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Truncation does not abrogate transcriptional downregulation of the c-*myc* gene by sodium butyrate in Burkitt's lymphoma cells

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We have examined the effect of sodium butyrate, a potent inducer of differentiation in various cell systems, on the steady state RNA level and transcriptional activity of the c-*myc* gene in Burkitt's lymphoma cells. Following sodium butyrate treatment a rapid decrease of c-*myc* RNA was observed in all Burkitt's lymphoma cell lines studied, irrespective of the type of translocation, the location of the breakpoint relative to c-*myc* or of the association with EBV. Since cellular genes induced by interferon are suspected to play a role in c-*myc* regulation we have studied transcription of the 2-5A synthetase gene in sodium butyrate-treated Burkitt's lymphoma cells. Transcriptional activity and steady state mRNA levels of the 2-5A synthetase gene were induced by sodium butyrate. The time course of induction excluded, however, that the decrease of c-*myc* RNA is caused by induction of the 2-5A synthetase/RNase L endonuclease system. The reduction of c-*myc* RNA is caused, at least in part, by a reduced transcription rate, as shown by nuclear run-on analysis. The fact that sodium butyrate is capable of downregulating a truncated c-*myc* gene indicates that an important target site of transcriptional regulation is located outside the region encompassing the upstream regulatory sequences, the dual promoters and the leader region.

Key words: c-*myc* regulation/chromosomal translocation/2-5A synthetase

Introduction

Perturbation of the c-*myc* gene by gene amplification (Collins and Groudine, 1982; Dalla-Favera *et al.*, 1982) retroviral insertion (Hayward *et al.*, 1981; Corcoran *et al.*, 1984; Li *et al.*, 1984) or chromosomal translocation (for review see Klein and Klein, 1985; Cory, 1986) is believed to be an important step in the development of a variety of neoplasias. In Burkitt's lymphoma and mouse plasmacytoma, the c-*myc* gene is assumed to be deregulated by juxtaposition to an immunoglobulin gene, even though the precise mechanism(s) of c-*myc* deregulation is (are) still unclear. In many cases of Burkitt's lymphoma with t(8;14) translocation and murine plasmacytomas the c-*myc* gene is truncated without alteration of the coding part (Battey *et al.*, 1983; Rabbitts *et al.*, 1983; Wiman *et al.*, 1984; Stanton *et al.*, 1984) suggesting that separation of the body of the gene from its physiological promoters and upstream regulatory sequences is one of the important mechanisms leading to c-*myc* deregulation (Klein and Klein, 1985; Cory, 1986). The c-*myc* gene is expressed in proliferating cells and turned off in cells during terminal differentiation. The fact that c-*myc* mRNA disappears early after induction of differentiation in various cell systems suggests a regulatory role of the c-*myc* gene product for the option of the cell either to proliferate or to undergo differentiation (Westin *et al.*, 1982; Reitsma *et al.*, 1983; Gonda and Metcalf, 1984; Campisi *et al.*, 1984; Grosso and Pitot, 1984; Lachman and Skoultchi, 1984; Müller *et al.*, 1984). This is supported by the fact that overexpression of c-*myc* can prevent induction of differentiation in mouse erythroleukemia cells (Coppola and Cole, 1986; Dmitrovsky *et al.*, 1986; Prochownik *et al.*, 1986).

Burkitt's lymphoma cells show characteristic morphological changes in response to sodium butyrate (Anisimova *et al.*, 1982, 1984; Balana *et al.*, 1985), a potent inducer of differentiation in various cell systems (Leder and Leder, 1975; Collins *et al.*, 1978; Andersson *et al.*, 1979). We report here that treatment of Burkitt's lymphoma cells with sodium butyrate leads to transcriptional downregulation of the c-*myc* gene. Sodium butyrate exerts its effect even in cases where the c-*myc* gene is truncated by the chormosomal translocation and separated from its physiological promoters indicating that a target site for c-*myc* regulation is located outside the upstream regulatory sequences and dual promoter-leader region.

Results

Effect of sodium butyrate on the c-myc steady state RNA level in Burkitt's lymphoma cells

To study the effect of sodium butyrate on the c-*myc* gene expression in Burkitt's lymphoma cells, Raji cells were treated for various times and with various concentrations of sodium butyrate and the RNA was analyzed by Northern blotting. In Raji cells the breakpoint of the t(8;14) translocation is 5′ of the c-*myc* gene giving rise to transcipts from the physiological promoters P1 and P2 (Hamlyn and Rabbitts, 1983; Taub *et al.*, 1984; Dyson and Rabbitts, 1985). The time course of c-*myc* mRNA reduction induced by sodium butyrate in Raji cells is shown in Figure 1a. c-*myc* mRNA decreased substantially already after 1 h and was hardly detectable after 3 h.

