The EMBO Journal vol.6 no.10 pp.3043–3047, 1987

Inhibition of SV40 replicon function by engineered antisense RNA transcribed by RNA polymerase III

P.A.Jennings and P.L.Molloy

CSIRO Division of Molecular Biology, PO Box 184, North Ryde, NSW 2113, Australia

Communicated by G.G.Brownlee

Promoters recognized by RNA polymerase III were used to direct synthesis of RNAs of opposite polarity to the 5′ end of the mRNA for the large T-antigen of SV40. A construct was made utilizing the adenovirus (human type II) VA1 gene promoter linked to 163 bp of SV40 DNA sequences cloned in antisense orientation relative to the promoter. The SV40 sequence corresponds to the 5′ end of the large T-antigen gene. In addition to the antisense constructs control plasmids were utilized which either lacked both promoter and SV40 elements, lacked RNA polymerase III promoter elements but contained SV40 sequences or contained the VA1 gene promoter fused to SV40 sequences in the sense orientation. The function of the various gene fusions was demonstrated in an *in vitro* transcription system and *in vivo* by S1 nuclease 5′ end mapping following transfection into COS1 cells. Co-transfection of COS1 cells with the 'antisense' gene and a plasmid containing an SV40 origin of replication resulted in a substantial transient inhibition of SV40-replicon function when compared to control determinations (50% to nearly complete inhibition of large T-antigen dependent DNA replication for 18–36 h). These results show that an antisense RNA generated by RNA polymerase III can effectively block expression of a chromosomally located gene.

Key words: antisense RNA/RNA polymerase III/SV40 T-antigen

Introduction

Antisense RNA has been shown to have an important regulatory role in certain prokaryotic systems (Simons and Kleckner, 1983; Tomizawa, 1984; Mizuno *et al.*, 1984) and it has also been suggested that naturally occurring antisense RNAs might be involved in gene regulation in eukaryotes (Adeniyi-Jones and Zasloff, 1985). In addition, engineered RNAs complementary to certain messenger species (antisense RNA) have been used to impose a novel control on gene expression in a number of prokaryotic and eukaryotic systems (Coleman *et al.*, 1984; Izant and Weintraub, 1984; Kim and Wold, 1985; Melton, 1985; Rosenberg *et al.*, 1985; Weintraub *et al.*, 1985).

A potential application of the technology is the generation of cells or organisms in which the viral replicative cycle is disrupted. Virus-resistance has been achieved in *Escherichia coli* (Coleman *et al.*, 1985) and partial inhibition of virus production obtained for a non-lytic RNA virus, Rous sarcoma virus, in a eukaryotic system (Chang and Stoltzfus, 1985). We use here the lytic and transforming DNA virus SV40 to study the efficacy of an RNA polymerase III promoter in the generation of antisense RNAs. SV40 provides a good model system since its biology is well understood (Acheson, 1981) and its complete DNA sequence is known (Reddy *et al.*, 1978; Fiers *et al.*, 1978). Additionally, various viral functions have been segregated onto plasmids or into cell lines enabling their convenient experimental manipulation (Gluzman, 1981).

SV40 virus exhibits a lytic cycle in permissive cells, which can be divided into two phases, early and late. Large T-antigen (T-ag) is the major product of the early phase of the viral lytic cycle and is required for replication of viral DNA and effective stimulation of late gene expression. Inhibition of its production might therefore be expected to disrupt SV40-replicon function. Accordingly we have made DNA constructs which produce mRNAs complementary to the 5′-end region of the mRNAs for SV40 T-ag and tested them in COS1 cells in which T-ag provided in *trans* permits replication of plasmids containing the SV40 origin of replication.

