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Scaffold-Attached Regions (SAR Elements) Mediate Transcriptional Effects Due to Butyrate†

Dagmar Klehr, Thomas Schlake, Karin Maass, and Jürgen Bode*

GBF, Gesellschaft für Biotechnologische Forschung mbH, Genetik von Eukaryoten, W-3300 Braunschweig-Stöckheim, Germany

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ABSTRACT: The expression of certain genes has been reported to respond positively to sodium butyrate. This study demonstrates the same feature for two marker genes under the control of five different promoters. In all examples, the stimulatory effect is largest if one or especially two scaffold/matrix-attached regions (SAR/MAR elements) are present adjacent to the gene, and in one case, the stimulation depends entirely upon this situation. These results are observed with several SAR sequences including those obtained by oligomerizing short stretches of DNA surrounding a core motif. It is suggested that butyrate exerts important actions at the level of the chromatin structure.

Recent evidence suggests that the eukaryotic genome is organized into topologically constrained domains enabling a differential gene expression (Gasser & Laemmli, 1987; Gross & Garrard, 1987; Kellum & Schedl, 1991). In *Drosophila*, it has been demonstrated that the size of a domain is inversely related to its transcriptional activity (Gasser & Laemmli, 1987). Following this rationale, various groups have tried to isolate the DNA sequences mediating the attachment to the nuclear scaffold or matrix (called scaffold- or matrix-attached regions, SARs or MARs)[1] and to use them alone or in combination in order to create artificial domains with improved transcriptional properties (Klehr et al., 1991; Phi-Van & Strätling, 1988; Phi-Van et al., 1990; Stief et al., 1989).

Some domain borders are constituted by a class of (A + T)-rich DNA elements which were initially detected by differential melting of samples prepared for electron microscopy (Moreau et al., 1981, 1982; Scherrer & Moreau, 1985). It is probably the same class of elements which have been

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* Address correspondence to this author at the following address: GBF, Gesellschaft für Biotechnologische Forschung mbH, Genetik von Eukaryonten, Mascheroder Weg, D-3300 Braunschweig, Germany.

[1] Abbreviations: SAR, scaffold-attached region; MAR, matrix-attached region; IFN, interferon; huIFN-β, human interferon-β; muIFN, murine interferons; muHMG, murine hydroxymethylglutaryl-CoA reductase; neo^r, neomycin-resistance gene (Tn5 aminoglycoside 3′-phosphotransferase); LIS, lithium 3,5-diiodosalicylic acid; PCR, polymerase chain reaction.

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identified either by a halo-mapping procedure using lithium diiodoacetic acid (LIS) to localize such attached regions in a "nuclear scaffold" or by a reassociation approach in which the affinity of labeled fragments to nuclear scaffolds or matrices (isolated by LIS- or salt-extraction procedures, respectively) is quantitated (Farache et al., 1990).

The same procedures detect a related but functionally different type of element which is located within introns, usually close to enhancers. Such an element, termed MAR, was first detected close to the Ig heavy and light (κ) chain genes (Cockerill & Garrard; 1986, Cockerill et al., 1987). More recent examples are elements in the large intron of the β-globin gene (Jarman & Higgs, 1988; Klehr & Bode, 1988), in the second and third introns of the interleukin-2 gene (Straube and Bode, unpublished results), and in the first and second introns of the light-inducible ST-LS1 gene from potatoes (Willmitzer and Bode, unpublished results). Since these SAR/MAR-like sequences do not impede the passage of RNA polymerase II, their occupation must be regulated (Cockerill & Garrard, 1986; Cockerill et al., 1987). The finding of a soluble factor that recognizes sites within the IgH MAR supports this concept (Scheuermann & Chen, 1989).

Our present studies concentrate on a SAR element detected upstream from the human interferon-β (huIFN-β) gene. The element has the following properties. On its 5'-side (position −1730) it is flanked by the predominant DNaseI hypersensitive site of the domain. This site is followed, in the upstream direction, by an extended nucleosome-free, protected region (Bode et al., 1986). The underlying DNA has an affinity for the nuclear scaffold as determined by both the halo-mapping and the reassociation approaches using isolated scaffold or matrix preparations (Bode & Maass, 1988; Mielke et al., 1990). A 2.2-kb fragment (E) harboring the sequences which bind most tightly to the scaffold has been linked to marker genes and was found to increase their expression in an orientation- and position-independent fashion after and *only* after integration of the construct into the genome of the host cell. In contrast, during the phase of transient expression, the element behaved neutral or even antagonistic, which clearly distinguishes its action from that of the classical enhancers (Klehr et al., 1991). We have ascribed these observations to the element's capability of reducing the effective domain size after integration, assuming that the second domain border is provided by the recipient cell. In accord with this concept, the element's effect could be potentiated by attaching a second SAR element (P) to the other side of the marker gene (Klehr et al., 1991). In control experiments, this situation (E–gene–P) is clearly superior to an alternative construct (PE–gene) in which the entire SAR potential is concentrated at one end (Klehr et al., unpublished results).

Gene activation causes the apparent loss of a nucleosome ahead of the upstream SAR element of huIFN-β, and a similar change could also be induced by the action of butyrate (Bode et al., 1986). This effect could be explained by the predominant action of the short fatty acid which inhibits histone deacetylases shifting nucleosomes to a highly acetylated and thereby relaxed state (Bode et al., 1983; Bode, 1984). In the context of a chromatin domain, the loss of nucleosomes would have topological consequences, i.e., would induce negative superhelicity and possibly promote transcription [cf. Norton et al. (1989, 1990)]. Since the components involved in the turnover of histone acetyl groups appear to be parts of the internal nuclear matrix (Hendzel et al., 1991; Loidl, 1990; and personal communications), we became interested in possible influences of butyrate on the action of SAR elements. Here we report the novel observation that SARs and butyrate act synergistically, and this finding may shed light on the function and possible regulation of these elements.

