Gene Expression within a Chromatin Domain: The Role of Core Histone Hyperacetylation†

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ABSTRACT: Scaffold-attached regions (SAR elements) increase transcriptional rates for integrated but not episomal templates, and this effect can be potentiated by using an epigenetically active reagent, butyrate. The action of butyrate is a direct one, not involving *de novo* protein synthesis, and can be mimicked by using a novel and highly specific inhibitor of histone deacetylases, (*R*)-trichostatin A. This leads to a model in which SAR elements serve to stabilize the chromosomal topology arising as a consequence of hyperacetylation of histone cores. The synergistic effects of histone hyperacetylation and SARs are mediated by promoter upstream elements since, for a simple TATA box, the response to both parameters is an additive one.

The eukaryotic genome is organized into a series of discrete and topologically independent higher order domains. This conclusion was originally based upon *in vitro* experiments which selectively extracted histones and certain other proteins from either interphase nuclei or mitotic chromosomes. Under these conditions, independent loops could be observed which, due to the removal of nucleosomes, contained unrestrained negative supercoils. The bases of the loops were found to be attached to a scaffold or a matrix consisting of protein and hnRNA components. Accordingly, the class of DNA sequences that displays an affinity for this nuclear substructure has been termed MAR[1] or SAR [i.e., matrix- or scaffold-attached region; reviewed by Freeman and Garrard (1992)]. All S/MAR elements characterized to date are AT-rich sequences that bind topoisomerase II, histone H1, HMG I/Y [Käs et al. (1993) and references cited therein] and a number of less abundant proteins such as ARBP (von Kries et al., 1991), SAFA/SP120 (Romig et al., 1992; Tsutsui et al., 1993), and SATB1 (Dickinson et al., 1992). The recognition is not based on a singular motif or structure, but involves a multisite association with segments bearing a narrow minor groove and/or nucleation sites for DNA unwinding (Kohwi-Shigematsu & Kohwi, 1990; Bode et al., 1992); plectonemic structures are also bound (Kay & Bode, 1993).

Although SAR elements are prime candidates to function as domain boundaries, this issue has remained controversial. Currently, a prototype border element is thought to have the following basic properties: (1) It should coincide with those regions of a chromatin domain where the general accessibility to nucleases decreases, indicating limited accessibility. Such a correlation has been established for the chicken lysozyme gene (Fritton et al., 1988), the human apolipoprotein B gene (Levy-Wilson & Fortier, 1989), and the human interferon-β gene (M. Rios and J. Bode, unpublished results). A similar situation is also found at the 5′-end of the human β-globin domain (Dillon & Grosveld, 1993), and it is likely to occur in the cases of other gene clusters or genes for which a delimitation has been shown on the basis of either SARs (e.g., the chicken α-globin domain; cf. de Moura Gallo et al., 1992) or DNAse accessibilities (e.g., the ovalbumin gene family; cf. Lawson et al., 1982). (2) It should shield a gene from the influences of chromosomal surroundings and should, hence, confer the property of position-independent (i.e., copy number-dependent) expression on transgenes. Available data suggest that at least some of the SAR elements have this property (Stief et al., 1989; Bonifer et al. 1990; Phi-Van et al., 1990; J. Schlake and J. Bode, unpublished results). (3) The shielding function should limit the action of enhancer and upstream elements to interactions with promoters in the same but not adjacent domains. This again appears to be borne out for some (Stief et al., 1989; Fishel et al., 1993) but not all SAR elements (Kellum & Schedl, 1992). In addition, in *Drosophila* there seems to exist a related group of elements (scs and scs′) that block enhancer activities in transgenic animals of this species, but are not SARs according to the classical definition (Kellum & Schedl, 1991, 1992).

Originally unexpected, and unlike the scs elements of *Drosophila*, SARs increase the transcription of a linked gene if it is anchored in the genome and has assumed its final chromatin structure. This "domain-opening" function (Laemmli et al., 1992) is clearly different from the action of classical enhancers, and there is no such effect on episomal templates (Stief et al., 1989; Klehr et al., 1991). Given their potential role as border elements, SARs could conceivably stabilize a topology within a domain that is appropriate for transcription. Such a topology can be created by butyrate, an epigenetically active reagent that causes preactivation of genes that have been linked to SAR elements, supposedly by massive hyperacetylation of histone cores (Klehr et al., 1992). This postulated mode of action leads to a number of predictions that are tested in the present contribution. This series of experiments will establish that the action of butyrate is a direct

† This publication is dedicated to Professor Fritz Wagner (University of Braunschweig) on the occasion of his 65th birthday.

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[1] Abbreviations: CHX, cycloheximide; HMG, 3-hydroxy-3-methylglutaryl-CoA reductase; IFN, interferon; huIFN-β, human interferon-β; MAR, matrix-attached region; MT, metallothionein; SAR, scaffold-attached region; S/MAR, consensus term covering SARs and MARs; TSA, (*R*)-trichostatin A.

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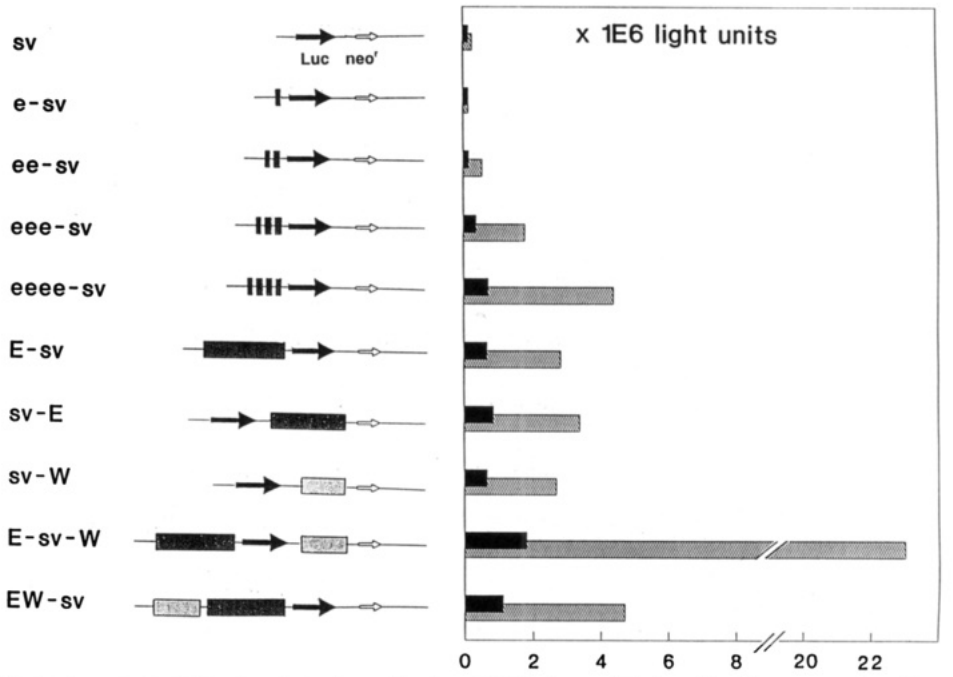

