The EMBO Journal vol.5 no.13 pp.3553-3562, 1986

The first exon of the c-*myc* proto-oncogene contains a novel positive control element

Jian-Qing Yang, Elaine F.Remmers and Kenneth B.Marcu

Biochemistry Department, S.U.N.Y. at Stony Brook, Stony Brook, NY 11794, USA

Communicated by E.Klein

We have identified a positive modulator within the c-*myc* first exon downstream of the gene's transcription initiation sites, P_1 and P_2. We introduced *myc*-CAT (chloramphenicol acetyltransferase) hybrid genes into three cell lines (BJAB, COS and HeLa) and measured their expression by either CAT enzymatic activity, S1 nuclease protection or by a nuclear 'run-on' transcription assay. Removal of 46 bp from the 3' end of the first exon results in a decrease of *myc*-CAT expression and P_2 activity. A 438-bp exon 1 segment, lacking the normal *myc* promoters, efficiently drives the expression of SV40 early promoters. We find that this first exon segment efficiently functions as a positive modulator only in its sense orientation, 3' of a nearby promoter. The positive effects of the *myc* first exon and the SV40 enhancer are complementary.

Key words: c-myc gene/first exon positive modulator/S1 nuclease mapping/CAT assays/nuclear run-on

Introduction

The c-*myc* proto-oncogene is highly conserved in vertebrate evolution (Colby *et al.*, 1983; Stanton *et al.*, 1983; Watt *et al.*, 1983; Battey *et al.*, 1983; Bernard *et al.*, 1983; Gazin *et al.*, 1984). The mammalian c-*myc* gene contains three exons, the first of which is a large non-coding sequence (Stanton *et al.*, 1983, 1984; Battey *et al.*, 1983; Bernard *et al.*, 1983; Watt *et al.*, 1983). *Myc* mRNAs are normally initiated from two promoters (P_1 and P_2) (Leder *et al.*, 1983; Stewart *et al.*, 1984a; Yang *et al.*, 1984, 1985) and are known to be highly unstable (Dani *et al.*, 1984). The function(s) of the c-*myc* gene product remains unknown though c-*myc* has been implicated in cellular proliferation (Kelley *et al.*, 1983; Campisi *et al.*, 1984; Armelin *et al.*, 1984; Kaczmarek *et al.*, 1985; Persson *et al.*, 1985), cellular transformation (Land *et al.*, 1983; Mougneau *et al.*, 1984; Vennstrom *et al.*, 1984; Keath *et al.*, 1984; Bechade *et al.*, 1985; Lee *et al.*, 1985; Zerlin *et al.*, 1986) and in the development of a variety of neoplasms (Bishop, 1983; Marcu *et al.*, 1984; Klein and Klein, 1985; Stewart *et al.*, 1984b; Adams *et al.*, 1985).

Myc expression is affected by a host of environmental phenomena in different cell types. The c-*myc* gene is activated in response to mitogenic stimuli (Kelley *et al.*, 1983; Reed *et al.*, 1985; Persson *et al.*, 1985; Dean *et al.*, 1986b), inducers of protein kinase C (Coughlin *et al.*, 1985) and double-stranded RNAs (Zullo *et al.*, 1985). Radical fluctuations in c-*myc* mRNA levels have also been noted in a murine B lymphoma treated with anti-IgM antibodies (McCormack *et al.*, 1984). Alterations in c-*myc* RNA levels have also been noted in HL-60 leukemia cells treated with vitamin D analogues (Reitsma *et al.*, 1983; Grosso and Pitot, 1985) and in mouse erythroleukemia cells treated with differentiation inducers (Lachman *et al.*, 1985). The c-*myc* gene is expressed at the RNA and protein levels throughout the cell cycle, though c-*myc* activity would seem to reflect the competency of cells to enter and progress through the cycle (Thompson *et al.*, 1985; Hann *et al.*, 1985; Kaczmarek *et al.*, 1985).

Myc expression is regulated at the transcriptional and post-transcriptional levels. The induction of *c-myc* expression in fibroblastic cells by growth factors has been documented to involve transcriptional and post-transcriptional phenomena though their relative contributions remain somewhat controversial (Greenberg and Ziff, 1984; Blanchard *et al.*, 1985; Greenberg *et al.*, 1986; Dean *et al.*, 1986b). Expression of c-*myc* is down-regulated at the post-transcriptional level in interferon-treated Daudi-Burkitt lymphoma cells (Jonak and Knight, 1984; Knight *et al.*, 1985; Dani *et al.*, 1985) and in F9 teratocarcinoma cells induced to differentiate with retinoic acid (Dony *et al.*, 1985; Dean *et al.*, 1986a), while a block in transcriptional elongation is mostly responsible for c-*myc* repression in differentiating HL60 cells (Bentley and Groudine, 1986). Post-transcriptional mechanisms contribute to the high level of rearranged *myc* RNAs expressed by broken *myc* genes in plasmacytomas (Piechaczyk *et al.*, 1985) and Burkitt lymphomas (Eick *et al.*, 1985; Rabbitts *et al.*, 1985). The normal c-*myc* gene (unrearranged allele) is not expressed in plasma cell tumors (Adams *et al.*, 1983; Stanton *et al.*, 1983; Bernard *et al.*, 1983; Fahrlander *et al.*, 1985) and in Burkitt lymphomas (Nishikura *et al.*, 1983; Taub *et al.*, 1984; Denny *et al.*, 1985) which contain *myc*-involved chromosome translocations (Klein and Klein, 1985). Endogenous c-*myc* expression is also extinguished in B lymphomas of *myc* transgenic mice (Adams *et al.*, 1985) and in myeloid cell lines transformed by murine retroviruses expressing a v-*myc* gene (Rapp *et al.*, 1985). The repression of c-*myc* in these malignant cells has been proposed to be an autoregulatory phenomenon which may be mediated by a repressor at the level of the first exon (Leder *et al.*, 1983; Dunnick *et al.*, 1983; Rabbitts *et al.*, 1984). The expression of c-*myc* can also be potentiated by protein synthesis inhibitors suggesting that c-*myc* is controlled by labile negative factors (Kelley *et al.*, 1983; Dani *et al.*, 1984; Greenberg *et al.*, 1986; Dean *et al.*, 1986b). We have recently identified a *cis*-acting negative control element upstream of the murine c-*myc* first exon which inhibits *myc* promoter and SV40 enhancer-driven gene expression at a distance (Remmers *et al.*, 1986).

In spite of the large volume of information available on alterations in the quality and quantity of c-*myc* expression in cell growth, differentiation and malignant transformation, the molecular mechanisms of normal c-*myc* regulation remain largely unknown. In this report, we have begun to elucidate the molecular requirements for c-*myc* positive control. By employing *myc*-CAT (chloramphenicolacetyltransferase) hybrid gene vectors, we show that the c-*myc* first exon contains a novel positive modulator of gene expression.

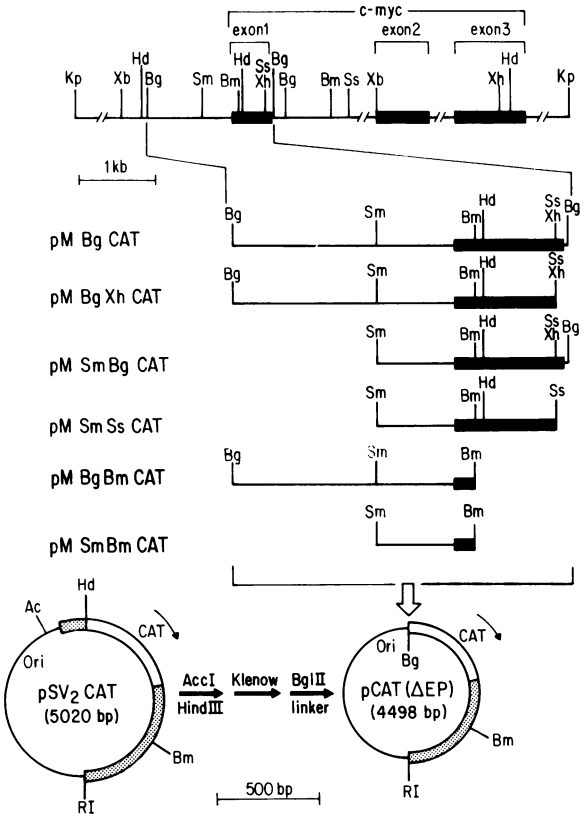

Fig. 1. The *myc* sequences inserted into pCAT(ΔEP) (an enhancer–promoter negative derivative of pSV2CAT) were derived from a plasmid subclone of a 10.5-kb *c-myc KpnI* fragment derived from a λ phage genomic clone of a BALB/c embryo *c-myc* gene (Battey *et al.*, 1983). Details of vector constructions are described in Materials and methods. The stippled boxes in pSV2CAT and pCAT(ΔEP) correspond to SV40 sequences (Gorman *et al.*, 1982). Restriction enzyme sites are labeled as follows: Ac, *Acc*I; Bm, *Bam*HI; Bg, *Bgl*II; Hd, *Hin*dIII; RI, *Eco*RI; Sm, *Sma*I; Ss, *Sst*I; Xh, *Xho*I.