Materials and Methods

(*a*) *SAR-Containing Plasmids.* The constructions of the pAG60-based plasmids pAG·F, pAG·B′, and pAG·A′B′ and the pLu-based plasmids sv (pLu-E), SV (pLu-E-P), and hi (HIV) (see Figure 1) have been described (Klehr et al., 1991; former designations in brackets). All constructs are based on a derivative of pAG60 permitting exchange of promoters via the adjacent *Hin*dIII sites and introduction of SAR elements via a *Bam*HI site (upstream) and a *Nru*I site (downstream).

Plasmids sv′, sv″, sv‴, and sv⁗ are analogous to sv but contain an increasing number of 166-bp units derived from the 2.2-kb SAR E [cf. fragment VIII in Mielke et al. (1990)]. The preparation of 166_n oligomers by the polymerase chain reaction (PCR) has been described (Mielke et al., 1990). Briefly, two 20-bp primers were used to isolate the relevant sequence from 400 ng of human placenta DNA by PCR. Amplification was performed in 40 cycles (1 min, 94 °C; 1 min, 26 °C; 1.5 min, 72 °C). The resulting 317-bp PCR fragment was repaired by Klenow polymerase, phosphorylated by polynucleotide kinase, and cloned into the *Sma*I site of the pTZ-18R polylinker. The 213-bp *Hin*dIII–*Xmn*I fragment was cleaved at the distal and an internal *Sph*I site and cloned (as a 166-bp subfragment) directly into the *Sph*I site of pTZ-18R (Biolabs) to yield monomeric or oligomeric inserts. Oligomers were excised by *Bam*HI plus *Hin*dIII and cloned between the *Bam*HI and *Sal*I sites of pLu.

Construct hm used the *Not*I–*Pvu*II promoter fragment of mouse hydroxymethylglutaryl-CoA reductase which was excised as a *Not*I–*Hin*dIII fragment from vector HMGGal (M. Rentrop, EMBL Heidelberg) and cloned between the *Hin*dIII sites of pLu constructs. The 1.8-kb *Eco*RI–*Xba*I fragment of the murine metallothionein-I promoter was cloned into the multiple cloning site of plasmid pTZ18R and excised as the *Eco*RI–*Hin*dIII fragment. This fragment was again cloned between the *Hin*dIII sites of the respective pLu derivative.

(*b*) *Cell Culture and Gene Transfer.* Mouse L cells and BHK-21 cells were grown as described (Bode et al., 1986). The transfection protocol has been optimized to yield low copy numbers (cf. Figure 4 and Table I) and to avoid head-to-tail integrates (Klehr et al., 1991). 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.

(*c*) *Copy Numbers.* Cells from a 69-cm^2 culture dish were washed twice with PBS. They were incubated for 2 h in 2 mL of a solution containing 0.1 mg/mL of proteinase K in 15 mM EDTA/80 mM Tris-HCl (pH 8.5)/1% SDS. DNAs were then precipitated by the addition of 4 mL of a 0.6 M ethanolic LiCl solution. The precipitates were dissolved in 2 mL of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), extracted by phenol (2×) and $CHCl_3$/isoamyl alcohol (3×), and dialyzed against several changes of TE. After sonication, the resulting solutions were adjusted to A_{260} = 3.0 and denatured by the addition of NaOH to a final concentration of 0.4 M. A series of 1:1 dilutions in 0.4 M NaOH were prepared on a microtiter plate, and 50-μL aliquots were transferred to a Nylon membrane (Bio-Rad Zeta-probe) in a slot-blot apparatus (Millipore). After neutralization in 2×SSC, the membranes were hybridized against plasmids pAG·F (cf. Figure 1) or pLu (Klehr et al., 1991). Where applicable, a subsequent hybridization against an 805-bp fragment from the endogenous murine interferon-β gene served to account for small variations in the

