T-Antigen expression by polyoma mutants with modified RNA splicing

Stefan V. Nilsson and Göran Magnusson*

Medical Nobel Institute, Department of Biochemistry, Karolinska Institute, Box 60400, S-104 01 Stockholm, Sweden

Communicated by P. Reichard

Received on 4 May 1983; revised on 11 July 1983

Polyoma virus mutants were constructed that could not express all the three T-antigens. The mutagenesis was directed to the two 5' splice sites utilized in the maturation of early RNA. The mutant *bc1051* had a base change at the splice site of large T-antigen mRNA, and the mutants *d/1061* and *d/1062* had deletions at the corresponding splice point of small and middle T-antigen mRNA. The site was removed in mutant *d/1061* and altered by fusion to upstream sequences in mutant *d/1062*. Analysis of viral RNA showed that *d/1061* and *d/1062* formed only large T-antigen mRNA, whereas *bc1051* did not produce this RNA-species. However, the biological properties of *d/1062* suggested that it also produced mRNA directing the synthesis of a small T-antigen-related polypeptide, at least in low amounts. Only mutant *bc1051* could induce transformation of rat cells. In mouse 3T3 cells *d/1062* multiplied to a limited extent, while *bc1051* and *d/1061* failed to produce virus. However, *d/1061* DNA was synthesized at a low rate which could be increased to normal levels by co-transfection with mutant *bc1051*. This result suggests that polyoma small and middle T-antigen have a previously unrecognized function in the early phase of the infection process, or in viral DNA-synthesis.

Key words: polyoma virus/productive infection/RNA splicing/T-antigen/transformation

Introduction

The early region of the polyoma virus genome encodes three proteins: small, middle and large T-antigen. The polypeptides are readily identified by immunochemical techniques (Hutchinson *et al.*, 1978; Schaffhausen *et al.*, 1978; Smart and Ito, 1978) and their primary structures have been deduced from the base sequence of polyoma DNA (Friedmann *et al.*, 1979; Soeda *et al.*, 1979). However, little is known about the molecular functions of the proteins. Their role in the infection process has been explored mainly by the analysis of virus mutants (Tooze, 1980).

In the productive infection of mouse cells, large T-antigen is required for the initiation of viral DNA synthesis (Francke and Eckhart, 1973) and the control of early transcription (Cogen, 1978). The protein is also involved in the establishment (Fried, 1970), and possibly the maintenance (Rassoulzadegan *et al.*, 1982), of cellular transformation. The function of small and middle T-antigen in the productive infection cycle is obscure. However, the expression of the two proteins appears to be necessary for virus multiplication in some established cell lines, like mouse 3T3 cells (Goldman and Benjamin, 1975). Furthermore, transformation of abortively infected cells is absolutely dependent on the expression of middle T-antigen (Ito, 1979; Treisman *et al.*, 1981b).

The interpretation of genetic data (Griffin and Maddock, 1979; Magnusson and Berg, 1979) is complicated by the partial overlap of the DNA sequences that code for the T-antigen polypeptides (Figure 1). However, these problems can be solved by the use of mutants that are unable to express all three T-antigens.

The expression of the early genes depends on the differential splicing of a primary transcript in the formation of mRNA (Kamen *et al.*, 1979; Treisman *et al.*, 1981a). Mutants expressing only one T-antigen species have been constructed by the substitution of a genomic DNA segment with cDNA sequences (Tyndall *et al.*, 1981). Although these mutants express the correct polypeptides, it is unclear whether their gene expression is sufficiently high to sustain a normal productive virus infection. Similar mutants of SV40 have been shown to express the affected genes very poorly, probably as a result of the abolished splicing event (Gruss *et al.*, 1979; Hamer and Leder, 1979; Gruss and Khoury, 1980).

Another way of restricting the polyoma early gene expression would be to inactivate one of the splice sites (Figure 1) by a mutation. It has been shown that a single base substitution in the highly conserved DNA sequences at splice junctions is sufficient to prevent RNA maturation (Baird *et al.*, 1981; Montell *et al.*, 1982; Treisman *et al.*, 1982; Wieringa *et al.*, 1983). Such a mutation would probably not affect the alternative maturation pathways of early polyoma RNA that do not utilize the mutated site in the splicing reaction.

Results

Construction of splice point mutants

The 5' splice sites at nucleotide 409 and 746 were the targets of mutagenesis. Since nucleotide 746 is located in the intervening sequences of the large T-antigen gene, a deletion which removes the splice site would not affect the large T-antigen coding information. In contrast, the splice site at nucleotide 409 is located in the coding sequences of small and middle T-antigen. To avoid substantial alterations of the protein structures, the removal of this splice site should be done by changing a base pair. Cleavage sites of restriction endonuclease *Bst*NI at nucleotide 406 and 718 provided suitable starting points for the mutagenesis of the two 5' splice sites. Since polyoma DNA contains a large number of *Bst*NI recognition sites, a subgenomic fragment (*Bam*HI-*Eco*RI), joined to plasmid pAT153, was used for the *in vitro* mutagenesis.

