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Requirement for the C-terminal region of middle T-antigen in cellular transformation by polyoma virus

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ABSTRACT

Deletions in polyoma virus DNA around the region that codes for the C-terminus of the viral middle T-antigen were created using a transforming fragment (BamH I/EcoR I) of viral DNA cloned in the plasmid vector pAT153. These species were recloned and assayed for their ability to transform Rat-1 cells in culture. Our results showed that whereas the DNA sequence between the presumed translational termination codon for the viral middle T-antigen and the single viral EcoR I site could be removed with no apparent effect on transformation, the removal of the termination codon itself or any amino acid coding sequences of this protein caused a drastic decrease in the transforming ability of the DNA. Transfection of Rat-1 cells with plasmids that contained viral DNA with deletions which corresponded to the last fourteen or more amino acids of the middle T-antigen never gave rise to cellular transformation.

INTRODUCTION

The early region of polyoma virus DNA, that is, the region transcribed in lytically infected cells before the onset of viral DNA replication, codes for at least three proteins. These have been designated small, middle and large T-antigens. In cells transformed by polyoma virus, all three T-antigens are normally expressed (1). The large T-antigen has mainly been associated with a function in DNA replication (2), with control of transcription of the viral early region (3), and with integration and excision of viral DNA from host cell DNA in transformed cells (4). Until recently, it was thought that the viral large T-antigen was important at least for the initiation of transformation, the observation of various truncated forms of this protein in some transformed cell lines, in addition to studies on tsa mutants (5), having suggested that it probably was not required

for the maintenance of the transformed phenotype (1,6). However, recent studies with fragments of the early region of polyoma virus DNA which are incapable of expressing a functional large T-antigen, since they lack a large part of the gene that codes for this protein, strongly suggests that the large T-antigen is not essential either for viral DNA mediated initiation or maintenance of transformation (7-11). In further support of this notion, a re-examination of the temperature sensitive mutants of polyoma virus which have lesions in large T-antigen, the tsa mutants, has shown that the DNA isolated from these mutants can transform cells at both the permissive and non-permissive temperatures (12,13). Thus a direct function of large T-antigen in transformation appears unlikely.

There is as yet no role known for the polyoma virus small T-antigen. Several viral mutants (middle and large T-antigen, mlt, mutants, 14) which express a normal small T-antigen, but truncated middle and large T-antigens, transform very poorly (15-18). In addition, Rat-1 cells in which small T-antigen is the only detectable virally coded protein, do not express the fully transformed phenotype (L. Lania, personal communication). Therefore, cellular transformation probably does not depend primarily on the small T-antigen.

Current data on viral mutants, or on the transformed cells themselves, strongly implicate the polyoma middle T-antigen in cellular transformation. That is, some polyoma virus mutant (mlt) strains having deletions within the region that codes for the C-terminal part of middle T-antigen have altered transformation properties (15-18), and host range transformation (hrt) mutants which lack middle T-antigen are non-transforming (19,20). Moreover, all transformed cell lines examined to date retain this protein (1,8,16). The presence of middle T-antigen in transformed cells appears to be always associated with the presence of small T-antigen. This is not, however, unexpected since all but the last four amino acids of small T-antigen are predicted to be present also in middle T-antigen (21). Due to a different pattern of RNA splicing (22), the information corresponding to the C-terminal 60% of middle T-antigen is obtained by translation in a different reading frame, thereby

avoiding the termination codon for small T-antigen.

Since the middle T-antigen may be the key protein for understanding cellular transformation by polyoma virus, we have been examining further that portion of the genome which encodes it with the aim of defining the minimum viral sequence required for transformation. Data from DNA sequence studies (21) suggest that in the A2 strain of the virus, the coding region of middle T-antigen extends from an initiation codon at nucleotide position 173-175 through position 1397, that is, sixty-three nucleotides from the single EcoR I restriction site at position 1560 in the DNA. In earlier studies, we showed by transfection experiments that the fragment of polyoma virus DNA which extended from the single Bcl I restriction site (at position 5021) to the EcoR I site was fully competent for transformation (8). For the purpose of our present study, a fragment of polyoma virus DNA which extends clockwise on the physical map from the BamH I site (nucleotide position 4632) through the viral origin of DNA replication to the EcoR I site, cloned in the plasmid pAT153, was used. By limited exonuclease digestion, deletions were introduced into the DNA, all originating from the EcoR I site (see Fig. 1). Plasmids carrying polyoma virus DNA fragments of different sizes have then been screened for their ability to transform Rat-1 cells *in vitro*, and cells transformed in this fashion subsequently tested for their tumorigenicity *in vivo*.