Studies of antisense inhibition of gene expression in eukaryotes have thus far utilized either RNAs synthesized *in vitro* and microinjected into cells or embryos, or endogenous transcription of antisense RNAs from RNA polymerase II promoters. The constructs used in the present study contain the adenovirus VA1 RNA gene promoter, an RNA polymerase III promoter. RNA polymerase III promoters (reviewed, Ciliberto *et al.*, 1983) have certain features potentially advantageous to their use for the *in vivo* production of small antisense RNAs. In general they are active in all cell types, their normal RNA products (e.g. tRNAs, 5S rRNA) are synthesized in large amounts and the sequences of their promoters are highly conserved. Additionally it has been shown that the promoter of a tRNA gene carried on an SV40 vector is highly active in a transient assay system following its introduction into cells (Lassar *et al.*, 1985). Promoters recognized by RNA polyermase III therefore provide an attractive alternative to the more commonly used RNA polymerase II promoters.

We show that the adenovirus VA1 RNA gene promoter is transcriptionally active when linked to SV40 sequences, and that in transient transfections of COS1 cells an RNA transcript antisense to the 5′-end of T-ag mRNA suppresses T-ag-dependent replication of a plasmid carrying an SV40 origin of replication.

Results

Antisense gene construction

Antisense gene and control constructs are represented diagrammatically in Figure 1 and their construction described in Materials and methods. The antisense construct chosen for use in studies of T-ag suppression is designed to transcribe, from the intragenic adenovirus VA1 gene promoter, a small RNA of ~230 nucleotides in length containing 145 bases complementary to the 5′ end of T-ag mRNA (residues 5092–5237 of SV40, first AUG commencing at residue 5163 — Genbank, 1986).The engineered gene VASVL contains the VA1 promoter (VA) linked to SV40 sequences (SV) in antisense orientation as well as additional formation in the RNA transcript through 'panhandle' base pairing of the 5′ and 3′ ends of the RNA. The additional hexadecamer sequence is the inverted complement of the 5′ end sequence of VA1 RNA and was included in order to protect the ends of the

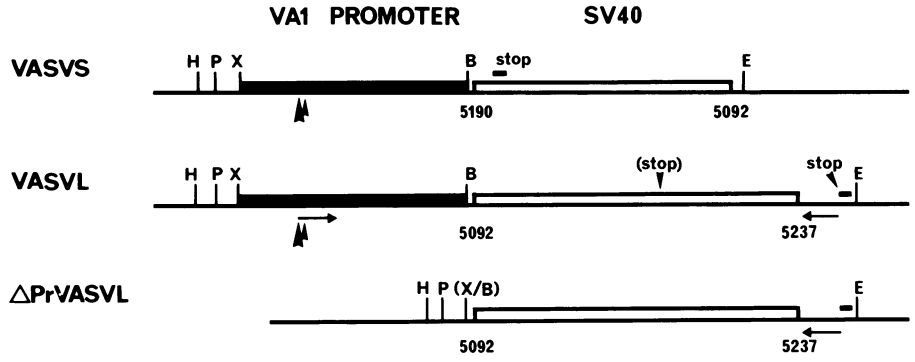

Fig. 1. Gene constructs. The arrangement of the components (VA1 RNA gene promoter and SV40 sequences) of the RNA polymerase III transcription units is shown. Sites of initiation of transcription are indicated (▲) as are RNA polyermase III stop signals. The adenovirus type 2 VA1 promoter was cloned as a *Xba*I−*Bam*HI fragment into *Xba*I, *Bam*HI-cut M13mp10w. For VASVS the SV40 sequences include bases 5092−5190. For VASVL the region of SV40 extends from base 5092−5237 with removal of the previous stop signal (by a T to G base change at position 5177). At the end of the SV40 sequences a hexadecamer with reverse complementarity to the 5′ end generated by VA transcription and a new RNA polymerase III stop site (four successive T's) were also incorporated into the construct. The arrows used to indicate the hexadecamer sequences also indicate scale. The control construct ΔPrSVL was made by deletion of the *Bam*HI or *Xba*I (VA1 gene promoter) fragment of VASVL with loss of both restriction sites. Inserts were re-cloned into pUC12 as *Eco*RI−*Pst*I fragments. Restriction sites are indicated: H, *Hin*dIII; P, *Pst*I; X, *Xba*I; B, *Bam*HI and E, *Eco*RI.

engineered RNA, via base-pairing, from digestion by single-strand exonucleases.