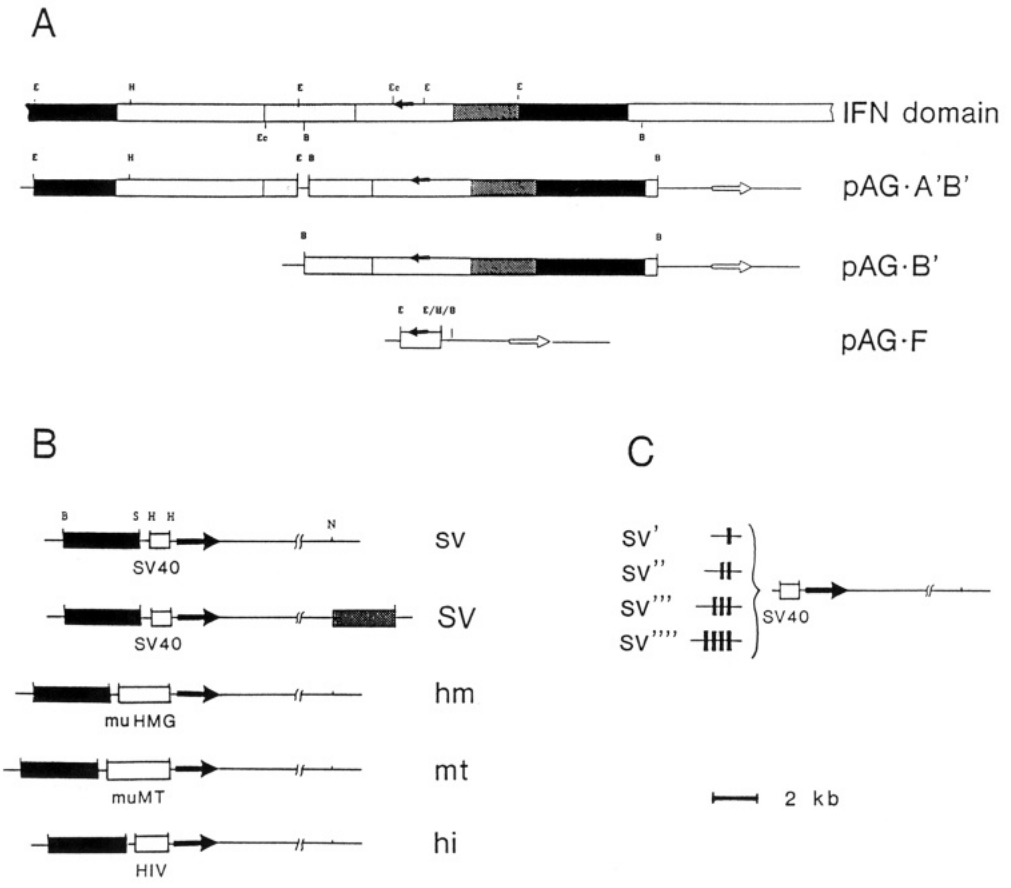

FIGURE 1: SAR constructs used in the present study. (A) Constructs based on huIFN-β (solid arrow) and the neo^r resistance gene (light arrow) are as described (Klehr et al., 1991). Filled boxes adjacent to the 14-kb IFN domain mark regions with a strong (black) or intermediate (hatched) affinity for the nuclear scaffold (Bode & Maass, 1988). The black part of the upstream SAR element has been termed E. (B) Constructs based on firefly luciferase (solid arrow) under the control of various promoters cloned between the *Hin*dIII sites of pLu (Klehr et al., 1991). SAR elements, cloned into the *Bam*HI and *Nru*I sites of pLu, were derived from huIFN-β (black) and the ST-LS1 gene (hatched). They correspond to elements E and P as described in Klehr et al. (1991). For each of the promoters, a SAR-free control was also available (sv^o, hm^o, mt^o, and hi^o). (C) Constructs related to sv but using oligomers (up to the tetramer) of a 166-bp sub-SAR fragment located between positions 781 and 946 of the 2.2-kb SAR element E. Abbreviations: B, *Bam*HI; E, *Eco*RI; Ec, *Eca*I; H, *Hin*dIII; N, *Nru*I; S, *Sal*I.

Table I: Number of Integrated Copies Obtained after Transfection of the Constructs of Figure 1[a]

	pAG.A'B'	pAG.B'	pAG.F	hm	mt	hi	sv^o
L cells	3.4	1.2	1.2	2.3/1.7	1.7/1.3		1.7
BHK cells						2.3/2.0	2.7

	sv	SV	sv'	sv''	sv'''	sv''''	sv^7
L cells	2.7	3.3	1.3	1.7	2.3	2.3	7.5
BHK cells	3.0	4.7					

[a] In the case of the constructs hm, mt, and hi, data refer to the respective constructs in the presence and absence of a SAR element (separated by a slash). For constructs based on the SV40 promoter, sv^o refers to the SAR-free construct; the remainder are the SAR constructs depicted in Figure 1.

amount of DNA submitted to hybridization.

Results of this assay on 30 clonal lines derived from construct hm and its SAR-less analogue are reproduced in Figure 4; copy numbers of 1 were verified by an analysis of bordering fragments via Southern blotting. Average copy numbers for mixed populations of clones are compiled in Table I. Except for the data in Figure 4, the relevant expression levels refer to a single copy; i.e., they have been normalized in the cases of Figures 2, 5, and 7.

(*d*) *Treatment with Stimulatory Reagents.* Semiconfluent cells were kept for 48 h in standard DME/10% FCS medium at the reagent concentrations indicated in the legend to Figure 3. They were then briefly washed with PBS and submitted to the following tests.

(*e*) *Assays for Gene Expression. Interferon*: The methods used for determining the inducible expression of interferon were essentially those described by Dinter and Hauser (1987). For the present experiments, induction of confluent monolayers of transformed L-cell pools was achieved by a 1-h treatment with Sendai virus in butyrate-free media. Cells were provided with new (butyrate-free) medium which was analyzed, after 20 h, for the interferon titers. Titers were determined on Vero cells (huIFN-β) or mouse-Ltk$^-$ cells (muIFN) after challenging with vesicular stomatitis virus and referenced to 10^6 cells and 1 mL of supernatant.

Luciferase: Extracts were directly prepared from a defined number of cells which had been grown in the presence or absence of butyrate and tested for their luciferase activity (Klehr et al. 1991). Usually 10^5–10^6 cells were lysed in 300–1000 μL of extraction buffer (0.1 M KH_2PO_4, pH 7.8, 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.

