Synthesis of rabbit β-globin in cultured monkey kidney cells following infection with a SV40 β-globin recombinant genome

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Rabbit β-globin complementary DNA (cDNA) has been inserted into SV40 DNA in place of the gene coding for the virus' major capsid protein, VP1. The recombinant genome, SVGT5–RaβG, multiplies efficiently in CV1 monkey kidney cell cultures and is transcribed to yield cytoplasmic, polyadenylated hybrid mRNAs containing the β-globin coding sequence. Cells propagating SVGT5–RaβG produce substantial quantities of rabbit β-globin polypeptide.

MAMMALIAN cells infected with SV40 virus carrying cloned eukaryotic genes provide one system in which the expression of eukaryotic genes can be studied[1–3]. SV40 is particularly attractive as a transducing vector for various reasons. The virus genome consists of a single, small, covalently closed circular DNA molecule, whose entire nucleotide sequence has recently been determined[4,5]; the viral DNA is obtainable in large quantities and relatively pure form; the genomic regions responsible for the various viral functions have been accurately located with respect to a detailed physical map of the DNA[4–7]; the viral genome can multiply vegetatively or as an integral part of cellular chromosomes; and a wealth of information exists on the replication and expression of the viral genome and their regulation in different host cells.

To date a number of genes have been recombined *in vitro* with SV40 vectors and propagated in cultured monkey kidney cells; these include segments of λ phage DNA[1,2], *Escherichia coli* DNA coding for $tRNA^{Tyr}$ (ref. 3), thymidine kinase (S. P. Goff and P. Berg, in preparation), guanine phosphoribosyl transferase (R. C. Mulligan and P. Berg, unpublished) and *Drosophila melanogaster* DNA containing the genes for H2A and H4 histones (H. Hofstetter and P. Berg, unpublished). Although the exogeneous DNA segments are transcribed during infections with each of these recombinant genomes, discrete, mature functional mRNAs have not been detected.

We report here the construction and propagation of a recombinant genome in which virtually all of the coding sequence for the major capsid protein, VP1, located between map coordinates 0.945 and 0.145 (see Fig. 1*a*) has been replaced by a cDNA that specifies the entire amino acid sequence of rabbit β-globin[8,9]. Cells infected with the recombinant genome

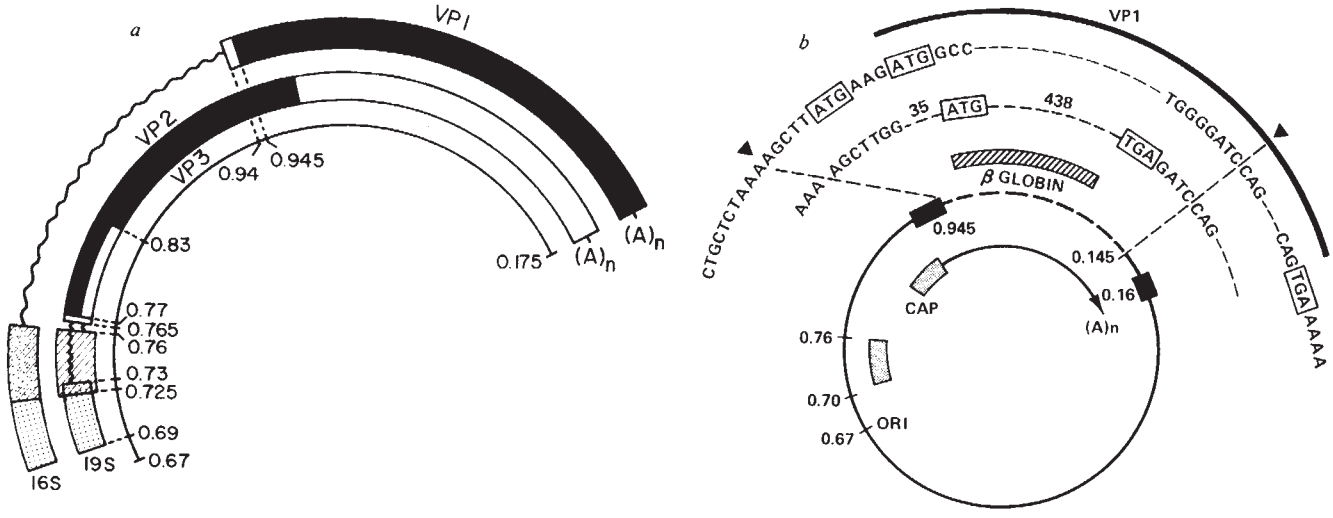

Fig. 1 *a*, The composite structure of SV40 late mRNAs. The map of SV40 DNA from the origin of DNA replication (0.67) to the end of the late region (0.175) is shown on the inner circle. The regions coding for the structure of VP1, VP2 and VP3 appear as shaded regions within bars that define the 'bodies' of the 19S and 16S mRNAs. The 'leader' segments are shown as bars spanning map coordinates 0.69–0.76. The cross-hatched and stippled regions of the 16S mRNA leader, for example, are intended as symbolic, rather than literal representations of more than one type of leader segment; one leader segment spans the region from 0.725 to 0.76 ▨ and another the region from 0.69 to 0.76 □. For the 19S mRNA species, one class of leaders spans map coordinates 0.69 to 0.73 □ and is joined to the body at 0.76; another class of leaders extends from 0.725 to 0.76 ▨. *b*, SV40 and rabbit β-globin cDNA nucleotide sequences relevant to the construction of SVGT5–RaβG. The origin of SV40 DNA replication at map position 0.67 is denoted as ori. SV40 16S late mRNA, with its 'capped' 5' leader sequence derived from 0.72 to 0.76 map units (stippled box), and 3' poly(A), is represented inside the circle. The region of the map coding for VP1 is represented by the heavy black line labelled VP1. The regions of SV40 DNA implicated in splicing and polyadenylation of the 16S mRNA are shown as black rectangles on the circular map. The dashed line extending leftward from map position 0.945 indicates the position of a *Hind*III endonuclease cleavage site in SV40 DNA (the outer sequence) and in pBR322–βG2 DNA (the inner sequence). The dashed line extending rightward from map position 0.145 locates the unique *Bam*HI restriction site of SV40 DNA (outer sequence) and the *Bgl*II endonuclease cleavage site of pBR322–βG2 (inner sequence). The nucleotide sequences between these dashed lines show the β-globin cDNA sequences inserted into SVGT5 and the SV40 sequences excised to construct SVGT5. The triplets enclosed in boxes show the initiation and termination signals for translation of VP1 and β-globin. The β-globin cDNA sequence in pBR322–βG2 contains 35 nucleotides proximal to the initiator AUG in β-globin mRNA. The small hatched bar spans the region containing the initiation β-globin codon, the 438 nucleotides specifying the entire β-globin protein amino acid sequence, and the translation terminator codon.

