Journal of Cellular Biochemistry 49:74–81 (1992)

Elevation of Large-T Antigen Production by Sodium Butyrate Treatment of SV40-Transformed WI-38 Fibroblasts

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Abstract The effects of sodium butyrate on simian virus 40 early gene expression were determined in SV40-transformed human embryonic lung fibroblasts (SVWI-38). Northern blot analysis and nuclear run-off transcription studies revealed that treatment of cells with millimolar concentrations of sodium butyrate (2.5 to 10 mM) resulted in increased levels of SV40 early gene transcripts, with a concomitant increase in their corresponding proteins (large-T and small-t antigens). Although sodium butyrate treatment enhanced the expression of the early genes, it was associated with a reduction in cell growth and total protein synthesis, as measured by cell number and incorporation of ^{3}H-leucine into macromolecules, respectively. Immunoprecipitation of ^{35}S-labelled cellular proteins with anti-p53 and anti-T antibodies revealed that the level of the cellular protein, p53, declined markedly in the presence of sodium butyrate. Furthermore, in control cells only 30% of the p53 was complexed with large-T antigen, whereas in butyrate-treated cells all the p53 was complexed with large-T antigen. The increased early gene expression was not due to altered methylation patterns, gene amplification, or rearrangement of the integrated SV40 genome. Sodium butyrate treatment did, however, result in the appearance of a new nuclear protein which bound specifically to a SV40 promoter fragment containing large-T antigen binding sites I and II.

Key words: SV40 integration, DNA-protein complex, transcriptional regulation, run-off transcription, p53 synthesis

Sodium butyrate is a four-carbon fatty acid which has numerous effects on cells in culture (Kruh, 1982). One of the best known effects is the inhibition of histone deacetylase, resulting in hyperacetylation of histones H3 and H4 (Boffa et al., 1978; Vidali et al., 1978). Sodium butyrate has also been implicated in cell cycle regulation by inducing a block in the G1 phase of the cell cycle (Wintersberger et al., 1983). A further effect of this agent is its ability to induce the expression of previously inactive genes, with the concomitant switching off of certain other genes, resulting in a more differentiated phenotype in several cell lines (Reeves and Cserjesi, 1979). Sodium butyrate may also have potential chemotherapeutic value, as has been shown clinically by treatment of a patient with acute myelogenous leukemia with intravenous administration of sodium butyrate; such treatment resulted in an improved peripheral blood smear with elimination of peripheral myeloblasts, as well as an increase in the number of mature myeloid cells (Novogrodsky et al., 1983).

SV40 is a small, circular, double-stranded DNA tumor virus (Tooze, 1981). Infection of non-permissive fibroblasts with SV40 results in integration of the virus in the host genome and the subsequent expression of the SV40 early genes coding for the large-T and small-t antigens. The large-T antigen is a multifunctional protein responsible for cell transformation (Kelly and Nathans, 1977); among other functions, it possesses ATPase activity, forms complexes with the cellular protein p53, initiates viral and cellular DNA synthesis, and specifically binds the SV40 origin of replication (Butel and Jarvis, 1986). The exact role that large-T-antigen plays in the transformation process remains unsolved. The SVWI-38 cell line used in this study has been characterized and the equivalent of 4 copies of SV40 DNA is stably integrated into the host genome as a tandem repeat separated by

Received December 2, 1991; accepted December 23, 1991.

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host DNA (unpublished observations). In addition, collagen synthesis is also affected in these SVWI-38 cells, although SV40 does not appear to have integrated within or near to these genes (Parker et al, 1989), it is believed that a transacting factor affects transcription of the procollagen genes (Parker et al., 1990).

In this study, the effects of sodium butyrate on SV40 early gene transcription were investigated. Treatment of SVWI-38 cells with sodium butyrate at concentrations which were sufficient to inhibit cell growth and protein synthesis enhanced the expression of both the SV40 early genes (large-T and small-t). This was accompanied by a concurrent decreased synthesis of the cellular protein, p53. Sodium butyrate treatment had no effect on SV40 integration, gene copy number, or methylation status of its unique CCGG site, but resulted in the synthesis of a new DNA-binding protein which may play a role in the regulation of SV40 gene expression by binding to the promoter region spanning T antigen binding sites I and II.