Results

Efficient expression of myc–CAT genes requires an intact myc first exon

Different segments of the murine c-*myc* gene were attached to the CAT gene (Gorman *et al.*, 1982) to provide us with a convenient, sensitive assay for regulatory elements. The enhancer and promoter sequences of the pSV2CAT expression vector (Gorman *et al.*, 1982) were replaced by different segments of the c-*myc* locus. The first series of such *myc*–CAT hybrid genes are shown in Figure 1. pMBgCAT, the largest *myc*–CAT vector, contains ~1.14-kb of 5′-flanking *myc* sequences, an intact first exon and the 5′ 14 bp of intron 1. The remainder of the *myc*–CAT vectors contain different portions of the 1.71 kb *myc* gene segment in pMBgCAT. CAT transfections were performed by either the DEAE–dextran (BJAB) or calcium phosphate (HeLa) techniques in triplicate with a constant number of cells and DNA to control for variations in transfection efficiencies (see data analysis in Table I). These data demonstrate that: (i) efficient expression of *myc*–CAT hybrid genes requires a c-*myc* first exon with an intact 3′ end, and (ii) 1.14 kb of c-*myc* 5′-flanking DNA including the first promoter, P_1, are insufficient for *myc*–CAT gene activity. These results suggest that a positive regulatory element(s) resides within the first exon downstream of the P_1 initiation site.

The diminished activity of pMBgCAT compared with pMSmBgCAT in BJAB cells is due to a negative element within the upstream 716-bp *Bgl*II–*Sma*I segment (Remmers *et al.*, 1986). The negative effect of this upstream segment has also been observed in other B lymphoid cell lines but is not apparent in NIH3T3 and HeLa cells (E.F.Remmers and K.B.Marcu, unpublished results) (Table I).

Table I. Comparative activities of *myc*–CAT hybrid genes in BJAB and HeLa cells

CAT vector	Rel. CAT activity[a]	
	HeLa	BJAB
pSV2CAT	100	100
pCAT(ΔEP)	1.4	1.0
pMBgCAT	18.0	9.0
pMBgXhCAT	2.3	1.8
pMSmBgCAT	20.5	22.0
pMSmSsCAT	4.4	1.2
pMBgBmCAT	0.7	3.2
pMSmBmCAT	3.5	3.2

[a]CAT activities are expressed relative to pSV2CAT and are the average of at least three independent transfections with results differing by no more than 25%. Variations in transfection efficiencies were also controlled for in some experiments by quantitation of plasmid DNAs in Hirt lysates (Hirt, 1967) of transfected cells.

Active myc–CAT genes employ the major myc promoter, P_2

The transcriptional start sites utilized by pMBgCAT and pMSmBgCAT were first determined with stable transformed lines of HeLa cells which were prepared by co-transfection of *myc*–CAT genes and pSV2NEO (Southern and Berg, 1982) followed by selection in media supplemented with G-418 (Geneticin, GIBCO). The results of S1 nuclease protection assays with a uniformly labeled mouse exon 1 probe, mpR*S10, and RNAs isolated from mixtures of stably transfected HeLa cells are shown in Figure 2A. S1 nuclease assays performed with mpR*S10 detect expression only from the transfected *myc*–CAT gene due to the divergence of the murine and human *myc* exon 1 sequences (Bernard *et al.*, 1983). pMSmBgCATP and pMBgCATP are mixtures of 20–30 independent stable transformants. As shown in Figure 2, the major normal *myc* promoter, P_2, is the predominant transcription start site employed by cells expressing pMBgCAT and pMSmBgCAT. The P_2 promoter would be expected to be preferentially used in HeLa cells in an analogous fashion as shown for the NIH3T3 control which has a P_1:P_2 usage ratio of ~0.3 (Nepveu *et al.*, 1985). The preferential usage of P_1 over P_2 in PC3741 reflects abnormal expression of c-*myc* in this plasma cell tumor line which is presumably caused by a 12;15 chromosome translocation (Yang *et al.*, 1985). S1-protected bands corresponding to *myc* P_1 are not apparent in any of these transfected lines. The absence of significant P_1 activity suggests that these promoters are independently regulated. Several additional minor start sites, which are less prominent than P_2, are present 5′ of P_1 and 3′ of P_2. It is interesting to note that several of these minor start sites are also observed in longer exposures of the endogenous *myc* transcripts expressed in NIH3T3 and PC3741 cells implying that these are not simply a consequence of gene transfer.

We next compared the activities of pMSmBgCAT and pMSmSsCAT in transiently transfected HeLa cells by S1 nuclease mapping of total cellular RNAs. The removal of the 3′-terminal 60 bp of *myc* sequences from pMSmBgCAT results in the loss of P_2-initiated transcripts (Figure 2B) and a commensurate drop in the level of CAT RNAs (Figure 2C). A rabbit β globin gene

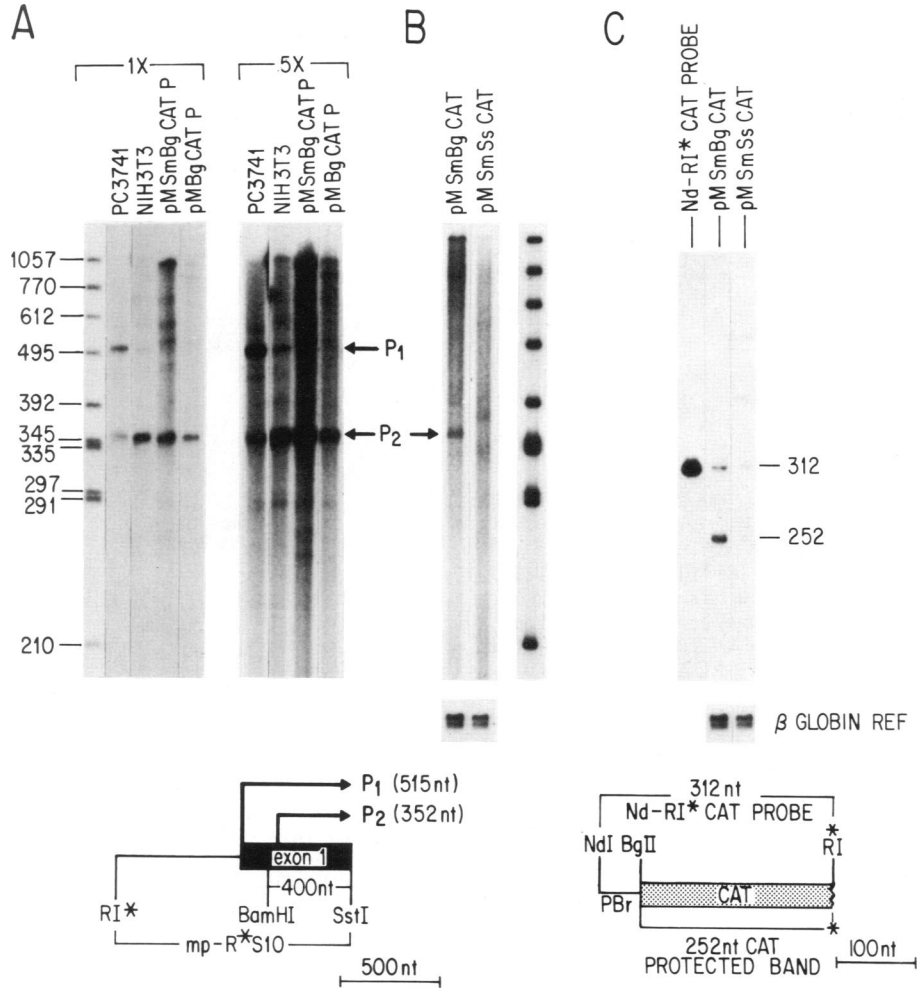

Fig. 2. S1 nuclease protection analysis of CAT RNAs expressed by HeLa cells stably and transiently transfected with *myc*−CAT vectors. S1 mapping of the indicated samples in **panels A** and **B** was performed with a uniformly labeled, single-stranded *myc* exon 1 probe, mp-R*S10 (Yang *et al.*, 1985). 1X and 5X are 1 and 5 day exposures with intensifier screens, respectively. Samples denoted with a P are stably transformed populations of 20−30 clones. PC3741 is a murine plasmacytoma which displays preferential usage of the 5′ *myc* promoter P_1 (Yang *et al.*, 1985). **Panels B** and **C** are the results of S1 mapping assays on total cell RNAs prepared 48 h after transfection of HeLa cells (2×10^6 cells seeded on 15 cm plates the day before the transfection) with 25 μg of the indicated CAT vectors. S1 mapping of CAT RNAs in **panel C** was performed with a 5′ end-labeled CAT probe. A plasmid (5 μg) containing a rabbit β globin gene regulated by the cytomegalovirus enhancer (pβHC41) (Boshart *et al.*, 1985) was co-transfected as a reference standard. β globin RNAs were detected with a 5′ end-labeled rabbit β globin probe prepared from the pSV2βG vector (see Materials and methods). The major SI-protected band corresponds to the 5′ end of the β globin sequences in the pSV2βG probe and the minor one is due to a cryptic start site within the β globin 5′ non-coding sequence (Boshart *et al.*, 1985). CAT S1 analysis in **panel C** was exposed to X-ray film with an intensifier screen for 5 days and β globin for 16 h without a screen.

regulated by the strong cytomegalovirus enhancer (Boshart *et al.*, 1985) was co-transfected with each of these *myc*−CAT genes as a reference control. These results demonstrate that the lower CAT activities of *myc*−CAT vectors, which lack an intact *myc* first exon, are mostly a consequence of reduced P_2 activity. In the remainder of this report, we characterize the properties of this positive element within the *myc* first exon.