Covalently closed circular DNA molecules were nicked at single *Bst*NI sites and the resulting open circular molecules were treated with exonuclease, to produce short single-strand regions.

Induction of base pair substitutions was performed by treatment of the gap-containing DNA molecules with bisulfite. In this procedure, exposed cytosine residues are deaminated, resulting in the formation of transition mutants.

For the construction of deletion mutants, DNA molecules with single-stranded regions of ~30 nucleotides were used. The molecules were linearized by digestion of the single-stranded segment with nuclease S1, and then recircularized by

*To whom reprint requests should be sent.

treatment with DNA ligase.

After transformation of *Escherichia coli* cells with mutagenized DNA, recombinant plasmids from individual bacterial colonies were isolated. Mutants were identified by gel electrophoresis of plasmid DNA digested with restriction endonuclease *Bst*NI. Of the 82 recombinant plasmids isolated from cells transformed by bisulfite-treated DNA, nine had lost the cleavage site at nucleotide 406. The corresponding frequency of plasmids that had the site at 746 deleted was 2/72.

Before additional tests of the mutants, the polyoma DNA segment (*Bam*HI-*Eco*RI) of the recombinant plasmids was used to reconstruct complete viral genomes containing the respective mutations.

Base sequence analysis

After a preliminary phenotypic characterization, two mutants of each category were selected for analysis of the base sequences at the mutations, using the chemical degradation technique.

DNA of the two bisulfite generated mutants had identical base sequences at the missing *Bst*NI site. In both cases a G-C base pair at nucleotide 410 in wild-type DNA was replaced by an A-T base pair (Figure 1). One of the mutants, *bc1051*, was further characterized.

Determination of the nucleotides missing from the genomes of the two deletion mutants, *d11061* and *d11062*, showed that they had lost 53 and 31 bp, respectively. The *d11061* deletion (nucleotides 711–763) included the splice site at nucleotide 746, whereas the smaller *d11062* deletion (nucleotides 715–745) had its distal endpoint 1 bp to the 5' side of the splice site.

Synthesis of viral early RNA

Since the mutations of *bc1051* and *d11061* changed the consensus sequence of 5' splice sites, the early gene expression of the mutants was expected to be restricted. Consequently, early RNA produced by the mutants *bc1051*, *d11061* and *d11062*, respectively, was analyzed.

HeLa cells were used for the transfections because of their high competence of DNA uptake. These cells support a transient expression of the early viral genes (Tyndall *et al.*, 1981). Isolated nuclear and cytoplasmic RNA was hybridized with a ³²P-labeled fragment of polyoma DNA (nucleotide 555–829) which included the positions of the 3' splice junctions of the three early mRNA species. The resulting hybrids were incubated with nuclease S1, to degrade those parts of the DNA probe that were not co-linear with RNA molecules. Analysis, on denaturing polyacrylamide gels, of S1-resistant [³²P]DNA showed that cytoplasmic RNA from wild-type DNA-transfected HeLa cells, or wild-type virus-infected mouse 3T6 cells, protected DNA fragments of three size classes, ~38, 35 and 20 nucleotides in length (Figure 2). These fragments had the predicted lengths of DNA molecules hybridized to RNA encoding large, small and middle T-antigen, respectively (Treisman *et al.*, 1981a). Although the same 3' splice site – at nucleotide 794 – is utilized in the formation of mRNA directing the synthesis of large and small T-antigen, the DNA-fragment produced by large T-antigen mRNA was 38 instead of 35 nucleotides long. This difference was a result of the identical trinucleotides preceding the 5' and 3' splice junctions (Figure 1).

Figure 2 also shows that cytoplasmic RNA produced by the mutant *bc1051* did not include detectable amounts of the

Fig. 1. Genetic map of the early region of the polyoma genome. The location on the physical map (Soeda *et al.*, 1979) of the coding sequences of small (t), middle (τ) and large (T) T-antigens are represented by thick lines, and the intervening sequences by thin slanted lines. The base sequences at the splice junctions of early RNA are shown and their positions on the genome are indicated by nucleotide numbers. The boxes represent *Bst*NI cleavage sites adjacent to the 5' splice sites.

large T-antigen mRNA species. Conversely, the cytoplasmic RNA produced by the mutants *d11061* and *d11062* contained only the large T-antigen encoding species. Similar results were obtained with nuclear RNA preparations. However, the nuclear RNA fraction also contained unspliced RNA which protected the whole DNA probe, or in the case of RNA produced by *d11061* and *d11062* the sequences on the 3' side of the deletions.