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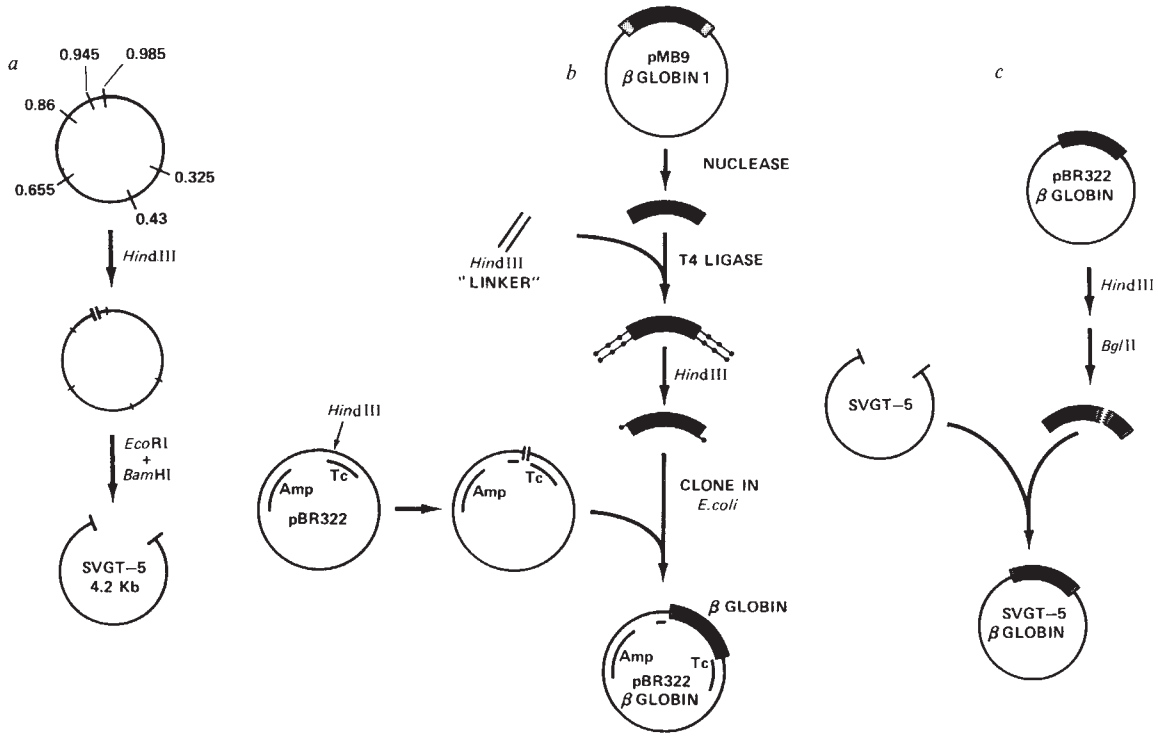

Fig. 2 Construction of SV40-RaβG recombinant genome. *a*, Construction of SVGT5 vector. Approximately 100 μg of SV40 DNA were incubated with 100 U of *Hin*dIII endonuclease (Boehringer) for 50 min at 30 °C in 10 mM Tris, *p*H 7.5, 5 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT. The partial digest was electrophoresed on a 0.8% agarose gel in Tris-borate buffer [6] to obtain full-length linear DNA molecules. The full-length linear DNA band was eluted electrophoretically from an excised gel slice, digested with an excess of *Bam*HI endonuclease (N.E. Biolabs) in 6 mM Tris, *p*H 7.5 containing 6 mM β-mercaptoethanol and 6 mM $MgCl_2$; then, after adjusting the Tris to 100 mM, the mixture was digested with an excess of *Eco*RI endonuclease (N.E. Biolabs). The digest was electrophoresed on a 0.7% agarose gel and the largest band (4.2-kilobase) was excised and electroeluted. To remove residual agarose, the DNA was absorbed to a 0.5 ml DE-52 column equilibrated with TE (10 mM Tris, *p*H 7.5, 1 mM EDTA); after washing the column with TE the DNA was eluted with TE containing 1 M NaCl. *b*, Subcloning of β-globin cDNA. PβG1 DNA was incubated with 1,200 U of S_1 nuclease (Miles) for 30 min at 50 °C in buffer containing 30 mM sodium acetate (*p*H 4.5), 1 mM $ZnSO_4$, 0.2 M NaCl and 45% (w/v) formamide [17]. The digestion was terminated by the addition of 10 mM EDTA, the DNA was precipitated with ethanol and electrophoresed in a 5% acrylamide gel in Tris-borate buffer for 3 h at 125 V. The ethidium bromide stained band containing the β-globin cDNA (0.5–0.58 kilobases) was excised and electroeluted. One μg of the β-globin cDNA was incubated with 3 μg *E.coli* polymerase I (provided by A. Kornberg) in 25 μl containing 60 mM Tris, *p*H 7.5, 8 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM ATP, and 0.2 mM of each of the deoxynucleoside trisphosphates for 10 min at 12 °C to convert 'ragged' into 'blunt' ends [16]. ^{32}P-labelled CCAAGCTTGG oligonucleotide (obtained from Collaborative Research) was added in a 50-fold molar excess relative to the cDNA termini and the reaction adjusted to contain 1 mM ATP. T4 DNA ligase (Bethesda Research Laboratory) (50 U ml^{-1}) was added, the mixture (50 μl) incubated for 1 h at 20 °C and then heated at 80 °C for 5 min. After adjusting the salt concentration to 50 mM, *Hin*dIII endonuclease (15 units) was added and after 4 h at 37 °C, the digest was electrophoresed on a 5% acrylamide gel. The β-globin cDNA band was located by autoradiography, excised, and electroeluted. Approximately 0.7 μg of the recovered DNA was mixed with 1.2 μg of *Hin*dIII endonuclease-cleaved pBR322 DNA in 50 mM Tris buffer, *p*H 7.8, containing 10 mM $MgCl_2$, 20 mM DTT, and 1 mM ATP (ligase buffer), 5 units of T4 DNA ligase were added and after an overnight incubation at 12 °C, suitable dilutions of the ligated mixture were introduced into HB101 (ref. 20). Transformants were selected on agar medium containing 20 μg ml^{-1} ampicillin. Approximately 10^4 Amp^r transformants were obtained per μg of ligated DNA. Amp^r colonies were picked onto agar medium containing 50 μg ml^{-1} tetracycline to identify recombinant plasmids with insertions at the *Hin*dIII restriction site [21]. Ten of 22 Tet^s clones carried plasmids containing the β-globin insert. One isolate pBR322–βG2, served as the source of β-globin cDNA for insertion into SV40 DNA. *c*, Construction of SVGT5–RaβG. pBR322–βG2 DNA was cleaved sequentially with an excess of *Bgl*II and *Hin*dIII endonucleases in the buffers and conditions described above. The products were electrophoresed on a 4% acrylamide gel and the 485-base pair β-globin cDNA band was excised and electroeluted. Approximately 12 ng of the cDNA were incubated with 100 ng of SVGT5 DNA and T4 DNA ligase (25 U ml^{-1}) in ligase buffer overnight at 12 °C. Dilutions of the ligated DNA and *ts*A58 DNA were used to transfect CV-1P cells as already described [22]. Fifty plaques were picked and (their viral DNAs) screened for the presence of β-globin cDNA sequences by a modification of the plaque hybridisation method of Villarreal and Berg [23]. A plate of confluent CV-1P cells (100 cm) was overlaid with 40 ml of agar medium and wells, 0.5 cm in diameter, were punched to expose small areas of the cell monolayer. An aliquot (10 μl) of each plaque suspension was added to a well, and after a 2 h incubation at 37 °C, the wells were filled with agar medium. After 3 d at 41 °C, the plate was overlaid with agar medium containing 0.01% Neutral Red and incubated overnight at 39°C. The agar layer was removed and the cell monolayer transferred to a nitrocellulose disk [23]; the disk was annealed with [^{32}P]–pBR322–βG2 DNA, washed and autoradiographed. Forty-six of the 50 plaques picked showed unmistakable evidence of hybridisation, thereby indicating the presence of β-globin cDNA sequences.