Internal controls: Where butyrate had major effects on the transfected marker genes, expression levels were also determined for the endogenous murine interferon, pyruvate kinase (PK), or actin gene(s); muIFN was quantified by the biological test (see above), and PK/actin was quantified by Northern blotting. muIFN served as a responsive control (8-fold stimulation in butyrate; cf. insert of Figure 2) and PK/actin served as unaffected controls (not shown). Since these internal controls were indistinguishable for cells prior to and after

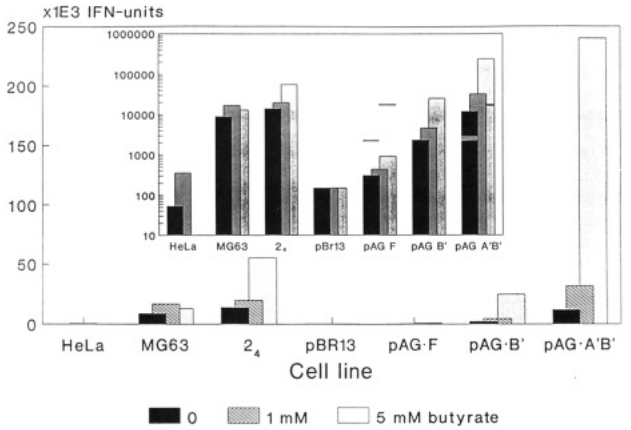

FIGURE 2: Enhancement of interferon expression due to the presence of butyrate. HeLa and MG63 are the nonmanipulated human cell lines assayed for the expression of the intrinsic interferon genes. Line 2_4 has been established by transfecting a 36-kb segment of human DNA into mouse L cells (Hauser et al., 1982); this segment was later found to contain the SAR element upstream of the huIFN gene (Bode & Maass, 1988); in this respect it resembles pAG·B′ (Figure 1). pBR13 and pAG·F are constructs based on plasmids pBR322 and pAG60′ (Klehr et al., 1991) and include only the immediate regulatory elements of huIFN-β. pAG·B′ and pAG·A′B′ were obtained by providing pAG·F with one or two SAR elements as shown in Figure 1. If expression levels at 0, 1, and 5 mM butyrate are normalized for construct pAG·F (=1), the enhancement factors for pAG·B′ and pAG·A′B′ are 7.5 and 38.3, respectively, without butyrate, 10.3 and 71.1 at 1 mM butyrate, and 27.1 and 259.7, respectively at 5 mM butyrate. Insert: Representation of the same expression figures on a logarithmic scale. Vertical bars mark levels of the endogenous muIFN activity which coinduced in cell lines pAG·F and pAG·A′B′.

transfection, no further correction was considered necessary.

RESULTS

Effects of Butyrate on the Inducible Interferon Promoter. Several groups have reported the observation that a variety of structurally unrelated substances increase the production of human lymphoblastoid interferon by Namalva cells after an induction by Sendai virus (Adolf & Swetly, 1979; Bode et al., 1982; Johnston, 1980; Morser et al., 1980). Active substances include the established inducers of erythropoietic differentiation in mouse erythroleukemic spleen cells (MEL or Friend cells). Within this group, the short-chain fatty acids (C_3–C_5), particularly butyrate, emerged as the most powerful reagents (Adolf & Swetly, 1979). This principle has been used for the large-scale production of lymphoblastoid interferon (Johnston, 1979; Swetly et al., 1979).

In the past we have investigated a variety of cell lines for a similar effect of butyrate. Figure 2 demonstrates a significant increase (7-fold) in the titer of interferon caused by this compound in HeLa cells and a smaller one (2-fold) in human MG63 osteosarcoma cells. Murine L-cells yield an 8-fold stimulation of the endogenous muIFN genes which served as a valuable control in the experiments to follow.

The interferon (IFN) produced when human cells are treated with viruses or dsRNA is usually a mixture of IFN-α and IFN-β which are not discriminated in the biological test. Transfection of a defined human gene into a rodent cell line is one way to study effects specific for its expression because the test discriminates between the human and the coinduced murine interferon activities. We have reported that after transfection of a 36-kb fragment of human DNA including the huIFN-β gene, a butyrate-induced enhancement of IFN-β expression can be monitored (Bode & Maass, 1988; and cell line 2_4 in Figure 2). However, we have also found that this effect is lost when only the immediate regulatory sequences of huIFN-β, as contained in the 1.9-kb *Eco*RI fragment, are used for transfection. Figure 1A shows the localization of this fragment within the IFN domain which has been inserted into the *Eco*RI site of plasmid pBR322 to yield construct pBR13 (Figure 2).

Recently, we have performed a reconstruction of the huIFN-β domain (cf. Figure 1) in order to elucidate the relative role of regions with an affinity for the nuclear scaffold (Klehr et al., 1991). The major steps of this series are represented by constructs pAG·F (no SAR character), pAG·B′ (upstream SAR element), and pAG·A′B′ (complete chromatin domain including the upstream and downstream SAR elements). The results depicted in Figure 2 (black bars) show that within this series the expression increases with the extension of SAR elements as already reported for the induction of IFN-β by poly(rI)·(rC) (Klehr et al., 1991).

Beyond these findings, Figure 2 shows that the enhancement due to a SAR element is strengthened further if cells are pretreated with 1 mM (cross-hatched bars) or even more so with 5 mM butyrate (stippled bars). Obviously, this effect increases with SAR length being most pronounced for the entire chromatin domain (pAG·A′B′) which enhances the expression level 38-fold for cells grown in regular medium and 260-fold for cells in medium supplemented with 5 mM butyrate.