FIGURE 1: Effects of butyrate potentiated by the strategic positioning of SAR element(s). A series of constructs based on the SV40 promoter/enhancer is shown. Solid arrows mark the position of the luciferase (Luc) test gene, and open arrows show the position of the neomycine resistance marker (neo^r). Data refer to averages of at least three transfections in Ltk$^-$ and BHK cells, respectively. Data for constructs E–sv and e–sv through eeee–sv from Klehr et al. (1991) are included for correlation (see their Figures 6 and 7, constructs sv, SV, and sv′ through sv′′′′, respectively). e is a 150 bp subfragment of the 2200-bp major SAR fragment (E). W is a 1300-bp major fragment from the tobacco ST-LS1 gene.

one not requiring *de novo* protein synthesis and that it is based on the hyperacetylation of histones, as suggested before. The use of various SAR positions and promoters will reveal aspects of the actions of butyrate within artificial chromatin domains.

MATERIALS AND METHODS

(a) SAR-Containing Plasmids. The construction of the pLu-based plasmids of the "sv" series (Figures 1–3) has been described (Klehr et al., 1991, 1992). All constructs are based on a derivative of pAG60, permitting the introduction of SAR elements via a *Bam*HI site (upstream) and an *Nru*I site (downstream). Promoters could be introduced or exchanged using the *Hind*III site(s). All T7-promoter constructs are based on the pGEM-1 plasmid (Stratagene). First, a *Hind*III/*Sma*I fragment containing the luciferase gene was cloned into the *Hinc*II site of the polylinker. Then an *Eco*RI–SAR fragment (E) was cloned into the upstream *Pvu*II site or the downstream *Nae*I site, respectively. For the downstream position, both orientations of E were used.

(b) Cell Culture and Gene Transfer. Mouse-L cells and BHK cells were grown as described (Bode et al., 1986). For stable expression experiments, the transfection protocol has been optimized to yield low copy numbers (Klehr et al., 1991, 1992). This has been achieved by omitting carrier DNA and by transferring linearized DNA (as opposed to supercoiled DNA), which reduced average copy numbers about 5-fold.

For the transient expression experiments in Figure 3, 10^5 cells were transfected in a six-well plate using a calcium(2+) phosphate coprecipitate from 4 μg of the respective pLu derivative together with 2 μg of pSBC2-SEAP and 4 μg of carrier DNA (corresponding to the host cell). pSBC2-SEAP, which is based on a secretory alkaline phosphatase indicator gene (Berger et al., 1988), served as an internal reference to correct for variations in the transfection efficiency.

(c) Copy Numbers. All data from stable expression experiments were referenced to the number of integrated copies, which was determined as described before (Klehr et al., 1992).

(d) Treatment with Stimulatory Reagents. Semi-confluent cells were kept for 48 h in standard DME/10% FCS medium at 1–5 mM butyrate or 165–800 nM TSA, as indicated in the figure legends. They were then briefly rinsed with PBS and submitted to the test of luciferase activity (section f).

(e) Treatment with Cycloheximide. Semi-confluent cells were kept in medium containing 10 μg/mL cycloheximide with or without butyrate. The medium was changed after 7 h and the incubation continued for another 17 h. Cells were washed with PBS and tested for luciferase (section f).

(f) Luciferase Tests. Extracts were prepared directly from a defined number of cells. Usually, 10^5–10^6 cells were lysed in 300–1000 μL of extraction buffer (0.1 M KH_2PO_4 (pH 7.8) and 1 mM dithiothreitol). The bioluminescence of 10–50 μL was quantified in a Berthold Biolumat Model LB 9500c by integrating the output over the first 10 s and correcting this value for the number of cells in the assay.

(g) Northern Blots and Internal Controls. Where butyrate had a major effect on the transfected marker genes, expression levels were also determined for the endogenous murine interferon, pyruvate kinase (PK), or actin gene(s), as in Figure 2. In detail, cells from two 69-cm^2 culture dishes were washed twice with PBS and then lysed with 10 mL of GuaSCN solution (4 M guanidinium thiocyanate and 0.1 M Tris-HCl, pH 7.5, was adjusted to 0.5% *N*-laurylsarcosine and 1% 2-mercaptoethanol prior to use). Lysed materials were collected using a rubber policeman, dispersed by sonification, and pelleted by layering 28 mL of the solution onto a cushion of 12 mL of 5.7 M CsCl/0.1 M EDTA followed by an 18-h centrifugation at 25 000 rpm in an SW27 rotor. After successive removal of the supernatant layers, the RNA pellet was dissolved in 500 μL of hot (70 °C) water and precipitated by adding 2 vol of 0.6 M LiCl (ethanolic solution). The suspension was kept for 20 min at −20 °C, and the pellet was recovered by centrif-