In addtion to the antisense construct control plasmids were utilized: (i) lacking both promoter and SV40 elements (pUC8), (ii) lacking RNA polymerase III promoter elements but containing SV40 sequence (ΔPrSV) and (iii) containing the VA1 gene promoter fused to SV40 sequence in the sense (S) orientation (VASVS).

Transcriptional activity of 'antisense' genes

The function of the various gene fusions was first assayed in an *in vitro* transcription system (Figure 2A) using a cell-free extract of human Hela cells (Weil *et al.*, 1979). For the various RNA polymerase III transcription units, the RNA products of the expected size were synthesized (Figure 2A), although in the case of VASVL significant amounts of both longer and shorter transcription products were seen. The longer transcripts were apparently due to read through of the engineered transcription terminator, while the shorter ones probably resulted from premature termination at three successive thymine residues in the template. S1 nuclease mapping of the 5′ end of the *in vitro* VASVL transcript showed only the correctly initiated transcripts (data not shown).

The antisense transcript units were further tested for transcriptional activity in COS1 cells. COS1 cells, a monkey kidney cell line transformed with SV40 defective in its origin of replication, were chosen because they are both permissive to SV40 infection and support replication of plasmids containing an SV40 origin of replication via the endogenous production of T-ag (Gluzman, 1981). S1 nuclease 5′ end mapping for VA promoter constructs (Figure 2B) and dot hybridization (not shown) demonstrated the presence of antisense RNAs in COS1 cells transfected with the antisense gene constructs.

SV40 replicon function is inhibited by 'antisense' genes

The effect of 'antisense' RNA production on SV40-replicon function in COS1 cells was tested following DNA transfection by a modified DEAE−dextran transfection procedure (Sussman and Milwan, 1984). Direct assay of the level of T-ag is not useful in a transient assay system as only a proportion of cells is actually transfected; therefore, significant T-ag suppression in the transfected cells may have little apparent effect on the level of T-ag in the total cell population. 'Antisense' or control constructs were co-transfected, in various ratios, with a dam-methylated plasmid pPJSV40.4T containing an SV40 origin of replication and the activity of T-ag in COS1 cells was measured by the capacity of these cells to replicate the test plasmid. As newly replicated (non-methylated) DNA is senstive to cleavage by *Mbo*I (McClelland and Nelson, 1985), the amount of replicated DNA in cell extracts was determined by *Mbo*I cleavage, Southern blotting and hybridization. Quantitation of replicated DNA was relative to samples of serially diluted *Sau*3A-cut test plasmid electrophoresed on the same gel with subsequent autoradiographic exposure for several different time intervals.

For VASVL a substantial inhibition of plasmid replication was seen. In Figure 3 the levels of replicated DNA in the presence of the antisense construct, compared with controls, were ~15% (18 h, panel 1) and <10% (24 h, panel 2). In different experiments this suppression varied between 50% to nearly complete (>95%) and persisted for 18−36 h after transfection. At later times, levels of replicated DNA became similar to those observed in control transfections, indicating that similar numbers of cells were transfected. Co-transfection with the control 'sense' construct VASVS did not produce an inhibition of replication of the test plasmid pPJSV40.4T (Figure 3, panel 1), demonstrating a requirement for the antisense orientation of the SV40 DNA segment. Similarly co-transfection with the promoter deletion mutant ΔPrSV did not lead to a reduction in replication of the test plasmid pPJSV40.4T (Figure 3, panel 2) showing that inhibition is dependent on the presence of the RNA polymerase III promoter and cannot be due to synthesis of an RNA initiated within vector DNA sequences.