The minimal concentration of sodium butyrate causing c-*myc* mRNA reduction was found to be 1–2 mM (Figure 1b), the same concentration as defined by Luka *et al.* (1979) for the optimal induction of EBV genes. Sodium butyrate treatment does not cause a general degradation of messenger RNA as shown by rehybridization of the same blot with a constant μ specific probe, revealing an unaltered constant μ mRNA level upon sodium butyrate treatment within the time course of the experiment (Figure 1a). However, because of the stability of the μ message, the unaltered steady state RNA level does not reflect the transcriptional activity of the μ heavy chain gene (see below).

Reversibility of sodium butyrate action

To test whether the effect of sodium butyrate on the level of c-*myc* mRNA is reversible, Raji cells were treated with sodium butyrate for 20 h, washed once with medium and reseeded in fresh medium containing 10% fetal calf serum (FCS) in the absence of sodium butyrate. RNA was prepared from cells collected after 1, 5 and 24 h. The level of c-*myc* mRNA was completely restored

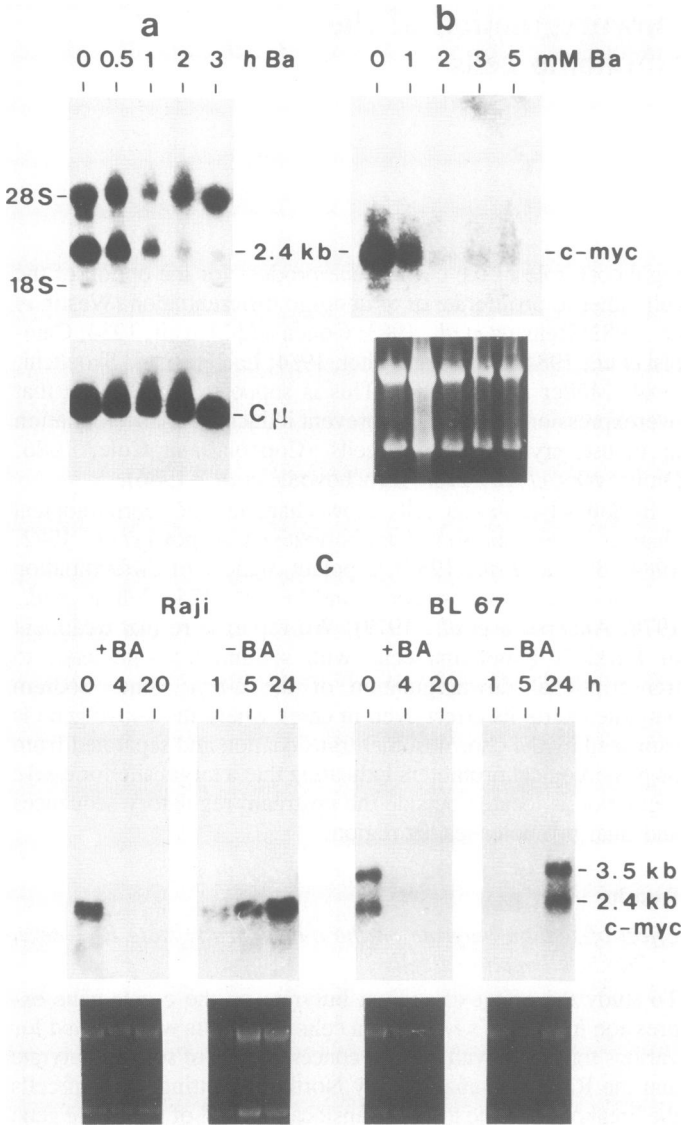

Fig. 1. c-*myc* RNA levels in Raji and BL67 cells after sodium butyrate treatment analyzed by Northern blot. **(a)** Kinetics of the decrease of c-*myc* mRNA in Raji cells at the times indicated in the presence of 3 mM sodium butyrate. The c-*myc* exon 3 labeled by SP6 *in vitro* transcription was used as a probe. The additional bands are due to cross hybridization with ribosomal RNA and are only visible with SP6-labeled, but not with nick-translated probes (see also Figure 2b). The rehybridization of the same blot with a constant μ specific probe (C75R5) (Rabbitts *et al.