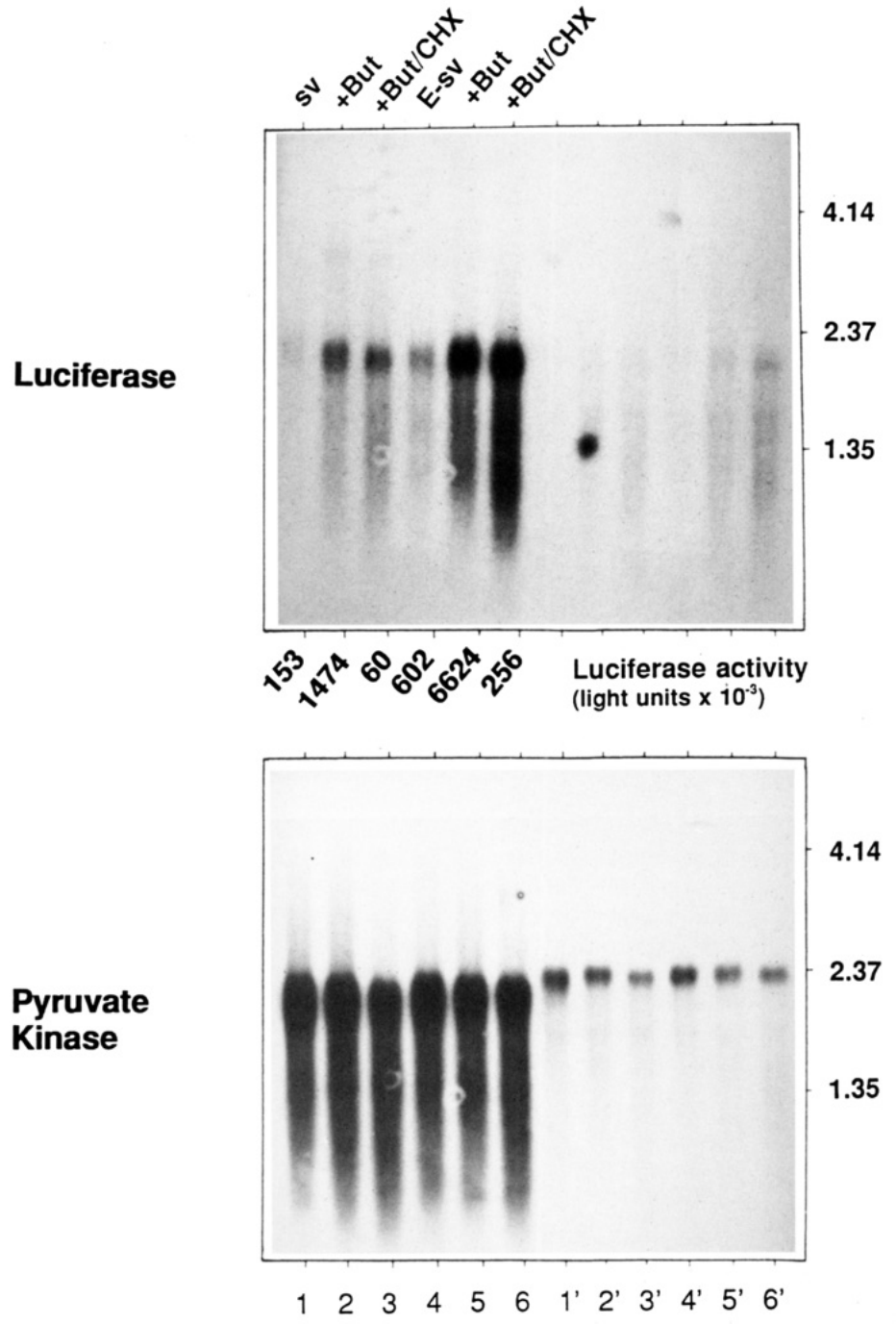

FIGURE 2: Action of butyrate independent of new protein synthesis. Constructs sv and E–sv (cf. Figure 1) were transfected into Ltk⁻ cells. Cells were treated with either 5 mM butyrate or 5 mM butyrate plus 10 μg/mL cycloheximide and analyzed on Northern blots. Traces 1–6, oligo(A)RNA; traces 7–12, the analogous analyses on total cellular RNA. Luciferase activities obtained in the presence of cycloheximide confirm significant inhibition of translation. The same blots were subsequently reprobed with DNA complementary to the endogenous pyruvate kinase (bottom part) and β-actin genes (not shown).

ugation. After it was washed with ice-cold 70% ethanol, RNA was dissolved on a Vortex mixer by adding 200 μL of hot (70 °C) water.

(h) Oligo(A)RNA. RNA (300 μg) was purified by the Dynabeads procedure (Dynal Inc.) and processed according to the manufacturer's recommendations to yield about 3 μg of oligo(A)mRNA.

(i) Electrophoresis and Blotting. Oligo(A)mRNA (3 μg) or 10 μg of total RNA was dried and dissolved in a mixture of 2 μL of H_2O, 5 μL of deionized formamide, 2 μL of deionized formaldehyde, 1 μL of 0.2 M MOPS/1 mM EDTA/10 mM NaOAc (pH 7), 1 μL of ethidium bromide (400 μg/mL), and 1 μL of 0.2% xylene cyanol/0.2% bromophenol blue/1 mM EDTA (in 50% glycerol). The sample size was sufficient for two trays of 1.2% agarose gel containing 6.6% formaldehyde. Electrophoresis proceeded for 3–5 h in 20 mM MOPS/1 mM EDTA/1 mM NaOAc. The gel was successively washed in water (1×) and 10× SSC (2×), blotted onto GeneScreenPlus (DuPont) with 10× SSC, prehybridized, and hybridized with nick-translated probes according to the manufacturer's protocol.

RESULTS

Arrangement of SARs Supporting the Stimulatory Actions of Butyrate. All SAR elements used in this study were originally detected and classified by an *in vitro* assay that demonstrated their affinity to the nuclear scaffold [roman numerals (in parentheses) refer to the original designation by

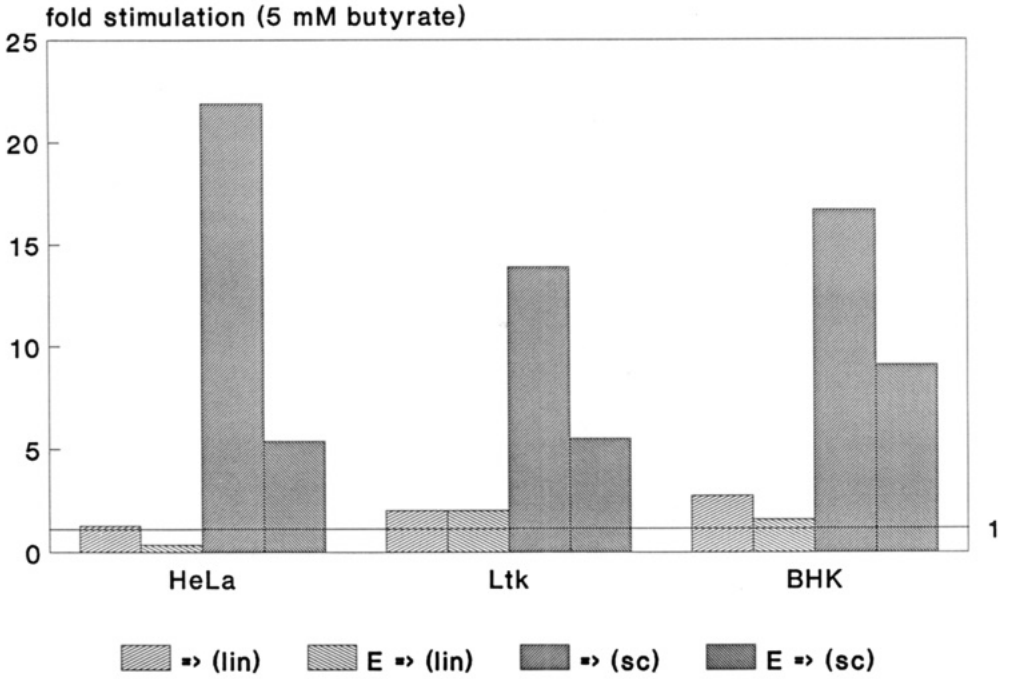

FIGURE 3: Butyrate-stimulated transcription from supercoiled but not linearized templates. Constructs sv and E–sv were introduced by transfection into three different cell lines (for nomenclature, see the legend to Figure 1). Templates were either linearized (light bars) or supercoiled plasmids (dark bars). Gene expression was monitored during the transient phase, i.e., 2 days post transfection.