MATERIALS AND METHODS

Preparation of viral and recombinant DNA

The large plaque A2 strain of polyoma virus was used as wild type virus. Supercoiled viral DNA was prepared by standard procedures (23). A clone of the viral DNA fragment that extends clockwise on the physical map from the BamH I site at position 4632 to the EcoR I site at position 1560 (21) inserted into the BamH I and EcoR I sites of the plasmid pAT153 (24) was obtained from Dr. J. Jenkins and designated pA_2. The plasmid was propagated in *E.coli* strain HB 101 in media containing ampicillin (100µg/ml). Plasmid synthesis was amplified by addition of 170µg/ml chloramphenicol when the cultures reached an O.D. of 1.0 at 590 nm and growth was continued for 12 to 15 hrs. Plasmid DNA

was prepared following a modification of the procedure of Birnboim and Doly (25).

Construction of recombinant DNA molecules that carry polyoma virus fragments of reduced size

DNA of the recombinant plasmid pA_2 was cleaved to completion with the restriction endonuclease EcoR I under standard conditions. Linearised molecules (250µg/ml) were added to a reaction mixture (25µl) containing 0.6 M NaCl, 12 mM $CaCl_2$, 12 mM $MgCl_2$, 20 mM Tris pH 8.1, 1 mM EDTA and 0.1% BSA. The exonuclease, BAL 31 (1 unit) (Bethesda Research Laboratories), was added and the solution incubated for 2 or 4 minutes at 30°C. Under these conditions, BAL 31 removed about 30-50 bp per minute from either end of the molecule. The reaction was terminated by addition of EDTA to 40 mM. After phenol extraction, single stranded termini of the DNA were filled in to yield blunt ends using E.coli DNA polymerase I (Klenow fragment, Boehringer Mannheim). The resulting DNA, which lacked variable amounts of polyoma virus and pAT153 sequences adjacent to the EcoR I site, was then ligated overnight with T_4 DNA ligase (greater than 10-fold excess) (a gift from Dr. N. Smolar) at 4°C. E.coli strain HB 101 were transfected with such plasmid DNA, and bacteria carrying recombinant DNA were selected for their ability to grow on media containing ampicillin.

Characterisation of deletion mutants

A preliminary determination of the size and location of the deletions, relative to pA_2, was carried out by enzyme mapping, using restriction endonucleases Hpa II, Hae III, Ava II (Biolabs), and Hha I and Sst I (prepared by established procedures) (see Fig. 2). The extent of the deletions were determined at the nucleotide level by DNA sequence analysis (24). Supercoiled plasmid DNA (10µg) was cleaved with Ava II, labeled with [^{32}P]-γ-ATP (5000 Ci/mmole, Radiochemical Centre Amersham) using T4 polynucleotide kinase (PL Laboratories) and then cleaved with Hinc II (Biolabs). The resulting fragments were separated by electrophoresis on a 4% polyacrylamide gel. The band corresponding to the fragment that extended from the Hinc II site

at position 3908 in pAT153 DNA to the Ava II site at position 1412 in polyoma virus DNA was eluted and its sequence determined by the Maxam-Gilbert procedure (26).

Biological activity of recombinant DNA in vitro

An established line (Rat-1) of rat cells from the Fischer strain F2408 (27), grown in Dulbecco's modified Eagle's medium containing 5% foetal calf serum, was used in cellular transformation studies. The cells were transfected using the calcium method as described by van der Eb and Graham (28). That is, precipitated DNA in Hepes buffer (0.5 ml., containing 1µg super coiled plasmid DNA, 20 µg salmon sperm carrier DNA and calcium chloride, final conc., 125 mM) was added to subconfluent Rat-1 cells without prior removal of growth media. The cells were media changed after 5 hrs at 37^{0} and thereafter twice weekly. Dishes were scored for foci after 2 weeks and dense foci picked and grown individually. All cell lines established from foci were tested for their ability to grow in soft (0.35%) agar (15).