Infectivity of mutant DNA

The transformation of rat cells by polyoma virus depends on the expression of middle T-antigen. Consequently, mutant *bc1051* was expected to be positive, and the two deletion mutants *d11061* and *d11062* negative in transformation tests.

Growing Rat-1 cells were transfected with mutant DNA which had been excised from the recombinant plasmids and recircularized. One day later the transfected cells were detached from the Petri dishes and seeded in semi-solid medium. Colonies of transformed cells appeared only in cultures of cells transfected with wild-type and mutant *bc1051* DNA (Table I). In cultures of cells transfected with the deletion mutants no colonies of transformed cells were observed, even after an extended incubation period.

The properties of the mutants in productive infection was tested by plaque assay. In addition to the three splice point mutants, the well characterized mutant NG-18 was included in the experiment. The latter mutant which produces truncated forms of small and middle T-antigen, multiplies only in some types of mouse cells (Goldman and Benjamin, 1975).

Mouse 3T3 cells were transfected with recircularized viral DNA. Two weeks later plaques appearing in the cell monolayers were counted. In this assay 1 ng of wild-type DNA produced ~1000 large plaques (Table I). Also mutant NG-18 DNA was infectious, although the specific infectivity was lower and the plaques were much smaller, demonstrating that the host-range phenotype of the mutant was only partially expressed in these 3T3 cells. Unexpectedly, the mutant *d11062* resembled NG-18 in its phenotype. Since the two defective mutants *d11061* and *bc1051* could complement each other, the result suggested that *d11062* expressed a protein functionally related to small and middle T-antigen. Hence, the mutant must be able to synthesize at least low levels of the corresponding mRNA species.

Fig. 2. Splicing of early region RNA. RNA was extracted from HeLa cells at 27 h after transfection with recombinant plasmids containing polyoma wild-type, *bc1051*, *dl1061*, and *dl1062* DNA, respectively. Nuclear and cytoplasmic RNA was purified and hybridized with a ^{32}P -labeled DNA fragment containing the 3' splice site of the early mRNA species. After nuclease S1 digestion, radioactively labeled DNA was analyzed by polyacrylamide gel electrophoresis. The control lane shows DNA hybridized with RNA isolated from virus-infected mouse 3T6 cells. The size markers, designated m, consisted of ^{32}P -labeled polyoma DNA cleaved with restriction endonuclease *DdeI*.

Table 1. Infectivity of splice point mutants

DNA type	Lytic infectivity ^a (plaques/ng DNA)	Transformation frequency ^b (colonies/ 10^5 cells)
Mock	—	1
Wild-type	680	140
<i>dl1061</i>	<0.02	1
<i>dl1062</i>	21	1
NG-18	90	N.D.
<i>bc1051</i>	<0.02	46
<i>dl1061</i> + <i>bc1051</i>	65	N.D.

^aLytic infectivity was measured by plaque formation on monolayers of 3T3 cell. The cells were transfected with serially diluted DNA and then overlaid with agar-containing medium. In the complementation test, the cells were transfected with dilutions of *dl1061* DNA mixed with 50 ng of *bc1051* DNA.

^bGrowing Rat-1 cultures of $\sim 3 \times 10^5$ cells were transfected with 0.2 μg of polyoma DNA. One day later the cells were trypsinized and portions of 10^5 cells were seeded in soft-agar medium. Visible colonies were counted after 3 weeks at 37°C.

N.D., not determined.

Fig. 3. Analysis of progeny polyoma DNA by gel electrophoresis. Cultures containing $\sim 8 \times 10^5$ 3T6 cells were transfected with 0.1 μg of polyoma wild-type or mutant DNA. At 40 h post-transfection, viral DNA was selectively extracted and purified. Half of the DNA was analyzed by electrophoresis in an agarose gel containing 0.5 $\mu\text{g}/\text{ml}$ of ethidium bromide. DNA was visualized by its fluorescence. The three lanes to the left contained reference samples with known amounts of covalently closed circular polyoma DNA.

Replication of viral DNA

To investigate the involvement of the polyoma T-antigens in the synthesis of viral DNA, the replication properties of the three splice point mutants were analyzed.