Mielke et al. (1990)]: E(I) is the 2200-bp, major part of the SAR upstream of the human interferon-β gene; e(VIII) is the 150-bp subfragment of E which, due to its small size, has lost any SAR character; SAR properties can be recovered by its oligomerization [Figure 2D in Mielke et al. (1990)]; W(X) is the 1300-bp, SAR element derived from a light-inducible potato gene; in spite of the shorter length, its binding strength is comparable to that of E. A second, 1800-bp SAR element from the same gene (P) shows reduced binding strength.

Subsequent studies have indicated a direct correlation between the affinity of the SAR–scaffold interaction *in vitro* and the biological activity of the respective SAR element. This correlation was valid across species in the sense that SARs from plants or mammals displaying an affinity for nuclear scaffolds from rodent cell nuclei were active in transcriptional assays that are routinely performed in rodent cells (Mielke et al., 1990; Klehr et al., 1991, 1992).

In a recent publication, we showed that SARs not only support the transcriptional levels of transfected genes after their integration but also potentiate the transcriptional stimulation caused by butyrate (Klehr et al., 1992), now a widely used medium component for cultured mammalian cells (Palermo et al., 1991). These basic facts are summarized in Figure 1 (compare, for instance, constructs sv and E–sv). If artificial SAR elements are created by oligomerizing short sub-SAR sequences, their effects clearly increase with the number of monomer units (constructs e–sv through eeee–sv). SAR fragments E (human) and W (plant) yield similar effects, regardless of whether they are positioned upstream or downstream from the indicator gene (Figure 1, constructs E–sv, sv–E, and sv–W), and this again was expected from their comparable affinities in the *in vitro* assay (J. Bode, unpublished results).

Typically (but not exclusively, see Figure 4), the action of single SAR elements is independent of their orientation and their position relative to the gene. A significant difference arises only in cases where the same elements (E and W) are used to create a minidomain, i.e., in a situation where the gene is flanked by two scaffold-attached elements. While their effect on basal transcriptional levels is roughly additive (construct E–sv–W, black bar), the transcription in the presence of butyrate increases in a synergistic manner (same construct, hatched bar). This synergism appears to be typical for a minidomain since it cannot be reproduced by accumulating the entire SAR potential on one side of the gene (construct EW–sv).

Butyrate Exerts a Direct Effect Independent of de Novo Protein Synthesis. Several investigators have studied the influence of the translational inhibitor cycloheximide (CHX) upon the stimulatory action of butyrate. In a few systems CHX prevents stimulation, such as in the case of germ cell alkaline phosphatase (Pan et al., 1991) and the expression of immediate–early genes of HSV (Kemp & Latchman, 1989). Here, butyrate appears to function in an indirect manner, probably by promoting the synthesis of a mediator protein. More typically, butyrate acts in the absence of protein biosynthesis. Prominent examples are constructs driven by the HIV long terminal repeat (Bohan et al., 1989), the metallothionein promoter (Birren & Herschman, 1986; Thomas et al., 1991), and the α-gonadotropin gene (Darnell, 1984). The only explanation that has been advanced so far for such a direct action of the fatty acid is the modification of a preexisting factor, although knowledge about the nature of such a process is lacking.

Figure 2 compares the effect of 5 mM butyrate in the presence and absence of CHX for the luciferase transgenes sv and E–sv under the control of the SV40 promoter/enhancer. The results of Northern blots are presented. At a concentration of 10 μg/mL, CHX causes at least a 20-fold reduction of luciferase activity, but not mRNA levels, demonstrating the expected biological effect of the inhibitor. On the other hand, mRNA levels are strongly increased by the presence of both an SAR element and the epigenetically active agent butyrate; this effect is not impaired by CHX. If the blot is rehybridized with probes against the endogenous housekeeping genes pyruvate kinase (Figure 2) or β-actin (not shown), these genes are found to be refractory to the actions of butyrate. Therefore, its activity depends on either the structure of the promoter,

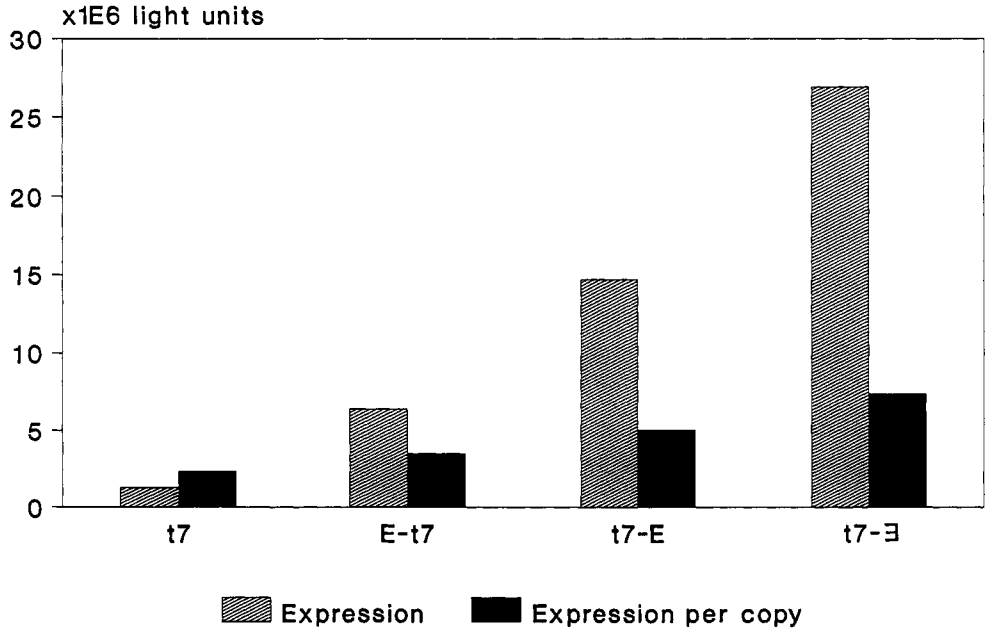

FIGURE 4: Simple TATA box (i.e., the T7 promoter) sufficient for mediation of basal SAR effects. T7-luciferase constructs were complemented by standard SAR element E, derived from the huIFN-β upstream region. Direct expression data (hatched bars) and expression levels referenced to a single gene copy (solid bars) are compared. E and Ǝ represent opposite orientations of the SAR element referring to the gene. Position-dependent differences for the constructs have been explained in the text.

the properties of the particular chromosomal locus, the presence of scaffold-attached regions that enable the fixation of a particular topology, or any combination of these factors. In this situation we first performed a model experiment demonstrating the relevance of template topology and then addressed the question of the relative role of promoter elements.