In other control experiments we investigated the effect of transfection of COS1 cells with the test plasmid pPJSV40.4T on transcription of T-ag mRNA. The basal level of T-ag mRNA in COS1 cells increased significantly following transfection (Figure 4). This is consistent with the retention of the capacity of T-ag to regulate its own synthesis in COS1 cells. Such autoregulation is well described for SV40 virus (Acheson, 1981). In our experiments co-transfection of COS1 cells with pPJSV40.4T and carrier DNA produced a ten-fold increase in T-ag mRNA signal (as judged from different autoradiographic exposures). The level of stimulation of the early promoter in individual COS1 cells is presumably much greater than this since < 10% of the monolayer cells are usually transfected (unpublished results). It might therefore be expected that suppression of T-ag expression by 'antisense' RNAs and/or binding of T-ag by

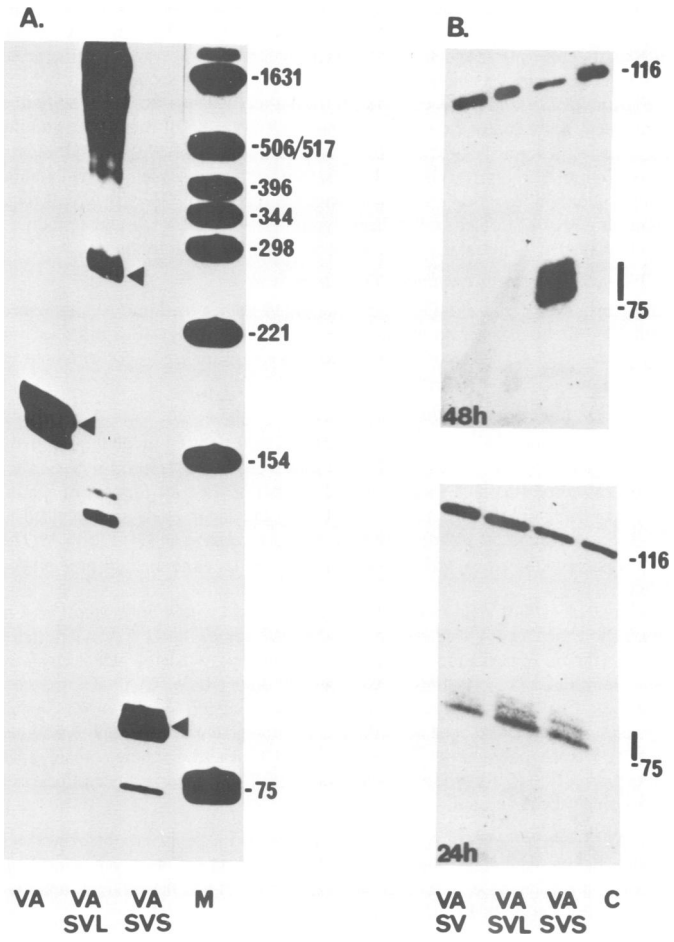

Fig. 2. Activity of transcription units. **Panel A.** *In vitro* transcription of constructs. Transcriptions were done using 0.5 μg of DNA of the designated plasmids. The track designations indicate genes VASVS, VASVL and VA, a control plasmid pHIIIB containing the adenovirus VA1 and VA2 genes (Manley and Colozzo, 1982). Track M contains marker DNAs (*Hin*fI-cut pBR322). (◀) indicates appropirate transcripts. **Panel B.** S1-nuclease analysis of RNAs. RNA was isolated from COS1 cells 24 or 48 h after transfection (lower and upper subpanels respectively) and 5 μg of RNA analysed for the presence of the VA RNA 5′ end (see Materials and methods). Track designations indicate RNA from transfections with genes VASVL, VASV (see Materials and methods), VASVS and C, control vector DNA. S1-nuclease-protected fragments and 75 and 116 base marker fragments are indicated.