Efforts have been undertaken to demonstrate that these effects do correlate with the SAR properties: (i) no transcriptional enhancement and no butyrate effect are seen after insertion of an equally (A + T)-rich non-SAR control [fragment XI in Mielke et al. (1990)] between the huIFN and neo^r genes, respectively; (ii) SAR elements from huIFN-β have been cloned in either orientation and at various distances relative to huIFN-β and neo^r [Figure 3 in Klehr et al. (1991)]. The expression of all these constructs has been reinvestigated in the presence of 5 mM butyrate and in all cases SAR-specific effects could be increased about 10-fold. On the other hand, the shielding effect observed when neo^r was inserted between the SAR element and the huIFN marker gene was reproduced also for butyrate-containing media (data not shown).

Adolf and Swetly have reported that IFN expression levels in Namalva cells respond to the whole spectrum of Friend cell inducing substances (Adolf & Swetly, 1979). Therefore, we have also tested other prototypes of this series, i.e., propionate (5 mM), hexamethylenebisacetamide (10 mM), and dimethyl sulfoxide (280 mM) which in their report yielded a 33-, 5-, and 16-fold increase of IFN titers, respectively, for Namalva. The corresponding data, derived for mouse cells after transfection with construct pAG·A′B′, are summarized in Figure 3, which demonstrates an activity for the short fatty acids butyrate and propionate upon the endogenous muIFN genes and upon the transfected copy of pAG·A′B′. DMSO and HMBA had some activity for pAG·A′B′ but not for muIFN. Bromodeoxyuridine (80 nM BrdU) was totally inactive; its apparent activity in Namalva (Shuttleworth et al., 1983) could be explained by the induction of C-type viruses.

Other Promoters. Next we asked whether the action of Friend cell inducing substances is restricted to the interferon system. Expression of IFN might be considered a special case since it requires induction and the strict absence of butyrate during the 1-h induction period (Adolf & Swetly, 1979; Bode et al., 1982). For the data in Figure 3 (right side), we chose the promoter of a housekeeping gene, hydroxymethylglutaryl-CoA reductase (HMG), coupled to luciferase as the detector gene. In the absence of a SAR element, all compounds counteracted gene expression. If the gene was provided

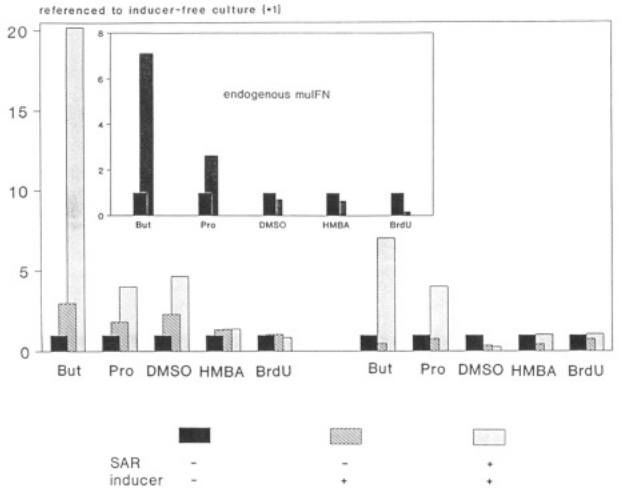

FIGURE 3: Among the MEL cell inducing compounds, only the short fatty acids butyrate (But) and propionate (Pro) have a broader activity. Left series: action of various MEL inducers and BrdU on the expression of the transfected huIFN-β construct pAG·A′B′. Insert: the corresponding muIFN levels arising from the endogenous interferon genes of the recipient murine L cells. Right series: action of the same compounds on the expression of luciferase, controlled by the HMG promoter (construct hm). Abbreviations: But, 5 mM butyrate; Pro, 5 mM propionate; DMSO, 280 mM dimethyl sulfoxide; HMBA, 10 mM hexamethylenebisacetamide; BrdU, 80 nM bromodeoxyuridine.

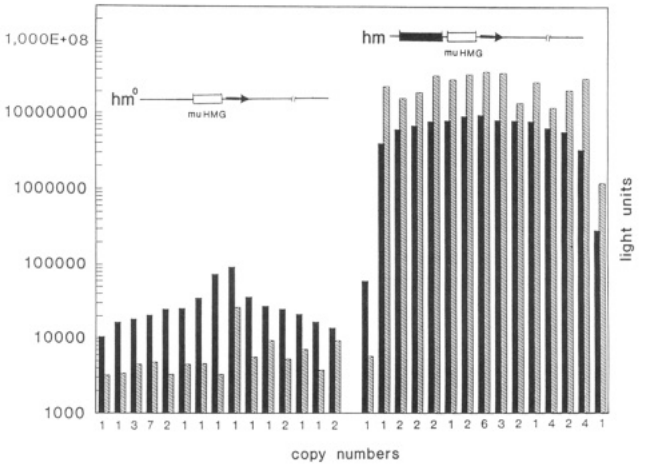

FIGURE 4: Clonal cell lines obtained after transfecting construct hm (right part) or its SAR-free counterpart hm° (left part) into L cells. Solid bars mark the expression in the absence and hatched bars mark the expression in the presence of 5 mM butyrate. In the presence of butyrate, the luciferase activity is decreased for any of the 15 SAR-free clones whereas it is potentiated in 14 out of 15 clones derived from the SAR construct. As an exception, these data do not refer to the single-copy level. The number of integrated copies is found below the graph.

with a SAR at an upstream position (construct hm; cf. Figure 1B), only the short fatty acids butyrate and propionate displayed a significant ability to stimulate expression from the HMG promoter. Incidentally, these are the only substances that affect the turnover of acetyl groups leading to nucleosomes with hyperacetylated histones (see below).