Tumourigenicity studies

Young adult syngeneic Fischer rats were each injected subcutaneously with 10^{6} cells from established cell lines in phosphate buffered saline (1 ml). The rats were examined for tumours twice weekly.

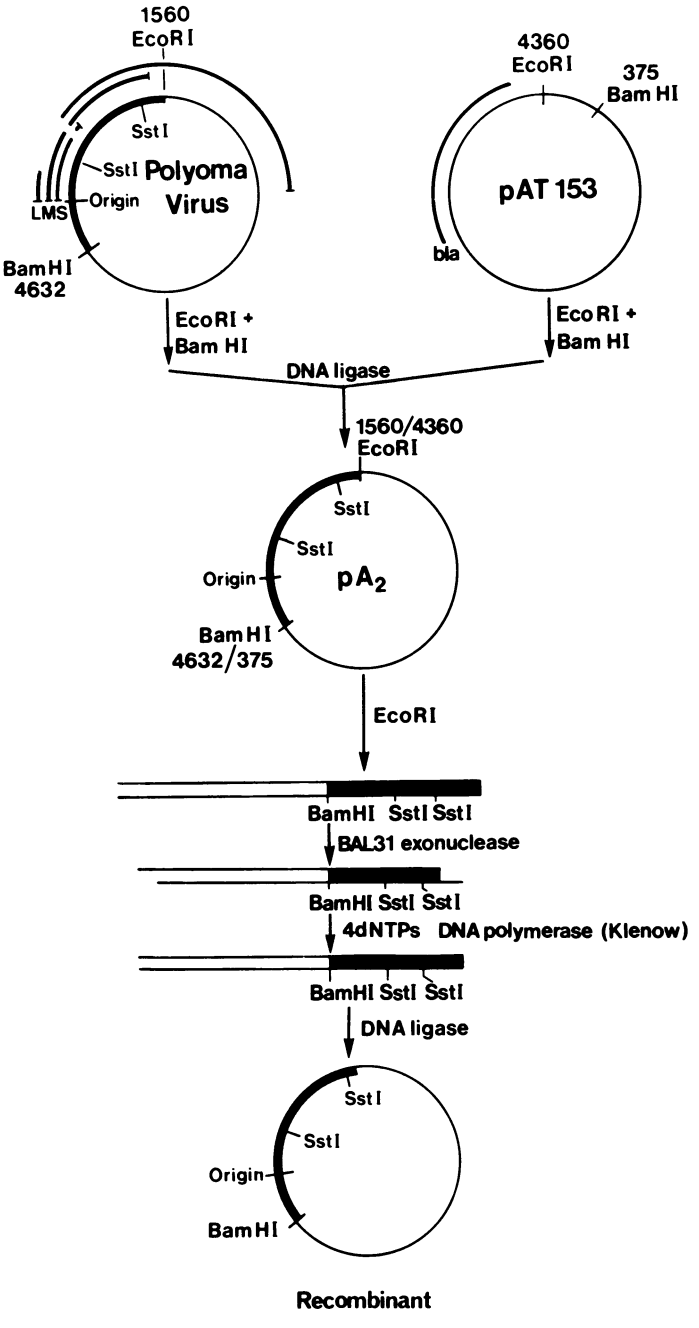
Immunoprecipitation of viral proteins

Cells were labeled with [^{35}S]-methionine for 4 hrs in methionine free, Dulbecco's modified Eagle's medium as described (29). Viral proteins were isolated by immunoprecipitation and separated by electrophoresis on 8.5% polyacrylamide gels.