MATERIALS AND METHODS

Cell Culture

SVWI-38 human lung embryonic fibroblasts were grown to confluency in 150 cm^2 flasks in Eagle's basal medium containing 10% heat-inactivated fetal calf serum supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml), as previously described (Parker et al., 1986). Cells received either no sodium butyrate or were treated for 18 hr with complete medium containing 2.5 mM, 5.0 mM, or 10 mM sodium butyrate.

Immunoprecipitation

Antibodies to large-T antigen and p53 were purchased from Oncogene Science (Manhasset, NY, USA). For immunoprecipitation experiments, approximately 10^6 cells were plated in 100 mm diameter dishes, incubated for 18 h in medium containing the required concentration of sodium butyrate and labelled with 10 μCi/ml of ^{35}S-methionine during the last 4 h of the treatment period. Alternatively, cells were treated with sodium butyrate for only 4 h, in which case the label was present during the entire treatment period. Cells were harvested by scraping with a rubber policeman and washed in 150 mM NaCl, 50 mM Tris-HCl pH 7.4, lysed at 4°C in 150 mM NaCl, 20 mM Tris-HCl, pH 8.0, containing 1% Nonidet P-40 and clarified by centrifugation. The supernatant was reacted overnight at 4°C with normal rabbit serum to remove any proteins which bound non-specifically to IgG (Harlow et al., 1981). After centrifugation at 12,000 *g*, the supernatant was incubated either with anti-T or anti-p53 monoclonal antibodies, or with normal rabbit serum. The amount of antibody required for maximum precipitation was optimized by sequential immunoprecipitation in order to ensure that sufficient antibody was used. Immune complexes were adsorbed with a crude cell suspension of protein A (Sigma), washed in 150 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.02% NaN_3, 50 mM Tris, pH 7.4, eluted with 2% SDS, 100 mM DTT, 10% glycerol, 50 mM Tris, pH 6.8, and analysed on 7–15% gradient polyacrylamide gels. The gels were fixed in 10% acetic acid for 30 min, soaked in 1 M sodium salicylate for 1 h, and exposed to X-ray film for 3 to 4 days.

DNA and RNA Analysis

Cellular DNA and RNA were extracted from SVWI-38 cells using previously described procedures (Davis et al., 1989). DNA was digested with various restriction endonucleases as recommended by the suppliers. DNA (10 μg) was digested with 2 units of enzyme/μg DNA for 2 h at 37°C. The digested DNA was fractionated on 1% agarose gels and transferred to Hybond-N membranes (Amersham) using standard procedures (Maniatis et al., 1982). Filters were prehybridized for 4 h at 68°C in 6 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na Citrate), 5 × Denhardt's solution (1 × Denhardt's = 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% BSA), 0.5% SDS, and 50 μg/ml denatured herring-sperm DNA. Hybridization was in the above solution containing ^{32}P-labelled SV40 DNA (Sigma), nick-translated to a specific activity of 1.2×10^8 cpm/μg DNA. Post-hybridization washes were performed at 65°C: two 15-min washes in 2 × SSC, 0.1% SDS, and one 30-min wash in 0.1 × SSC, 0.1% SDS.

Total cellular RNA was subjected to formaldehyde agarose gel electrophoresis (Lehrach et al., 1977). After fractionation, the RNA was transferred to Hybond-N without any further treatment of the gel (Maniatis et al., 1982). Post-transfer fixation of both DNA and RNA was mediated by UV irradiation. Filters were prehybridized for 4 h at 42°C in 5 × SSC, 5 × Denhardt's, 0.1% SDS, 50 mM Na phosphate,

10% dextran sulphate, and 100 μg/ml denatured herring-sperm DNA. Hybridization was in the above solution (16–20 h) containing ^{32}P-labelled SV40 DNA. Post-hybridization washes were performed as described for DNA, except that the temperature was kept at 42°C. After washing the filters, autoradiography was performed at −70°C for 16 to 24 h for both DNA and RNA blots.