Direct Actions of Butyrate Can Be Mimicked by Using Circular, Supercoiled Templates. Several lines of evidence suggest that transcriptionally poised chromatin is negatively supercoiled or underwound [summarized by Cockerill and Garrard (1986)]. Butyrate, by its action on histone deacetylases, mediates the hyperacetylation of the nucleosome core, creating unconstrained negative supercoils (Norton et al., 1989, 1990). The negative superhelicity would be fixed on an extrachromosomal circular template (which under these circumstances resembles a minidomain), but not on a linear template.

Figure 3 compares the effect of 5 mM butyrate on the transient expression of a vector in its supercoiled and linear forms, respectively. For three separate cell lines, the action of the reagent on linear templates is negligible, whereas the transcriptional stimulation with circular templates varies between 8- and 22-fold for the SAR-free construct. The presence of an SAR, which would support the effect of butyrate for integrated, stably expressed genes, counteracts the reagent in the case of the episomal templates. This observation is in line with earlier studies demonstrating the opposing effects of SARs on integated and extrachromosomal DNA (Stief et al., 1989; Klehr et al., 1991).

The Interplay of SARs and Butyrate in the Context of a Minimal Promoter. Transgenes under the control of the bacteriophage T7 promoter were originally designed to test their transcription in engineered eukaryotic cells expressing a transfected T7-polymerase gene (cf. Lieber et al., 1993). These studies included a number of controls which clearly demonstrated a predominant contribution of RNA polymerase II to the overall transcriptional levels (Lieber et al., 1993; Sandig et al., 1993; T. Schlake, unpublished results). Since besides an imperfect TATA box (TATAGG) the promoter contains no other sequences supporting initiation by this enzyme, we used the same constructs in order to investigate the activity of SARs and butyrate on a minimal promoter for RNA polymerase II.

Due to the short extension of the T7 sequence used, there is an almost direct apposition of the attachment region and the TATA box for construct E–t7 (spacer, 28 bp). As in other related cases, such an arrangement may cause a steric impediment, limiting the positive transcriptional effect of an SAR (K. Maass and J. Bode, unpublished results). In line with such an interpretation, the SAR effect increases as the SAR potential is positioned at the downstream end (t7–E), and it increases further if the prominent binding motifs are shifted for another 900 bp by an inversion of element E (construct T7–Ǝ; see Figure 4).

The interpretation of the raw data is complicated further by the fact that SAR element E increases the transcription of a T7-driven construct on two levels: (i) a 1.5–3 fold transcriptional enhancement referring to a single copy of the gene or SAR–gene combination, respectively (this is the way data are generally presented; Figure 4, solid bars); (ii) a 5–21 fold overall rise in transcriptional levels, which could be ascribed to the fact that element E tends to increase the number of integrated copies (Figure 4, hatched bars). This intrinsic feature of element E is reminiscent of the properties of other AT-rich, amplification-promoting sequences, such as the muNTS elements described by Wegner et al. (1989), and will be the subject of a forthcoming contribution (T. Schlake and J. Bode, manuscript in preparation). We can confirm that the copy number effect is a property of some but not all SAR elements and that it can be separated from the standard SAR functions. Element W, for instance, is a prototype SAR element without such a side effect.

Figure 5 demonstrates that the expression of non-SAR and SAR constructs based on the T7 TATA box is moderately but uniformly increased by butyrate. Depending on the cell type used for the transfection experiments, the stimulation factor is about 2 (L cells) or 4 (BHK cells). In conclusion, a minimal promoter responds to the individual actions of SAR elements

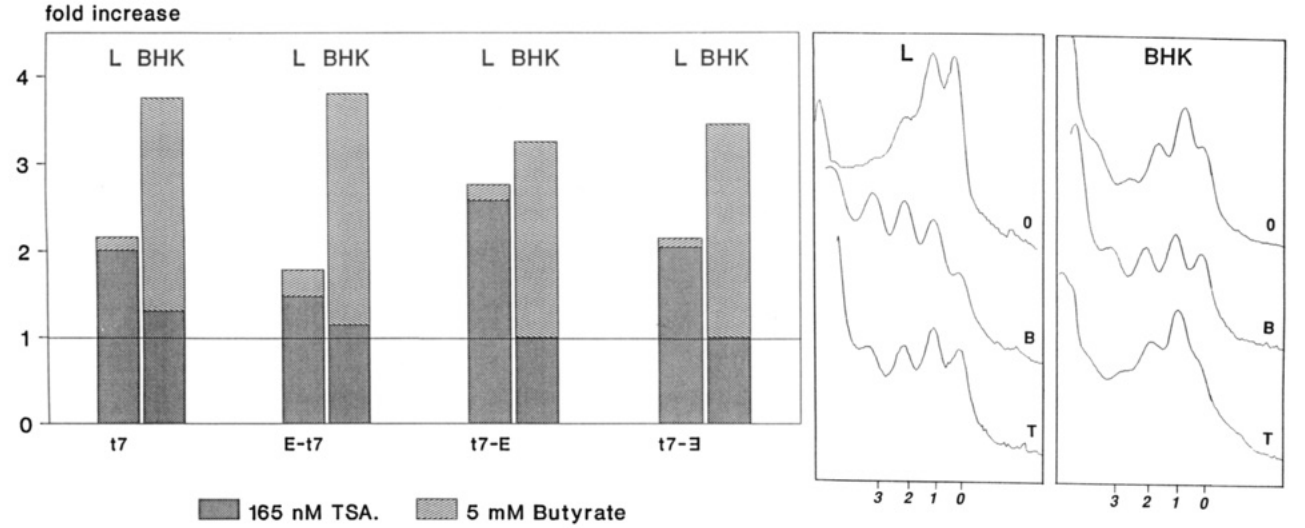

FIGURE 5: Effect of butyrate matched by the specific histone deacetylase inhibitor trichostatin A (TSA) in L cells. Bar diagram: Transcriptional stimulation. Butyrate stimulates the transcription of T7-luciferase-based constructs whether they contain an SAR element or not. TSA, on the other hand, is active only in L cells and is essentially inactive in BHK cells. Densitograms: The overall acetylation state of BHK and L cells. The potential of butyrate (B) or TSA (T) to act as a deacetylase inhibitor is determined by analyzing the acetylation states of histone H4 (numbers 0–3 mark the 1–3-fold acetylated forms). Analyses were performed by the two-gel system of Schröter et al. (1981) and were evaluated by densitometry.