Positive effect of the myc first exon exhibits a strong position and orientation dependence

We prepared a second set of *myc*−CAT hybrid genes that contain the SV40 early promoter region in place of the normal *myc* promoters. As shown in Figure 3, the SV*Myc*−CAT vector series was prepared by inserting a 438-bp *Hin*dIII−*Bgl*II *myc* segment, which contains most of exon 1 but lacks the P_1 and P_2 promoter regions, in either orientation in each of three different positions within pSV2CAT(ΔE) (an enhancer deletion derivative of pSV2CAT). Vectors containing the sense orientation of this 438-bp fragment are denoted with an R and those with the anti-sense orientation by an O subscript (see Figure 3). The results of CAT assays performed with cellular extracts of BJAB and HeLa cells transiently transfected with the SV*Myc*−CAT vectors are shown in Table II. The 438-bp *Hin*dIII−*Bgl*II fragment efficiently drives CAT expression in its sense orientation, downstream of the transcription initiation sites of the SV40 early promoters in pSVM(H)$_R$CAT. This positive effect is not observed in its anti-sense orientation nor when positioned in either orientation at a distance (3.2 kb 5′ and 1.7 kb 3′) from the SV40 early promoters [see pSVM(B)CAT vectors in Figure 3]. It is a weak stimulator of CAT activity in HeLa cells when positioned in its sense orientation 5′ of the SV40 promoters [compare activities of pSVM(H)$_R$CAT and pSVM(N)$_R$CAT in HeLa cells in Table II]. However, this latter effect is more apparent in HeLa cells where the intact first exon is more active.

The SV*Myc*−CAT vectors require an intact 438-bp *Hin*dIII−*Bgl*II *myc* segment to efficiently drive CAT expression. pSVM(H)$_R$ΔXhBgCAT and pSVM(H)$_R$ΔHdXhCAT are derivatives of pSVM(H)$_R$CAT that only contain the 5′ 378 and

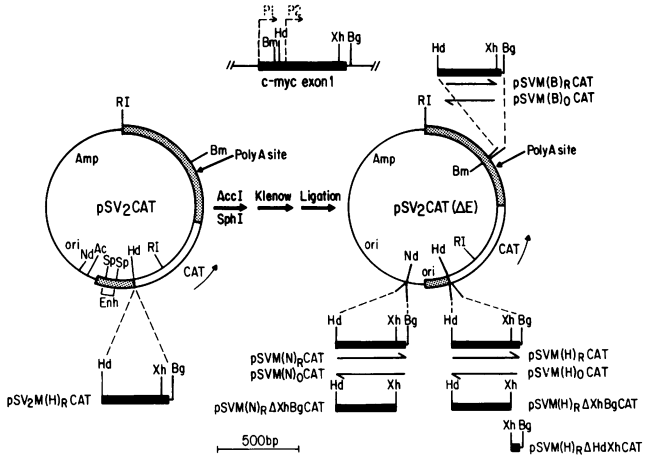

Fig. 3. SV*Myc*-CAT hybrid gene constructions. The indicated 438-bp *Hin*dIII−*Bgl*II *myc* fragment was inserted in either orientation in three different sites (*Nde*I, *Hin*dIII, *Bam*HI) within pSV2CAT(ΔE) as described in Materials and methods. Deleted derivatives of pSVM(H)$_R$CAT are indicated. The *Hin*dIII−*Bgl*II segment was also inserted in its sense orientation into the *Hin*dIII site of pSV2CAT. R and O subscripts designate sense and anti-sense orientations. Restriction enzyme sites are labeled as follows: Ac, *Acc*I; Bm, *Bam*HI; Bg, *Bgl*II; Hd, *Hin*dIII; Nd, *Nde*I; RI, *Eco*RI; Xh, *Xho*I.

Table II. Comparative activities of SV*myc*−CAT hybrid genes in HeLa and BJAB cells

CAT vector	Rel. CAT activity[a]	
	HeLa	BJAB
pSV2CAT	100	100
pSV2CAT(ΔE)	3.8	3.4
pSVM(B)$_R$CAT	5.0	2.3
pSVM(B)$_O$CAT	3.8	2.3
pSVM(N)$_R$CAT	13.0	5.8
pSVM(N)$_R$ΔXhBgCAT	8.0	4.7
pSVM(N)$_O$CAT	3.8	1.1
pSVM(H)$_R$CAT	46.0	25.0
pSVM(H)$_R$ΔXhBgCAT	14.0	7.1
pSVM(H)$_R$ΔHdXhCAT	8.0	4.7
pSVM(H)$_O$CAT	2.5	4.7
pSV2M(H)$_R$CAT	ND	214

[a]CAT activities are expressed relative to pSV2CAT and represent the average of at least three independent transfections with results differing by no more than 25%. Variations in transfection efficiencies were also controlled for in some experiments by quantitation of plasmid DNAs in Hirt lysates (Hirt, 1967) of transfected cells.
ND = Not determined.

3′ 60-bp of the *Hin*dIII−*Bgl*II *myc* fragment, respectively (see Figure 3). As shown in Table II, these two vectors produce weak CAT activity compared with pSVM(H)$_R$CAT. pSVM(N)$_R$CAT activity in HeLa cells is also reduced upon removal of the 60 bp XhBg fragment [see pSVM(N)$_R$ΔXhBgCAT in Table II].

To determine if these CAT activities are indicative of RNA levels, S1 nuclease protection experiments were performed with a [γ-^{32}P]-5′ end-labeled CAT DNA probe and total cellular RNAs isolated 48 h after transfection of COS cells with SV*Myc*−CAT vectors (Figure 4). The presence of an SV40 replication origin in the SV*Myc*−CAT vectors results in their amplification in COS cells thereby enhancing the sensitivity of S1 nuclease assays. An SV40 promoter-driven rabbit β globin gene (pSV2βG) (Mulligan *et al.*, 1979) was co-transfected along with each SV*Myc*−CAT vector as a reference control for transfection efficiency and RNA

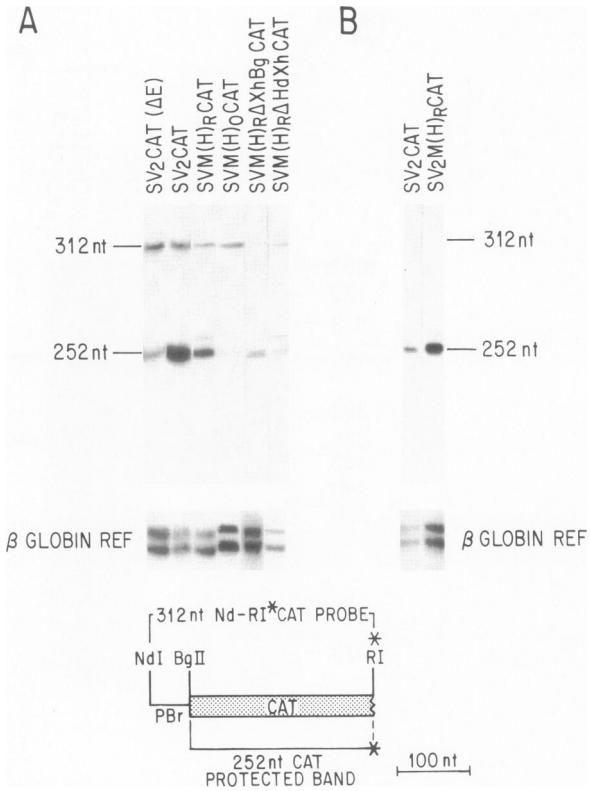

Fig. 4. Quantitative comparisons of CAT RNAs in COS cells transfected with pSV2CAT, pSV2CAT(ΔE) and various SV*Myc*-CAT vectors. (**A**) 50 μg of total cellular RNAs from each transfected cell population was annealed to 2 × 10^5 c.p.m. of a γ-^{32}P 5′ end-labeled DNA probe that contains 252 bases of CAT coding sequence. Minor S1-protected bands of slightly higher mol. wt than 252 bases are partial S1 digestion products. Annealing and S1 digestion conditions are described in Materials and methods. Each sample was also analyzed for the expression of rabbit β globin RNAs from pSV2βG, a co-transfected pSV2 β globin reference vector, with a 5′ end-labeled β globin probe. The β globin RNAs are expressed from the two SV40 early promoters. The autoradiographic exposure times for the CAT and globin RNAs were 5 days and 18 h with intensifier screens, respectively. (**B**) pSV2CAT and pSV2M(H)$_R$CAT expression were compared in an independent COS cell transfection. CAT and β globin autoradiographs in **panel B** were exposed for 48 and 18 h with intensifier screens, respectively.

integrity. pSVM(H)$_R$CAT is almost as active as the pSV2CAT positive control (Table III). However, SV*Myc*−CAT genes containing the 5′ 378 bp or the 3′ 60 bp of the *myc* sequences in pSVM(H)$_R$CAT are only ~2-fold more active than a pSV2CAT (ΔE) background control. A vector containing the complete 438-bp exon 1 sequence in its anti-sense orientation, pSVM(H)$_O$CAT, is inactive. For the most part, these observations agree fairly well with CAT assay data in other cell lines. However, CAT RNA levels in COS cells transfected with pSV2CAT(ΔE) and SV*Myc*−CAT vectors are ~2- to 4-fold higher than expected from CAT enzymatic assays with transfected HeLa and BJAB cells (Table II). This discrepancy could be caused by a background level of plasmid read-through transcription which is more apparent in COS cells due to vector replication. The low level of SV40 promoter usage in COS cells transfected with pSV2CAT(ΔE) supports this explanation (see Figure 7B and Table III).