(SVGT5–RaβG) produce substantial quantities of discrete hybrid mRNAs that contain the β-globin coding sequence flanked by 5′–'leader' and 3′–terminal sequences characteristic of late SV40 mRNAs. The infected cells also synthesise rabbit β-globin polypeptide in quantities nearly equivalent to the amount of VP1 produced by the co-infecting helper virus.

Construction of SVGT5–RaβG

The mRNAs which code for SV40's three capsid proteins, VP1, VP2 and VP3 are composite structures, that is, the mRNAs contain nucleotide sequences transcribed from non-adjacent DNA segments [10,11]. Each mRNA contains a 5′–terminal segment (the leader) transcribed from the l-strand between map coordinates 0.69 and 0.76 (see Fig. 1*a*). Joined to the leader sequence, the VP2 and VP3 mRNAs (the 19S class) have a segment of RNA homologous to the l-strand between map coordinates 0.77 and 0.175 (the 19S body); the body of the VPl mRNA (16S class) is transcribed from the l-strand of the DNA between map coordinates 0.94 and 0.175.

Deletion mutants have been obtained which lack the 3′ boundary of the late region leader segments [12]. RNA transcribed from these mutants is not processed into mature 16 or 19S mRNA, presumably, because splicing cannot occur. Previously constructed SV40 vectors resemble these deletion mutants in that they have lost most or all sequences required for splicing. Very possibly the failure to obtain discrete mRNAs with the earlier recombinants stems, at least in part, from the consequent disruption of post-transcriptional splicing reactions. We have therefore sought to construct a SV40 vector which would retain all the regions implicated in transcriptional initiation and termination, splicing and polyadenylation of 16 and 19S mRNAs (see Fig. 1*a*).

Inspection of the SV40 restriction map suggested two restriction endonuclease-cleavage sites that could be used to generate such a vector. The first, a *Hin*dIII restriction site at map position 0.945, is six nucleotides proximal to the initiation codon for VP1 (refs 4,5) and about 50 nucleotides distal to the site at which the leader sequence is joined to the body of the VP1 mRNA [13,14] (see

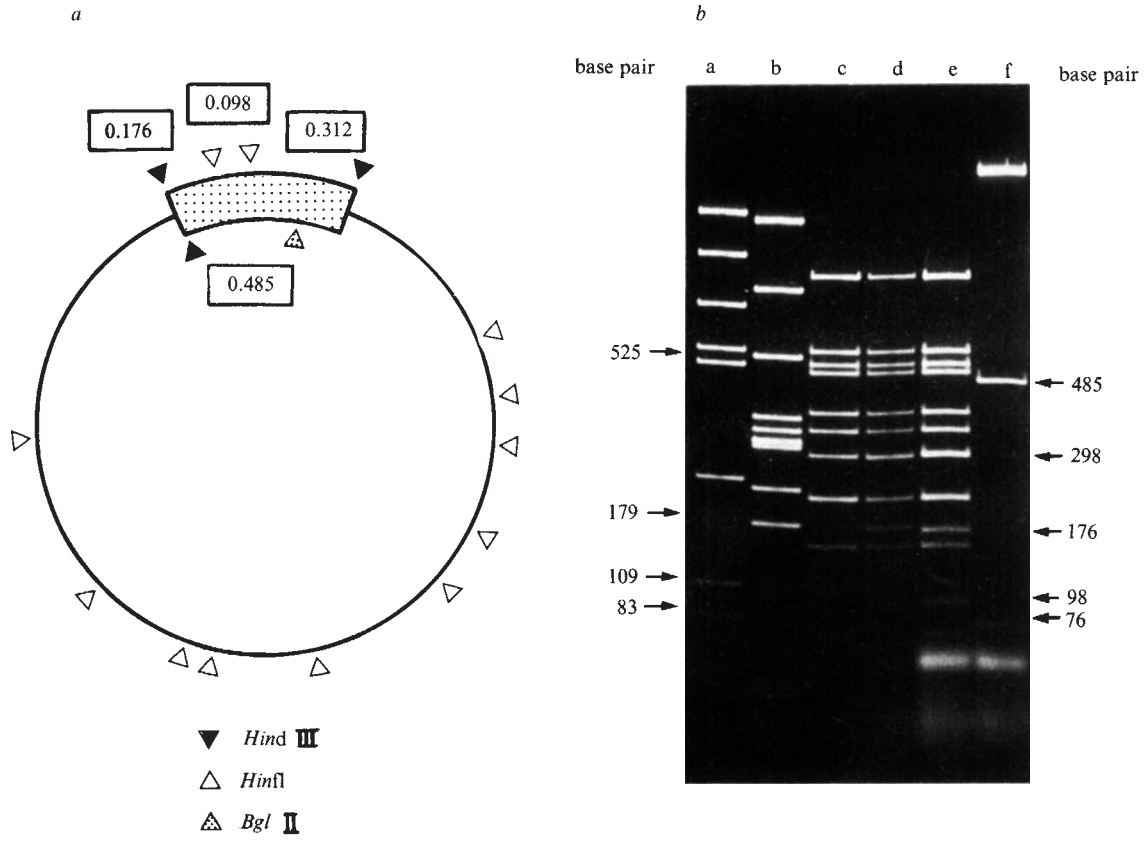

Fig. 3 Restriction analysis of pBR322–βG recombinants. *a*, The numbers in boxes are the expected sizes of restriction fragments, assuming the recovery of the complete cDNA sequence present in PβG1. The stippled region denotes the position of the β-globin cDNA insert and the various symbols indicate the particular restriction endonuclease cleavage sites. *b*, pBR322–βG DNAs were isolated as described by Katz *et al.*[40], digested with an excess of the indicated enzymes and electrophoresed on a 3–7% gradient acrylamide gel[6]. The gel was stained with ethidium bromide (1 μg ml^{-1}) and photographed under short wave ultraviolet light, using Polaroid 665 film and an orange filter. a, *Hin*fI endonuclease digested SV40 DNA. b, *Hae*III endonuclease digested SV40 DNA. c–e, *Hin*dIII plus *Hin*fI endonuclease digested pBR322, pBR322–βG2, pBR322–βG3 DNAs, respectively. f, *Hin*dIII plus *Bgl*II digested pBR322–βG2 DNA.