Table 1: Transcriptional Stimulation of Various Promoters by SARs and/or Butyrate[a,b]

					distal element		fold stimulation		
promoter	CCAAT	GC	TATA	enhancer	SAR	LCR	SAR	But.	SAR + But.
HIV	+	+	+	+			8×	4×	**50×**
huIFN	–	–	+	+	+[c]		8×	1×	**30×**
muHMG	–	+	–				3×	5×	**20×**
muMT	–	+	+	+		+[d]	6×	80×	**1900×**
T7	–	–	+	–			3×	2×	**6×**

[a] Promoters have been classified according to various combinations of regulatory sequences. In the case of the distal elements, SARs and LCRs have to be distinguished. The nature of the distal element of the muMT promoter has yet to be determined. The isolated effects of an SAR element (in the absence of butyrate) and of butyrate (on the SAR-free construct) are summarized and compared with the effect of the fatty acid on an SAR construct. For all examples but T7, a synergistic action of both factors is evident since the combined effect exceeds the product of the isolated contributions (boldface numbers). [b] Single SAR-element (E), 5 mM butyrate. [c] Bode and Maass (1988). [d] Palmiter et al. (1993).

or butyrate in a way similar to that of the highly evolved analogues. However, it will be shown later that there is a distinct difference if both parameters are combined (Table 1). For an interpretation of these effects, it is indispensable to support the common view that the main action of butyrate is mediated by histone hyperacetylation. This has become feasible due to the recent discovery of a different agent affecting the turnover of histone acetyl groups, (*R*)-trichostatin A (TSA).

TSA, a Novel and Highly Specific Inhibitor of Histone Deacetylases. (*R*)-Trichostatin A (TSA) was originally reported as a fungistatic antibiotic (Tsuji et al., 1976). Studies on the cellular targets of this agent showed that nanomolar concentrations were sufficient for mimicking one of the numerous primary effects of butyrate, i.e., the inhibition of histone deacetylase(s) (Yoshida et al., 1990). Interestingly, this agent triggers a number of secondary effects that have been also observed for the pleiotropic reagent butyrate, such as the induction of Friend erythroleukemia cell differentiation and the inhibition of the cell cycle at G1 and G2. For TSA, all known effects are strictly stereospecific as they are not found for the (*S*) enantiomer, suggesting that this reagent can be utilized to dissect and classify those effects of butyrate that are due to histone hyperacetylation.

Along these lines, we have replaced 5 mM butyrate with 165 nM TSA. We could observe a comparable action of the drug for L but not BHK cells (Figure 5) and therefore decided to perform an analysis of the acetylation states of histone H4 using the conventional techniques developed in this laboratory (Schröter et al., 1981). The results (right-hand densitograms in Figure 5) demonstrate comparable H4 acetylation by either butyrate (B) or TSA (T) in the case of the L cells. This result contrasts with the analyses on BHK cells, which are affected by butyrate but not by TSA. Since the presence of functional histone deacetylase(s) is clearly documented by the use of butyrate, the most likely explanation for the missing effects of TSA is a failure of the agent to be transported to the nuclear compartment. For these reasons, BHK cells were excluded from all subsequent experiments that involved a comparison between TSA and butyrate.

Response of Various Promoters to Histone Hyperacetylation. In the case of the HIV-1 long terminal repeat, the elements responding to butyrate have been traced, by mutagenesis, to a triplet of Sp1 binding sites (GC boxes). For HeLa cells, the destruction of these boxes nearly abolished the butyrate-mediated, approximately 30-fold transcriptional stimulation of an LTR-CAT construct (Bohan et al., 1989). On the other hand, it was reported by the same authors that both the SV40 promoter (containing two GC boxes per 21-bp repeat) and the HMG promoter (erroneously quoted as lacking Sp1 sites but containing three GC boxes in the proximal 5′-region; cf. Reynolds et al., 1984) respond poorly to butyrate.

Incidentally, among our examples, the only case where there is no net effect of butyrate is an huIFN-β construct covering

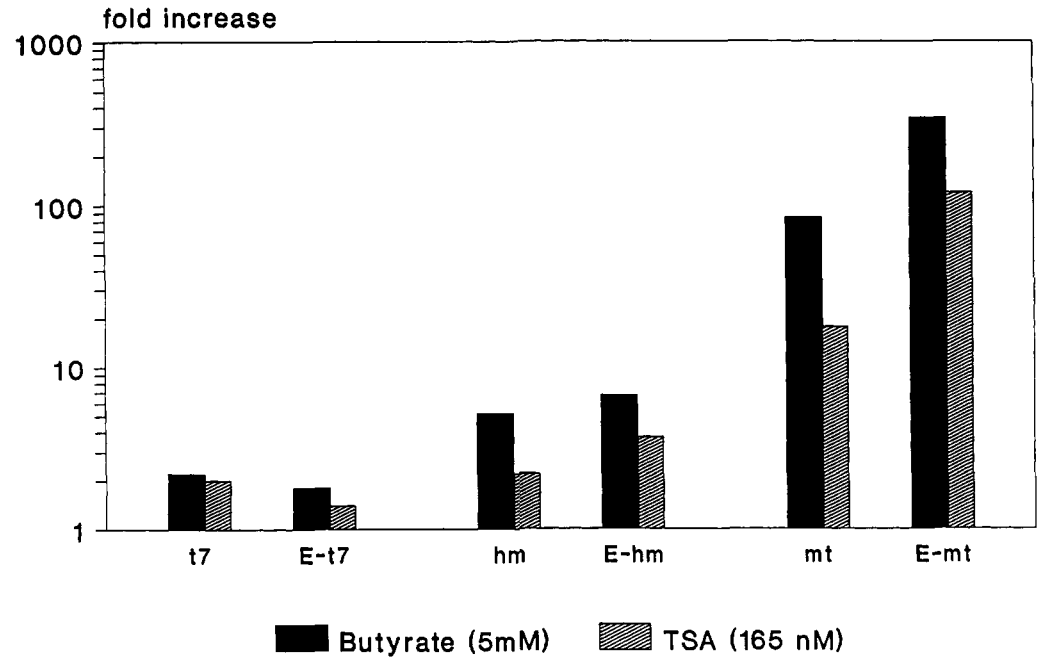

FIGURE 6: Histone hyperacetylation stimulated transcription of non-SAR and SAR constructs. The extent of stimulation depends on the nature of the promoter. L cells were transfected with a luciferase-based construct under the control of the T7 (t7), HMG (hm), or metallothionein (mt) promoters, respectively. Transcriptional stimulation factors due to butyrate or TSA were determined for the basal constructs and the analogous constructs supplemented by SAR element E.

the immediate 284 bp of regulatory upstream sequences, which are devoid of a GC box (Klehr et al., 1992; data included in Table 1). In its natural context, the huIFN-β gene domain comprises more remote sequences, i.e., SAR elements (Bode & Maass, 1988), the regulatory relevance of which have been demonstrated (Klehr et al., 1991). If these elements are added to the basic construct, a 30-fold stimulation by butyrate can be monitored, which closely matches the respective figure for the gene in its natural environment, i.e., in human Namalva lymphoma (Adolf & Swetly, 1979) and human MG63-osteosarcoma cells (Bode et al., unpublished results). Observations of this type led to the conclusion that SAR elements are the basis for at least some actions of butyrate (Klehr et al., 1992).

