homologous to AP1 (45), AP2 (46), NF°B (47) and to SV40 and Moloney murine leukemia virus enhancer core (48). The presence of these genetic elements may contribute to butyrate-sensitivity of the region. Indeed, some of them represent recognition blocks for transcription factors whose activity has been shown to be modulated post-translationally. AP1 is the target site for fos and jun which are modified following phosphorylation events (49). NFκB sequences binds NFκB which is activated after its release from a complex in which it is bound to inhibitor IκB. Phosphorylation reactions mediate this release (50). These sites could also bind other factors which would be sensitive to butyrate's action.

As yet, there are no reports demonstrating unequivocally that sodium butyrate causes changes in the binding or function of any cellular transcription factors. However, acetylation and phosphorylation events of nuclear proteins have been noted following butyrate treatment (51,52,16,22). Thus, sodium butyrate may alter directly or indirectly the post-translational modifications of C11 transcriptional factors or proteins that act upon these for their activity, and change the fate of gene expression.

The region of C11 between −682 and −427 contain sequences homologous to AP1, an octamer (OCT) motif (53), a conserved lymphokine element (CLE1) binding site (54) and the purine box of the IL2-specific nuclear factor binding site NFAT (55,56). This area of the 5′-end of C11 exerted a significant effect on butyrate induction when placed in the c-fos promoter context (compare Figures 3 and 4). A possible explanation for such an effect could be that the C11 DNA-binding factors are sensitive to modifications induced by butyrate and participate in the formation of transcriptional complexes along with other proteins that recognize the basal fos promoter. In this instance, the combination of the C11 and fos transcriptional factors induces the level of *cat* activity and the response to butyrate.

Sodium butyrate has been shown to stimulate HIV-1 gene expression (57). This specific induction was recently attributed to at least two LTR inducible regions, a distal site −117 to −103 and a proximal site −65 to −17 present within the 3′-LTR (28). Figure 6 (panel A) shows the homologies between the butyrate-inducible sequences found within HIV-1 3′-LTR and the C11 5′-flanking regions. It was very interesting to find that the C11

region comprising −243 to −112 sequences gave the highest degree of identity with HIV-1 sequences. This region was butyrate-sensitive in both contexts examined i.e. natural and cellular fos. These areas of homology between these two unrelated genes i.e. AGCTTG (−243 to −238), GGGACTNNG (−230 to −222), CNNGCCCT (−179 to −172) point to novel butyrate-inducible consensus sequences. No homology was detected within the C11 region delineated by nucleotides −682 to −427. In HIV-1, the butyrate-sensitive sequences map to the Sp1 binding site I and II and within the TATA box binding region suggesting the importance of Sp1 and TATA box binding factors in sodium butyrate induction of HIV-1 gene expression.

Tang and Taylor (29) were able to convert the butyrate-insensitive APRT promoter into a sensitive one by positioning the MSV enhancer in its vicinity. By comparing the Mo-MSV enhancer (58) with butyrate-sensitive regions from the HIV-1 3′ LTR and C11, we identified a common 17 bp sequence in all three nucleotide segments (Figure 6, panel B). This element appears to be comprised of two parts, a nonamer and an octamer which can be separated by a different number of nucleotides, 6 in the case of HIV-1, 42 in C11 and none in Mo-MuSV. It was interesting to note that in HIV-1 3′ LTR and in C11, the nonamer and octamer sequences were found in the same orientation i.e. head to tail while in Mo-MuSV, both elements were facing each other. In addition, in the Mo-MuSV enhancer, the nonamer sequence was found in the reverse orientation i.e. reading 3′ to 5′ instead of 5′ to 3′ as in C11 and HIV-1. We are not aware of any precedent for the importance of a sequence. The presence of this 17 nucleotide sequence, found at two different locations within the Mo-MuSV and once within HIV-1 3′ LTR and 5′-end of C11, suggests an important role in butyrate-responsiveness. Further experiments are required to determine the mechanism(s) by which these elements could promote butyrate-stimulation when placed within different genomic contexts.

In summary, we have shown that two members of the serine protease multigene family, namely C11 and B10, have a different level of sensitivity to the inducer sodium butyrate. We have further demonstrated that this differential stimulation of C11 and B10 is attributed to the presence of distinct responsive sequences located within the 5′-flanking region which confer butyrate responsiveness to heterologous promoters. In the natural promoter context, the inducible regions for C11 were delineated between −243 and −112 and −682 and −427. The same region found to sensitize C11 in its natural context (−243 to −112) was functional in fos. We have also shown that these butyrate-sensitive sequences share homologies with HIV-1 3′ LTR butyrate-responsive regions. Finally, we have identified a 17 nucleotide sequence common to elements that confer butyrate responsiveness to heterologous genes.

The identification of a distinct butyrate-sensitive areas within the 5′-ends of one member of the serine protease family is very interesting. It emphasizes the concept that these two cytotoxic T cell products are regulated in a different manner and their 5′-upstream regions bind different factors. It also confirms the results obtained from earlier studies which indicated that C11 and B10 share minimal sequence homology within their 5′-upstream regions and contain distinct negative and positive regulatory sequences (36,14). The experiments performed in this report and the previous ones (14,15) used the MTL2.8.2 type II cell line which represents a biological system where constitutive expression of C11 and B10 prevails. Based on the results accumulated so far, it is possible that the proximal regulatory butyrate-sensitive regions of the C11 gene would be implicated in basal regulation in the MTL2.8.2 cells. On the other hand, the distal regulatory butyrate-responsive sequence could be involved in inducible regulation of C11 in the type I cytotoxic T cell clones which are both IL2- and antigen-dependent. These studies and those aimed at looking at butyrate sensitivity in this system are in progress.

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