Nuclear Run-Off Transcription

Nuclear run-off transcription was performed essentially as described by Greenberg and Ziff (1984) and Ausubel et al. (1988). Nuclei were incubated with ^{32}P-UTP and the labelled transcripts hybridized to slot blots containing 250, 500, or 1000 ng of the indicated cloned cDNA probe.

Electrophoretic Mobility Shift Assays

Nuclear proteins from control and butyrate-treated cells were isolated as described by Dignam et al. (1983). SV40 DNA fragments were labelled to a specific activity of 10^7 dpm/μg by Klenow filling, using ^{32}P-dCTP and used to perform the DNA-binding studies essentially as previously described (Ausubel et al., 1988). DNA-protein complexes were analysed by electrophoresis on a 5% non-denaturing polyacrylamide gel at 150 volts at 4°C, dried, and exposed to X-ray film for 10 to 16 h.

South-Western Blotting

Nuclear proteins were fractionated on an 8% SDS polyacrylamide gel and transfered to nitrocellulose membranes at 4°C for 16 h at 100 mA (Ausubel et al., 1988). Duplicate lanes on the filter were cut off and stained with amido black in order to assess the transfer efficiency. The rest of the filter was incubated in blocking buffer (1% skimmed milk powder in 10 mM HEPES pH 7.9) for 2–3 hours and incubated with 10^6 dpm/ml end-labelled SV40 DNA in binding buffer (20 mM HEPES pH 7.9, 50 mM KCl, 0.2 mM EDTA, 1 mM $MgCl_2$), containing 0.25% skimmed milk powder for 3 h at room temperature. The filter was washed for 15 min in binding buffer at room temperature and exposed to X-ray film for 1 to 16 h.

RESULTS

SV40 Large-T Antigen and Cellular p53 Synthesis

Immunoprecipitation of ^{35}S-methionine-labelled proteins with the monoclonal antibody, PAb 419, precipitated the SV40 large-T and small-t antigens and trace amounts of other cellular proteins (Fig. 1A). PAB 419 was raised against the amino-terminal region, which is common to both large-T and small-t antigens, and would therefore bind to both these proteins (Harlow et al., 1981). In the experiments reported here, the major co-immunoprecipitated cellular protein was p53, which is known to form a tight complex with large-T antigen. Thus immunoprecipitation with an antibody to either large-T antigen or p53 will precipitate both of these proteins if they are complexed with each other. Control non-immune rabbit serum did not immunoprecipitate either of these proteins (Fig. 1A). Treatment of cells with sodium butyrate

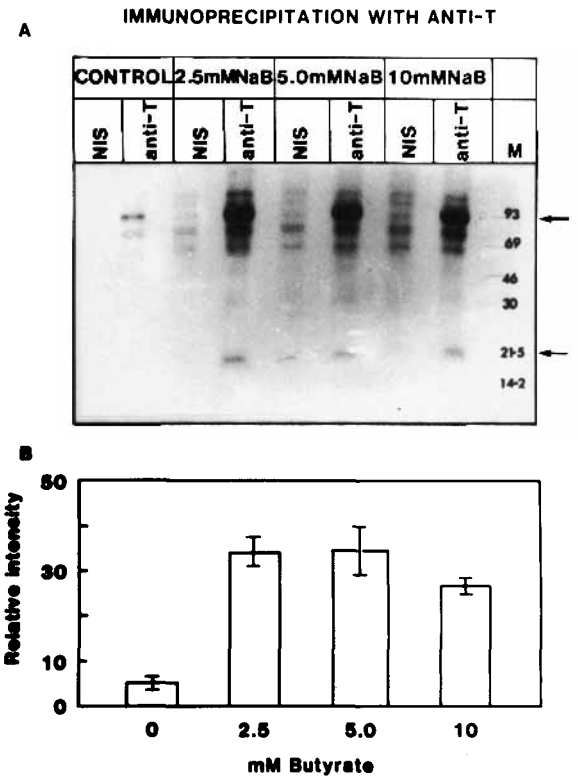

Fig. 1. Immunoprecipitation of small-t and large-T antigens in control and sodium butyrate-treated cells. **(A)** 10^6 cells were plated in a 100 mm petri dish and treated with the indicated concentrations of sodium butyrate for 18 h in a final volume of 10 ml. All dishes received 100 μCi of ^{35}S-methionine during the last 4 h before harvesting and were immunoprecipitated as described in Materials and Methods. Proteins were analysed on 7–15% gradient polyacrylamide gels. The thick and thin arrows indicate the positions of the large-T and small-t antigens, respectively, while NIS indicates immunoprecipitation with non-immune serum. **(B)** Quantitation of the data shown in (A). The autoradiographs in (A) were scanned on a Helena densitometer, and the relative quantities of the large-T antigen in the control and sodium butyrate-treated samples are indicated.