In Figure 6 we compare the effects of 165 nM TSA and 5 mM butyrate for three widely different promoters: one consisting of a TATA box (T7), one containing three proximal Sp1 boxes but no TATA box (HMG; cf. Reynolds et al., 1984), and a composite one containing a variety of elements such as GC boxes, TATA boxes, and inducible enhancer elements (MT). In its natural surroundings, the MT gene also responds to distal elements which mediate a position-independent and elevated expression. These features would qualify them as being either SARs or LCRs (locus control regions; Palmiter et al., 1993). Many studies have been devoted to the butyrate response of the MT promoter, but to our knowledge no causal relationship has yet been delineated. Our measurements add to the subject the following important aspects: the inhibition of histone deacetylases, and, hence most likely histone hyperacetylation is causal to the increase of transcript levels, which can be up to 80-fold; the promoter is strongly supported by the presence of an SAR (a single element of this type (E) yields a 6-fold enhancement; if both parameters (presence of an SAR and hyperacetylation) are combined, they act synergistically, causing a 1900-fold stimulation (cf. Table 1).

These properties of the metallothionein promoter made it an ideal example for a closer correlation of effects caused by the pleiotropic reagent, butyrate (Boffa et al., 1981), and the specific one, TSA (Yoshida et al., 1990; Figure 7). Earlier studies have shown that 5 mM concentrations of butyrate are sufficient to achieve the maximum degree of both acetylated histones (Schröter et al., 1981) and transcriptional levels (Klehr et al., 1992). It is demonstrated in Figure 7D that, for the SAR construct E–mt–luc, the effect of 5 mM butyrate can be closely approximated by TSA concentrations exceeding 300 nM. More importantly, the presence of 0–800 nM TSA in addition to 5 mM butyrate is without any further effect. If, on the other hand, butyrate is applied at suboptimal concentrations (1 mM, cf. Figure 7B), additional 200 nM TSA will restore the maximum response and, hence, substitute for the fatty acid. These observations suggest that in the case of SAR constructs the effect of butyrate is fully accounted for by histone hyperacetylation.

For a non-SAR construct (mt–luc), TSA approaches the effect of 5 mM butyrate to about 70% (Figure 7C). The action of 1 mM butyrate is surpassed by even the lowest concentrations of TSA, and similar end values are reached at 800 nM TSA, irrespective of the presence of fatty acid (Figure 7A). These observations permit the following conclusions: (1) 1 mM butyrate causes some histone acetylation and no major side effects, since the results for 800 nM TSA or 800 nM TSA plus 1 mM butyrate are comparable; (2) 5 mM butyrate causes an effect in addition to histone acetylation since its stimulatory level is approached but not attained by TSA. This additional effect is much less pronounced in the case of the SAR construct because of the synergism of SAR action and hyperacetylation, which results in a ca. 4-fold higher stimulation of expression by butyrate in comparison to the non-SAR construct.

DISCUSSION

One of the earliest actions that was ascribed to butyrate as a medium component was its effect upon the differentiation of Friend murine erythroleukemia (MEL) cells, i.e., transformed erythropoietic cells that were released from an arrest

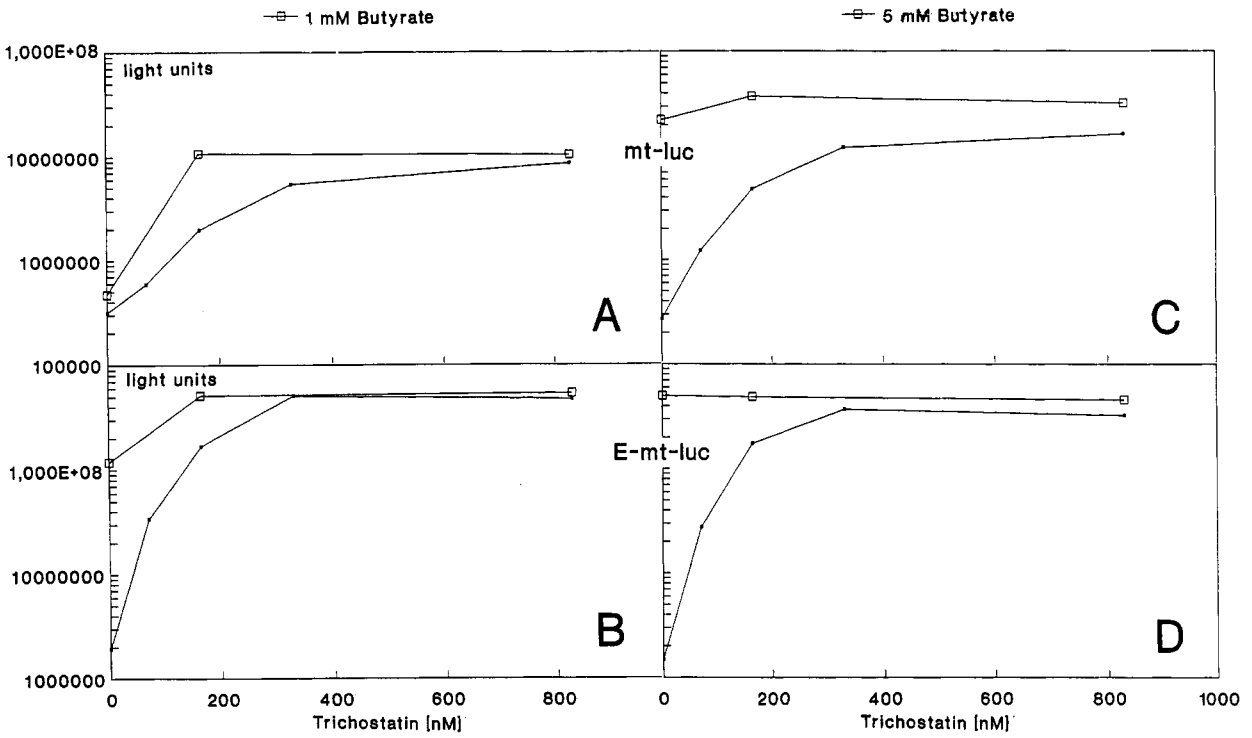

FIGURE 7: Butyrate and trichostatin A acting along the same pathway, exerting transcriptional stimulation. For parts A and B, cells harboring constructs mt–luc or E–mt–luc were treated with 0–800 nM TSA in the presence (upper traces) or absence (lower traces) of 1 mM butyrate. Similarly, for parts C and D the same cells were treated with TSA in the presence or absence of 5 mM butyrate.

at the proerythroblast stage (Reuben et al., 1980). An early clue as to its molecular action came from reports by Riggs et al. (1977) stating that butyrate causes massive hyperacetylation of the core histones, an effect that was later assigned to its function as a noncompetitive inhibitor (K_i = 60 μM) of histone deacetylase(s) (Vidali et al., 1978; Boffa et al., 1978; Cousens et al., 1979).