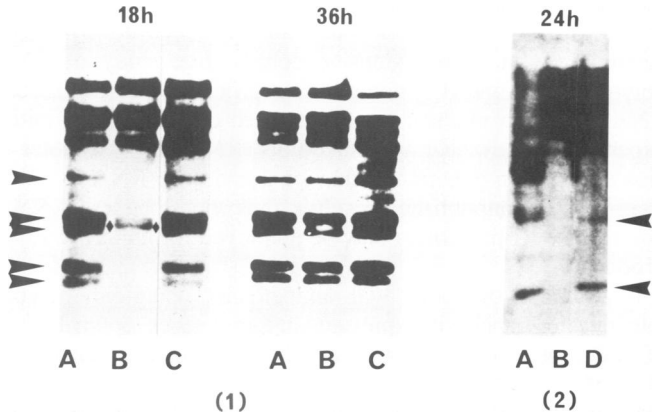

Fig. 3. Assay for T-antigen-dependent replication. Duplicate 60 mm dishes of COS1 cells were transfected with 200 ng of plasmid pPJSV40.4T and 2 μg of the following DNAs: **A**, pUC8; **B**, pUCVASVL; **C**, pUCVASVS and **D**, pUCΔPrSV. Blots of *Mbo*I-digested DNA recovered from different transfection experiments (**panels I and 2**) were hybridized with either nick-translated pUC8 DNA (panel 1) or with a specific RNa probe (panel 2) (see Materails and methods). An end-labelled DNA fragment (♦) was added to cell lysates in panel 1 to monitor DNA recovery. Filters were autoradiographed prior to hybridization analysis to verify equivalent DNA recovery (not shown). Arrows indicate *Mbo*I digestion products, corresponding to replicated DNA. In the majority of electrophoresis experiments several dilutions of *Sau*3A-cut test plasmid were also run in parallel (on the same gel) to *Mbo*I-cleaved DNA samples, in order to quantitate levels of replicated plasmid (data not shown). For example the signal seen for the *Mbo*I digestion product in a control track (lane A, 18-h time point, panel 1) represents a level of replicated plasmid DNA of 1.5−2 ng DNA from the tissue culture dish.

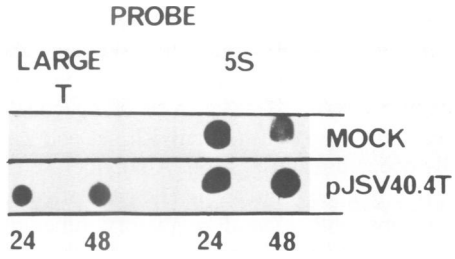

Fig. 4. Induction of large T mRNA in COS1 cells. COS1 cells were either mock transfected or transfected with 2 μg of pPJSV40.4T DNA. RNA was isolated from cells 24 and 48 h post-transfection and RNA samples (5 μg) were spotted onto nitrocellulose and hybridized with a probe specific for the 5′ end of large t-antigen mRNA. The probe was synthesized by primer extension on an M13 clone containing the *Bst*NI to *Stu*I fragment (bases 5092−5190, Genbank) of SV40 DNA. Control hybridizations were done using a 5S RNA probe made by end-filling of two overlapping oligonucleotides corresponding to the promoter region of the 5S rRNA gene.

input plasmid-borne SV40 origin sequences would lead inevitably to de-repression of early promoter activity. Such an effect, coupled with waning levels of the non-replicating 'antisense' plasmid, might account for the transient nature of the inhibition.

Consistent with this, S1 nuclease-analysis of 'antisense' RNAs generated by VASVL in COS1 cells showed significant levels of the antisense RNA at 24 h but failed to detect such species at 48 h (Figure 2, panel B). By contrast the control 'sense' RNA (from VASVS) was still present at this time. This suggests that the antisense RNA either has an intrinsically lower stability than the sense RNA or that the antisense RNA is sequestered by hybridization to increasing levels of mRNA from the de-repressed T-ag gene.

Discussion

A number of parameters may dictate the effectiveness of 'antisense' inhibition (reviewed by Weintraub *et al.*, 1985), some of which may vary between RNA polymerases II and III promoter systems. In general it has been found that antisense RNAs are more effective if directed against the 5′ region of mRNA (Weintraub *et al.*, 1985) as used here, though in some cases effective inhibition has been seen using RNAs directed against other regions. A significant difference between the RNA polymerase II-promoted antisense genes and those used by other workers (RNA polymerase II) lies in the nature of the RNA transcripts. They are neither 'capped' nor polyadenylated and are substantially shorter than those antisense RNAs so far transcribed from other introduced DNAs (Weintraub *et al.*, 1985; Green *et al.*, 1986). In general these latter transcripts have been long, though the region of complementarity to the target RNA may have been quite short. Small RNA products may have a kinetic advantage, both in accessibility and hybridization to target sequences. The stability and cellular localization of the RNA polymerase III