RESULTS

Physical characterisation of deletion mutants of plasmid pA_2

The DNA of the recombinant plasmid designated pA_2, which contains the 5'-portion of the early region of polyoma virus DNA inserted at the BamH I and the EcoR I sites into plasmid pAT153


1560
EcoR I
SstI
SstI
Origin
LMS
Polyoma
Virus
BamHI
4632
4360
EcoRI
375
Bam HI
pAT 153
bla
EcoRI +
Bam HI
EcoRI +
Bam HI
DNA ligase
1560/4360
EcoRI
SstI
SstI
Origin
pA2
BamHI
4632/375
EcoRI
BamHI SstI SstI
BAL31 exonuclease
BamHI SstI SstI
4dNTPs DNA polymerase (Klenow)
BamHI SstI SstI
DNA ligase
SstI
SstI
Origin
BamHI
Recombinant

DNA, was cleaved with the restriction endonuclease EcoR I and then incubated with the exonuclease BAL 31 (30). The protocol used to obtain specific truncated fragments of viral DNA is illustrated in Fig. 1. Selection of transformed bacteria for ampicillin resistance puts constraints upon the deletion. Deletions in the plasmid DNA larger than about 150-200 base pairs affect the ß-lactamase gene and therefore destroy the ampicillin resistance encoded by the plasmid (31).

An analysis of the recombinant plasmids was carried out with several restriction endonucleases that are known to cleave near the EcoR I site in the plasmid and viral DNA sequences (31,32). The DNA fragments obtained after cleavage of various mutant plasmids with one of these, Ava II, is shown in Fig. 2a. Detailed restriction maps of the truncated species indicated that the exonuclease activity of BAL 31 had not been wholly synchronous since deletions from specific time points varied in size: Plasmids p37, p40, p42, p43, p46, p52 and p56 were derived from 2 min incubations with BAL 31; plasmids p2, p4, p5, p7, p11 and p12

Figure 1

Schematic representation of the method used to clone fragments of polyoma virus DNA and construct deletion mutants of the recombinant plasmids.

Polyoma virus (strain A2) and plasmid pAT153 DNAs were cleaved to completion with restriction endonucleases EcoR I and BamH I. Fragments were ligated at concentrations of 20µg each/ml. Recombinant molecules were selected for their ability to grow on medium containing ampicillin and inability to grow on medium containing tetracyclin. A plasmid containing the smaller of the two polyoma virus DNA fragments, obtained following cleavage with EcoR I and Bam H I, was selected and designated pA_2. Plasmid pA_2 DNA was then cleaved with EcoR I and the resultant linear species treated with exonuclease BAL 31 for either 2 or 4 minutes. BAL-31 is a multifunctional enzyme; it contains exonuclease activity that simultaneously degrade both the 3' and 5'-termini of duplex DNA (30). 5'-extended ends of the DNA were filled to blunt ends using DNA polymerase, and the resultant species religated to form circular molecules. E.coli strain HB 101, transfected with these DNA molecules, were grown on medium containing ampicillin.

The sequences coding for the polyoma virus proteins, small (S), middle (M), and large (L) T-antigens, and for the plasmid coded protein ß-lactamase (bla) are indicated. The heavy line represents the portion of polyoma virus DNA found within the pA_2 plasmid and deletion mutants derived from pA_2. For reference, two of the Sst I sites in polyoma virus DNA are shown.

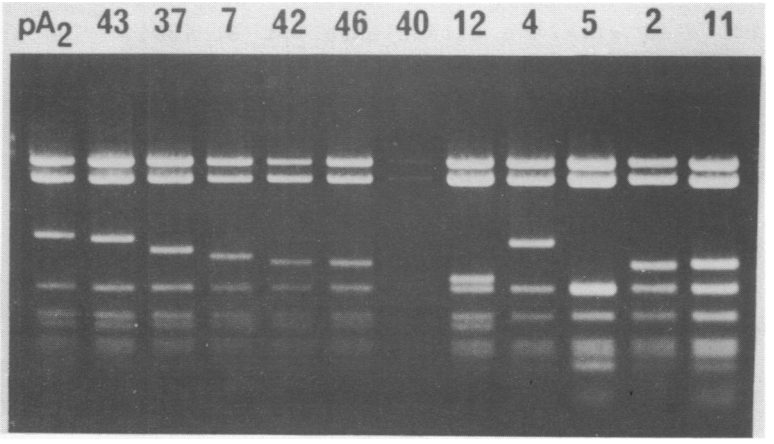


Figure 2a

Analysis of deletion mutants of plasmid pA_2.
For determining the sizes of the various deletions, in a typical experiment plasmid pA_2 and recombinant deletion mutants (as indicated) were cleaved with the restriction endonuclease Ava II under standard conditions. Resulting fragments were separated in Tris-acetate buffer by electrophoresis in a 1.4% agarose slab gel containing 1µg/ml ethidium bromide and visualized under ultraviolet light. Deletion sizes were estimated from size changes in the third and sixth largest bands.