*, 1981) is shown below. **(b)** Relative levels of c-*myc* RNA in Raji cells treated with the indicated concentrations of sodium butyrate for 4 h. The ethidium bromide stained lanes are shown underneath each autoradiogram. **(c)** The effect of sodium butyrate on c-*myc* expression is reversible. Raji and BL67 cells were treated for 0, 4 and 20 h with 3 mM sodium butyrate (+BA), washed once with RPMI 1640 medium after 20 h and reseeded in fresh medium containing 10% FCS for 1, 5 and 24 h (−BA). The ethidium bromide stained lanes are shown underneath each autoradiogram. For (b) and (c) the exon 3 *Cla*I−EcoRI fragment labeled by nick translation was used as a probe.

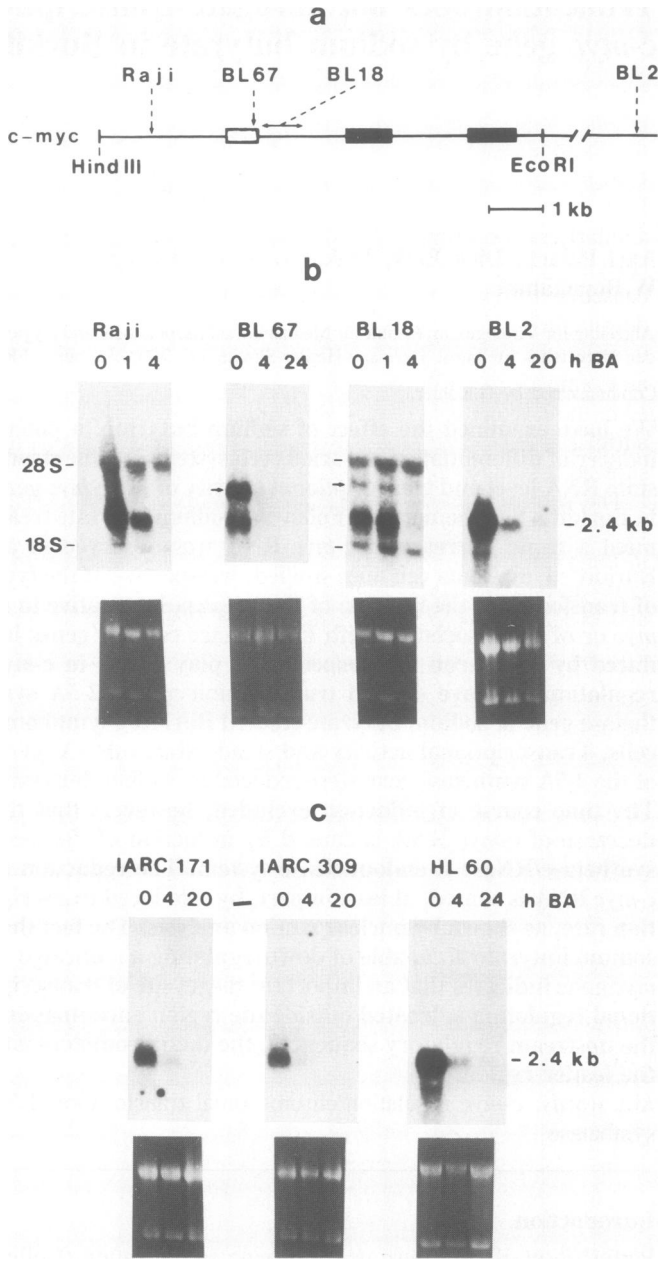

Fig. 2. Relative amounts of c-*myc* RNA in various cell lines after different times of sodium butyrate treatment. **(a)** Schematic drawing of the human c-*myc* gene. The filled boxes represent the coding exons 2 and 3, the open box the noncoding exon 1. The arrows mark the breakpoints of the translocations in the cell lines analyzed in **(b)** relative to the c-*myc* gene. The precise localization of the breakpoints is known only for Raji (Dyson and Rabbitts, 1985) and BL67 (Eick *et al.*, 1985). **(b)** c-*myc* RNA in Burkitt's lymphoma cells treated with 3 mM sodium butyrate for the times indicated. The aberrant 3.5-kb c-*myc* RNAs of BL67 and BL18 are marked with arrows. **(c)** The effect of sodium butyrate on c-*myc* RNA in two EBV-immortalized cell lines (IARC 171 and IARC 309) and the promyelocytic leukemia cell line HL60. For Raji and BL18 the probe of the third exon was labeled by SP6 *in vitro* transcription, for the other blots by nick translation.

to pretreatment levels (Figure 1c) within 24 h after the removal of sodium butyrate in Raji cells (as well as in BL67 cells, see below). This indicates that the continuous presence of sodium butyrate is necessary to abolish expression of the c-*myc* gene.