transcripts (which are likely to differ significantly from RNA polymerase II transcripts) may also influence their effectiveness. Accordingly, a more extensive comparative study of RNA polymerase III and II systems for the production of engineered RNAs has been initiated to address such questions. Our initial experiments show that a synthetic 5S rRNA gene promoter is also effective in generating functional antisense RNA to SV40 T-ag mRNA (unpublished results); thus, both classes of RNA polymerase III promoters may be employed for antisense RNA production.

In a number of studies with animal cells, using either stably integrated or transiently expressed 'antisense genes' optimal levels of inhibition of expression of specific proteins has varied from 80 to 90% (Chang and Stoltzfus, 1985, Crowley *et al.*, 1985; Izant and Weintraub 1984; Kim and Wold, 1985; McGarry and Lindquist, 1986; Sandri-Goldin *et al.*, 1987). It is difficult to compare our results on antisense RNA-mediated inhibition of DNA replication with these studies involving measurement of suppression of synthesis of a specific protein. Nevertheless at least 95% suppression of T-ag dependent DNA replication was observed in some experiments at early time points. The level of inhibition of DNA replication need not be directly proportional to that of T-ag synthesis. It is possible that there is an amplification of the effect due to inhibition of each successive round of DNA replication. That the effect was transient may relate both to the autoregulatory capacity of the SV40 early promoter and to the incapacity of the input 'antisense' construct to replicate. it is also possible that a proportion of the COS1 cells received only the test plasmid, which then was able to replicate in the absence of VASVL.

The use of antisense RNA has importance, as a tool for molecular-genetic analysis and as a means of modifying the phenotype of cells or whole organisms. We have shown that an RNA polymerase III promoter is potentially useful in the generation of antisense RNAs effective in blocking gene expression. This supports the suggestions (Adeniyi-Jones and Zasloff, 1985) that naturally occurring antisense RNA polymerase III transcripts may have a role in gene regulation. The gene affected in the present study is one with a vital regulatory function in a viral replicative cycle; this and the work of others (Chang and Stoltzfus, 1985) offers the prospect that eukaryotic cells or organisms may be engineered to a state of constitutive resistance to given viral diseases.

Materials and methods

Restriction endonucleases, T_4 polynucleotide kinase, T_4 DNA ligase, *E. coli* DNA polymerase I (and DNA polymerase I Klenow fragment) and S1 nuclease were purchased from International Biotechnologies, Inc., New England Biolabs and Boehringer-Mannheim. ^{32}P-labelled compounds were purchased from BRESA, South Australia. Oligonucleotide synthesis was conducted using an Applied Biosystems 180B DNA synthesizer using reagents purchased from the manufacturer. Nylon membranes, Biodyne A, were purchased from Pall Ultrafine Filtration Corporation. Bacterial strains TG1 and RR1 were employed in this study.

Vector construction

DNA constructs. The adenovirus VA1 gene promoter was asymmetrically cloned on a *Bam*HI, *Xba*I fragment (bases 10 579–10 685 of Adenovirus, type II, Genbank 1987) into M13mp10W and an end-repaired *Bst*NI–*Stu*I fragment of SV40 (bases 5092–5192, Genbank 1987) was then inserted into the adjacent *Sma*I site in both orientations to give MVASV (antisense orientation) and MVASVS (sense orientations); MVASV had a RNA polymerase III stop signal within the SV40 insert (five successive thymines, 5175–5179). The antisense RNA would thus cover the first AUG but not extend appreciably further towards the 5′ end of T-ag mRNA. Accordingly a 101-nucleotide synthetic DNA 'insertion' primer was used to direct mutagenesis (Nisbet and Beilharz, 1985) and thus generate MVASVL. The primer was complementary at its 5′ end to cloned SV40 sequence (bases 5168–5192, with a G for T substitution at position 5177) and at its 3′ end complementary to 13 residues of the M13 vector in the region of the commonly used 'reverse-priming' site. Sequences thus inserted included further SV40 sequence (5193–5239) and immediately upstream of the M13 sequences, a run of four thymines, (transcription terminator) preceded by the hexadecameric inverted complement of the adenovirus VA1 RNA 5′ end sequence.