resulted in a five fold increase in the amount of large-T antigen (Fig. 1A,B). This increase was observed at sodium butyrate concentrations as low as 2.5 mM, and was maintained at concentrations as high as 10 mM. This effect was not due to a generalized increase in protein synthesis, since total protein synthesis was inhibited between 30 and 45% when the cells were treated with sodium butyrate at concentrations between 2.5 and 10 mM (data not shown).

Conversely, it was clearly shown that this sodium butyrate treatment resulted in a 50% reduction in p53 synthesis (Fig. 2). Since the anti large-T antibody immunoprecipitates both free large-T as well as large-T complexed with p53, while the anti-p53 antibody precipitates only the large-T antigen complexed with p53,

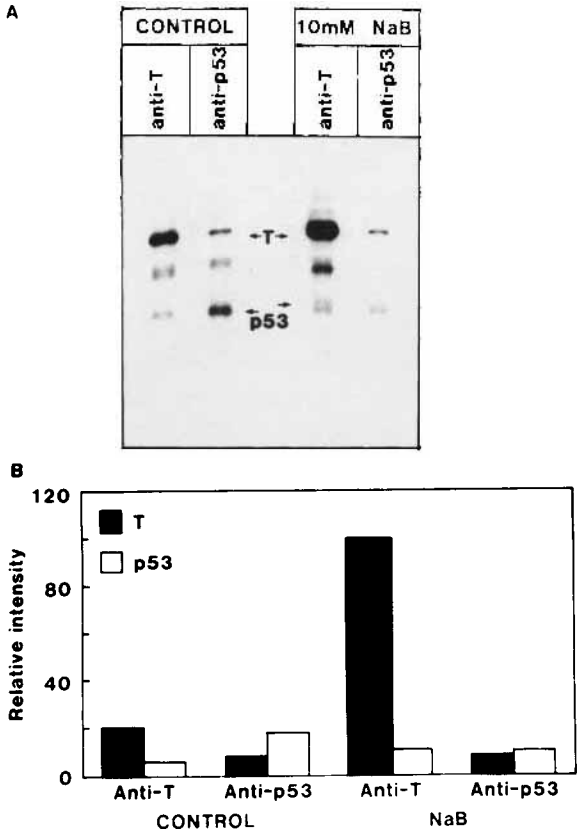

Fig. 2. Analysis of large-T antigen and p53 levels in control and sodium butyrate-treated cells. (A) Cells were treated as described in the legend to Figure 1 and ^{35}S-methionine-labelled proteins immunoprecipitated with either anti-large-T antigen or anti-p53 monoclonal antibodies. Proteins were fractionated on SDS polyacrylamide gels and the dried gels were exposed to X-ray film as described in Materials and Methods. NaB indicates cells treated with 10 mM sodium butyrate. (B) The radioactivity in the large-T and p53 bands was quantitated as described in the legend to Figure 1B and is shown as shaded and open bars, respectively.

the data could be used to determine the proportion of complexed and free molecules. Quantitation of the immunoprecipitated proteins in control cells (Fig. 2B) showed that only 40% of the available large-T antigen was associated with p53 (anti-p53 lane), while 30% of the available p53 was complexed with large-T antigen (anti-T lane). Clearly, a fair amount of free p53 and large-T antigen was therefore present in the control cells. In sodium butyrate-treated cells, however, the p53 levels had declined and all of it was complexed with large-T antigen (anti-p53 lanes). Since the amount of large-T antigen increased five-fold, most of it was therefore in the free form.