Analysis of Burkitt's lymphoma lines with different breakpoints

To study whether downregulation of c-*myc* by sodium butyrate is related to particular structural features of the gene, the effect of sodium butyrate was studied on cell lines carrying the translocation at different sites with respect to c-*myc*. In Raji cells the breakpoint of the translocation is upstream of the c-*myc* gene (Figure 2a) (Hamlyn and Rabbitts, 1983; Dyson *et al.*, 1985), however, a number of mutations are present in the first and second exon leading to 16 amino acid changes in the c-*myc* protein (Rabbitts *et al.*, 1983).

BL67 and BL18 represent cell lines with t(8;14) translocations

carrying a truncated c-*myc* gene with the breakpoint in the first exon or intron of the c-*myc* gene respectively (Eick *et al.*, 1985) (Figure 2a). The truncation of the gene leads to initiation of transcription from nonphysiological promoters in the first intron giving rise to RNA of 2.4 kb. In both lines an additional RNA of 3.5 kb is transcribed from the Ig heavy chain antisense strand across the breakpoint into the c-*myc* gene (Eick *et al.*, 1985). Both c-*myc* RNAs have an extended half life (Eick *et al.*, 1985), similarly to aberrant c-*myc* RNAs described for other cell lines (Piechaczyk *et al.*, 1985; Rabbitts *et al.*, 1985). Sodium butyrate caused a drop of c-*myc* RNA levels in BL18 and BL67 cells despite the fact that the c-*myc* gene giving rise to the transcripts is separated from its physiological promoters (Figure 2b). As an example of a cell line with the breakpoint downstream of c-*myc*, the EBV negative Burkitt's lymphoma cell line BL2 with a t(8;22) translocation (Bernheim *et al.*, 1981; Croce *et al.*, 1983) showed a similar response to sodium butyrate.

The shut-off of the c-*myc* gene by sodium butyrate is not restricted to Burkitt's lymphoma cell lines with a translocation in the vicinity of c-*myc*, but is also observed in nonmalignant EBV immortalized B lymphocytes (IARC 171 and IARC 309) and in HL60 cells (Grosso and Pitot, 1985) (Figure 2c).

Induction of the 2-5A synthetase gene by sodium butyrate

Sodium butyrate is known to enhance induction of interferon by other inducers in various hematopoetic cells including Burkitt's lymphoma cells (Adolf and Swetly, 1982). Furthermore, interferon induces arrest of proliferation and selective reduction of c-*myc* mRNA in Daudi cells (Jonak and Knight, 1984; Dani *et al.*, 1985; Einat *et al.*, 1985). It has been speculated that the interferon induced 2-5A synthetase/RNase L endonuclease system might be involved in the degradation of c-*myc* mRNA (Knight *et al.*, 1985; Dani *et al.*, 1985). To ask whether the reduction of c-*myc* mRNA by sodium butyrate is mediated by activation of the 2-5A synthetase pathway, 2-5A synthetase mRNA was analyzed in Northern blots at different times after sodium butyrate treatment of the cells. In fact, the 2-5A synthetase mRNA was strongly induced by sodium butyrate, at a time when c-*myc* mRNA had already decreased to hardly detectable levels (Figure 3a). The increase in 2-5A synthetase mRNA is mediated, at least in part, by an increase of the transcriptional activity of the gene (Figure 3b). The time course of induction excludes a causal relationship between 2-5A synthetase mRNA increase and sodium butyrate induced reduction of c-*myc* mRNA.