Rapidly growing mouse 3T6 cells were transfected with DNA of the mutants and then incubated for 40 h at 37°C. Low mol. wt. DNA was extracted and analyzed by agarose gel electrophoresis (Figure 3). The result shows that DNA of mutant *dl1061* replicated in the rapidly growing cells. However, the quantity of progeny DNA was significantly lower than in the cells transfected with wild-type DNA. In accordance with the result of the plaque assay, the synthesis of *dl1062* DNA was intermediate between the wild-type and

Fig. 4. Time course of viral DNA-synthesis. Cultures of 8×10^5 3T6 cells were transfected with $0.2 \mu\text{g}$ of wild-type (●—●), *dl1061* (▽—▽), *dl1062* (□—□), NG-18 (■—■), or *bc1051* (○—○) DNA, or with a mixture of *dl1061* and *bc1051* DNA (▼—▼), containing $0.1 \mu\text{g}$ of each DNA type. Viral DNA was extracted at 30, 40 and 50 h, respectively, post-transfection. Purified DNA was analyzed by gel electrophoresis and quantitated.

dl1061 levels. As expected, the mutant *bc1051* did not produce detectable amounts of viral DNA, corroborating the result of the RNA analysis that the mutant failed to express large T-antigen which is essential for viral DNA replication.

In a second experiment, the time course of viral DNA-synthesis was determined (Figure 4). The replication of mutant *dl1061* was very slow, and did not amount to more than 10% of the wild-type value at 50 h post-transfection. At this time point the net synthesis of wild-type DNA had ceased. The mutants NG-18 and particularly *dl1062* replicated more efficiently than *dl1061*. However, maximal rates of mutant DNA synthesis were only obtained by co-transfection with *bc1051* which produced normal small and middle T-antigens. Restriction endonuclease cleavage of viral DNA synthesized in the cells transfected with the 1:1 mixture of *dl1061* and *bc1051* DNA showed that the two types of molecules replicated at equal rates (data not shown).

Effect of RNA splicing on gene expression

The phenotypes of the splice point mutant *dl1061* and the cDNA mutant LT-1 (Tyndall *et al.*, 1981) were compared. The genome of the latter mutant has a deletion, removing the intervening sequences of the gene encoding large T-antigen that limits the early gene expression to the production of an unspliced large T-antigen mRNA.

Cultures of 3T6 cells were transfected with LT-1 and *dl1061* DNA, respectively, either alone or mixed with *bc1051* DNA. At the end of the first infection cycle viral DNA was extracted and analyzed by gel electrophoresis (Figure 5A). Although the synthesis of LT-1 DNA was enhanced in the presence of *bc1051*, it was much lower than the *dl1061* synthesis under corresponding conditions. To exclude that LT-1 DNA had a *cis*-acting defect in replication, it was mixed with wild-type DNA before transfection. In the analysis of progeny viral DNA, LT-1 DNA which has a 385-bp deletion, could be separated from full length molecules. The result of the experiment shows that LT-1 and wild-type DNA replicated at similar rates in co-transfected cells.

The expression of large T-antigen was analyzed using

Fig. 5. Effect on large T-antigen expression of RNA splicing. Mouse 3T6 cell cultures were transfected as described in the legend to Figure 4 with *dl1061*, LT-1, *bc1051* or wild-type DNA, either alone or in combination. At 33 h post-transfection the cells were harvested. From one set of cultures viral DNA was isolated and after purification it was analyzed by agarose gel electrophoresis (A). I denotes the position of covalently closed circular polyoma DNA of full length. From parallel cultures labeled with ^{32}P for 30 min soluble proteins were extracted. Polyoma T-antigens were isolated from the extracts using rat anti-T serum and analyzed by SDS-polyacrylamide gel electrophoresis (B). Labeled proteins were identified by autoradiography for 5 h. HC denotes the position of immunoglobulin heavy chains.

parallel cell cultures. Phosphoproteins, including large T-antigen, were labeled with ^{32}P for 30 min before harvest of the cells. The polyoma T-antigens were then isolated from cell extracts by immune precipitation and analyzed by SDS-polyacrylamide gel electrophoresis. The result (Figure 5B) shows that *dl1061* synthesized phosphorylated large T-antigen at a higher rate than mutant LT-1. Co-transfection with *bc1051* DNA did not increase the production of large T-antigen by either mutant, suggesting that the effect of *bc1051* on viral DNA replication was mediated by another mechanism.

Discussion

To construct mutants that express large T-antigen alone, and mutants that do not express this protein, but rather the other two T-antigens, the 5' splice sites were mutagenized. Bisulfite mutagenesis of DNA at the position corresponding to the proximal splice site utilized in the formation of large T-antigen mRNA yielded a mutant, *bc1051*, with an AT dinucleotide instead of the normal GT dinucleotide at the 5' end of the intervening sequence. It is not likely that the mutated DNA segment contained other base substitutions, since the procedure for site-directed bisulfite mutagenesis is very specific (Shortle and Nathans, 1978). The single base pair change of *bc1051* led to a conservative amino acid replacement in the small and middle T-antigen polypeptides, substituting a valine with an isoleucine residue.