were derived from 4 min incubations (see Figs. 2a and b). The precise location of the deletion in some of the species was determined by sequencing the DNA (data summarised in Figs. 2b and 3, and that for p46 shown in Fig. 4).

Biological characterisation of deletion mutants of plasmid pA_2

Subconfluent Rat-1 cells were transfected with plasmid DNA using the calcium method (28). Dishes were scored for foci two weeks after transfection. With 1µg DNA per 90 mm dish, recombinant plasmid pA_2 produced an average of 350 foci per dish. By similar procedures, other recombinants were assayed for their ability to produce foci, and the figure 350 used as a standard (100%) in calculating transforming efficiencies relative to pA_2. At least ten independent assays were carried out for each plasmid. Within any experiment, the number of foci on any particular dish did not vary to a significant extent from those

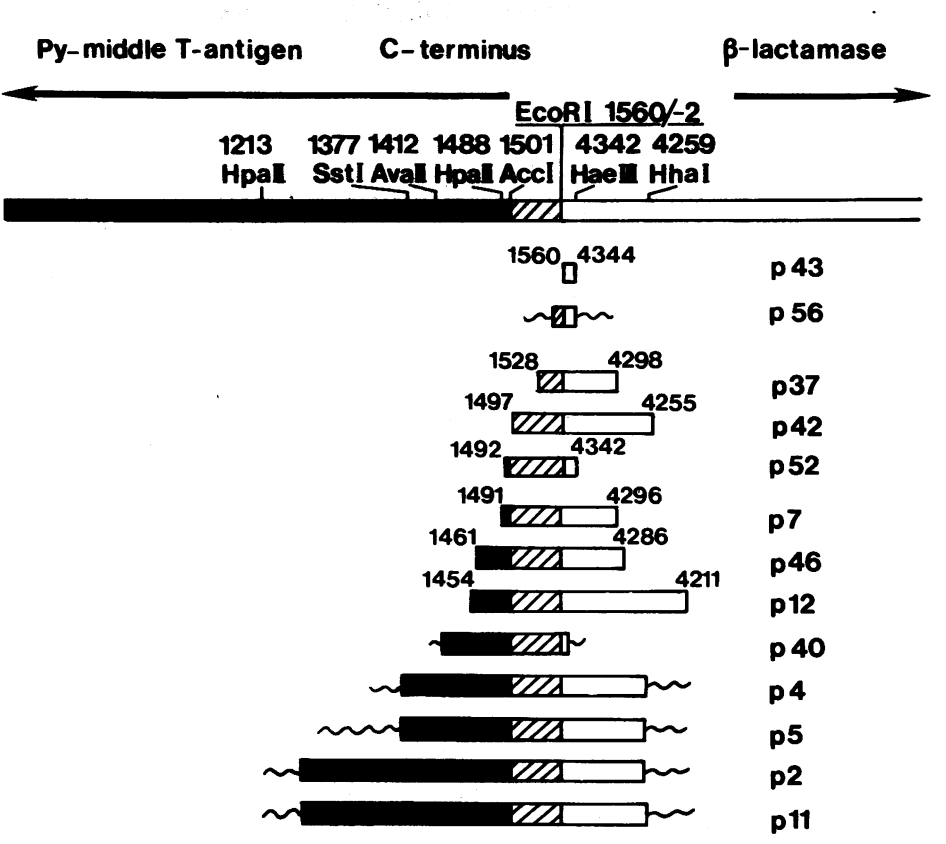


Figure 2b

Schematic representation of the deletions within the pA_2 derivatives.