Transcriptional downregulation of c-myc by sodium butyrate

To ask whether sodium butyrate exerts its effect at a transcriptional or post-transcriptional level, the transcriptional rate of the c-*myc* gene was studied in a nuclear run-on assay, in which in-vivo initiated RNA is elongated *in vitro* in the presence of ^{32}P-labeled UTP. As shown in Figure 4, the RNA polymerases are not equally distributed along the c-*myc* gene in untreated Raji, BL18 and BL67 cells (panels c, e and g). The pattern is similar in the two cell lines with a truncated c-*myc* gene, BL18 and BL67, and shows a high density of RNA polymerases on intron 1 and exons 2 and 3 (fragments E, F, G and H). In Raji cells, the transcriptional activity is higher on the 3′ part (fragments F, G and H) than on the 5′ part of the c-*myc* gene. In all three cell lines transcription is also observed in the region upstream of the dual promoters (fragments A, B and C). This might be due to transcription originating from the PO promoter of c-*myc* or to antisense transcription recently described for this region (Bentley and Groudine, 1986a, b; Nepveu and Marcu, 1986). Alternatively in the case of BL18 and BL67 this region could be transcribed

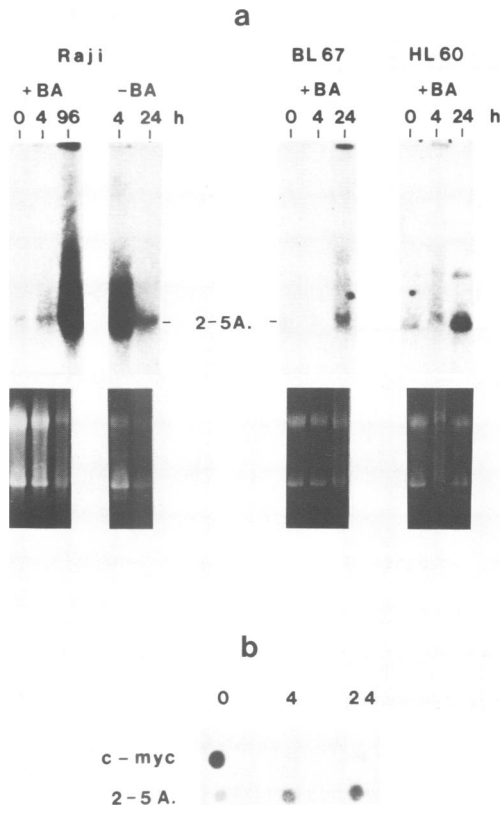

Fig. 3. (**a**) Relative levels of 2-5A synthetase mRNA in Raji, BL67 and HL60 cells treated for the times indicated with 3 mM sodium butyrate (+BA). After 96 h, Raji cells were washed once in RPMI 1640 and reseeded in fresh medium with 10% FCS for 4 and 24 h (−BA). The probe hybridized to the Northern blot was the 2-5A synthetase cDNA clone 9-21 (Benech *et al.*, 1985). (**b**) Transcriptional activity of the 2-5A synthetase gene and of the third c-*myc* exon in nuclear extracts of BL67 cells treated for 0, 4 and 24 h with 3 mM sodium butyrate. ^{32}P-labeled RNA synthesized in isolated nuclei (for details see Materials and methods) was hybridized to dot blots containing 5 μg DNA of phmycCR and 2-5A synthetase c-DNA clone 9-21.

from the immunoglobulin heavy chain promoter which is fused to the upstream region of c-*myc* on chromosome 8q−. Thus, transcription originating from the immunoglobulin heavy chain promoter could run across the breakpoint into the antisense strand of c-*myc*.

Four hours after treatment of the cells with sodium butyrate c-*myc* transcription was considerably reduced in all three cell lines (panels d, f and h). In Raji cells, the transcriptional activity was diminished along the total c-*myc* gene including the region upstream of P1 and P2. In BL18 and BL67 the high density of polymerases on fragments E, F, G and H was dramatically reduced after sodium butyrate treatment. The transcriptional activity of the first exon (fragment D) was also reduced, however to a lesser extent, compared to that of the downstream region. Transcription of the 5′ region of c-*myc* upstream of P1 and P2 was, to a variable degree, sensitive to sodium butyrate treatment.