Plasmid pPJSV40.4T was constructed from a recombinant pUC-based plasmid containing, additionally the phage F_1 single-strand origin of replication and the SupF gene (generously provided by J.Karn Laboratory of Molecular Biology, MRC Centre, Cambridge, UK) by ligating into polylinker sequences a eukaryotic transcription unit containing the SV40 origin of replication (*Hin*dIII–*Pvu*II fragment), a cDNA of influenza viral haemagglutinin — A/PR/8/34, and a modified SV40 polyadenylation signal (derived from *Bam*HI–*Bcl*1 fragment).

COS1 cells — growth conditions/DNA transfection

COS1 cells were grown in Dulbecco's modified Eagles medium, supplemented with 10% foetal calf serum at 37°C and 7.5% CO_2. Cells were passaged the day prior to DNA transfections and were split to ensure a 50–70% monolayer at the time of DNA transfection.

DNA preparation and Southern blot analysis

Cells were washed twice with Hepes buffered saline, lysed by addtion of 1 ml of 50 mM Tris–HCl, 10 mM EDTA, 10 mM β-mercaptoethanol, 1% SDS; after 5 min 250 μl of 4 M KCl was added and the mix held on ice for at least 5 min. After centrifugation for 5 min at 11 000 × *g* the supernatant was adjusted to 0.5% Sarkosyl and 50 μg/ml proteinase K and incubated at 37°C for 1 h. The mix was extracted twice with phenol:chloroform (1:1) and ethanol precipitated twice.

The DNA (1/4 of yield from a dish) was incubated with 4 units *Mbo*I for 4 h, electrophoresed on 1.4% agarose gels and transferred to Biodyne A nylon filters. Filters (Figure 3, panel 1) were hybridized with nick translated pUC8 DNA (as per Church and Gilbert, 1984). Alternatively a specific RNA probe was used (panel 2). The RNA probe to an insert in the vector Bluescribe (+) (Vector Cloning System) was made and used according to the manufacturers' recommendations, using T7 RNA polymerase. The recombinant Bluescribe vector contained the cloned *Hin*dIII–*Pvu*II origin fragment of SV40 DNA, and had been linearized at its single *Sca*I site.

In vitro transcription

Transcriptions were done using 0.5 μg of DNA in 20 μl reactions containing 10 μl of a HeLa cell lysate as described (Weil *et al.*, 1979) and the products analysed on 6% acrylamide, 8 M urea denaturing gels.

Analysis of RNAs

RNA was isolated from COS1 cells 24 or 48 h after transfection with the various transcription units, using the guanidine isothiocyanate, hot phenol method (Feramisco *et al.*, 1982) followed by digestion with DNAseI in the presence of RNAsin and further phenol extraction and ethanol precipitation. Five micrograms of RNA was annealed with a 5′-end labelled single-stranded DNA fragment, running from the *Pst*I site in the polylinker, 5′ to the transcription start site, to the *Bam*HI site of the cloned VA1 promoter region (116 bases), in 10 mM Tris–HCl ph 7.9, 1 M NaCl, 1 mM EDTA, for 2 h at 55°C. It was then subjected to S1 nuclease digestion in 300 mM NaCl, 30 mM $NaCH_3COO$ pH 4.5, 1 mM $ZnSO_4$ (Sharp *et al.*, 1980) and the products analysed on an 8% acrylamide 8 M urea denaturing gel. RNA analysis using dot-hybridization was performed as in the legend to Figure 4.