Fig. 1*b*). The second, a *Bam*HI restriction site at map coordinate 0.145, is 50 nucleotides proximal to the termination codon for VP1 translation and 150 nucleotides before the poly(A) sequence at the 3′-end of VP1 mRNA[4]. The 4.18 kilobase DNA segment bounded by these two restriction sites (SVGT5) contains, in addition to the regions at which splicing and polyadenylation of late mRNAs occur, the origin of DNA replication and the entire early region; hence, it can be propagated by complementation with an appropriate *ts*A mutant[15].

The rabbit β-globin cDNA sequence was selected as the transducible gene because it is small enough to be accomodated in SVGT5 and because the protein can be isolated and characterised readily. Furthermore, the cDNA had already been cloned in *E. coli* and shown to contain the complete coding sequence for β-globin, as well as additional 5′ and 3′-terminal nucleotide sequences present in β-globin mRNA[9]. Our aim was to excise the β-globin cDNA segment from pMB9, modify and reclone it in another *E. coli* plasmid, pBR322 (ref. 16); in doing this we could obtain the β-globin cDNA segment with a *Hin*dIII cohesive end just proximal to β-globin's initiator codon (see Fig. 1*b*). Consequently, SVGT5 and the modified β-globin cDNA segment could be joined readily to form the desired recombinant genome.

Construction, propagation and characterisation of SVGT5–RaβG genomes

To obtain SGVT5, wild-type SV40 DNA was partially digested with *Hin*dIII endonuclease to produce full-length linear molecules (Fig. 2*a*). Full-length linear DNAs were isolated by electrophoresis in agarose gel and digested with *Bam*HI and *Eco*RI endonucleases. These cleavages, and the subsequent electrophoretic separation, yielded a SV40 DNA fragment of 4.18 kilobases which contained the nucleotide sequences between map coordinate 0.145, clockwise to the *Hin*dIII restriction site at map coordinate 0.945. The *Eco*RI endonuclease digestion was introduced to eliminate a potentially contaminating fragment of 4.28 kilobases formed by cleavages at map position 0.325 (a *Hin*dIII restriction site) and the *Bam*HI restriction site at 0.145.

The rabbit β-globin coding sequence was isolated from the PβG1 plasmid recombinant described by Maniatis *et al.*[8]. PβG1 was obtained by inserting a cDNA copy of purified rabbit β-globin mRNA at the *Eco*RI restriction site of the plasmid pMB9 using the poly(dA):(dT) joining method, and propagating the recombinant plasmid in *E. coli* (strain HB101)[8]. Nucleotide sequence analysis confirmed that PβG1 contained the entire β-globin coding sequence, all of the 3′ and most (43 of 56 bases) of the 5′-non-coding sequences in β-globin mRNA[9].

PβG1 DNA was digested with S_1 nuclease as described by Hofstetter *et al.*[17] to excise the β-globin cDNA segment from PβG1 (Fig. 2*b*). β-Globin cDNA fragments varying in size between 500–585 base pairs were recovered by electrophoresis in acrylamide gels and incubated with *E. coli* DNA polymerase I and the four deoxynucleoside triphosphates to convert 'ragged' into 'blunt' ends[16]. A decanucleotide 'linker', CCAAGCTTGG, containing the *Hin*dIII endonuclease recognition sequence, was phosphorylated at its 5-end with polynucleotide kinase and [$\gamma-^{32}$P]ATP[18] and then joined to the ends of the β-globin cDNA with T4 ligase[19]; subsequent digestion with *Hin*dIII endonuclease generated *Hin*dIII cohesive ends.

The β-globin cDNA with its *Hin*dIII cohesive ends was ligated to *Hin*dIII endonuclease-cleaved pBR322 with T4 DNA ligase, and introduced into *E. coli* K12 (strain HB101)[20]. Trans-

formants were selected for ampicillin resistance and pBR322–βG recombinants were screened for their sensitivity to high levels of tetracycline. (Cells containing pBR322 are resistant to ampicillin and tetracycline, but insertions at the *Hin*dIII restriction site eliminate resistance to high levels of tetracycline[21].) Small cultures of each putative pBR322-βG recombinant were grown, the plasmid DNA was isolated, cleaved with *Hin*dIII endonuclease and the products electrophoresed in agarose; 10 of 22 isolated plasmid DNAs yielded fragments 0.50 to 0.58 kilobases in addition to the linear pBR322 DNA (data not shown).

One of these, pBR322-βG2, produced an insert fragment of about 570 base pairs following *Hin*dIII endonuclease digestion. Two fragments, 485 and 76 base pairs long were detected by digesting this plasmid with *Hin*dIII and *Bgl*II endonucleases (Fig. 3 track f). These findings show that the expected *Hin*dIII joins are intact, that they are separated from each other by nearly the expected length of the β-globin cDNA segment and that the single internal *Bgl*II restriction site in the β-globin cDNA is present. These conclusions are reinforced by the double digestions of pBR322-βG2 and pBR322-βG3 with *Hin*dIII and *Hin*fI endonucleases (Fig. 3 tracks d and e). Fragments corresponding to the expected 176 base pair fragment (derived from the 5′-end of the β-globin coding sequence) and 98 base pair fragment (produced from an internal sequence in the cDNA) were readily apparent in the electropherogram. The predicted 312-base pair fragment, generated from the 3′-end of the globin coding sequence, is actually 298 base pairs and is obscured by a fragment of the same size derived from the vector DNA. This was deduced by inspection of the original film in which the staining of the 298-base pair band was more intense than the band migrating behind it. Determination of the nucleotide sequences[18] at the two *Hin*dIII join sites in pBR322-βG2 further supports the restriction enzyme analysis and establishes that 5 nucleotides at the 5′-end, 15 nucleotides at the 3′-end and all of the dA:dT sequences of the originally cloned β-globin cDNA are absent in the β-globin cDNA segment recloned in pBR322 (data not shown).

The β-globin cDNA segment of pBR322-βG2 was excised by sequential digestion with *Hin*dIII and *Bgl*II endonucleases (Fig. 2*c*). This yielded a fragment having the codon for initiating translation of β-globin 37 nucleotides beyond the *Hin*dIII endonuclease-generated cohesive end and the translation termination codon just proximal to the *Bgl*II endonuclease-generated cohesive end. Even though the *Bam*HI (GGATCC) and *Bgl*II (AGATCT) endonuclease recognition sites differ, the cohesive ends generated by the two endonucleases are identical (GATC). Accordingly, the 0.485-kilobase β-globin cDNA fragment could be ligated to SVGT5 with T4 ligase[24] (Fig. 2*c*). To propagate the SVGT5-βG2 genome, CV1-P cells were transfected with a mixture of the ligated DNA and *ts*A58 DNA[22]. *ts*A58 is a thermosensitive mutant of the SV40 early function and can not multiply at 41 °C (ref. 15). About 2.5×10^4 plaques were obtained per microgram of ligated DNA.