SV40 early promoters are differentially activated by the myc first exon

S1 mapping experiments performed on total cell RNAs isolated 48 h after transfection of COS cells with various CAT vectors

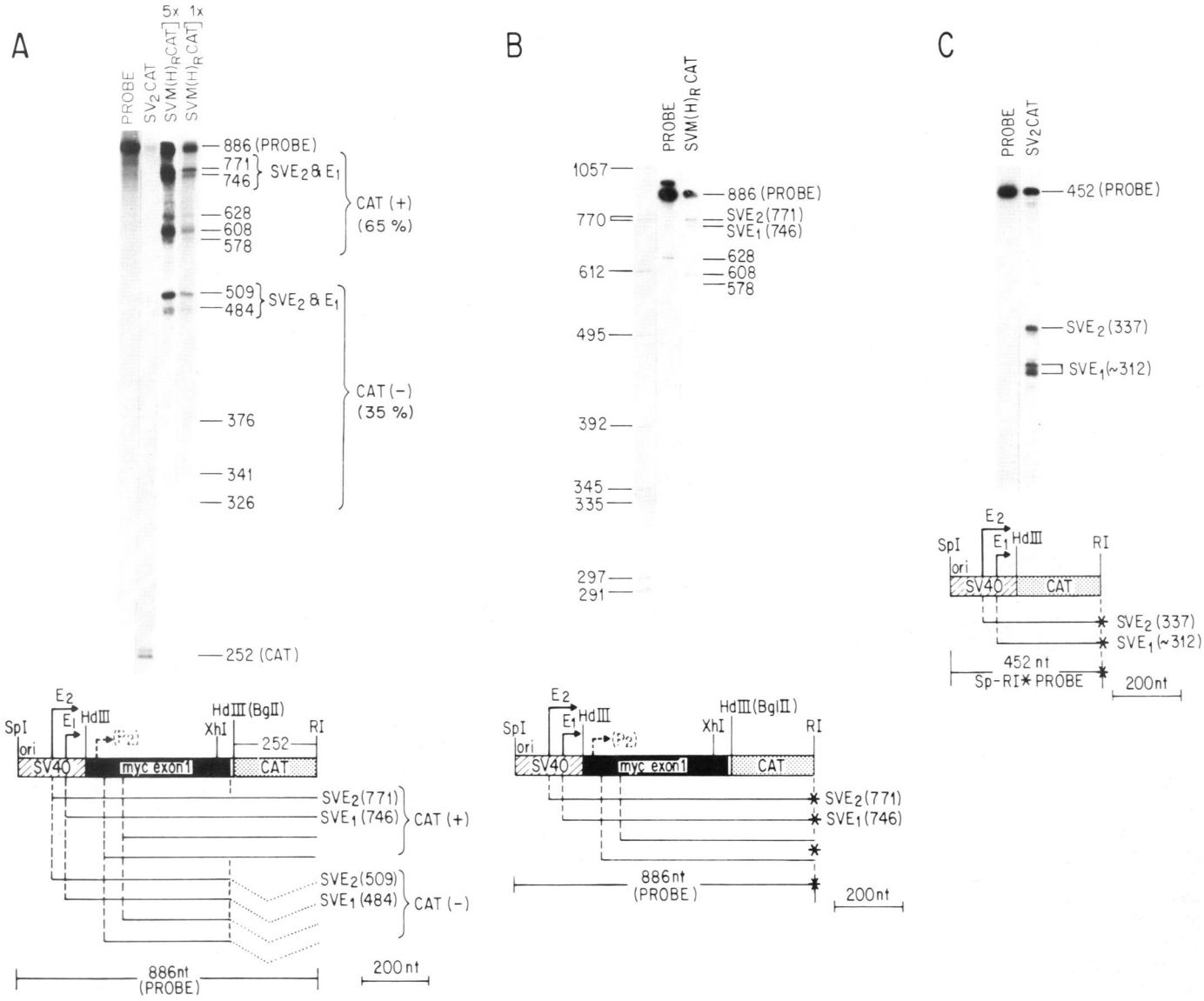

Fig. 5. S1 nuclease mapping of total cell RNAs isolated 48 h after transfection of COS cells with pSVM(H)$_R$CAT and pSV2CAT. (**A**) S1 mapping with a uniformly labeled pSVM(H)$_R$CAT probe. (**B**) and (**C**) S1 mapping with 5′ end-labeled pSVM(H)$_R$CAT and pSV2CAT probes, respectively. The origins of the S1-protected bands in **panels A–C** are indicated below the restriction maps of the probes (see text for discussion). Restriction site designations are the same as in Figures 1 and 4.

are presented in Figures 5–7. The usage of both SV40 early promoters (E2 and E1) are comparable in pSV2CAT transfected cells (Figures 5C and 7B). The SVE_1 site is somewhat heterogeneous but this has also been noted by others (Benoist and Chambon, 1981; Ghosh *et al.*, 1981; Buchman *et al.*, 1984). S1 nuclease assays on RNAs of pSVM(H)$_R$CAT-transfected COS cells were performed with uniformly labeled (Figure 5A) and 5′ end-labeled DNA probes (Figures 5B and 6). No S1-protected band corresponding to the *myc* P_2 site is evident. The *myc* *Hin*dIII–*Bgl*II fragment in pSVM(H)$_R$CAT contains 24 bp 5′ of the P_2 initiation site but lacks the TATA box positioned 28–32 bp 5′ of this start site (Stanton *et al.*, 1983, 1984). The lack of P_2 activity in pSVM(H)$_R$CAT-transfected cells suggests that the usage of this transcription initiation site requires >24 bp of 5′-flanking sequences in addition to a downstream element within the exon. The results in Figures 5–7 confirm that the dual SV40 early promoters SVE_2 and SVE_1 (also referred to as E_{up} and E_{do}) (Benoist and Chambon, 1981; Ghosh and Lebowitz, 1981; Ghosh *et al.*, 1981; Buchman *et al.*, 1984) are utilized in pSVM(H)$_R$CAT-transfected cells with a strong preference for SVE_2. The 3′-terminal 60-bp segment of the 438-bp *Hin*dIII–*Bgl*II exon I segment also drives the expression of the SV40 early promoters with a strong preference for SVE_2 [see pSVM(H)$_R$ΔHdXhCAT construct in Figure 3 and S1 analysis in Figure 7A]. The SV40 early promoters are much more active in cells transfected with pSVM(H)$_R$ΔHdXhCAT in comparison to the original pSV2CAT(ΔE) vector (Figure 7, Table III). Similar results were obtained with pSVM(H)$_R$ΔXhBgCAT which only contains the 5′ 378 bp of the first exon (data not shown, Table III). However, a comparison of Figures 6 and 7A (after correcting for differences in transfection efficiencies detected by SV2β globin reference controls in Table III) reveals that pSVM(H)$_R$ΔHdXhCAT is less active in driving the SV40 promoters than the pSVM(H)$_R$CAT construct which contains the intact 438-bp exon 1 fragment (Table III). A number of cryptic start sites within the *myc* first exon are also apparent in

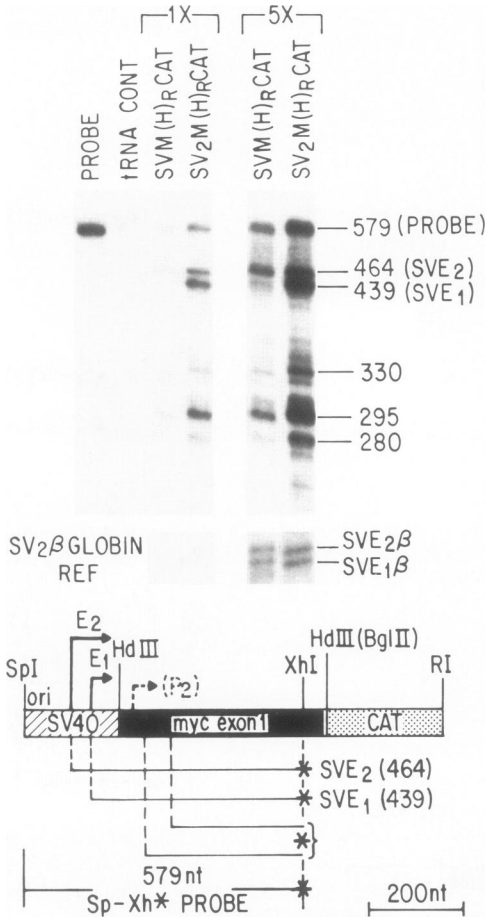

Fig. 6. Comparisons of SV40 early promoter usage in COS 1 cells transfected with pSVM(H)$_R$CAT and pSV2M(H)$_R$CAT vectors. S1 mapping with a 5′ end-labeled SV*Myc* probe (Sp-Xh*) reveals the relative contributions of the SVE_2 and SVE_1 start sites for CAT expression in pSVM(H)$_R$CAT and pSVM(H)$_R$CAT transfected cells. Exposure times were 1 and 5 days with intensifier screens from the 1X and 5X lanes, respectively, S1-protected bands corresponding to rabbit β globin transcripts expressed by the co-transfected pSV2 β globin reference control, are indicated.