The constructs based on hm were chosen for a closer inspection (Figure 4). Fifteen out of a total population of 200 clones were isolated and tested for their individual copy numbers and luciferase activities. A corresponding set of clones was also derived from the transfectants harboring the SAR-free control constuct (hm°). With a single exception, all individual clones exhibited the behavior expected from the cell populations; i.e., in the absence of a SAR the expression of the test gene was decreased by butyrate (average depression 4.7-fold) while in the presence of SAR element E it was increased (average enhancement 2.8-fold).

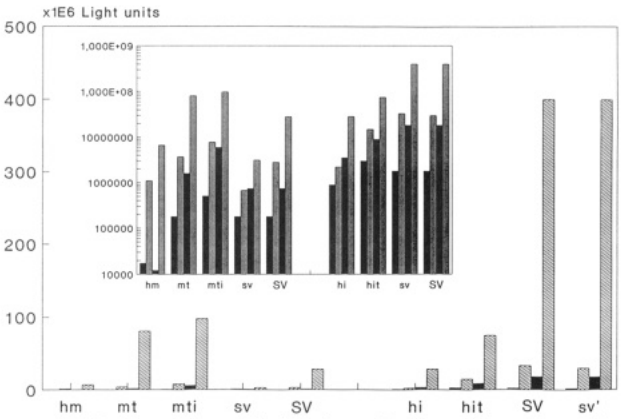

FIGURE 5: Promoter strength of various pLuc constructs depending on cis-acting SAR elements and the presence of sodium butyrate (5 mM). The constructs shown in Figure 1B and their SAR-free counterparts have been investigated for luciferase expression. Each experiment is characterized by a quadruplet of bars referring (from left to right) to the SAR-free construct, SAR construct, SAR-free construct + butyrate, and SAR construct + butyrate. Each couple of black bars corresponds to SAR-free plasmids and each couple of hatched bars corresponds to SAR plasmids. Left: Constructs expressed in mouse L cells. mti stands for mt but after a 48-h induction with 100 μM Zn^{2+} plus 6 μM Cd^{2+}. Right: Constructs expressed in BHK cells. hit stands for as hi but was trans-activated by the tat protein. Insert: same data represented on a logarithmic scale.

In Figure 5 we have extended the range of promoters to include SV40 with two or one SAR (constructs SV and sv), the metallothionein promoter in its induced (mti) and ground state (mt), and the HIV LTR transactivated (hit) or not (hi) by the tat protein. The expression with and without the SAR element(s) is compared. In this series, the HMG promoter (hm) remains the only case where a stimulatory effect of butyrate is strictly dependent on the presence of a SAR. In all other examples, we find a stimulation whether a SAR is present (compare the cross-hatched bars) or absent (compare the black bars). However, an important rule implicit in the data of Figure 5 is the fact that butyrate consistently acts more strongly on the SAR constructs (compare the set of black bars with the set of cross-hatched bars in Figure 5) or, conversely, that a SAR element becomes more potent in media containing butyrate (compare the first couple of black/cross-hatched bars within each quadruplet with the second couple of black/cross-hatched bars in Figure 5).

These features become more explicit by the representation chosen for Figure 6. Here the enhancement factors due to the presence of a SAR have been compared in regular and butyrate-containing media. The most extreme example is the HMG promoter for which our standard SAR element, the 2.2-kb E fragment derived from the 5′-border of the huIFN-β domain, yields a 63-fold enhancement in the absence and a 546-fold enhancement in the presence of 5 mM butyrate. In cases where single-SAR constructs are compared with double-SAR constructs, the phenomenon becomes more pronounced in the latter case (cf. the data for pAG·B′ as compared to those for the complete interferon domain pAG·A′B′ and those for sv as compared to those for the artificial minidomain SV).

SAR Function and SAR Length. SAR elements have thus far been investigated as rather large fragments (element E, 2.2 kb; element P, 1.8 kb). Their size resembles the SAR sequences (2.3 kb) within the 3-kb A elements adjacent to the chicken lysozyme gene which have been used in related studies (Phi-Van et al., 1990; Stief et al., 1989). The simple criterion by which all the sequences were originally selected is their

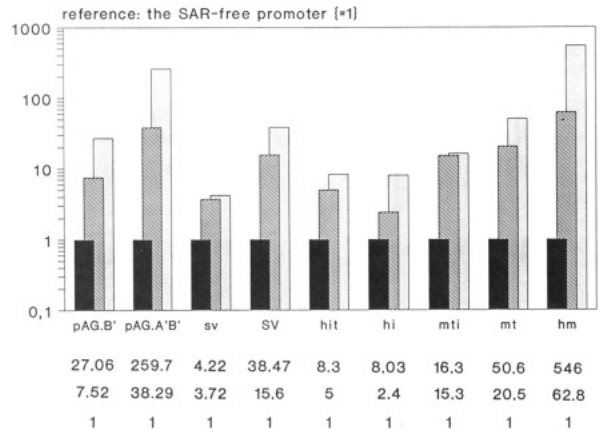

FIGURE 6: Synergistic action of butyrate and SAR elements: Summary of enhancement factors in media containing or not containing butyrate. Numbers in the first line below the figure designate the enhancement factors for the combined action of SAR elements and butyrate, i.e., the expression ratio of SAR construct to non-SAR construct determined in a medium containing 5 mM butyrate. Numbers in the second line are expression ratios of SAR construct to non-SAR construct obtained in a butyrate-free medium. Likewise, the enhancement by SAR + butyrate is characterized by the long, stippled bars and the enhancement due to a SAR is characterized by the shorter cross-hatched bars; all data have been normalized to the respective figures derived from the SAR-free constructs in media with or without 5 mM butyrate (black bars = 1).