Since the proximal splice site of small and middle T-antigen is located in the intervening sequences of the large T-antigen gene the mutagenesis was in this case performed by the removal of short DNA segments. One of the resulting mutants, *dl1061*, had lost all of the conserved sequences at

the splice junction, whereas another, *d/1062*, had a smaller deletion, removing only translated DNA sequences, with one of its endpoints 1 bp before the splice junction.

Analysis of early RNA produced by the mutants showed that both the deletion mutants produced only the mRNA species encoding large T-antigen. Since the *d/1062* deletion did not impinge on the highly conserved sequences of the splice site utilized in the formation of small and middle T-antigen mRNA, the failure of the mutant to produce these RNA species was unexpected. However, the biological characterization of the mutant indicated that proteins related to small and middle T-antigen indeed were expressed. It is therefore possible that the *d/1062* deletion decreased the synthesis of small and middle T-antigen mRNA to levels undetectable in the assay. Such an effect might result from an alteration of the secondary structure of the early region transcript (Khouri *et al.*, 1979), or of the primary structure at the splice junction. The *d/1062* DNA base sequence at this putative splice site is CTA/GT, instead of the wild-type sequence CAA/GT. These sequences should be compared with the consensus sequences at splice sites. CAG/GT or AAG/GT (Mount, 1982).

The mutant *bc1051*, with a transition mutation at the 5' site utilized in the splicing of large T-antigen mRNA, did not produce detectable quantities of this RNA species. The result is consistent with earlier observations in studies of globin RNA maturation and adenovirus early gene expression. Also in these cases mutations at the corresponding positions preclude RNA splicing (Baird *et al.* 1981; Montell *et al.*, 1982; Treisman *et al.*, 1982; Wieringa *et al.* 1983).

The inactivation of one of the proximal splice sites of these polyoma mutants did not inhibit the activity of the remaining site. Furthermore, the RNA mapping showed that the selection of 3' splice sites was normal, i.e., splicing of RNA from nucleotide 409 to 809 did not occur at detectable levels even after inactivation of the splice site at nucleotide 746.

The involvement of small and middle T-antigen in the productive infection cycle of polyoma virus has been demonstrated in studies of *hr-t* mutants. This class of mutants has a restricted range of host cells that can support virus multiplication, and is defective in the transformation of semi-permissive cells. The *hr-t* mutants produce a normal large T-antigen (Eckhart, 1977; Fluck *et al.*, 1977). The phenotype of the mutants results from alterations of small and middle T-antigen. DNA of the prototype mutant NG-18 has a deletion that leads to the production of an N-terminal fragment of small and middle T-antigen instead of the normal polypeptides (Soeda and Griffin, 1978; Carmichael and Benjamin, 1980).

The biological characterization of the splice point mutants showed that *bc1051* and *d/1061* had the phenotypes that could be predicted for mutants unable to express large T-antigen or small and middle T-antigen, respectively. Both mutants failed to multiply in mouse 3T3 cells, but were able to complement each other. The level of complementation was the same as previously observed (Luthman *et al.*, 1982). It is therefore unlikely that a significant proportion of the infectivity resulted from wild-type genomes generated by recombination. Mutant *bc1051* alone induced transformation of rat cells. However, the transformation frequency was significantly lower than after transfection with wild-type DNA. This observation is consistent with earlier reports of a function of large T-antigen in the establishment of transformation (Fried, 1970; Della Valle *et al.*, 1981).

The mutant *d/1062*, which did not produce detectable quantities of RNA coding for small and middle T-antigen after transfection of HeLa cells, nevertheless produced plaques on monolayers of 3T3 cells. Similar small plaques were formed after transfection of the cells with mutant NG-18 DNA. The result of the experiment suggests that the mutants *d/1062* and NG-18, but not *d/1061*, expressed a function related to small T-antigen. To produce such a polypeptide, mutant *d/1062* must be able to utilize the splice site adjacent to the deletion, at least at low levels. Properly spliced mRNA molecules would direct the synthesis of a truncated form of small T-antigen which had the 14 C-terminal amino acid residues replaced by the hexapeptide: Asn-Gly-Gly-Ala-Arg-Asn. Previous studies have shown that the slightly shorter small T-antigen-related polypeptide produced by mutant NG-18 is labile (Schaffhausen *et al.*, 1978). The result of the present experiments suggests that both these mutant polypeptides have biological activity.