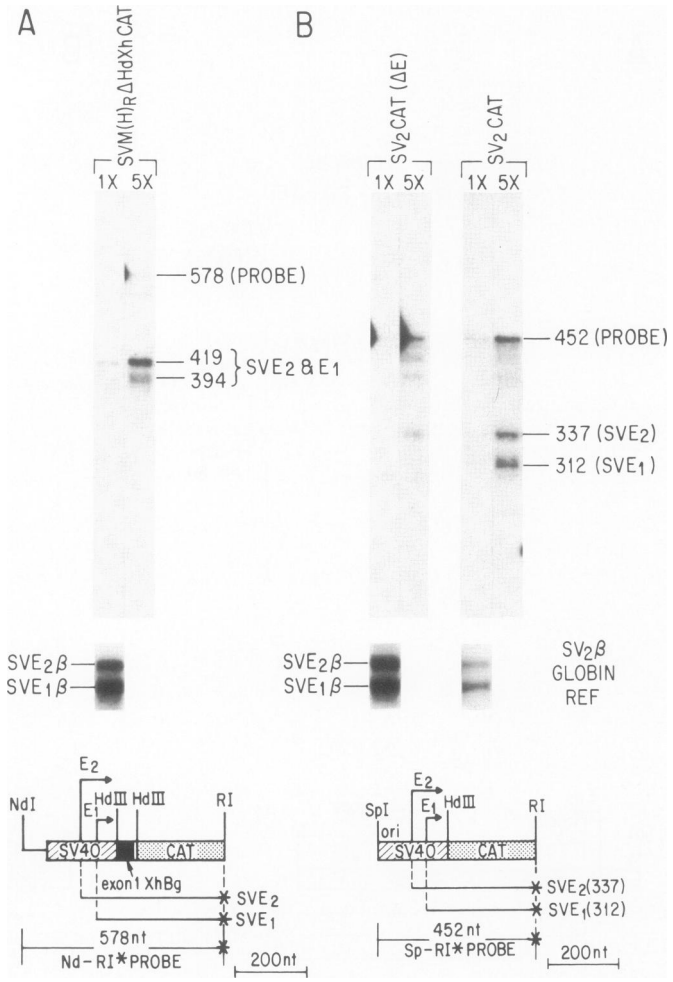

Fig. 7. Activation of the SV40 early promoters by a 60-bp exon 1/intron 1 junction segment. Transcription initiation sites employed by pSVM(H)$_R$ΔHdXhCAT, pSV2CAT(ΔE) and pSV2CAT were determined by S1 nuclease mapping with the indicated DNA probes. The pSV2βG vector was co-transfected as an internal reference control. 1X and 5X exposures are for 1 and 5 days, respectively.

pSVM(H)$_R$CAT-transfected cells. The usage of these cryptic start sites may be a consequence of removing the normal *myc* promoters.

A set of smaller S1-protected bands can be seen with a uniformly labeled pSVM(H)$_R$CAT probe [denoted CAT (−) in Figure 5A] which are not detected with a 5′ end-labeled probe (Figure 5B). The larger and smaller groups of bands in Figure 5A exhibit similar migration patterns with the higher mol. wt set being ~250 nucleotides larger. As expected, RNA from pSV2CAT-transfected cells protects only the 252-nucleotide CAT segment at the 3′ end of the uniformly labeled pSVM(H)$_R$CAT probe (Figure 5A). These findings are interpreted in Figure 5A to represent two alternative splicing pathways of the *myc*−CAT transcripts since the CAT coding region is 3′ of the *myc* exon 1/intron 1 splice donor signal in pSVM(H)$_R$CAT. Densitometric analyses of the autoradiographs in Figure 5A reveal that ~35% of the *myc* transcripts utilize the *myc* splice donor signal which would remove the CAT coding region from these mature transcripts. This result indicates that some of our CAT assays have somewhat underestimated the level of transcription obtained with *myc*−CAT vectors which retain the *myc* splice donor signal within the 60 bp XhBg segment [pMBgCAT, pMSmBgCAT, pSV2M(H)$_R$CAT, pSVM(H)$_R$CAT and pSVM(H)$_R$ΔHdXhCAT]. It would also follow that the utilization of this *myc* splice donor site could not enhance the stability of *myc*−CAT hybrid transcripts since such processed RNAs would lack CAT coding sequences.

Positive effects of the myc first exon and the SV40 enhancer are complementary

SV*Myc*−CAT hybrid genes which retain the SV40 enhancer are considerably more active than CAT vectors that only contain the *myc* exon or the SV40 enhancer. The 438-bp *Hin*dIII−*Bgl*II *myc* segment was also inserted into the *Hin*dIII site of pSV2CAT to generate pSV2M(H)$_R$CAT (see Figure 3). Experiments performed with BJAB cells demonstrate that pSV2M(H)$_R$CAT is considerably more active than either pSV2CAT or pSVM(H)$_R$CAT (Table II). This result is also confirmed by S1 nuclease mapping of CAT RNAs expressed in pSV2M(H)$_R$CAT-transfected COS cells (see Figures 4B and 6 and Table III). In addition to these quantitative increases in promoter activities, a shift from SVE_2 to SVE_1 usage is evident in COS cells transfected with pSV2M(H)$_R$CAT compared with pSVM(H)$_R$CAT (Figure 6). SVE_1 usage is also stronger in the pSV2M(H)$_R$CAT-transfected cells compared with pSV2CAT (Figures 6 and 7B).

Table III. Transcriptional activities of SV*Myc*−CAT vectors in COS cells

CAT vector	SV40 promoter activity[a]	Total CAT RNA level[b]	Transcription activity[c]
pSV2CAT	1.00	1.00	1.00
pSVM(H)$_R$CAT	0.75[d]	0.85[d]	1.10
pSVM(H)$_O$CAT	ND	0.05	0.20
pSVM(H)$_R$ΔHdXhCAT	0.15[d]	0.40[d]	0.45
pSVM(H)$_R$ΔXhBgCAT	0.25	0.38	0.40
pSV2M(H)$_R$CAT	3.00[d]	2.07[d]	2.20
pSV2CAT(ΔE)	0.03	0.15	0.20

[a]The combined activities of the two SV40 early promoters (SVE_2 and SVE_1) were determined from densitometric scans of the results in Figures 6 and 7 and from another independent experiment which also included pSVM(H)$_R$ΔXhBgCAT. Variations in transfection efficiencies were corrected by comparisons with SVβ globin reference standards and the data are expressed relative to pSV2CAT.
[b]Activities of CAT vectors were determined from densitometric scans of the S1 nuclease-resistant CAT bands in Figure 4. Variations in transfection efficiencies were controlled for by analyzing SV2β globin reference standards. CAT RNAs are expressed relative to the level in pSV2CAT-transfected cells. Some of these determinations are averages of two independent experiments [pSV2CAT, pSVM(H)$_R$CAT and pSV2M(H)$_R$CAT].
[c]Transcriptional activities were determined from densitometric scans of the *in vitro* nuclear 'run-on' assay shown in Figure 8. CAT transcription activities were corrected for variations in transfection efficiency by comparison with SV2β globin reference controls and were then expressed relative to the activity obtained with pSV2CAT.
[d]Values have been corrected for the fraction of transcripts (35%) which lost CAT sequences due to the usage of the *myc* exon 1/intron 1 splice donor signal (see Figure 5 and Discussion in text).

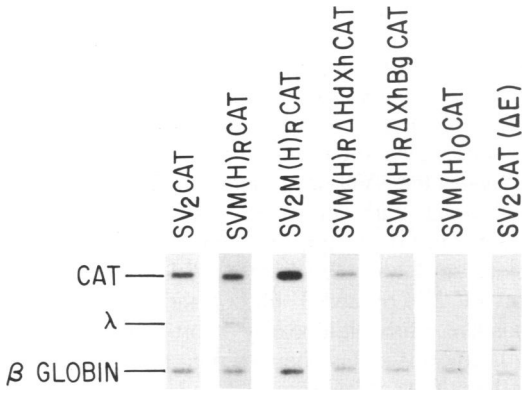

Fig. 8. Comparative transcriptional activities of various CAT vectors determined by nuclear 'run-on'. 2 × 10^6 COS cells were transfected with 20 and 10 μg of CAT and pSV2βG DNAs, respectively. Nuclei were prepared 48 h after transfection essentially as described (Schibler *et al.*, 1983). Nuclear 'run-on' was performed with 1 × 10^7 nuclei. Approximately 1.5 × 10^7 c.p.m. [α-^{32}P]UTP-labeled transcripts were hybridized to nitrocellulose filters previously spotted with 300 ng of purified restriction fragments of CAT [551-bp *Bgl*II−*Nco*I fragment from 5′ end of CAT gene in pCAT(ΔEP), Figure 1] (Gorman *et al.*, 1982; Alton and Vapnek, 1979) and rabbit β globin (437-bp *Hin*dIII−*Bgl*II fragment containing the first and part of the second exon of a rabbit β globin cDNA clone) (Mulligan *et al.*, 1979). An equivalent amount of *Hin*dIII-digested λ DNA (BRL) was also included to assess the degree of non-specific hybridization. Autoradiography was performed with an intensifier screen for 1 and 4 days for β globin and CAT, respectively.

Positive effect of the myc first exon is at the transcriptional level

Nuclear 'run-on' transcription assays were performed with nuclei prepared 48 h after transfection of COS cells with various CAT vectors. The results of this experiment are presented in Figure 8 and Table III and are summarized as follows: (i) pSV2CAT and pSVM(H)$_R$CAT have comparable transcriptional activities; (ii) pSVM(H)$_R$ΔHdXhCAT and pSVM(H)$_R$ΔXhBgCAT have low activity; (iii) pSVM(H)$_O$CAT is comparable with the background control pSV2CAT(ΔE); and (iv) pSV2M(H)$_R$CAT, which contains the SV40 enhancer and the intact 438-bp exon 1 sequence, has the highest transcriptional activity. We conclude from this analysis that the relative activities of SV*myc*−CAT vectors as determined by CAT enzymatic assays and quantitative S1 nuclease analyses of accumulated CAT RNAs are a good indication of their relative transcriptional activities (Table III). These results also demonstrate that the 438-bp *myc* sequence contains one or more *cis*-acting positive elements which function at the transcriptional level.