The linearized map shows the region that overlaps the join between polyoma virus DNA and plasmid pAT153 DNA around their respective EcoR I sites. The location of various restriction sites are shown (24,32). The regions that code for the C-terminus of polyoma virus middle T-antigen and N-terminus of the plasmid β-lactamase are indicated. Within any recombinant deletion mutant, losses of viral DNA between the termination codon of middle T-antigen and the EcoR I site (shaded blocks, ▨,), within the coding region for middle T-antigen (solid blocks, ■) and within the plasmid (open blocks, □) are indicated. Numbers above individual blocks show the position of the last base pair in either polyoma virus or plasmid DNAs which are joined, that is, still present within the recombinant molecule. Deletions which extend further into the coding region of the viral middle T-antigen have only been mapped with restriction enzymes; wavy lines indicate uncertainties about the exact location of these deletions.

```
                Thr Leu Leu Leu Val Thr Phe Leu Ala Ala Leu Leu Gly Ile Cys Leu Met Leu Phe Ile Leu
pA2     :  1413- ACC CTG CTC CTA GTG ACT TTC CTA GCA GCC TTA CTG GGT ATT TGT CTC ATG CTA TTT ATT CTA
                                                                  ▲p12      ▲p46
                Ile Lys Arg Ser Arg His Phe ---
                ATA AAA CGT TCC CGG CAT TTC TAG
                ———————      ▲p7 ▲p52  ▲p42

p43/37/56:      Amino acid sequence not altered


                Gly Thr Pro Ile Cys Leu Phe Phe ---
p42     :  1497- GGA ACC CCT ATT TGT TTA TTT TTC TAA

                Pro Arg Asp Thr Pro Ile Phe Ile Gly Ala Ser ---
p52     :  1492- CCT CGT GAT ACG CCT ATT TTT ATA GGG GCC TCG TGA

                Phe Leu Arg Arg Gln Val Ala Leu Phe Gly Glu Met Cys Ala Glu Pro Leu Phe Val Tyr
p7      :  1491- TTT CTT AGA CGT CAG GTG GCA CTT TTC GGG GAA ATG TGC GCG GAA CCC CTA TTT GTT TAT

                Phe Ser Lys Tyr Ile Gln Ile Cys Ile Arg Ser ---
                TTT TCT AAA TAC ATT CAA ATA TGT ATC CGC TCA TGA

                Val Arg Trp His Phe Ser Gly Lys Cys Ala Arg Asn Pro Tyr Leu Phe Ile Phe Leu Asn
p46     :  1461- GTC AGG TGG CAC TTT TCG GGG AAA TGT GCG CGG AAC CCC TAT TTG TTT ATT TTT CTA AAT

                Thr Phe Lys Tyr Val Ser Ala His Glu Thr Ile Thr Leu Ile Asn Ala Ser Ile Ile Leu
                ACA TTC AAA TAT GTA TCC GCT CAT GAG ACA ATA ACC CTG ATA AAT GCT TCA ATA ATA TTG

                Lys Lys Glu Glu Tyr Glu Tyr Ser Thr Phe Pro Cys Arg Pro Tyr Ser Leu Phe Cys Gly
                AAA AAG GAA GAG TAT GAG TAT TCA ACA TTT CCG TGT CGC CCT TAT TCC CTT TTT TGC GGC

                Ile Leu Pro Ser Cys Phe Cys Ser Pro Arg Asn Ala Gly Glu Ser Lys Arg Cys ---
                AAT TTG CCT TCC TGT TTT TGC TCA CCC AGA AAC GCT GGT GAA AGT AAA AGA TGC TGA

p12     :  1454-    Arg Ser ---
                C CGC TCA TGA
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Figure 3

The amino acid sequences of recombinant plasmid coded "middle T-antigens" as deduced from DNA sequences.