Mutants of the *hr-t* class have been reported to produce normal amounts of viral DNA in mouse 3T3 cells (Fluck *et al.*, 1977), the block in virus multiplication being at a later stage. We therefore tested the ability of the splice point mutants to replicate in growing 3T6 cells. A comparison of viral DNA synthesis in cells transfected with wild-type and *d/1061* DNA, respectively, showed that the yield of mutant DNA was reduced ~10-fold. The synthesis of mutant *d/1062* and NG-18 DNA was higher, but still far from the wild-type level. The minor variability between experiments (Figures 3 and 4) in the rate of mutant DNA replication was probably caused by differences in the physiological state of the cell cultures at the time of transfection. The synthesis of *d/1061* DNA could be increased to wild-type levels by co-transfection with mutant *bc1051* DNA. This result strongly suggests that small and middle T-antigen have a function in the early events of the infection cycle in 3T6 cells, or have a direct function in viral DNA synthesis. In this context, it is of interest that the production of phosphorylated large T-antigen by *d/1061* was not increased by the presence of *bc1051* (Figure 5).

The cDNA mutant LT-1 (Tyndall *et al.*, 1981) has a deletion in the early region that leads to the direct formation of large T-antigen mRNA, obviating the need for splicing and preventing the production of mRNA that directs the synthesis of small and middle T-antigen. This mutant did not produce detectable amounts of viral DNA in the standard assay. Although LT-1 DNA synthesis was stimulated by co-transfection with *bc1051* DNA, significant quantities could be isolated only when wild-type DNA was used as a helper. The limited replication of viral DNA can be explained by the low rate of T-antigen synthesis in the LT-1 transfected cells. The comparison between the two mutants, *d/1061* and LT-1 suggested that the splicing event promotes gene expression. Similar observations have been made in the SV40 system (Gruss *et al.*, 1979; Hamer and Leder, 1979; Gruss and Khouri, 1980).

Materials and methods

For many details on the source of materials and the methodology previous publications (Luthman *et al.*, 1982; Nilsson and Magnusson, 1982; Luthman and Magnusson, 1983) should be consulted.

Cells, virus and DNA

HeLa cells were obtained from R. Kamen. The sources of mouse 3T3 and 3T6 cells, as well as Fischer Rat-1 cells have been described. All cells were grown in Dulbecco's modification of minimal essential medium supplemented with 10% newborn calf or horse serum.

E. coli cells, strain HB101 (Boyer and Roulland-Dussoix, 1969) were grown on rich media.

The polyoma virus used for the preparation of DNA was a plaque-purified derivative of the A-2 strain (Griffin *et al.*, 1974). Nucleotide numbers refer to the polyoma DNA sequence of Soeda *et al.* (1980). A plasmid recombinant of the polyoma cDNA mutant LT-1 (Tyndall *et al.*, 1981) was obtained from R. Kamen.

Plasmid pAT153 (Twigg and Sherrat, 1980) was used as a vector for the propagation of polyoma DNA in *E. coli* cells.

All experiments involving recombinant DNA were performed according to the National Institutes of Health guidelines for recombinant DNA research adopted by the Swedish authorities.

Transfection and transformation procedures

The procedures for transfection of animal cells with viral DNA and transformation of bacterial cells with plasmid DNA have been described. HeLa cells and Rat-1 cells were transfected by the calcium phosphate co-precipitation method (Graham and van der Eb, 1973), whereas absorption of DNA to mouse 3T3 and 3T6 cells was done in the presence of DEAE-dextran (McCutchan and Pagano, 1968). In analyses of viral DNA synthesis, the cells were treated with 0.1 mM chloroquine diphosphate for 4–5 h subsequent to DNA absorption (Luthman and Magnusson, 1983), to increase the fraction of transfected cells. Measurement of DNA infectivity by plaque assay and by induction of cellular transformation was done as before.

Analysis of large T-antigen

Transfected 3T6 cells were labeled for 30 min with ^{32}P (0.15 mCi/ml of phosphate-free medium). After rinsing, the cells were lysed in buffer containing Nonidet P-40 (NP-40). This and subsequent operations were performed as described by Ito (1979). The cell extracts were freed from nuclei and rat anti-T serum was then added (20 μl per extract of 5×10^5 cells). The immune complexes were precipitated with formalin-fixed protein A-producing *Staphylococcus aureus* cells, eluted, and run on a 10% SDS-polyacrylamide gel (Laemmli, 1970). After electrophoresis, radioactively labeled proteins were identified by autoradiography.

Preparation of DNA and RNA

Polyoma DNA was isolated from 3T6 cells by the method of Hirt (1967) and plasmid DNA was isolated from *E. coli* cells by alkaline extraction (Birnbom and Doly, 1979). The DNA preparations were purified by treatment with proteinase K and phenol extraction. After ethanol precipitation of DNA, the samples were incubated with RNase. For preparative purposes, circular DNA was further purified by sedimentation in neutral sucrose gradients.