Discussion

Negative functions ascribed to the c-myc first exon

The c-*myc* first exon has been hypothesized to contribute to the extreme instability of c-*myc* RNAs (Dani *et al.*, 1984; Blanchard *et al.*, 1985). Two recent observations support this suggestion: (i) truncated *myc* transcripts, in murine plasma cell tumors and human Burkitt lymphomas with broken *myc* genes, lack the first exon and accumulate to higher levels (Piechaczyk *et al.*, 1985; Eick *et al.*, 1985; Rabbitts *et al.*, 1985); (ii) a long terminal repeat (LTR) driven human c-*myc* expression vector, which lacks the first exon, produces 2- to 3-fold higher steady-state levels of *myc* transcripts in COS cells (Butnick *et al.*, 1985). It is important to note that the c-*myc* expression vector in the latter study expressed authentic mRNAs in contrast to the *myc*−CAT vectors in the present study which only contained the *myc* first exon. The potential of the first and second exons to form a stable stem−loop structure (Saito *et al.*, 1984) may have some relevance for the possible role of the first exon in c-*myc* mRNA turnover. This secondary structure was originally proposed to modulate *myc* expression at the translational level (Saito *et al.*, 1984) and there now exists evidence for (Darveau *et al.*, 1985) and against (Butnick *et al.*, 1985) this suggestion. The *myc* first exon was also proposed to contain the binding site of a repressor (Leder *et al.*, 1983; Dunnick *et al.*, 1983; Rabbitts *et al.*, 1984) to account for the absence of normal c-*myc* expression in plasma cell tumors and Burkitt lymphomas (Adams *et al.*, 1983; Stanton *et al.*, 1983; Bernard *et al.*, 1983; Taub *et al.*, 1984; Nishikura *et al.*, 1983; Denny *et al.*, 1985; Fahrlander *et al.*, 1985). The data presented here do not address this latter hypothesis. Two recent reports have shown that sequence elements within the first exon or near the exon 1−intron 1 boundary are involved in the premature termination or pausing of *myc* transcription in differentiating and proliferating cells (Bentley and Groudine, 1986; Nepveu and Marcu, 1986). In this study, we provide strong evidence that this large non-coding exon also contains a positive control element.

Properties of a positive modulator within the c-myc first exon

A positive transcriptional modulator resides within the c-*myc* first exon, downstream of the gene's transcription initiation sites. A *myc*−CAT hybrid gene construct with 1.0 kb of c-*myc* sequences (including 423 bp of 5′-flanking DNA, the 563-bp first exon and the first 14 bp of intron 1) requires its 3′-terminal 60 bp for efficient usage of the major *myc* promoter, P_2. However, P_1, the 5′ c-*myc* promoter is not activated by the gene's first exon nor by 1.14 kb of 5′-flanking sequences, suggesting that P_1 and P_2 are regulated by independent control elements. We have localized a positive element (or elements) within a 438-bp *Hin*dIII−*Bgl*II segment that encompasses the 3′ 424 bp of exon 1 and the 5′

14 bp of intron 1. This intragenic positive modulator is necessary but not sufficient for the activity of the c-*myc* P_2 promoter since sequences 5′ of P_2 are also required for its activity. The 438-bp sequence efficiently drives gene expression only when positioned in its sense orientation, 3′ of a nearby promoter. It preferentially activates the SV40 upstream early promoter (SVE_2) over the downstream early promoter (SVE_1) providing additional evidence that this c-*myc* positive element possesses some degree of promoter selectivity. We divided the 438-bp sequence into two segments (5′ 378 bp and 3′ 60 bp) and found that each has a weak positive effect on a nearby 5′ promoter. We can suggest two explanations for this phenomenon: (i) a single positive element with multiple domains resides within the exon and (ii) there are multiple positive elements which have synergistic effects. Furthermore, we observe that the intact 438-bp *myc* segment does not appear to act as a conventional enhancer since it has different though complementary positive effects as the SV40 enhancer on the SV40 early promoters. Actinomycin D chase experiments indicate that the *myc* first exon does not significantly alter the stability of CAT transcripts (Piechaczyk *et al.*, 1986).

Intragenic positive modulators have been described for other genes transcribed by RNA polymerase II. Functionally rearranged immunoglobulin genes contain tissue-specific enhancers in their introns (reviewed in Calame, 1985) and possibly other positive elements within or nearby their heavy chain constant region gene segments (Grosschedl and Baltimore, 1985). The β globin (Charnay *et al.*, 1984) and chicken thymidine kinase genes (Merrill *et al.*, 1984) are also regulated in part by intragenic sequences. In addition to an enhancer element upstream of its promoter, the bovine leukemia virus (BLV) LTR also contains a second positive modulator downstream of its transcription initiation site (Derse and Casey, 1986). In analogy to the c-*myc* first exon positive effector, the latter BLV LTR positive element efficiently functions only downstream of a transcription initiation site. However, unlike the *myc* element, the BLV LTR sequence functions in both orientations. It remains to be determined whether this second BLV LTR positive element functions at the transcriptional or post-transcriptional level. Positive transcriptional elements have also recently been identified immediately downstream of the transcription start site of the *Drosophila hsp22* heat-shock gene (Hultmark *et al.*, 1986) and the adenovirus major late promoter (Mansour *et al.*, 1986).

A computer-assisted search of the 438-bp *Hin*dIII−*Bgl*II *myc* segment for enhancer- and promoter-like sequences did not reveal striking matches. An octamer sequence (GTTGGAAA), located 6−14 bp 5′ of the exon 1/intron 1 junction (Stanton *et al.*, 1983, 1984), bears some resemblance to an enhancer core motif ($GTGG^{TTT}_{AAA}G$) (Khoury and Gruss, 1983); and several GC-rich sequence motifs that have some similarities with the binding site of the Sp1 transcription factor (Dynan and Tjian, 1983) are also present. However, the strong position and orientation dependence of this *myc* positive element are not in keeping with the properties of the Sp1 transcription factor (Dynan and Tjian, 1983; Gidoni *et al.*, 1985). Future experiments will determine the precise molecular requirements of this *myc* first exon positive effector. It will be interesting to determine whether other 'housekeeping' genes that possess a large non-coding leader exon (like HMG-COA reductase) (Reynolds *et al.*, 1984) contain analogous positive modulators.

Materials and methods

Cell lines, transfections and RNA isolations

Myc-CAT vectors were introduced into COS 1 (Gluzman, 1981) and HeLa cells by the calcium phosphate procedure (Graham and Van der Eb, 1973; Remmers *et al.*, 1986) while the DEAE−dextran technique (Mosthof *et al.*, 1985) was used for BJAB cells (Zech *et al.*, 1976). For CAT assays, 10 μg of DNA was applied to a 10 cm plate seeded with 5×10^5 HeLa cells on the day before the transfection while 5×10^6 cells were used for BJAB. Preparative transfections for RNA isolations and subsequent S1 nuclease analyses were performed in duplicate with 25 and 5 μg, respectively of CAT and β globin DNAs applied to 2×10^6 cells seeded in a 15 cm plate on the day before transfection for HeLa and COS 1 cells. CAT lysates and total cell RNAs were prepared 48 h after transfection. Total cellular RNAs were prepared by lysing cells in 4 M guanidinium thiocyanate followed by phenol/chloroform extraction and ethanol precipitation (Chirgwin *et al.*, 1979; Maniatis *et al.*, 1982).

CAT enzyme assays

To quantitate CAT activity, 20 μl of each cell lysate was employed to acetylate [^{14}C]chloramphenicol (Lopata *et al.*, 1984). Labeled chloramphenicol and its resultant acetylated derivatives were separated by ascending silica gel chromatography ($CHCl_3$:methanol, 95:1) and detected by autoradiography. Reactions were quantitated by cutting out the radioactive spots and counting them in a scintillation counter.

Vector constructions

Myc−CAT series. The SV40 enhancer and early promoter were removed from pSV2CAT (Gorman *et al.*, 1982) by double digestion with *Acc*I and *Hin*dIII followed by Klenow polymerase treatment and insertion of a *Bgl*II linker to generate pCAT(ΔEP). The largest *myc*−CAT vector, pMBgCAT, contains a 1.71-kb *myc Bgl*II fragment which encompasses 1.14 kb of 5′-flanking c-*myc* sequence, the entire first exon and the 5′ 14 bp of intron 1. pMSmBgCAT was prepared by deleting the 5′ 716-bp *Bgl*II−*Sma*I segment in pMBgCAT. pMBgXhCAT and pMSmSsCAT were generated by deleting the 3′-terminal *Xho*I−*Bgl*II and *Sst*I−*Bgl*II fragments from pMBgCAT and pMSmBgCAT, respectively. pMBgBmCAT was prepared by inserting the 5′ 1.3-kb *myc Bgl*II−*Bam*HI fragment directly into pCAT(ΔEP). pMSmBmCAT was prepared from pMBgBmCAT by deleting the 5′-flanking 716-bp *Bgl*II−*Sma*I fragment. Deletions were resealed by Klenow polymerase treatment and blunt end ligated unless otherwise indicated.