The predicted C-terminal amino acid sequence of wild type A2 strain polyoma virus middle T-antigen (38) is the same as that shown for plasmid pA_2. A putative polyadenylation signal (AATAAA) within the viral DNA sequence is underlined. The significance (if any) of this signal is unclear since in p46 it is not present, and there is little difference between the transforming ability of this recombinant species and others (p42, p52 and p7) which retain it. The locations (nucleotide number) (21) of the DNA deletion endpoints for p42, p52, p7, p46 and p12 within the coding sequence of polyoma virus are indicated. The nucleotide sequences which follow start at the join of polyoma virus and plasmid DNAs and were determined by DNA sequencing methods (see Fig. 4); amino acids shown are plasmid-coded and linked to the respective truncated viral middle T-antigens. Plasmids p43, p37 and p56 have deletions which do not affect the coding region of middle T-antigen.

on the other dishes. An average value was taken to represent transforming efficiency. A summary of the results are given in Table I. In brief, p43, p37 and p56, with losses of less than 60 viral nucleotide pairs relative to pA_2, had the same transforming efficiency as the latter. Recombinants p42, p52, p7, and p46, with losses of between 63 and 99 viral base pairs, had

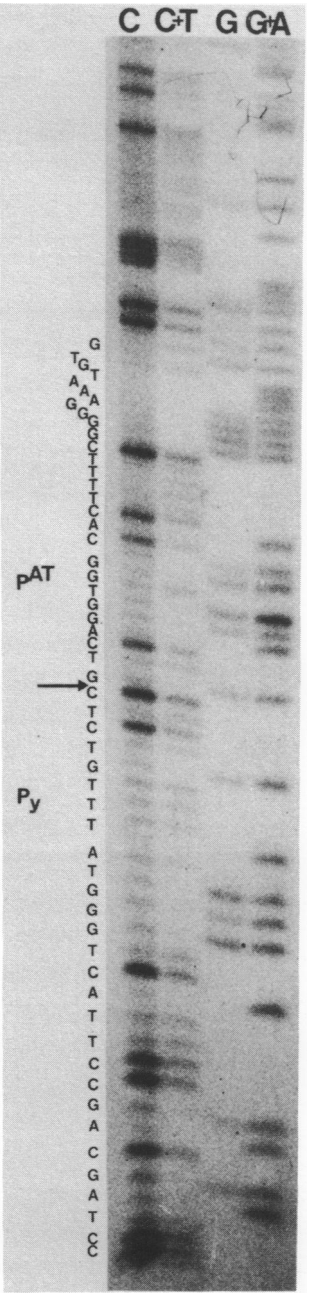

Figure 4

The sequence of the join between polyoma virus and plasmid vector DNAs in the recombinant p46.

The DNA sequence was determined by the Maxam-Gilbert method (26), as described in Materials and Methods. Plasmid derived sequences (pAT) lie above the arrow (at left) (see Fig. 3) and polyoma virus (Py) derived sequences lie below.

Table I: Properties of Fragments of Polyoma Virus DNA

Designation	Transformation* Efficiency (%)	Total number of viral nucleotides lost++	Number of amino acids lost from viral middle T-antigen	Number of Plasmid amino acids added to middle T-antigen+++
pA_2	100	0	0	0
p43	100	0	0	0
p56	100	ND	0	0
p37	100	32	0	0
p42	4	63	0(term.codon)	8
p52	8	68	2	11
p 7	1	69	2	31
p46+	2.5	99	12	78
p12+	<0.1	106	14	2
p40,2,4,5, 11+	<0.1	>150	ND	ND

* As deduced from dense focus assay, relative to pA_2 (350 foci per µg DNA per 90mm dish).

\+ Removes a putative polyadenylation signal.

++ From DNA sequence, relative to pA_2. Subtract 63 to get number of nucleotides lost from the coding sequence of middle T-antigen.

+++ Preceding a termination codon (see Fig. 3).

ND Not determined

transforming efficiencies of 1 to 8%. No foci were detected in assays using recombinant plasmids p2, p4, p5, p11, p12 or p40 which lack more than 106 nucleotide pairs. Their relative transforming efficiencies are therefore smaller than 0.03%. Some continuous cell lines, produced from individual foci following only a few passages in culture, were tested for their ability to grow in 0.35% agar. Cell lines from pA_2, p43, p37, p42, p7, and p46 were able to grow in soft agar although those from p42 (p42f2) or p46 (p46f2) or p46 (p46f1, p46f3) grew with reduced efficiency and increased doubling time relative to lines derived from the other recombinant plasmid induced foci (data not shown). Studies of tumor formation in whole animals produced results which paralleled the soft agar assays.