Before transfection of cells with polyoma DNA prepared as a recombinant plasmid, viral DNA was excised by digestion with restriction endonuclease *Bam*HI and then recircularized by treatment with T4 DNA ligase at a low DNA concentration.

RNA was isolated from cultures of HeLa cells transfected with intact recombinant plasmids (20 $\mu\text{g}/5 \times 10^6$ cells) as described by Favaloro *et al.* (1980). Briefly, the cells were lysed by the addition of 0.5% (v/v) NP-40. Cell nuclei and the cytoplasmic fraction were separated. Both fractions were then incubated with proteinase K, extracted with phenol and ethanol precipitated. DNA was removed by treatment with pancreatic DNase. Following another phenol extraction and ethanol precipitation, RNA was dissolved and selectively precipitated in 3 M LiCl, 0.1 M sodium acetate, pH 5.3.

Mutagenesis procedures

Covalently closed circular DNA was nicked at single *Bst*NI sites by cleavage with the restriction endonuclease at 0°C in the presence of 20 $\mu\text{g}/\text{ml}$ of ethidium bromide. The nicks were converted to short gaps by digestion with DNA polymerase I (Luthman *et al.*, 1982).

For the construction of deletion mutants, the gaps of the DNA molecules were extended by treatment with exonuclease III for 1 min at 18°C (Nilsson and Magnusson, 1982). The circular DNA was then directly linearized by digestion with nuclease S1. Recircularization was performed by incubation with T4 DNA ligase at 25°C in the presence of spermidine.

Base substitution mutants were generated by deaminating exposed cytosine residues in the DNA molecules with sodium bisulfite (Shortle and Nathans, 1978). Incubation in 3.0 M NaHSO₃ was done for 6 h at 37°C. After treatment at pH 9 (Luthman *et al.*, 1982), DNA was used directly for the transformation of *E. coli* cells.

Analysis of DNA and RNA

Polyoma DNA extracted from 3T6 cells was analyzed by electrophoresis in horizontal 0.7% agarose gels containing 0.5 $\mu\text{g}/\text{ml}$ of ethidium bromide. DNA was visualized in u.v. light. Quantitation of DNA was done by densitometer tracing of photographic negatives.

Determinations of base sequences were done according to Maxam and Gilbert (1980). The base sequence at the mutation in *dI*1051 DNA was deter-

mined using a DNA fragment 5' end-labelled with ^{32}P at nucleotide 399 (*Hpa*II site). For determinations of the base pairs missing in *dI*1061 and *dI*1062 DNA, fragments labeled at nucleotide 658 (*Ava*I site) were used. The base sequences were established from four base-specific reactions (G, A plus G, C and C plus T).

Mapping of RNA was done by the nuclease S1 technique (Weaver and Weissmann, 1979; Favaloro *et al.*, 1980; Kamen *et al.*, 1982). The DNA fragment used as a probe was ^{32}P -labeled at the 5' end and the complementary strands of the fragment were separated by gel electrophoresis (Maxam and Gilbert, 1980). In the hybridization reactions which were performed for 16 h in 80% formamide, 0.4 M NaCl, 0.04 M PIPES (pH 6.4), 0.001 M EDTA at 42°C, the DNA strand complementary to early mRNA was used. After digestion with nuclease S1, labeled DNA was analyzed by electrophoresis on a denaturing 20% polyacrylamide gel.

Acknowledgements

We thank Robert Kamen for hospitality and expert advice on the analysis of RNA. This work was supported by grants from the Swedish Cancer Society and the Swedish Medical Research Council, and by a grant to P. Reichard from the Wallenberg Foundation.