Remarkably, the transcriptional activity of the μ heavy chain gene decreased substantially in Raji and BL18 cells and, to a lesser extent, also in BL67 cells. The change in transcriptional activity was not detected at the steady state RNA level in the experiment shown in Figure 1 because of the high stability of the μ heavy chain message. In BL67 cells, the extent of downregulation of the myc upstream region resembled more that

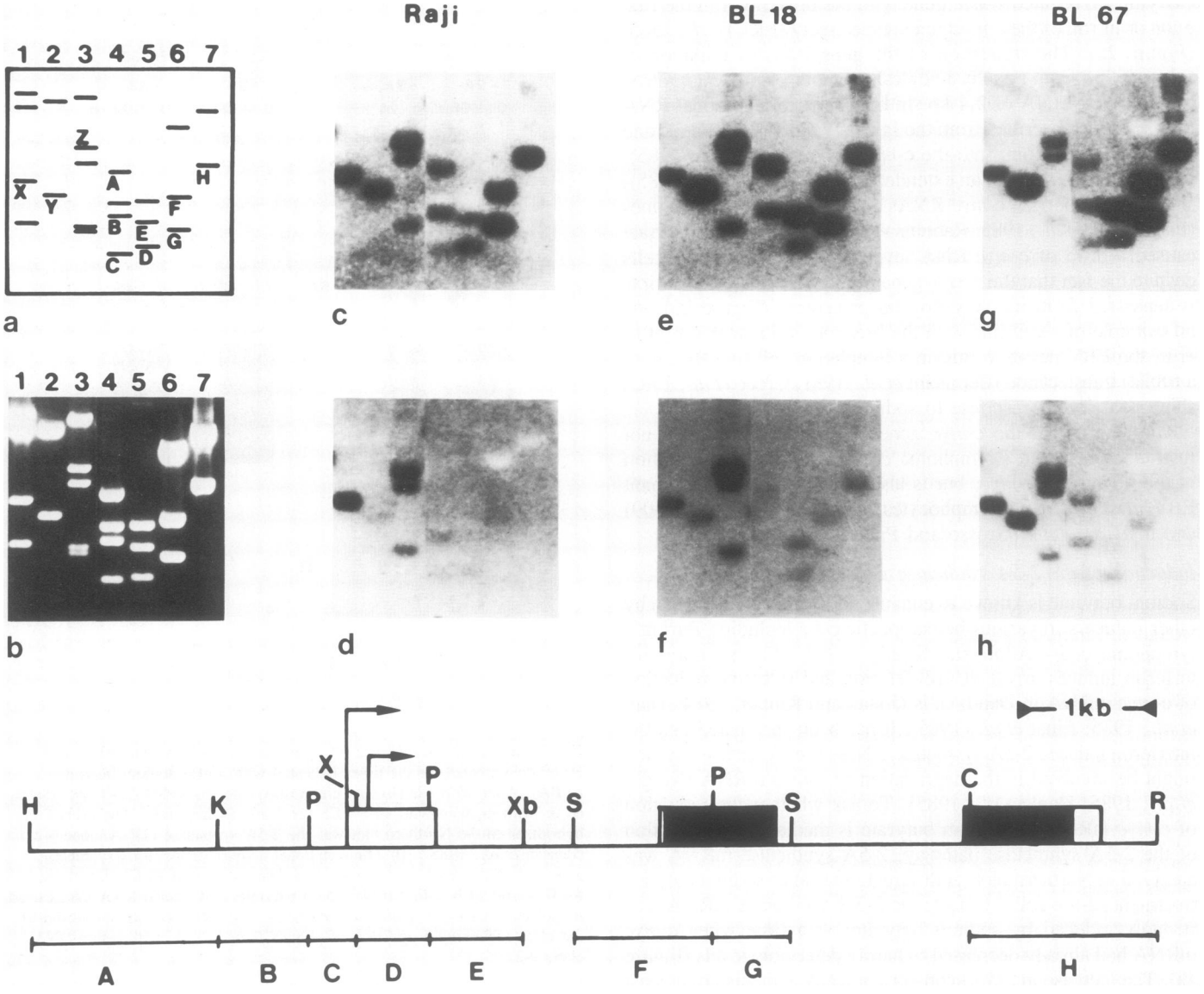

Fig. 4. Run-on transcription of c-*myc* in Raji, BL18, and BL67 cells untreated or treated for 4 h with 3 mM sodium butyrate. 3 μg DNA of plasmids containing bcr (fragment X, lane 1), μ heavy chain (fragment Y, lane 2), β-actin (fragment Z, lane 3), and c-*myc* fragments A−H (lane 4-7) (for details see Materials and methods) were separated on a 1.8% agarose gel and transferred to Zeta-Probe (Biorad) by Southern blotting (Southern, 1975). Schematic drawing (**a**) of the ethidium bromide stained gel. (**b**) Hybridization with ^{32}P-labeled nuclear run-on transcripts, from untreated cells (c, e, g) or cells treated for 4 h with sodium butyrate (d, f, h). Abbreviations of restriction enzymes: H, *Hin*dIII; C, *Cla*I; P, *Pvu*II; X, *Xho*I; S, *Sac*I; R, *Eco*RI; K, *Kpn*I.

of the immunoglobulin locus than that of the rest of the c-*myc* gene. This might indicate that the transcriptional activity of the c-*myc* upstream region of BL67 cells is reflecting the transcriptional activity of the immunoglobulin promoter on the 8q− chromosome. However, since c-*myc* upstream transcription may be derived from both strands of both chromosomes (8 and 8q−), other possibilities cannot be excluded.