These plaques were screened for virus containing the β-globin cDNA using a modification of the *in situ* plaque hybridisation technique[23](see legend to Fig. 2*c*). Forty-six of fifty plaques examined contained DNA that hybridised specifically to the β-globin cDNA. Recombinant virus from independent clones were purified by plaque isolation and high-titre virus stocks and purified viral DNA were prepared in CV1 cells. Judging from the relative yields of the two DNAs from such mixed infections, we estimate that the recombinant and helper genomes multiply equally well.

As expected from its somewhat smaller size (4.7 compared with 5.2 kilobases), SVGT5-βG DNA is separable from the helper *ts*A58 DNA by electrophoresis in agarose (Fig. 4, tracks a–c). Restriction endonuclease digestions and electrophoresis of the products helped to confirm the structure of the hybrid DNA. The locations of the *Hin*dIII and *Mbo*I restriction sites in SVGT5 and the β-globin cDNA sequence are indicated in Fig. 4*a*. Correct joining of the β-globin cDNA to SVGT5 regenerates the *Hin*dIII restriction site at map position 0.945. Therefore, digestion of SVGT5-βG DNA with *Hin*dIII endonuclease should generate a fragment of 1.43 kilobases from cleavages at the *Hin*dIII join sequence and the *Hin*dIII restriction site at map position 0.325 in the vector; the 1.43-kilobase fragment is produced in the *Hin*dIII endonuclease digests of two independent recombinants, SVGT5-βG 10A and SVGT5-βG 12A (Fig. 4, tracks e and f), but is absent from a comparable digest of SV40 DNA (track d). After transfer of the DNA in the gel to a nitrocellulose sheet[25] and hybridisation with [^{32}P] PβG1

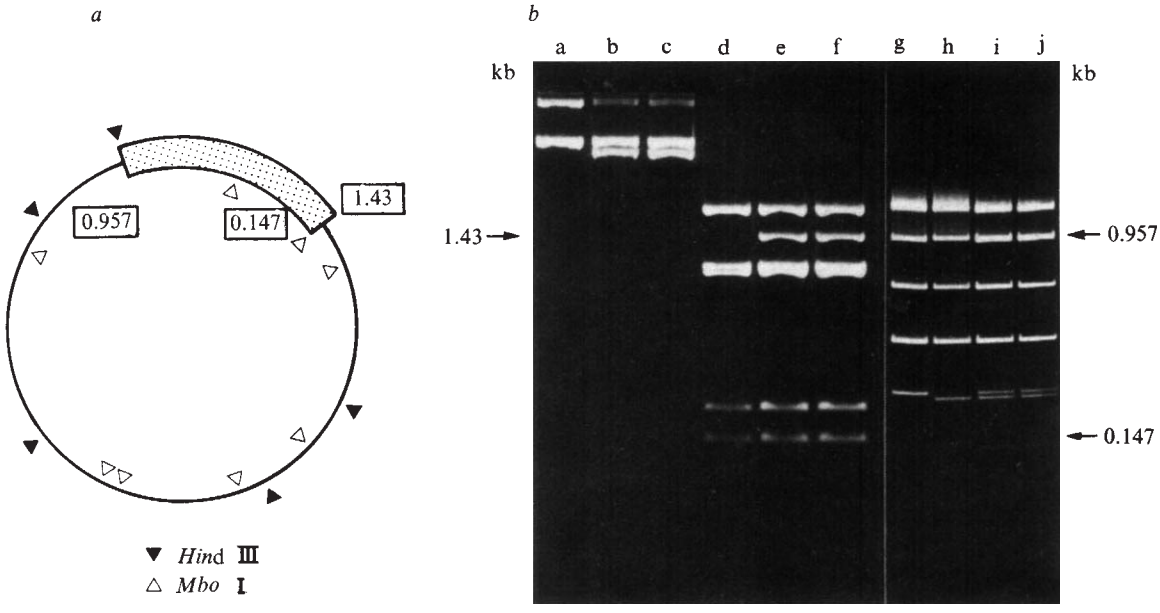

Fig. 4 Restriction analysis of SVGT–RaβG. *a*, The stippled region denotes the β-globin cDNA sequence and the numbers enclosed in boxes are the expected fragment sizes based on the sequence of the β-globin cDNA in pBR322–βG2 and the known locations of the *Hin*dIII and *Mbo*I restriction sites. *b*, Viral DNA was extracted by Hirt's method[27] from CVl cells that had been infected with plaque-purified SVGT5–βG and *ts*A58 virus (M.O.I. 0.005 PFU per cell). The DNA was centrifuged to equilibrium in ethidium bromide–caesium chloride gradients[28]; aliquots were then digested with the appropriate enzymes and electrophoresed on either 0.8% agarose gels (tracks a–f) or on 3–7% gradient acrylamide gels (tracks g–j). Tracks a–c: undigested SV40, SVGT5–βG10A and SVGT5–βG12A DNAs. Tracks d–f: the same three DNAs digested with *Hin*dIII. Tracks g–h: *Mbo*I endonuclease digests of SV40, *ts*A58, SVGT5–βG10A and SVGT5–βG12A DNAs.

DNA, only the 1.43-kilobase fragment was labelled (data not shown); hence, all of the β-globin cDNA sequence is contained within that fragment.

Ligation of *Bam*HI and *Bgl*II endonuclease-created cohesive ends should generate a sequence which is resistant to cleavage by these two enzymes but sensitive to *Mbo*I endonuclease (recognition sequence NGATCN). Indeed, *Mbo*I endonuclease digestion of SVGT5-βG 10A and SVGT5-βG 12A DNAs produced the expected array of fragments (Fig. 4*b*). The 0.957 fragment, which spans the *Hin*dIII join site, comigrates with one of the vector DNA fragments (compare tracks i and j with g and h); the 0.147-kilobase fragment, formed from cleavages at a *Mbo*I restriction site within the β-globin cDNA and the *Mbo*I site formed by the join of the *Bgl*II and *Bam*HI cohesive ends is produced from both recombinants (tracks i and j) but is absent from SV40 DNAs (tracks g and h). Note that the 0.240 fragment produced from the SV40 DNA used to make the vector (track g) is slightly larger than the corresponding fragment cleaved from the *ts*A58 helper DNA (track h). The mixture of recombinant and helper DNA yielded both fragments in about equal amounts indicating that here too the recombinant and helper genomes were equally represented in the infection from which these DNAs were isolated.