SVMyc−CAT series. The SV40 enhancer region was removed from pSV2CAT by *Acc*I−*Sph*I double digestion followed by sequential Klenow and T4 DNA polymerase treatments then blunt end ligation to generate pSV2CAT(ΔE). This leaves the entire SV40 early promoter region intact including the 21-bp repeats. A 438-bp *Hin*dIII−*Bgl*II *myc* sequence (containing the 3′ 424 bp of exon 1 and the 5′ 14 bp of intron 1) was inserted in both orientations (designated by an R for sense and an O for anti-sense) into each of three sites within pSV2CAT(ΔE). $pSVM(N)_{R\&O}CAT$ were prepared by converting the *Nde*I site 5′ of the SV40 promoter to *Bgl*II followed by insertion of the 438-bp *Hin*dIII−*Bgl*II fragment after converting its ends to *Bgl*II. $pSVM(H)_{R\&O}CAT$ were constructed by converting the ends of the *myc Hin*dIII−*Bgl*II fragment to *Hin*dIII followed by insertion into the *Hin*dIII site between the SV40 promoter and the CAT gene in pSV2CAT(ΔE). $pSVM(B)_{R\&O}CAT$ were generated by converting the ends of the *myc Hin*dIII−*Bgl*II fragment to *Bgl*II followed by its insertion into the *Bam*HI site 3′ of the poly (A) addition site in pSV2CAT(ΔE). Deleted derivatives of some of these vectors [$pSVM(N)_R\Delta XhBgCAT$, $pSVM(H)_R\Delta HdXhCAT$ and $pSVM(H)_R\Delta XhBgCAT$] were prepared essentially as described for the above *myc*−CAT series. Finally, $pSV2M(H)_RCAT$ was generated by insertion of the *Hin*dIII-converted *myc Hin*dIII−*Bgl*II fragment into the *Hin*dIII site of pSV2CAT.

pSV2βG was prepared by replacing the CAT gene in pSV2CAT with a rabbit β globin cDNA derived from the vector pU3R−IIIβ that was kindly provided by Dr Craig Rosen (Rosen *et al.*, 1985).

S1 nuclease protection assays

S1 mapping (Berk and Sharp, 1977) of murine *myc*−CAT RNAs in stably transfected HeLa cells was performed with an α-^{32}P uniformly labeled *myc* exon 1 probe mp-R*S10, (see Figure 2) (Yang *et al.*, 1985). Annealing and S1 nuclease digestions were performed as previously described (Yang *et al.*, 1985).

Uniformly labeled and 5′ end-labeled CAT probes were employed to measure the expression of pSV2CAT and SV*Myc*−CAT vectors in transfected COS cells. SV*Myc*−CAT hybrid RNAs were detected with a uniformly labeled SV*Myc*−CAT probe prepared from an 886-nucleotide *Sph*I-RI fragment of $pSV2M(H)_RCAT$ cloned into M13 as previously described (Stanton *et al.*, 1983) (see Figure 5A). SV*Myc*−CAT RNAs were also analyzed with the same 886-nucleotide probe from $pSV2M(H)_RCAT$ that was 5′ end-labeled at the RI site within the CAT gene (Figure 5B) and with a smaller 5′ end-labeled probe derived from $pSV2M(H)_RCAT$ (Sp-Xh* probe in Figure 6) to determine the relative usage of the SV40 early promoters. The expression of all CAT vectors was also determined in some experiments with a CAT gene probe that was 5′ end-labeled at the RI site of a 312-bp *Nde*-RI CAT fragment of pCAT(ΔEP) (Figure 4). pSV2CAT RNAs were measured with a 5′ end-labeled, 452-nucleotide *Sph*I-RI probe (Figure 5C). 5′ end labeling was performed with polynucleotide kinase and [γ-^{32}P]ATP (Maxam and Gilbert, 1980). 50 μg of total cellular RNAs from

transfected COS cells was annealed to 2 × 10^5 c.p.m. of each of the probes at 42°C for 4 h in 80% formamide (Fluka), 0.4 M NaCl, 0.4 M Pipes pH 6.4, 1 mM EDTA except for the Sp-Xh* pSVM(H)$_R$CAT probe which was annealed at 56°C for 5.5 h followed by slow cooling to 42°C for 10 h. Digestion of the resultant RNA:DNA hybrids was performed with 100 units of S1 nuclease (BRL) for 60 min at room temperature.

S1 mapping of rabbit β globin RNAs was performed with a 5′ end-labeled 561-nucleotide *Sph*I−*Bam*HI* fragment derived from pSV2βG. Annealing was performed with 40 μg of total cell RNA from transfected COS cells at 56°C for 15 h.

Nuclear 'run-on' assays

Nuclear 'run-on' transcription assays were performed essentially as described (Schibler *et al.*, 1983) with minor modifications. Completed reactions were treated with 200 μg/ml of proteinase K for 30 min at 42°C. Nascent transcripts were precipitated in 10% trichloroacetic acid (TCA) and the precipitates washed in 5% TCA. 1.5 × 10^7 c.p.m. of [α-^{32}P]UTP-labeled, purified nuclear transcripts were hybridized to nitrocellulose strips containing 300 ng of purified DNA fragments. Pre-hybridizations and hybridizations were carried out for 24 and 48 h, respectively at 42°C in 50% formamide buffer as described (Schibler *et al.*, 1983). Filters were washed in 0.1 × SSC, 0.1% SDS for 1.5 h with six changes at 65°C prior to autoradiography.

Acknowledgements

We thank Dr Philip Leder for a λ phage genomic clone of a BALB/c embryo *c-myc* gene, Dr Walter Schaffner for PβHC41 and Dr Paul Fahrlander for critical reading and suggestions about the manuscript. We gratefully acknowledge Ms Barbara Springhorn, Mary Ann Huntington and Chris Helmke for their help with the manuscript, figures and photography. This research was supported by a grant from the National Cancer Institute awarded to K.B.M. who is the recipient of an NIH research career development award.

References

Adams,J.M., Gerondakis,S., Webb,E., Mitchell,J., Bernard,O. and Cory,S. (1983) *Proc. Natl. Acad. Sci. USA*, **80** 6966−6970.

Adams,J.M., Harris,A.W., Pinkert,C.A., Corcoran,L.M., Alexander,W.S., Cory,S., Palmiter,R.D. and Brinster,R.L. (1985) *Nature*, **318**, 533−538.

Alton,N.K. and Vapnek,D. (1979) *Nature*, **282**, 864−869.

Armelin,H.A., Armelin,M.C.S., Kelly,K., Stewart,T., Leder,P., Cochran,B.H. and Stiles,C. (1984) *Nature*, **310**, 655−660.

Battey,J., Moulding,C., Taub,R., Murphy,W., Potter,H., Lenoir,G. and Leder,P. (1983) *Cell*, **34**, 779−787.

Bechade,C., Calothy,G., Pessac,V., Martin,P., Coll,J., Denhez,S., Saule,S., Ghysdael,J. and Stehelin,D. (1985) *Nature*, **316**, 559−562.

Benoist,C. and Chambon,P. (1981) *Nature*, **290**, 304−310.

Bentley,D. and Groudine,M. (1986) *Nature*, **321**, 702−706.

Berk,A.J. and Sharp,P.A. (1977) *Cell*, **12**, 721−732.

Bernard,O., Cory,S., Gerondakis,S., Webb,E. and Adams,J. (1983) *EMBO J.*, **2**, 2375−2383.

Bishop,J.M. (1983) *Annu. Rev. Biochem.*, **52** 301−354.

Boshart,M., Weber,F., Jahn,G., Dorsch-Hasler,K., Fleckenstein,B. and Schaffner, W. (1985) *Cell*, **41**, 521−530.

Blanchard,J.-M., Piechaczyk,M., Dani,C., Chambard,J.-C., Franchi,A., Pousseger,J. and Jeanteur,Ph. (1985) *Nature*, **317**, 443−445.

Buchman,A.R., Fromm,M. and Berg,P. (1984) *Mol. Cell. Biol.*, **4**, 1900−1914.

Butnick,N.Z., Miyamoto,C., Chizzonite,R., Cullen,B.R., Ju,G. and Skalka,A.M. (1985) *Mol. Cell. Biol.*, **5**, 3009−3016.

Calame,K. (1985) *Annu. Rev. Immunol.*, **3**, 159−212.

Campisi,J., Gray,H.E., Pardee,A.B., Dean,M. and Sonenshein,G.E. (1984) *Cell*, **36**, 241−247.

Charnay,P., Treisman,R., Mellon,P., Chao,M., Axel,R. and Maniatis,T. (1984) *Cell*, **38**, 251−263.

Chirgwin,J.M., Przybyla,A.E., MacDonald,R.J. and Rutter,W.J. (1979) *Biochemistry*, **18**, 5294−5299.

Colby,W., Chen,E., Smith,D. and Levinson,A. (1983) *Nature*, **301**, 722−725.

Coughlin,S.R., Lee,W.M.F., Williams,P.W., Giels,G.M. and Williams,L.T. (1985) *Cell*, **43**, 243−251.

Dani,Ch., Blanchard,J.-M., Piechaczyk,M., El Sabouty,S., Marty,L. and Jeanteur,Ph. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7046−7050.

Dani,Ch., Mechti,N., Piechaczyk,M., Lebleu,B., Jeanteur,Ph. and Blanchard, J.-M., (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4896−4899.

Darveau,A., Pelletier,J. and Sonenberg,N. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 2315−2319.

Dean,M., Levine,R.A. and Campisi,J. (1986a) *Mol. Cell. Biol.*, **6**, 518−524.

Dean,M., Levine,R.A., Ran,W., Kindy,M.S., Sonenshein,G. and Campisi,J. (1986b) *J. Biol. Chem.*, **261**, 9161−9166.

Denny,C.T., Hollis,G.F., Magrath,I.T. and Kirsch,I.R. (1985) *Mol. Cell. Biol.*, **5**, 3199−3207.

Derse,D. and Casey,J.W. (1986) *Science*, **231**, 1437−1440.

Dony,C., Kessel,M. and Gruss,P. (1985) *Nature*, **317**, 636−639.