SV40 Early mRNA Synthesis

Northern blots of RNA from control cells or cells treated with sodium butyrate for 18 h were hybridised with ^{32}P-labelled SV40 DNA to distinguish the two SV40 early mRNAs (i.e. the small-t and large-T mRNAs of 2.7 kb and 2.4 kb, respectively). Treatment with sodium butyrate resulted in an increase in both large-T and small-t mRNA's, which reached a maximum eight-fold enhancement at a concentration of only 2.5 mM sodium butyrate (Fig. 3). No late gene transcripts were detected either in the presence or absence of sodium butyrate. In order to elucidate the mechanism resulting in increased early gene expression, ^{32}P-labelled nuclear run-off transcripts were hybridised to slot blots containing immobilised SV40, β-actin or c-fos DNA (Fig. 3C). Sodium butyrate treatment (10 mM) clearly resulted in increased SV40 and c-fos transcription rates, whereas the β-actin transcription rate was unaffected. The induction of c-fos gene expression by sodium butyrate is consistent with the results obtained by Naranjo et al. (1990) in PC12 cells.

SV40 Copy Number, Rearrangement and Methylation Status

DNA isolated from control and sodium butyrate treated SVWI-38 fibroblasts were digested with the indicated restriction enzymes and the Southern blots hybridized to ^{32}P-labelled SV40 DNA. Restriction endonuclease digestion patterns were the same for sodium butyrate-treated and the untreated control cells (Fig. 4A,B). The MspI/HpaII isoschizomers were employed to detect the extent of methylation of the unique CCGG site within the promoter of the integrated SV40 genome, as well as the

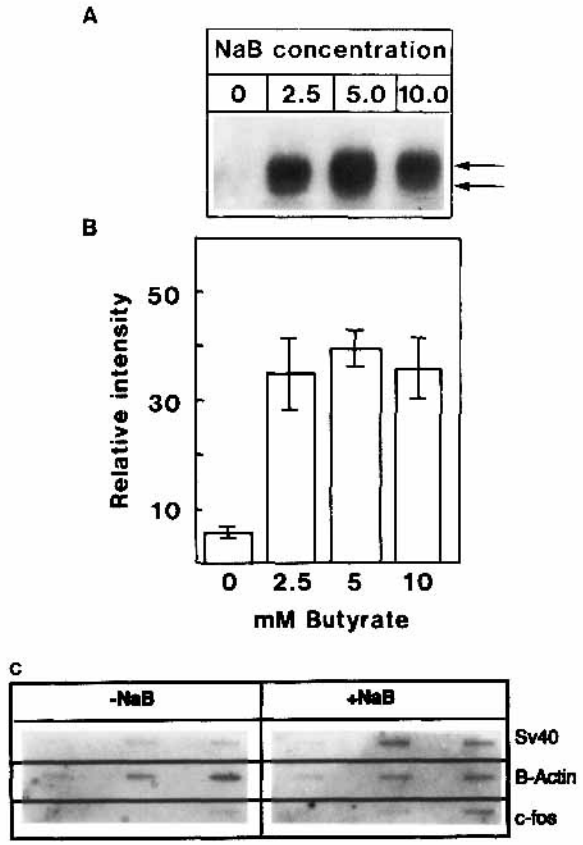

Fig. 3. Effect of sodium butyrate on small-t and large-T mRNA levels. (A) Cells were treated with sodium butyrate for 18 h, the mRNA isolated, fractionated on formaldehyde agarose gels, transferred to Amersham Hybond-N nylon membranes, and hybridized to ^{32}P-labelled nick-translated SV40 DNA, as described in Materials and Methods. The large-T and small-t mRNAs of 2.4 and 2.7 kilobases, respectively, are indicated. (B) Densitometric analysis of the autoradiograph in (A). Each box represents the sum of both small-t and large-T antigen mRNAs. The bars indicate standard deviations between different experiments. (C) ^{32}P-labelled nuclear run-off transcripts were prepared from control and sodium butyrate treated cells and hybridised to slot blots containing 250, 500, or 1,000 ng of cloned SV40, c-fos, or β-actin DNA.