Although not shown, 8 of 14 other independent clones of SVGT5-βG gave restriction digests comparable to the two documented here. These data indicate that the β-globin cDNA cloned in pBR322 has been introduced into SVGT5 in the expected way.

Formation of SV40-Ra β-globin hybrid mRNAs

Considering the structure of SVGT5-βG and present ideas about SV40 late mRNA formation, two cytoplasmic SV40–β-globin hybrid mRNAs should be formed following infection with SVGT5-βG. These should be about 0.5 kilobases smaller than the normal SV40 19S and 16S late mRNAs. To test these expectations, CV1 cells were infected with *ts*A58 and each of two SVGT5-βG recombinant viruses (MOI 10–50 PFU per cell) at 37 °C and, after 48 h, cytoplasmic, poly(A)containing RNA (freed from DNA) was isolated (see legend to Fig. 5); similar RNA preparations were isolated from SV40 and mock-infected cultures. Each RNA preparation was denatured with glyoxal[26], electrophoresed on 1.5% agarose gel and transferred to diazotised benzyloxymethyl paper[12,30]. The imprints were annealed with ^{32}P-DNA to reveal the positions of the separated RNAs (Fig. 5).

With [^{32}P] PβG1 DNA as hybridisation probe, two discrete β-globin mRNA species are detectable in the RNA from cells infected with either recombinant (Fig. 5, tracks c and d). These RNAs are missing in the RNA from mock or SV40-infected cells (tracks a and b). With DNA fragments of known size as length standards (tracks e and f) we estimate the sizes of these mRNAs as 1.8 and 1 kilobases. When duplicate tracks were hybridised with [^{32}P]-SV40 DNA containing the late mRNA leader sequences, two labelled bands are visible in the SV40-infected cell RNA (track i), but not in the RNA from mock-infected cells (track j). The faster migrating, more heavily labelled band is the 16S mRNA (1.5 kilobase) and the slower, less heavily labelled band the 19S mRNA (2.3 kilobase). There are four labelled bands when RNA from cells infected with the two SVGT5-βG recombinants is hybridised with the labelled SV40 leader probe (tracks g and h). Two (the 1.5 and 2.3-kilobase species) are the 16S and 19S mRNAs produced by the *ts*A58 helper genome. Another has the mobility of the 1-kilobase RNA species that hybridised to the globin cDNA probe. The fourth, which is only barely discernable in this exposure, has a mobility very close to that of the 1.8-kilobase RNA detected with the β-globin cDNA probe. Thus the 1 and 1.8-kilobase polyadenylated RNAs contain the β-globin cDNA sequence and the leader segment characteristic of SV40 late mRNAs. Comparing the labelling intensities of the 1-kilobase β-globin mRNA and the 1.5-kilobase SV40 mRNA (in Figs, tracks g and h) and taking account of the ratio of recombinant to helper DNA in these infected cultures (0.3 recombinant), we surmise that the steady state levels of the two mRNAs are within a factor of two of each other.

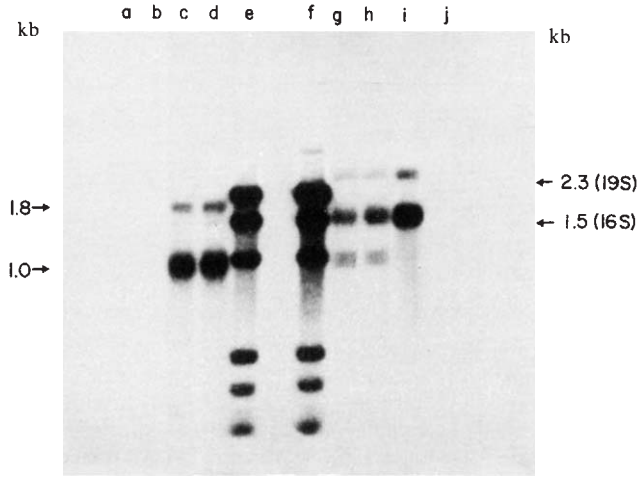

Fig. 5 Analysis of mRNAs made in cells infected with SVGT5-βG. Cytoplasmic poly(A)-containing RNA was isolated from CV1 cells 48 h after infection with a mixture of SVGT5-βG and *ts*A58 virus (>10 PFU per cell) as follows. Cells were washed twice with ice-cold Tris-buffered saline and once with a cold solution containing 10 mM Tris, *p*H 7.5, 5 mM $MgCl_2$, 10 mM NaCl. The cell monolayer, in the latter buffer containing 0.1% NP40 and 0.05% deoxycholate (1 ml per plate), was scraped off the plate with a rubber policeman and centrifuged for 2 min at 1,500 r.p.m. to pellet the nuclei. The supernatant was mixed with an equal volume of solution containing 0.3 M NaCl, 0.1 M Tris, *p*H 8.0, 10 mM EDTA, and 0.15% SDS. Cell aggregates were dispersed by pipetting and the suspension was extracted two times with an equal volume of water-saturated phenol-chloroform (1:1). After ethanol precipitation, the nucleic acid was digested with iodoacetate-treated DNase[29] (Worthington) (20 μg per mg nucleic acid), at room temperature for 45 min in a small volume of 20 mM Tris, *p*H 7.5, 10 mM NaCl, and 10 mM $MgCl_2$. The mixture was extracted twice with phenol-chloroform and passed over poly(U)-Sepharose[41] to isolate poly(A)-containing RNA. Aliquots of the poly(A)-containing RNA were denatured with glyoxal[26] and electrophoresed in a 1.5% agarose gel in 10 mM sodium phosphate *p*H 7.0. The fractionated RNA was then transferred to diazotised benzyloxymethyl paper[30] as described by Villarreal, White and Berg[12]. The paper was annealed with about 4×10^6 c.p.m. of the indicated ^{32}P-nick-translated DNA probe for 24 h, then washed[12] and autoradiographed for 2 d. ^{32}P-labelled pBR322-βG2 DNA fragment (from map position 0.67–0.76, produced by cleavages with *Bgl*I and *Hpa*I endonucleases) was the hybrisation probe for tracks g–j. Tracks e and f contained a *Hin*dII digest of [^{32}P] SV40 DNA as size standards. The lengths of the fragments (kilobases) in decreasing order are: 1.96, 1.54, 1.07, 0.369, 0.240. Tracks a, j; RNA from non-infected cells; b, i SV40 RNA; c, h SVGT5-βG10A RNA; d, g SVGT5-βG12A RNA.