The differentiating activity of butyrate is shared by a variety of other so-called "Friend-cell inducers", which do not belong to the classes of fatty or dicarboxylic acids (hexamethylenebis-(acetamide) (HMBA), DMSO, nicotinamide) and do not affect the overall acetylation state (Candido et al., 1978; Schröter et al., 1981). It was therefore difficult to postulate a direct correlation between these effects, although we could demonstrate that a prototype stimulator, HMBA, supported histone acetylation in an indirect manner (Bode et al., 1982). With the advent of the highly specific deacetylase inhibitor (*R*)-trichostatin A (K_i = 3.4 nM), which mimicked the effects of butyrate and HMBA at nanomolar concentrations, a direct correlation of these events has become likely (Yoshida et al., 1990). In the present study, TSA is used in an analogous way to support recent data suggesting a role for SAR elements in the stimulation of transcriptional levels by histone hyperacetylation.

Our previous work was concerned with the stable expression of interferon and luciferase indicator genes under the control of five different promoters. The response of these promoters to butyrate was variable, ranging from negligible (huIFN-β) to stimulation factors of 80 (MT, cf. Table 1). After the addition of a single SAR element (E), a positive response was monitored in all cases. Interestingly, for most promoters this response was synergistic, exceeding the product of the stimulatory factors for an SAR element and butyrate (Table 1).

After transfection, transgenes integrate more or less at random into a chromatin domain of the recipient cell. Active domains are rather small (5 kb for the histone gene cluster in *Drosophila*, 14 kb for the human interferon-β gene), but domain sizes vary and can reach up to 200 kb. The addition of a single SAR element to a transgene will reduce the effective domain size and, hence, support transcription. Although the molecular reasons for an inverse relationship of transcriptional activities and domain sizes have not been fully established, the possibility that small domains efficiently stabilize superhelical states of DNA appears intriguing. Along these lines, we have constructed transgenes that are flanked by an SAR element on either side, forming minidomains of about 5 kb. Without exception, two flanking SARs supported the response to butyrate more strongly than a single fragment. Early constructs of this type were composed of SARs E and P (tobacco ST-LS1 gene, weakly binding), whereas the present series of constructs comprises an example with fragments E and W (tobacco ST-LS1 gene, strongly binding). In perfect agreement with expectations, the stimulation factors increased from 2 (sv) to 4 (E–sv), 8 (E–sv–P), and 13 (E–sv–W) (Figure 1; Klehr et al., 1992).

We have previously suggested a simple model based on the sole assumption that butyrate triggers the hyperacetylation of the core histones followed by a number of topological changes (Klehr et al., 1992). The present work includes studies on episomal templates, which are able to stabilize changes in linking numbers in a way resembling chromosomal domains. The results (Figure 3) clearly emphasize the role of template topology. Using TSA as a novel tool we could confirm the notion that the predominant effects of butyrate are due to its influence on acetyl turnover (cf. Figure 7). Hyperacetylation of nucleosomal cores has been shown to generate unconstrained negatively supercoiled DNA *in vitro* (Norton et al., 1989, 1990) and has the same properties *in vivo* (Thomsen et al., 1991). It should be realized in this context that the majority of transfections that have been undertaken to localize the site responsive to butyrate are in fact based on supercoiled templates, since these studies investigated the properties of uncut plasmids during the phase of transient expression (i.e.,

prior to integration; cf. Bohan et al., 1987, 1989; Deng et al., 1992). These circular templates are packaged into nucleosomes that will constrain part or all of the negative supercoils. Histone hyperacetylation would reverse this situation and provide DNA in a superhelical or underwound state, which supports the function of a variety of eukaryotic promoters (Hirose et al., 1988). Recent evidence indicates that a transient and localized occurrence of underwinding suffices for largely facilitated transcriptional initiation (Dunaway & Ostrander, 1993).

What is the connection between a negative superhelical state and promoter function? Weintraub et al. (1986) have demonstrated that supercoiled DNAs yield markedly higher levels of expression than linearized DNA when transfected into L or CV-1 cells. Quite remarkably, the influence of supercoiling was most profound in plasmids containing enhancer sequences. While the results suggested that supercoiling did not influence the binding of enhancer factors *per se*, it nevertheless had an effect upon the recruitment of some limiting transcription factor, e.g., by facilitating the transition to an open complex. Earlier work had already shown that, under the negative superhelicity of a plasmid, S1 hypersensitive sites arise at the precise location in 5′-flanking regions where they also occur *in vivo* (Schon et al., 1983). These sites covered predominantly homopurine–homopyrimidine stretches but also Sp1 sites (Evans et al., 1984), which have been implicated by some authors in the actions of butyrate (Bohan et al., 1989). The topology-dependent structure of these sites is not clear, although there are arguments favoring a non-Z-type left-handed helix (Cantor & Efstratiadis, 1984). Due to their tendency to separate strands, SARs could function as localized sinks for negative superhelicity in the same way that they did in previous *in vitro* experiments (Bode et al., 1992). This would divert topology-dependent changes from the promoter to the SAR, explaining the negative action of these elements during the phase of transient expression (Figure 3). These arguments would not pertain to the integrated state where SARs become fixed due to their attachment to nuclear substructures.

Considering the group of promoters investigated here, the correlation between an effect of butyrate and the presence of an Sp1 (GC) box is not clearcut (Table 1). In stable expression assays, all promoters display significant transcriptional stimulation if they are linked to an SAR element. Moreover, all natural promoters of RNA polymerase II show a synergistic action of SARs and butyrate, and all of them contain one or several upstream elements. Only for the artificial (T7) promoter do the effects of the SAR (3×) and butyrate (2×) combine in a nonsynergistic fashion, yielding a total enhancement of 6×, which may be taken as an indication that the efficiency of upstream elements depends on DNA topology. These results lead to several conclusions that would require further experiments for their verification, as follows.

(1) Butyrate has some basal effect upon the formation of an initiation complex around the TATA box, which is ascribed to a general relaxation of the chromatin structure following histone hyperacetylation (T7). (2) SARs may stabilize the existence of a twin domain structure during transcription and thereby favor reinitiation in the negatively supercoiled wake behind the transcription complex, and this may be effective even for the T7 promoter. (3) The existence of a direct butyrate effect (not involving *de novo* protein synthesis) on transcription is readily explained by the action of negative superhelicity on upstream elements. So far, there is no direct evidence for a common alternative explanation that implies the modification (phosphorylation or acetylation) of a preexisting transcription factor. This alternative had to be considered since butyrate has well-documented effects on phosphate incorporation into histone and nonhistone nuclear proteins (Boffa et al., 1981), which could include phosphorylatable transcription factors or RNA polymerase itself. (4) Maximal synergism between the presence of an SAR and butyrate exists for huIFN-β and metallothionein, i.e., two genes for which the existence of distal elements (domain borders) has been demonstrated. We consider it an interesting possibility that these genes are designed in such a way that makes them maximally responsive to topological changes.

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