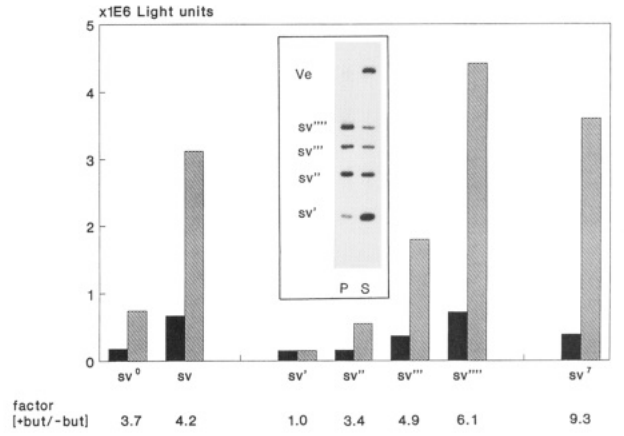

FIGURE 7: SAR functions can be recovered by oligomerizing sub-SAR sequences. Left: SAR-free standard (sv^o) and SAR standard (sv). Center: The oligomers of a 166-bp segment from SAR element E have been assayed for their relative affinity to a LIS-extracted scaffold preparation (insert). The monomer (sv′) is indistinguishable from a nonbinding control (Ve, the 2.9-kb *Hin*dIII–*Eco*RI fragment of vector pTZ). The higher oligomers show an increasing contribution to the P fraction as opposed to the S fraction, indicating an increased affinity for the scaffold (sv″, 60%; sv‴, 80%; sv⁗, 90% in the pellet fraction, P). In the context of plasmid pLu, the trimer (sv‴) and the tetramer (sv⁗) are seen to enhance the expression of the luciferase gene under the control of the SV40 promoter if compared with the SAR-free control (sv^o); this is true both for the cells kept in butyrate (hatched bars) and for cells kept in the absence of the stimulator. Moreover, butyrate becomes increasingly more efficient for the higher oligomers (cf. the relative expression in butyrate-containing and butyrate-free media ranging between 1 and 6.1). Right: A 25-bp sequence next to the IgH enhancer has been oligomerized to yield a heptameric SAR-like element, the properties of which have been tested in plasmid pLu [sv^7; cf. Bode et al. (1992)].

affinity for nuclear matrix/nuclear scaffold preparations, and this of course does not rule out the presence of other functional elements. A further restriction of the sequence appeared hence desirable. Unfortunately, experiments along these lines have consistently failed because the SAR character in vitro and its transcriptional effects in vivo are simultaneously lost below a critical length of 200–300 bp. This has been ascribed to the mechanism of SAR scaffold interactions which involves multiple attachment sites and occurs in the cooperative mode (Mielke et al., 1990).

An alternative possibility to reducing the sequence content of the studied element is the oligomerization of small sub-SAR fragments. We have realized this approach for two adjacent sequences (166 and 154 bp) from the huIFN-β upstream SAR E and one sequence (25 bp) from the MAR next to the immunoglobulin heavy chain (IgH) enhancer, each of which contains a single motif of the ATATTT-type implicated in SAR/MAR function (Mielke et al., 1990; Bode et al., 1992). Typical results are illustrated in Figure 7.

The insert shows increasing proportions of the 166-bp oligomers partitioning with the P (i.e., scaffold) fraction beginning with the dimer (sv″, 60%; sv‴, 80%; sv⁗, 90%). The expression data are in complete accord with these relative affinities although the 166-bp sub-SAR (sv′) appears to have an antagonistic effect if it is compared with the control plasmid sv^o (no insert ahead of the SV40 promoter–luciferase construct). The dimeric insert (sv″) behaves rather neutral, and at higher degrees of oligomerization the SAR-specific properties manifest themselves. Indeed, the tetrameric construct (664 bp) displays stronger SAR character than even the entire 2.2-kb element E (cf. construct sv). An artificial SAR element composed from seven repeats of 25 base pairs surrounding the aatATATTT motif next to the IgH enhancer also shares all SAR-specific characteristics including an extreme stimulation by butyrate [see construct sv^7 in Figure 7 and Bode et al. (1992)].

Discussion

The data reported here are compatible with a simple model which could explain the synergism between SAR elements and the action of butyrate.

On a molecular level, the most evident targets of butyrate are the histone deacetylases which are inhibited noncompetitively; the agent also affects the phosphorylation of histones H2A and H1 in a more indirect manner (Boffa et al., 1981; Schröter et al., 1981; Kruh, 1982). Blocking of deacetylating activities enables acetyltransferases to hyperacetylate the four nucleosomal core histones at distinct lysine residues within the N-terminal tails. Hyperacetylated nucleosomes in turn undergo structural changes similar to those seen in active chromatin (Bode et al., 1983; Bode, 1984, Oliva et al., 1990; Boffa et al., 1990; Chen et al., 1991; Gross & Garrard, 1987).