Formation of β-globin

One goal of our experiment was to determine whether replacing the VP1 coding sequence with the β-globin sequence would permit the formation of a discrete, cytoplasmic SV40–β-globin hybrid mRNA. An additional goal was to determine whether such SV40-β-globin hybrid mRNAs could be translated *in vivo* to produce β-globin. CV1 cells were infected with *ts*A58 and either of the two SVGT5-βG recombinants (MOI 10-50 PFU per cell) and, after 48 h (37 °C), the cultures were labelled with ^{3}H-leucine and ^{3}H-valine for 30 min; SV40 and mock-infected cultures were labelled in a comparable way. An extract of each culture was prepared by sonication and treatment with NP40-deoxycholate; aliquots were electrophoresed on a SDS 15–20% gradient acrylamide gel (see legend to Fig. 6). The autoradiograms of each electrophoresed extract had a complex array of labelled protein bands (Fig. 6 tracks a–d). But the patterns from the SVGT-βG infected cell extracts (tracks c and d) contain a protein band migrating at the position of added rabbit β-globin; this protein band is not discernable in either the SV40 or mock-infected cell extracts (tracks a and b). The formation of a β-globin-like protein in cells infected with SVGT5-βG is supported by the fact that a protein with the same mobility as authentic rabbit β-globin can be recovered from complexes

formed by incubating the cell extracts with purified goat anti-rabbit β-globin immunoglobulin (kindly provided by S. Boyer, Johns Hopkins) and heat killed, formalin-fixed *Staphylococcus aureus* cells[33]; the electrophoretic pattern of the labelled proteins eluted with SDS and urea from such complexes are shown in tracks e–h. β-globin bands are seen in the extracts from cells infected with the recombinant (tracks g and h), but not in extracts from uninfected and SV40-infected cells (tracks e and f). If the extracts from SVGT5-βG infected cells are mixed with non-immune goat serum (tracks i and j) or anti-SV40 VP1 serum (track k), before adding the *S. aureus* cells, no β-globin is detectable in the electrophoresed samples.

In a preliminary experiment (carried out in collaboration with T. Hunter at the Salk Institute) the putative β-globin protein obtained from cells infected with SVGT5-βG was immunoprecipitated with the immune serum and *S. aureus* cells mentioned above, eluted with SDS, digested with trypsin and analysed by the two-dimensional electrophoretic-chromatographic procedure[36]. The tryptic peptide maps of the putative β-globin and labelled rabbit β-globin synthesised *in vitro*[36] were strikingly similar; at least 8 of 10 peptide spots in the two maps matched (data not shown). Further experiments to characterise the β-globin, particularly the amino and carboxy terminal amino acid sequences, are in progress.

In experiments to be published later we have compared the amounts of β-globin and VP1 synthesised in cells infected with SVGT5-βG and the helper virus *ts*A58. ^{35}S-methionine labelled infected cell extracts were mixed with either anti-β-globin or anti-SV40 VP1 serum, both immune complexes were adsorbed to *S. aureus* cells, washed, eluted with SDS, electrophoresed in SDS–polyacrylamide gel and autoradiographed. The amount of ^{35}S-label in β-globin and VP1 was quantitated by densitometry of their respective bands in the autoradiograms. Taking into account the difference in the number of methionine residues per mole of β-globin and VP1 (1:9) and the ratio of SVGT5-βG to *ts*A58 genomes during the period of protein labelling (1:3), we estimate that β-globin and VP1 are synthesised at nearly equal rates.

Discussion

These experiments establish that an exogenous gene, in this case the coding segment for rabbit β-globin, can be recombined with SV40 DNA *in vitro* to yield a hybrid genome that expresses the incorporated gene. The principal conceptual innovation is the decision to leave intact the regions in the vector implicated in SV40 late mRNA processing: specifically, the late mRNA leader sequence, the sequence to which the leader is spliced during maturation of late mRNAs, and the region in which late transcripts terminate and are polyadenylated (see Fig. 1*a* and *b*). In the recombinant genome, virtually all of the coding sequence of SV40's major capsid protein VP1 has been replaced by a β-globin cDNA. β-globin's initiator codon, and presumably its ribosome-binding sequence, replace the corresponding initiation sequence of VP1; furthermore, β-globin's translation terminator codon is proximal to the VP1 termination codon remaining in the vector (Fig. 1*b*). This strategy was adopted so that the β-globin coding sequence would be incorporated into mature SV40-β-globin hybrid mRNAs and be translated.

The success of the experiment clearly demonstrates that processing of both the 16 and 19S late mRNAs does not require nucleotide sequences within the VP1 coding region: analogous (smaller) mRNAs with poly(A) and leader sequences are formed efficiently when most of the VP1 coding sequence is replaced by the β-globin cDNA. Detailed studies are needed to compare the nucleotide sequences of wild-type 16 and 19S mRNA leaders with SVGT5-βG's corresponding β-globin mRNA leader sequences; it may also be interesting to compare the relative rates of the various splicing reactions of normal and recombinant RNAs. Another intriguing question is whether the monkey cells can excise the two intervening sequences present in the rabbit β-globin genomic DNA segment (Maniatis, T. *et al.*, in preparation), a question which can be examined with recombinants in which the rabbit β-globin genomic DNA sequence has been inserted into analogous SVGT vectors.

VP1 is believed to be translated exclusively from the 16S mRNA although the protein's coding sequence is also contained within the 19S mRNA[37]. Therefore, β-globin is probably translated from the 1-kilobase SV40-β-globin hybrid mRNA (the 16S mRNA analogue) and not the 1.8-kilobase hybrid mRNA (the 19S mRNA analogue). Conceivably, however, β-globin may be translated from the 19S mRNA analogue as well, either because the altered nucleotide sequence of the large hybrid mRNA significantly affects its secondary structure or because the ribosome binding site for β-globin is functionally independent of its position within a message. Experiments are in progress to insert the β-globin coding sequence at several different locations in the late and early regions of SV40 DNA, making it possible to assess the effect of varying the distance of the β-globin coding sequence from the 5'-end of the mRNA, the role of neighbouring sequences and any regulatory influence on the efficiency of expression—transcription, processing and translation—of the β-globin 'gene'.

Of particular interest is whether other genes can be expressed if they are inserted into appropriate SV40 DNA vectors in a similar way. Hofstetter and Berg (unpublished results) have