References

- Baird, M., Driscoll, M.C., Schreiner, H., Sciaratta, G.V., Sarsone, G., Niazi, G., Ramirez, F. and Bank, A. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4218–4221.
- Birnbom, H.C. and Doly, J. (1979) *Nucleic Acids Res.*, **7**, 1513–1523.
- Boyer, H.W. and Roulland-Dussoix, D. (1969) *J. Mol. Biol.*, **41**, 459–472.
- Carmichael, G.C. and Benjamin, T.L. (1980) *J. Biol. Chem.*, **255**, 230–235.
- Cogen, B. (1978) *Virology*, **85**, 222–230.
- Della Valle, G., Fenton, R.G. and Basilico, C. (1981) *Cell*, **23**, 347–356.
- Eckhart, W. (1977) *Virology*, **77**, 589–597.
- Favaloro, J., Treisman, R. and Kamen, R. (1980) *Methods Enzymol.*, **65**, 718–749.
- Fluck, M., Staneloni, R. and Benjamin, T. (1977) *Virology*, **77**, 610–624.
- Francke, B. and Eckhart, W. (1973) *Virology*, **55**, 127–135.
- Fried, M. (1970) *Virology*, **40**, 605–617.
- Friedmann, F., Esty, A., LaPorte, P. and Deininger, P. (1979) *Cell*, **17**, 715–724.
- Goldman, E. and Benjamin, T.L. (1975) *Virology*, **66**, 372–384.
- Graham, F.L. and van der Eb, A.J. (1973) *Virology*, **52**, 456–467.
- Griffin, B.E., Fried, M. and Cowie, A. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 2077–2081.
- Griffin, B.E. and Maddock, C. (1979) *J. Virol.*, **31**, 645–656.
- Gruss, P. and Khoury, G. (1980) *Nature*, **286**, 634–637.
- Gruss, P., Lai, C.-J., Dhar, R. and Khoury, G. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4317–4321.
- Hamer, D.H. and Leder, P. (1979) *Cell*, **18**, 1299–1302.
- Hirt, B. (1967) *J. Mol. Biol.*, **26**, 365–369.
- Ito, Y. (1979) *Virology*, **98**, 261–266.
- Hutchinson, M.A., Hunter, T. and Eckhart, W. (1978) *Cell*, **15**, 65–77.
- Kamen, R., Favaloro, J., Parker, J., Treisman, R., Lania, L., Fried, M. and Mellor, A. (1979) *Cold Spring Harbor Symp. Quant. Biol.*, **44**, 63–75.
- Kamen, R., Jat, P., Treisman, R., Favaloro, J. and Folk, W.R. (1982) *J. Mol. Biol.*, **159**, 189–224.
- Khoury, G., Gruss, P., Dhar, R. and Lai, C.-J. (1979) *Cell*, **18**, 85–92.
- Laemmli, U.K. (1970) *Nature*, **227**, 680–685.
- Luthman, H. and Magnusson, G. (1983) *Nucleic Acids Res.*, **11**, 1295–1308.
- Luthman, H., Nilsson, M.-G. and Magnusson, G. (1982) *J. Mol. Biol.*, **161**, 533–550.
- Magnusson, G. and Berg, P. (1979) *J. Virol.*, **32**, 523–529.
- McCutchan, J.H. and Pagano, J.S. (1968) *J. Natl. Cancer Inst.*, **41**, 351–357.
- Maxam, A. and Gilbert, W. (1980) *Methods Enzymol.*, **65**, 499–560.
- Montell, C., Fisher, E.F., Caruthers, M.H. and Berk, A.J. (1982) *Nature*, **295**, 380–384.
- Mount, S.M. (1982) *Nucleic Acids Res.*, **10**, 459–472.
- Nilsson, S.V. and Magnusson, G. (1982) *Nucleic Acids Res.*, **10**, 1425–1437.
- Rassoulzadegan, M., Cowie, A., Carr, A., Glaichenhaus, N., Kamen, R. and Cuzin, F. (1982) *Nature*, **300**, 713–718.
- Schaffhausen, B.S., Silver, J.E. and Benjamin, T.L. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 79–83.
- Shortle, D. and Nathans, D. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 2170–2174.
- Smart, J.E. and Ito, Y. (1978) *Cell*, **15**, 1427–1437.
- Soeda, E., Arrand, J.R. and Griffin, B.E. (1979) *Nucleic Acids Res.*, **7**, 839–858.
- Soeda, E., Arrand, J.R., Smolar, N., Walsh, J.E. and Griffin, B.E. (1980) *Nature*, **283**, 445–453.

- Soeda,E. and Griffin,B.E. (1978) *Nature*, **276**, 294-298.
- Tooze,J., ed. (1980) *Molecular Biology of Tumor Viruses, Part 2*, published by Cold Spring Harbor Laboratory, NY.
- Treisman,R.H., Cowie,A., Favaloro,J.M., Jat,P. and Kamen,R. (1981a) *J. Mol. Appl. Genet.*, **1**, 83-92.
- Treisman,R., Novak,U., Favaloro,J. and Kamen,R. (1981b) *Nature*, **292**, 595-600.
- Treisman,R., Proudfoot,N.J., Shander,M. and Maniatis,T. (1982) *Cell*, **29**, 903-911.
- Twigg,A.J. and Sherrat,D. (1980) *Nature*, **283**, 216-218.
- Tyndall,C., La Mantia,G., Thacker,C.M., Favaloro,J. and Kamen,R. (1981) *Nucleic Acids Res.*, **9**, 6231-6251.
- Weaver,R.F. and Weissmann,C. (1979) *Nucleic Acids Res.*, **7**, 1175-1193.
- Wieringa,B., Meyer,F., Reiser,J. and Weissmann,C. (1983) *Nature*, **301**, 38-43.