The transcription rate of bcr and β-actin was unaltered in the presence of sodium butyrate, whereas transcription of the 2-5A synthetase gene (Figure 3) and of EBV genes (data not shown) was induced. This excludes that sodium butyrate causes a general transcriptional shut-off.

Discussion

We have shown that sodium butyrate induces downregulation of the c-*myc* gene in various human lymphoid cell lines at the level of transcription. The action at the level of transcription does, of course, not exclude that c-*myc* mRNA stability might also be affected by sodium butyrate. The analysis of transcription in isolated nuclei did not provide evidence of discontinous transcription of c-*myc* after sodium butyrate treatment. We thus have no indication that the recently described mechanism of regulation at the level of RNA elongation (Bentley and Groudine, 1986b; Eick and Bornkamm, 1986) is involved. Surprisingly, a truncated c-*myc* gene, separated from its physiological promoters and the noncoding first exon, responds equally to sodium butyrate with a decrease in c-*myc* RNA as the intact c-*myc* gene in EBV-immortalized lymphocytes.

There are two possibilities to explain this finding: firstly, it may be assumed that sodium butyrate acts at the same target site in cases with truncated and non-truncated c-*myc* genes. Such a target would have to be located in the coding part, the non-coding introns or downstream of the gene. Alternatively, sodium butyrate might have different targets in cases with intact versus truncated c-*myc* genes. In cases with an intact c-*myc* gene it may act by

interfering with transcription initiation or elongation at a site close to the dual promoters or first exon. In cases with a truncated c-*myc* gene, however, it may act at a target site in the immunoglobulin locus and may cause bidirectional transcriptional shut-off of the immunoglobulin heavy chain gene as well as of c-*myc*. Consistent with this interpretation is the observation that transcription across the breakpoint of the translocation from the heavy chain locus into the c-*myc* gene does in fact occur (Eick *et al.*, 1985) and that the μ heavy chain gene is downregulated at a transcriptional level concomitantly with c-*myc*. It is an attractive hypothesis that such an element in the immunoglobulin locus might be identical with the 'long-range enhancer', which, as postulated by Carlo Croce, is involved in driving immunoglobulin and c-*myc* transcription in Burkitt's lymphoma cells, and is turned off in somatic hybrids of Burkitt's lymphoma cells with mouse fibroblasts (Nishikura *et al.*, 1984; Croce and Nowell, 1985).

There is, however, one point which is difficult to reconcile with the second model. Downregulation of c-*myc* and of immunoglobulin genes could, of course, be two distinct phenomena which are not causally related to each other. This might be supported by the fact that the immunoglobulin locus in BL67 cells is downregulated by sodium butyrate to a lesser extent than the c-*myc* gene. The different behaviour of both genes may, in fact, argue that butyrate-induced downregulation of immunoglobulin genes and of c-*myc* are mediated by different mechanisms and through different target sites.

By studying the effect of sodium butyrate on cell lines carrying truncated and non-truncated c-*myc* genes with and without immunoglobulin gene elements on episomal vectors, we are now attempting to identify the target sequences which mediate c-*myc* downregulation by sodium butyrate.

Materials and methods

Northern blot analysis and radioactive labeling of hybridization probes

Total cellular RNA extraction and Northern blots were done according to standard methods (Auffray and Rougeon, 1980; Maniatis *et al.*, 1982). The third exon of the human c-*myc* gene cloned in pSP65 was labeled by SP6 *in vitro* transcription or nick translation. The filters were hybridized and washed as described (Eick *et al.*, 1985).