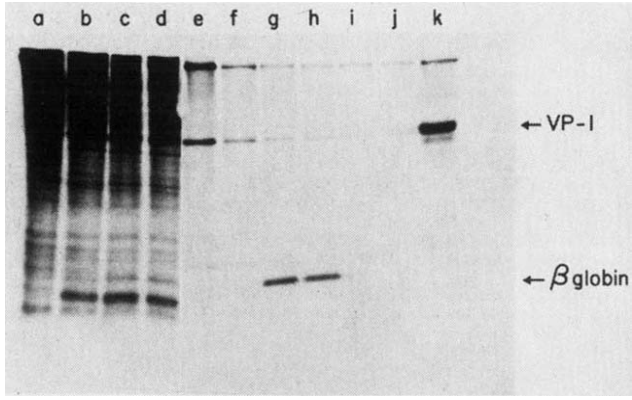

Fig. 6 Production of rabbit β-globin in cells infected with SVGT5-Raβ G. Subconfluent CV1 cells were infected with a mixture of SVT5-βG and *ts*A58 (>10 PFU per cell) for 48 h at 37 °C in a medium containing 4% fetal bovine serum. The cells were washed once with Tris-buffered saline and once with medium lacking leucine and valine. Cells were labelled for 30 min at 37 °C with 200 μCi each of ^{3}H-leucine and ^{3}H-valine (Amersham-Searle) in 1 ml of medium containing 4% dialysed calf serum but lacking leucine and valine. The labelled cells were washed twice in ice-cold Tris-buffered saline, scraped off the plate in Tris-buffered saline containing 10 mM DTT and 250 μg ml^{-1} PMSF (added fresh) (1 ml per 100 cm plate)[31] and then sonicated for 2 min in a water-cooled Heat Systems sonicator cup horn (maximum setting). Extracts were made 0.1% in NP40 and 0.05% in deoxycholate, incubated on ice for 10 min, centrifuged at 18,000 r.p.m. for 30 min, and the supernatants stored in aliquots at −80 °C. Aliquots of each extract were reacted with specific antisera or antibody proteins according to the method of Padgett *et al.*[32]. Extracts of approximately 2.5×10^6 cells were preincubated with 50 μl of a 10% suspension of heat-killed formalin-fixed *Staphylococcus aureus* Cowen I bacteria[33] in 25 mM Tris, *p*H 7.4, 10 mM EDTA, 0.35 M NaCl, and 0.15% Triton X-100 (TENT buffer) for 15 min on ice. After removal of the bacteria by centrifugation, purified rabbit β-globin (1 μg) and an excess of either purified goat anti-rabbit β-globin immunoglobulin (provided by S. Boyer, Johns Hopkins), normal goat serum (Microbiological Associates), or rabbit anti-VP1 serum (provided by J. Reiser, Stanford) was added and the extracts incubated overnight at 4 °C. Then 50 μl of *S. aureus* suspension (as described above) were added, the mixtures were incubated for 10 min at 4 °C, and centrifuged for 2 min at 8,000 r.p.m. The bacterial pellet was washed four times with TENT buffer and the immune complexes eluted from the bacteria for 1 h in a solution containing 10 mM Tris *p*H 7.4, 1% SDS, and 8 M urea. Electrophoresis of cell extracts or of immune complexes prepared from those extracts was for 3 h at 125 V in a 15–20% gradient acrylamide–SDS gel prepared according to the method of Laemmli[34]. The gel was stained and fluorographed[35] (3-day exposure shown). Tracks a–d are untreated cell extracts; e–h are immune complexes from extracts reacted with goat-anti-rabbit β-globin immunoglobulin; a,e, uninfected cell extract; b,f, SV40-infected cell extract; c,g, SVGT5-βG10A-infected cell extract; d,h, SVGT5-βG12A-infected cell extract. Tracks i,j: immune complexes prepared from SVGT5-βG10A (i) or SVGT5-βG12A (j) infected cell extract reacted with normal goat serum; k, immune complex prepared from SVGT5-βG10A-infected cell extract reacted with rabbit anti-VP1 serum.

inserted a *Drosophila melanogaster* (Dm) DNA segment containing the gene for histone H2B into SV40 DNA (between map positions 0.825 and 0.145) and observed the formation of both a cytoplasmic, polyadenylated SV40-Dm H2B hybrid mRNA and H2B protein during multiplication of the recombinant in monkey cells. Experiments are in progress to examine the expression of a variety of prokaryotic, lower eukaryotic and mammalian genes in mammalian cells using this approach.

SVGT5-RaβG may prove invaluable for testing the effects of mutational alterations on the regulation of SV40 transcription. Deletions could be introduced into those portions of the vector DNA that are implicated in viral mRNA formation and their consequences determined, even in the presence of helper genomes, by monitoring β-globin mRNA and protein synthesis. This approach will be particularly advantageous where the mutational changes either prevent or have no effect on the transcription of particular messages. More generally, this system may be used in conjunction with new methods for site-directed mutagenesis[38,39] to 'synthesise' altered eukaryotic mRNA structures at the DNA level, and to examine their function *in vivo*.

SVGT5-RaβG should be able to transform a variety of mammalian cells, such as mouse, rat and human. Since integrated SV40 genomes do not express the late region, SVGT5-βG transformed cells are not likely to make β-globin mRNAs or β-globin. However, other vectors, which permit transcription of the exogenous gene under early control are being explored for their ability to promote expression of the integrated genes.

β-Globin made during infection of CV1 cells by SVGT5-βG is not stable; following a 30-min labelling period most of the newly synthesised β-globin disappears during a 60 min chase (data not included). Several explanations come to mind: the newly synthesised polypeptide is an altered form of β-globin; factors in the monkey cell cultures actively promote breakdown of β-globin chains; cultured monkey kidney cells lack factors normally present in erythroid cells that stabilise β-globin. Our agenda includes experiments to characterise further the structure of rabbit β-globin synthesised in monkey cells and to determine if the β-globin can be stabilised, perhaps by the addition of haemin to the culture medium or the concomitant production of rabbit α-globin by co-infection with a SVGTαG recombinant genome.

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letters

Interstellar reddening and distance of Nova Cygni 1978

THE interstellar feature of KI(λ7699 Å) has been observed in the direction of Nova Cygni 1978 with an equivalent width of $W_\lambda \simeq 116$ mÅ and has been used here to deduce a colour excess of $E(B-V) = 0.38 \pm 0.08$ mag. The distance–reddening law to the nova is derived from a sample of stars in the nearby field surrounding the nova. The nova seems to be at a distance of $r \simeq 3.3 \pm 0.6$ kpc with a corresponding absolute visual magnitude at maximum light of $M_{V_0} \simeq -7.5 \pm 0.5$ mag. Nova Cygni 1978 is classified as a fast galactic nova on the basis of its absolute magnitude at maximum light and the rate of decline towards minimum light.

A composite light curve for Nova Cygni 1978 is shown in Fig. 1; the data are a mixture of visual, photographic (prediscovery) and photoelectric measurements[1–3]. A polynomial fit to the data yields a time of maximum light of $t_m = 12.20 \pm 0.04$ September 1978 (UT) when the nova attained a brightness of $m_v \simeq 6.2 \pm 0.1$ mag. Near maximum light and thereafter, high resolution spectra (220 mÅ) were obtained of the nova using the 2.7-m telescope of the McDonald Observatory with a self-scanned Reticon 1024B detector[4] and the coudé spectrograph.

In addition to monitoring various emission features, including Hα, Hβ, OI(λ8446 Å), and HeI(λ10830 Å), spectra were also obtained of the NaI(λ5890 Å) and KI(λ7699 Å) interstellar lines.

The interstellar NaI lines are very strong, having equivalent widths ~550 mÅ; NaI lines arising from the nova are also seen displaced by 626 km s^{-1} to the blue. The KI line, shown in Fig. 2, is weaker and seems to contain two unresolved components. From the measured equivalent width of the KI line ($W_\lambda \simeq 116$ mÅ) a column density of $N(KI) \simeq 9.3 \times 10^{11}$ cm^{-2} is

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