Dunnick,W., Shell,B.E. and Dery,C. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 7269−7273.

Dynan,W.S. and Tjian,R. (1983) *Cell*, **35**, 79−87.

Eick,D., Piechaczyk,M., Henglein,B., Blanchard,J.-M., Traub,B., Kofler,E., Wiest,S., Lenoir,G.M. and Bornkamm,G.W. (1985) *EMBO J.*, **4**, 3717−3725.

Fahrlander,P., Piechaczyk,M. and Marcu,K.B. (1985) *EMBO J.*, **4**, 3195−3202.

Gazin,C., Dupont de Dichenin,S., Hampe,A., Masson,J.-M., Martin,P., Stehelin,D. and Galibert,F. (1984) *EMBO J.*, **3**, 383−388.

Ghosh,P.K. and Lebowitz,P. (1981) *J. Virol.*, **40**, 224−240.

Ghosh,P.K., Lebowitz,P., Frisque,R.J. and Gluzman,Y. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 100−104.

Gidoni,D., Kadonaga,J.T., Barrera-Saldana,H., Takahashi,K., Chambon,P. and Tjian,R. (1985) *Science*, **230**, 511−517.

Gluzman,Y. (1981) *Cell*, **23**, 175−182.

Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044−1051.

Graham,F. and Van der Eb,A. (1973) *Virology*, **52**, 456−457.

Greenberg,M.E. and Ziff,E.B. (1984) *Nature*, **311**, 438−442.

Greenberg,M.E., Hermanowski,A.L. and Ziff,E.B. (1986) *Mol. Cell. Biol.*, **6**, 1050−1057.

Grosschedl,R. and Baltimore,D. (1985) *Cell*, **41**, 885−897.

Grosso,L.E. and Pitot,H.C. (1985) *Cancer Res.*, **45**, 847−850.

Hann,S.R., Thompson,C.B. and Eisenman,R.N. (1985) *Nature*, **314**, 369.

Hirt,B. (1967) *J. Mol. Biol.*, **26**, 365−369.

Hultmark,D., Klemenz,R. and Gehring,W.J. (1986) *Cell*, **44**, 429−438.

Jonak,G. and Knight,E. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1747−1750.

Kaczmarek,L., Hyland,J.K., Watt,R., Rosenberg,M. and Baserga,R. (1985) *Science*, **228**, 1313−1315.

Keath,E.J., Caimi,P.G. and Cole,M.D. (1984) *Cell*, **39**, 339−348.

Kelly,K., Cochran,B.H., Stiles,C. and Leder,P. (1983) *Cell*, **35**, 603−610.

Khoury,G. and Gruss,P. (1983) *Cell*, **33**, 313−314.

Klein,G. and Klein,E. (1985) *Immunol. Today*, **6**, 208−215.

Knight,E., Anton,E.D., Fahey,D., Friedland,B.K. and Jonak,G.J. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 1151−1154.

Lachman,H.M., Hatton,K.S., Skoultchi,A.I. and Schildkraut,C.L. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5323−5327.

Land,H.L., Parada,L. and Weinberg,R. (1983) *Nature*, **304**, 596−602.

Leder,P., Battey,J., Lenoir,G., Moulding,G., Murphy,W., Potter,H., Stewart,T. and Taub,R. (1983) *Science*, **222**, 765−771.

Lee,W.M.S., Schwad,M., Westaway,D. and Varmus,H.E. (1985) *Mol. Cell. Biol.*, **5**, 3345−3356.

Lopata,M.A., Cleveland,D.W. and Sollner-Webb,B. (1984) *Nucleic Acids Res.*, **12**, 5707−5717.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) In *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Laboratory Press, NY.

Mansour,S., Grodzicker,T. and Tjian,R. (1986) *Mol. Cell. Biol.*, **6**, 2684−2694.

Marcu,K.B., Yang,J.Q., Stanton,L.W., Mushinski,J.F., Fahrlander,P.D., Eckhardt,L.A. and Birshtein,B.K. (1984) In Wahren,B., Holm,G., Hammerstrom,S. and Perlmann,P. (eds), *Molecular Biology of Tumor Cells—Progress in Cancer Research and Therapy*. Raven Press, NY, Vol. **32**, 11−27.

Maxam,A. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499−580.

McCormack,J.E., Pepe,V.H., Kent,R.B., Dean,M., Marshak-Rothstein,A. and Sonnenshein,G.E. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5546−5550.

Merrill,G.F., Hauschka,S.D. and McKnight,S.L. (1984) *Mol. Cell. Biol.*, **4**, 1777−1784.

Mosthof,L., Pawlita,M. and Gruss,P. (1985) *Nature*, **315**, 597−600.

Mougneau,E., Lemieux,L., Rassoulzadegan,M. and Cuzin,F. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5758−5762.

Mulligan,R.C., Howard,B.H. and Berg,P. (1979) *Nature*, **277**, 108−114.

Nepveu,A. and Marcu,K.B. (1986) *EMBO J.*, **5**, 2859−2866.

Nepveu,A., Fahrlander,P., Yang,J.-Q. and Marcu,K.B. (1985) *Nature*, **317**, 440−443.

Nishikura,K., Ar-Rushdi,A., Erikson,J., Watt,R., Rovera,G. and Croce,C.M. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 4822−4826.

Persson,H., Gray,H.E. and Godeau,F. (1985) *Mol. Cell. Biol.*, **5**, 2903−2912.

Piechaczyk,M., Yang,J.-Q., Blanchard,J.-M., Jeanteur,Ph. and Marcu,K.B. (1985) *Cell*, **42**, 589−597.

Piechaczyk,M., Bonnieu,A., Eick,D., Remmers,E., Yang,J.-Q., Marcu,K.B., Jeanteur,Ph. and Blanchard,J.-M. (1986) *Curr. Top. Microbiol. Immunol.*, **132**, 331−338.

Rabbitts,T.H., Forster,A., Hamlyn,P.H. and Baer,R. (1984) *Nature*, **309**, 592−597.

Rabbitts,P.H., Forster,A., Stinson,M.A. and Rabbitts,T.H. (1985) *EMBO J.*, **4**, 3727−3733.

Rapp,U.R., Cleveland,J.L., Brightman,K., Scott,A. and Ihle,J.N. (1985) *Nature*, **317**, 434–438.
Reed,J.C., Nowell,P.C. and Hoover,R. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4221–4224.
Reitsma,P.H., Rothberg,P.G., Astrin,S.M., Barr-Shavit,Z., Hall,A., Teitelbaum,S.L. and Kahn,A.J. (1983) *Nature*, **306** 492–494.
Remmers,E.F., Yang,J.-Q. and Marcu,K.B. (1986) *EMBO J.*, **5**, 899–904.
Reynolds,G.A., Basu,S.K., Osborne,T.F., Chin,D.J., Gil,G., Brown,M.S., Goldstein,J.L. and Luskey,K.L. (1984) *Cell*, **38**, 275–285.
Rosen,C.A., Sodroski,J.G. and Haseltine,W.A. (1985) *Cell*, **41**, 813–823.
Saito,H., Hayday,A., Wiman,K., Hayward,W.S. and Tonegawa,S. (1984) *Proc. Natl. Acad. Sci. USA*, **80**, 7476–7480.
Schibler,U., Hagenbuchle,O., Wellauer,P.K. and Pittet,A.C. (1983) *Cell*, **33**, 501–508.
Southern,P.J. and Berg,P. (1982) *J. Mol. Appl. Genet.*, **2**, 327–341.
Stanton,L.W., Watt,R. and Marcu,K.B. (1983) *Nature*, **303**, 401–406.
Stanton,L.W., Fahrlander,P., Tesser,P. and Marcu,K.B. (1984) *Nature*, **310**, 423–425.
Stewart,T.A., Bellve,A.R. and Leder,P. (1984a) *Science*, **226**, 707–710.
Stewart,T.A., Pattengale,P.K. and Leder,P. (1984b) *Cell*, **38**, 627–637.
Taub,R., Moulding,C., Battey,J., Murphy,W., Vasicek,T., Lenoir,G.M. and Leder,P. (1984) *Cell*, **36**, 339–348.
Thompson,C.B., Challoner,P.B., Neiman,P.E. and Groudine,M. (1985) *Nature*, **314**, 363–366.
Vennstrom,B., Kahn,P., Adkins,B., Enrietto,P., Hayman,M.J., Graf,T. and Luciw,P. (1984) *EMBO J.*, **3**, 3223–3229.
Watt,R., Stanton,L.W., Marcu,K.B., Gallo,R.C., Croce,C.M. and Rovera,G. (1983). *Nature*, **303**, 725–728.
Yang,J.-Q., Bauer,S., Mushinski,J.F. and Marcu,K.B. (1985) *EMBO J.*, **4**, 1441–1447.
Yang,J.-Q., Mushinski,J.F., Stanton,L.W., Fahrland,P.D., Tesser,P.C. and Marcu,K.B. (1984) *Curr. Top. Microbiol. Immunol.*, **113**, 146–153.
Zech,L., Haglund,U., Nilsson,K. and Klein,G. (1976) *Int. J. Cancer*, **17**, 47–56.
Zerlin,M., Julius,M.A., Cerni,C. and Marcu,K.B. (1986) *Curr. Top. Microbiol. Immunol.*, in press.
Zerlin,M., Julius,M.A., Cerni,C. and Marcu,K.B. (1986) *Curr. Top. Microbiol. Immunol.*, **32**, 320–326.
Zullo,J.N., Cochran,B.H., Huang,A.C. and Stiles,C.D. (1985) *Cell*, **43**, 793–800.

Received on 29 August 1986