Acknowledgements

We would like to thank Jenny Tallon and Jan Edwards for excellent technical assistance, Dr G.Both for oligonucleotide synthesis and Professor G.Brownlee and Drs G.W.Grigg, G.Both and M.Sleigh for critical reading of the manuscript.

References

Acheson,N.H. (1981) In Tooze,J. (ed.), *DNA Tumor Viruses*, 2nd edn Part 2. Cold Spring Harbor Laboratory, New York, pp. 189–204.
Adeniyi-Jones,S. and Zasloff,M. (1985) *Nature*, **317**, 81–84.
Chang,L.-J. and Stoltzfus,C.M. (1985) *Mol. Cell. Biol.*, **5**, 2341–2348.
Church,G.M. and Gilbert,W. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1991–1995.
Ciliberto,G., Castagnoli,L. and Cortese,R. (1983) *Curr. Topics Dev. Biol.*, **18**, 59–88.
Coleman,J., Green,P.J. and Inouye,M. (1984) *Cell*, **37**, 429–436.
Coleman,J., Hirashima,A., Inokuchi,Y., Green,P.J. and Inouye,M. (1985) *Nature*, **315**, 601–604.
Crowley,T.E., Nellen,W., Gomer,R.H. and Firtel,R.A. (1985) *Cell*, **43**, 633–641.
Feramisco,J.R., Healhman,D.M., Smart,J.E., Burridge,K. and Thomas,G.P. (1982) *J. Biol. Chem.*, **257**, 11024–11031.
Fiers,W., Contreras,R., Haegeman,G., Rogiers,R., van de Voorde,A., Heuverswyn,H.Van, Heereweghe,J.Van, Volckaertand,G. and Ysebaer,M. (1978) *Nature*, **273**, 113–120.

Gluzman,Y. (1981) *Cell*, **23**, 175–182.
Green,P.J., Pines,O. and Inouye,M. (1986) *Annu. Rev. Biochem.*, **55**, 569–597.
Izant,J.G. and Weintraub,H. (1984) *Cell*, **36**, 1007–1015.
Kim,S.K. and Wold,B.J. (1985) *Cell*, **42**, 129–138.
Lassar,A.B., Hamer,D.H. and Roeder,R.G. (1985) *Mol. Cell. Biol.*, **5**, 40–45.
Manley,J.L. and Colozzo,M.T. (1982) *Nature*, **300**, 376–379.
McClelland,M. and Nelson,M. (1985) *Nucleic Acids Res.* **13** (Suppl.), r201–r207.
McGarry,T.J. and Lindquist,S. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 399–403.
Melton,D.A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 144–148.
Mizuno,T., Chou,M. and Inouye,M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1966–1970.
Nisbet,I.A. and Beilharz,M.W. (1985) *Gene Anal. Techniques*, **2**, 23–29.
Reddy,V.B., Thimmappaya,B., Dhar,R., Subramanian,K.N., Zain,B.S., Pan,J., Ghosh,P.K., Celma,M.L. and Weissman,S.M. (1978) *Science*, **200**, 494–502.
Rosenberg,U.B., Preiss,A., Seifert,E., Jackle,H. and Knipple,D.C. (1985) *Nature*, **313**, 703–706.
Sandri-Goldin,R.M., Sekulovich,R.E. and Leary,K. (1987) *Nucleic Acids Res.*, **15**, 905–919.
Sharp,P.A., Berk,A.J. and Berget,S.M. (1980) *Methods in Enzymol.*, **65**, 750–768.
Simons,R.W. and Kleckner,N. (1983) *Cell*, **34**, 683–691.
Sussman,D.J. and Milwan,G. (1984) *Mol. Cell. Biol.*, **4**, 1641–1643.
Tomizawa,J. (1984) *Cell*, **38**, 861–870.
Weil,P.A., Segall,J., Harris,B., Ng,S.-Y. and Roeder,R.G. (1979) *J. Biol. Chem.*, **254**, 6161–6173.
Weintraub,H., Izant,J.G. and Harland,R.M. (1985) *Trends Genet.*, **1**, 22–25.

Received on April 30, 1987; revised on June 29, 1987