If SAR elements function as the borders of a chromatin domain as suggested, the loss of a nucleosome would be a potential source of supercoiling releasing one negative superhelical turn of DNA (Patient & Allan, 1989; Stewart, 1990; Thoma, 1991). Such a loss has been observed in vivo, and it is promoted by histone hyperacetylation/butyrate treatment (Bode et al., 1986). In vitro, histone hyperacetylation generates unconstrained negative supercoils (Norton et al., 1989), and this effect is due to the modification of the H3 and/or H4 histones (Norton et al., 1990). The recent discovery of supercoiling factors acting in conjunction with topoisomerase II are another potential source of negative supercoiling which could promote formation of a preinitiation complex (and hence transcription) within the borders of the domain (Hirose & Ohta, 1990; Mizutani et al., 1991).

By binding to underwound regions, scaffold proteins can serve as an additional store of negative supercoils (Tsutsui et al., 1988; Kay and Bode, unpublished results). For an increasing number of SAR sequences, strand separation under superhelical tension has been demonstrated (Kohwi-Shigem-

atsu & Kohwi, 1990) and data on the mechanism of scaffold SAR recognition show that these features are involved in binding (Mielke et al., 1990; Bode et al., 1992). Moreover, minimal sequences optimized for this property yield an exceptionally strong transcriptional enhancement suggesting that these parameters are closely linked (Bode et al., 1992; construct sv^7 in Figure 7). According to the "twin-domain" model, positive supercoils are generated ahead of the transcription complex (Thoma, 1991) and these could be relaxed by using part of the underwound DNA structure fixed within the scaffold-attached regions.

If SAR DNA is not constitutively bound to the scaffold as claimed by some authors (Eggert & Jack, 1991; Jackson et al., 1990), its binding could be regulated by acetylation. SAR sequences contain long homopolymeric runs of dA or dT (Adachi et al., 1989) which is a form of DNA that is not easily assembled in nucleosomes (Travers, 1987). Hence, it appears conceivable that loosening histone–DNA interactions by the acetylation of lysines would favor alternative interactions with the scaffold.

Although we cannot strictly rule out other modes of action, we have collected data (not shown) to evaluate some of the alternatives. Mutational analyses by Bohan et al. (1989) suggested that butyrate acts on a route involving transcription factor SP1 since its transcriptional effects depended on the presence of intact GC boxes. This could explain our finding that all constructs including SP1-dependent promoters (hi, mt, and sv) respond to butyrate even in the apparent absence of a SAR while the promoters lacking a functional GC box (hm and huIFN-β) are more or less nonresponsive under these conditions. Although devoid of GC boxes, attached SAR elements clearly increase this basal effect (hi, mt, sv, sv″–sv⁗, sv^7, SV) or enable it in case of hm as monitored by the luciferase assay or mRNA levels. Moreover, butyrate-mediated stimulation acting via SAR elements (measured as mRNA) could be reproduced in the presence of 10 μg/mL of cycloheximide for 24–48 h. While this does not rule out butyrate-dependent modification/activation of some preexisting, abundant, and stable factor, it eliminates the possibility that the fatty acid induces a transcription factor which then binds to some as yet undefined sequence within the SAR.

Others have considered that the growth arrest caused by butyrate (Kruh, 1982) could be responsible for an enhanced gene expression in the sense that a high proportion of cells is shifted into the phase most amenable for transcription (Adolf & Swetly, 1979; Tichonicki et al., 1990). We have shown that all compounds applied for Figure 3 increased the population of cells at G1 although to different extents (control, 35%; butyrate, 80%; propionate, 70%; HMBA = DMSO = BrdU, 60%) while only the short fatty acids influenced gene expression in a consistent fashion (Figure 3). In our study, promoters of widely different origins responded similarly to butyrate although they should differ in their susceptibility to cell cycle position. Quite untypical for an housekeeping gene, HMG is upregulated in mid-G_1 (Pardee, 1989) but exactly in this case butyrate acts in an antagonistic fashion unless it is supported by a SAR element.

In conclusion, we have demonstrated that butyrate has a stimulatory effect on a wide range of promoters, especially if these are supported by the presence of a SAR(s). The role of our prototype SAR element E in this context depends upon the individual promoter, but even in cases where it appears marginal (sv) it becomes evident as more SAR potential is accumulated at the upstream position (cf. construct sv′ to sv⁗ and sv^7 in Figure 7) or even more so if it is flanked by two strong SAR elements (cf. construct SV in Figures 5 and 6).

The relation of genetic elements that stimulate transcription, i.e., the now-classical enhancers, the locus control regions (LCRs), and the SAR elements, is barely understood. The effect of butyrate may become an additional criterion to discriminate between their actions and add to the growing list of other criteria such as a copy-number-dependent transcription of transgenes (brought about by an LCR or two flanking SAR elements), distance/orientation independence (enhancer, SAR, LCR), or activity in the episomal (enhancer but not LCR and SAR) or integrated state (enhancer, LCR, SAR).

From a more practical point of view, we are in the position to construct cell lines in which a transfected gene is expressed more efficiently following the addition of butyrate to the medium. Considering the use of this medium component in biotechnology (Palermo et al., 1991), this may be of considerable interest.

Added in Proof

We have recently replaced 5 mM butyrate by 150 nM (*R*)-trichostatin A (TSA; kindly donated by T. Beppu, University of Tokyo) which produced comparable results. TSA is a highly efficient inhibitor of histone deacetylases (Yoshida et al., 1990) reinforcing our conclusions about the role of histone hyperacetylation for the action of SAR elements.

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Registry No. HMG, 37250-24-1; sodium butyrate, 156-54-7; propionic acid, 79-09-4.

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