adjacent host CCGG sites. Treatment of the cells with sodium butyrate did not alter the methylation status of the viral sequences which remained unmethylated and a CCGG site in the flanking host DNA remained 50% methylated (Fig. 4B). The copy number of the integrated SV40 genome was quantitated in slot blot experiments and shown to remain unchanged (data not shown). DNA was also digested with restriction enzymes which do not cleave within the SV40 genome (Bgl II, Sma I, and Xba I), with some which cleave only once (Bam HI, Bgl I, Eco RI, Eco RV, Hpa II, and Taq I) or some which cleave at several sites (Hind III, Pst I, and Pvu II). Identical restriction endonuclease fragments were obtained in all cases (data not shown). These results confirmed that no SV40 gene amplification or rearrangement had occurred upon treatment with sodium butyrate.

DNA-Protein Complex Analysis

In order to investigate the mechanism of sodium butyrate-enhanced transcription of the large-T antigen gene, we analysed the binding of regulatory proteins to the SV40 early promoter. DNA-protein complexes were formed using a 109 base-pair Hind III/NcoI fragment of the SV40 early promoter containing the Large-T antigen binding sites I and II. Nuclear proteins from control SVWI-38 cells formed three distinct DNA-protein complexes on this promoter fragment. In sodium butyrate-treated cells, however, an additional fourth complex was present (Fig. 5). In order to determine whether or not this protein was induced by sodium butyrate, cells were treated with 20 μg/ml cycloheximide concurrently with the butyrate pulse prior to the isolation of nuclear proteins. When protein synthesis was inhibited, complex 4 was not detectable in the electrophoretic mobility shift assays. Cycloheximide alone had no effect on any of the DNA-protein complexes. This new complex is therefore unlikely to be due to modification of a pre-existing protein, but rather the induction by butyrate of a new trans-acting factor which binds to the this 109 basepair fragment.

The sodium butyrate-induced protein was characterized by Western blotting of nuclear proteins onto nitrocellulose membranes and probing with the 109 base pair Hind III/Nco I early promoter fragment. The butyrate-inducible protein(s) is a doublet with molecular weights of 81 and 84 Kd (Fig. 6). This South-Western blot analysis also ruled out the involvement of other known proteins, such as p53 or super T antigen, and is further evidence for the induction of a cellular trans-acting activator by sodium butyrate. An increase in high molecular weight protein in the vicinity of the large-T antigen is also seen. This is maybe due to over-modification of large-T antigen, which would not be detected in the electrophoretic mobility shift assays shown in Figure 5.