Hybridization probes for Northern blot analysis

For the Northern blot analysis in Figures 1, 2, and 3 the following plasmids were used: c-*myc* exon 3 cloned as a *ClaI*–*EcoRI* fragment into pBR327 (phmycCR), subcloned as an *EcoRI* fragment into pSP65 (pBT43-22) for SP6 *in vitro* transcription, constant μ cloned as an 1.2-kb *EcoRI* fragment into pACYC184 (C75R5) (Rabbitts *et al.*, 1981) and 2-5A synthetase cDNA clone 9-21 (Benech *et al.*, 1985).

Nuclear run-on analysis

The nuclear extracts were made as described by Greenberg and Ziff (1984) with slight modifications. The cells grown in suspension were washed once in ice-cold phosphate buffered saline. The pellets of 1×10^8 cells were resuspended in 10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% (v/v) NP40 and incubated on ice for 5 min. The nuclear pellets were spun down at 500 *g* and washed by resuspension in 10 ml of the same buffer. The pelleted nuclei were resuspended in storage buffer [50 mM Tris–HCl, pH 8.3; 40% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA] and frozen in liquid nitrogen in portions of 100 μl corresponding to 10^7 nuclei. The nuclei were mixed with 100 μl reaction buffer (10 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 0.5 mM of each ATP, CTP, GTP and 100 μCi of (alpha-^{32}P) UTP (800 Ci/mmol, Amersham) and incubated for 30 min at 28°C. DNase I was added to a final concentration of 10 μg/ml and the incubation continued for 5 min at 28°C. After addition of 20 μl 10% SDS, 10 μl proteinase K (5mg/ml, preincubated at 37°C for 1 h) and 200 μl STE buffer (0.5% SDS, 100 mM Tris–HCl, pH 7.5, 50 mM EDTA) the samples were incubated for 1 h at 37°C. Nuclear transcripts were separated from unincorporated nucleotides on a Sephadex G50 column equilibrated with 10 mM Tris–HCl pH 7.5, 1 mM EDTA and 0.1% SDS. The labeled RNA was boiled for 10 min, chilled on ice and hybridized to DNA immobilized on Zeta Probe filters in 50% formamide, 5 × SSC (SSC is 0.15 m NaCl and 0.015 M trisodium citrate), 10 mM sodium phosphate, pH 6.5, 0.1% Ficoll, 500 μg/ml tRNA, 1 mg/ml salmon sperm DNA, 1% SDS) after preincubation of the filters in the same buffer. After hybridization the filters were washed once at 52°C in 0.1% SDS, 0.1 × SSC once in 2 × SSC, twice in 2 × SSC containing 10 μg/ml RNase A and subsequently 1 h in 0.1 × SSC, 0.1% SDS at 52°C. The filters were air dried and exposed to a Kodak XAR 5 film using an Dupont Cronex Lightning Plus intensifying screen.

Southern blots used for nuclear-run analysis

Recombinant plasmids of c-*myc* were digested with the enzymes indicated below, fragments separated on 1.8% agarose gels and transferred to Zeta-Probe (Biorad) filters (Southern, 1975). PhmycH3Xh containing the 2395-bp *HindIII-XhoI*-fragment, cloned into the *HindIII* and *SalI* sites of piAN7, was digested with *HindIII*, *KpnI*, *PvuII* and *EcoRI*, and gave rise to fragments A, B and C. phmycXhXb containing the 1113-bp *XhoI*–*XbaI* fragment was cloned into the *SalI* and *XbaI* sites of piAN7, and digested with *XbaI*, *PvuII* and *BamHI*, and gave rise to fragments D and E. S471-1 containing the 1533-bp *SacI* fragment was cloned into the *SacI* site of pUC12, digested with *SacI* and *PvuII*, and gave rise to fragments F and G. PhmycCR containing the 1405-bp *ClaI*-*EcoRI* fragment cloned into the *ClaI* and *EcoRI* sites of pBR327, was digested with *ClaI* and *EcoRI*, and gave rise to fragment H (Figure 4). The plasmids used as controls were immunoglobulin μ heavy chain gene (S418-22, 0.9-kb *SacI*–*EcoRI* fragment containing intron sequences and part of μ exon 1; Eick *et al.*, 1985) digested with *SacI* and *EcoRi* giving rise to fragment Y, bcr (third exon the bcr gene cloned as a *BamHI*–*HindIII* fragment in a pBR322 derivative; G.Grosveld, personal communication; Grosveld *et al.*, 1986) digested with *HindIII* and *BamHI* giving rise to fragment X, and β-actin (pHAC63A23; Moos and Gallwitz, 1983) giving rise to fragment Z.

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