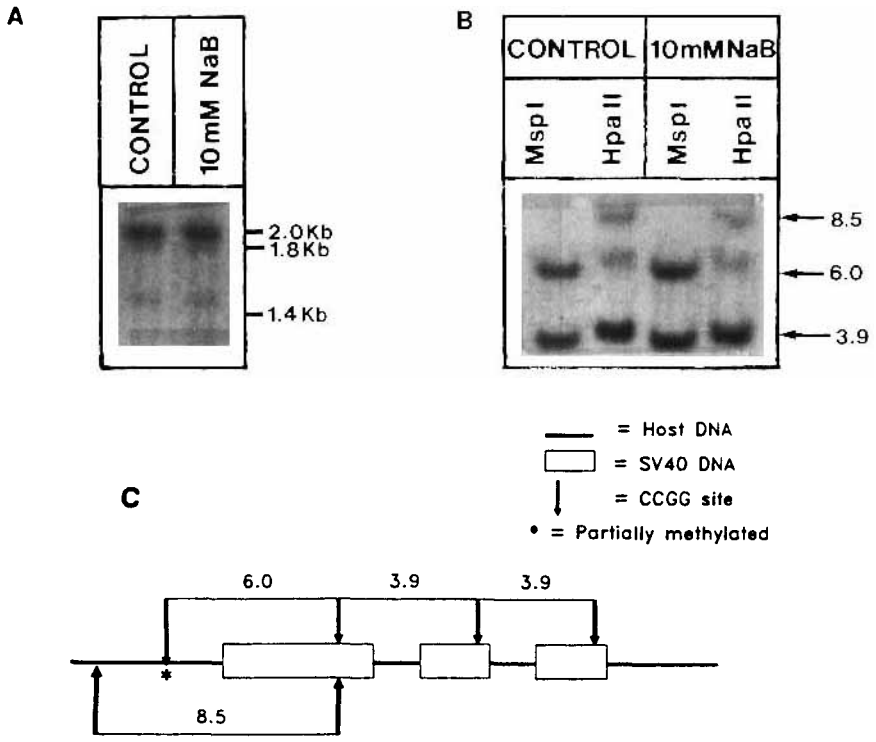

Fig. 4. Copy number and methylation status of the integrated SV40 DNA. **(A)** DNA was isolated, digested with Pvu II, fractionated on a 1% agarose gel, transferred to nylon membranes, and hybridized to ^{32}P-labelled, nick-translated SV40 DNA as described in Materials and Methods. **(B)** Methylation pattern analysis; the DNA was digested with either Msp I or Hpa II, and processed as described above. The sizes in kilobase pairs are indicated on the right hand side of the panels. The presence of the 8.5 kb band indicates methylation of a CCGG site within the flanking host DNA, as shown in **C**.

DISCUSSION

Sodium butyrate treatment of the SV40-transformed WI-38 cell line resulted in an eight-fold increase in SV40 early mRNA's, but only a five-fold increase in their respective proteins. This result suggests the involvement of both transcriptional and translational control mechanisms in SV40 early gene expression. That this increase was specific was shown by the concomitant decrease in both total protein synthesis and in p53 production. Nuclear run-off transcription experiments showed that the increased production of SV40 early mRNA demonstrated in this study was due to enhanced transcription of these genes. Gorman and Howard (1983) have demonstrated similar increased transcription of the CAT gene after sodium butyrate treatment of cells transfected with a construct containing the SV40 promoter.

Recent studies have shown that sodium butyrate treatment of cells results in hypermethylation of total cellular DNA (De Haan et al., 1986; Parker et al., 1986). Moreover, when active chromatin was fractionated from inactive chromatin by organomercurial affinity chromatography (Allegra et al., 1987; Sterner et al., 1987), sodium butyrate treatment was shown to result in increased methylation of the active gene fraction (L.C. Boffa and M.I. Parker, manuscript in preparation). SV40 contains a unique CCGG sequence near the center of a CpG cluster in a nucleosome-free region whose methylation has previously been shown to be associated with repression of late gene expression, without affecting early gene activity (Fradin et al., 1982; Graessmann et al., 1983). In the SVWI-38 cells, this site was completely unmethylated, while another site in the host flanking DNA was partially methylated. If it can be assumed that the methylation status of this HpaII site is representative of the other CpG sites within the cluster, then demethylation alone clearly is not sufficient to enhance transcription of the late or early genes.

Treatment of cells with various compounds, including chemical carcinogens, very often results in amplification of the integrated SV40 DNA (Lavi, 1981). Analysis of the SV40 copy

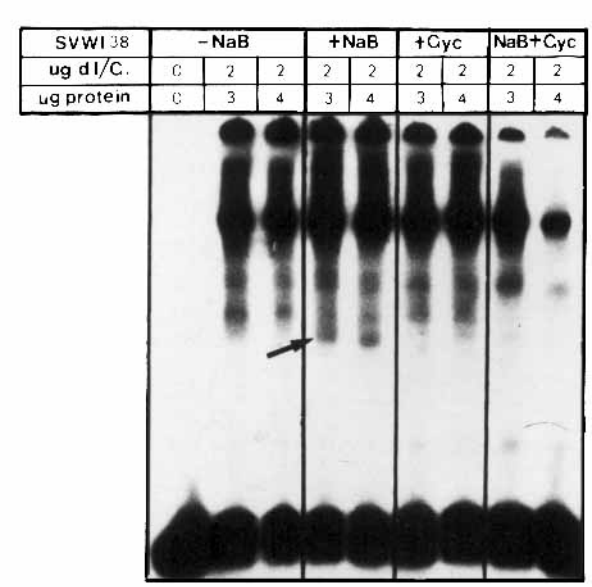

Fig. 5. Electrophoretic mobility shift assays of complexes between a 109 base pair fragment of the SV40 early promoter and SVWI-38 nuclear proteins. Nuclear protein extracts were prepared from control cells, cells treated for 16 h with 10 mM sodium butyrate (NaB), cells treated with 20 μg/ml cycloheximide (Cyc) or cells treated concurrently with 20 μg/ml cycloheximide and 10 mM sodium butyrate (NaB + Cyc). DNA-protein complex formation with the ^{32}P-labelled SV40 promoter fragment was performed as described in Materials and Methods. The complexes were fractionated on 5% non-denaturing polyacrylamide gels and exposed to Du Pont Cronex-4 X-ray film for 16 h. The arrow indicates the position of the additional DNA-protein complex in sodium butyrate treated cells.

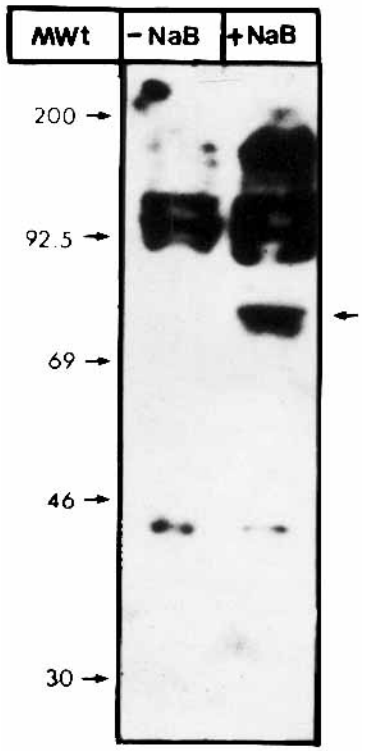

Fig. 6. South-Western blot analysis of the butyrate-inducible nuclear protein. Nuclear proteins isolated from control and butyrate-treated cells were fractionated on an 8% SDS polyacrylamide gel, electroblotted onto nitrocellulose membranes, and incubated with the ^{32}P-labelled 109 basepair Hind III/Nco I promoter fragment as described in Materials and Methods. The blots were exposed to Du Pont Cronex-4 X-ray film for 10–16 h. The arrow indicates the position of the sodium butyrate induced protein(s).

number in our control and sodium butyrate-treated cells ruled out the possibility that the increased transcriptional activity was due to amplification or rearrangement of the SV40 DNA.

Our previous studies have shown that sodium butyrate treatment of SVWI-38 cells has a growth inhibitory effect (De Haan et al., 1986; Parker et al., 1986), which may be due to G1 phase arrest in the cell cycle (Wintersberger et al., 1983). The cellular protein, p53, is a G1 phase nuclear phosphoprotein which turns over rapidly and is usually present in low amounts in normal cells; it has accordingly been implicated in cell cycle progression (Denhardt et al., 1986). Elevated levels and/or mutant forms of p53 have been detected in a wide variety of transformed cells, implicating a role in cell proliferation (for reviews see Crawford, 1983; Denhardt et al., 1986; Klein, 1987). Sodium butyrate treatment, however, decreases p53 production in non-transformed cells and to a lesser extent in transformed cells (Wintersberger and Mudrak, 1983), which may account for the observed growth inhibitory effects of this compound. Large-T antigen forms a tight complex with p53, resulting in a greatly prolonged half life of p53 (Deppert et al., 1987). Since sodium butyrate treatment specifically increased large-T antigen production, one would expect an increase in the stability of p53. The reduced levels of p53 in sodium butyrate treated SVWI-38 cells is therefore probably due to decreased transcriptional activity of this gene.

This study has shown that sodium butyrate treatment of SVWI-38 cells resulted in a specific increase in large-T antigen production. A striking finding was that this was accompanied by a concomitant decrease in the biosynthesis of a large-T associated protein, p53. The mechanism responsible for this alteration in gene expression appears not to be associated with altered

DNA methylation patterns, gene rearrangements, or amplification, but with the induction of a new DNA-binding protein which binds to either the large-T antigen binding sites I or II, or prevents large-T antigen from binding to these sites.

ACKNOWLEDGMENTS

This work was supported by grants from the National Cancer Association, the South African Medical Research Council, and the University of Cape Town.

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