Immunoprecipitation of polyoma virus proteins

[^{35}S]-Methionine labeled proteins were isolated from lines established from rat-1 cells that had been transformed by various recombinant plasmids and immunoprecipitated using either anti T-antigen serum or serum from normal rats. Characterization of the proteins by SDS gel electrophoresis is shown in Figure 5. All cell lines expressed polyoma virus small and middle T-antigens. The amounts and sizes of the truncated species of large T-antigen varied, however, among the lines. The size of the middle T-antigens appeared to vary slightly. The DNA sequences of the recombinants allow the sizes of the various middle T-antigen related proteins to be predicted, and in some cases (p42,p52), they should differ only slightly from the wild-type proteins. With p46 especially, a protein about 6K larger than the viral middle T-antigen was expected. This was not observed (see Table I). None of the cell lines appeared to express a very large fusion (viral/plasmid/host) protein, indicating that translational termination occurred at codons within the plasmid (see Fig. 3).

DISCUSSION

Several experiments using limited portions of the polyoma virus genome to transform hamster cells *in vivo* (7,11) or semi-permissive cells *in vitro* (8-10) have demonstrated that the viral

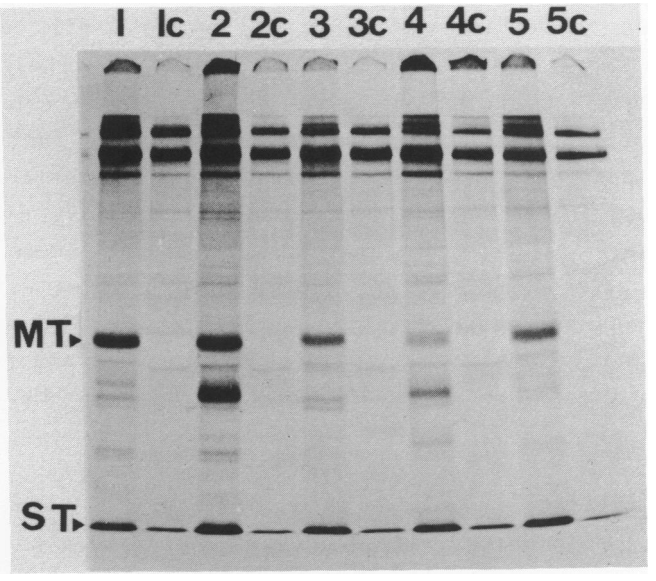

Figure 5

Comparison of viral early proteins expressed in cell lines transformed by different recombinant plasmids.

[^{35}S]-methionine labeled proteins were extracted from cells, immunoprecipitated and separated on an 8.5% acrylamide gel. Lane 1 contains proteins from the cell line pA_2f_1, precipitated with anti T-antigen serum, and lane 1c proteins precipitated using serum from normal rats. Similarly, lanes 2 and 2c contain proteins from $p37f_1$ cells, lanes 3 and 3c from $p42f_2$ cells, lanes 4 and 4c from $p7f_2$ cells, and lanes 5 and 5c from $p46f_1$ cells. In our nomenclature, p represents the plasmid, a capital letter or number the viral DNA and f, a particular focus from which the cell lines were derived. Thus, pA_2f_1 is a cell line derived from a single dense focus produced from Rat-1 cells transfected with parental recombinant plasmid pA_2, etc. The location of small (ST) and middle (MT) T-antigens are indicated. Some of the proteins which lie between these two antigens are presumably truncated forms of large T-antigen, as noted earlier (8). Plasmid coded "middle T-antigens" varied in size among the different species, but size changes were not large enough to give clear shifts of mobility on 8.5% gels.

DNA sequence between the EcoR I site at 0/100 units and 26 units on the physical map (33) is not required for the initiation or maintenance of cellular transformation. This sequence encodes only the C-terminus, or about 60% of the viral large T-antigen

(which in lytically infected cells and in many transformed lines is about 100K in size). All segments of viral DNA used in these experiments, however, apparently leave the coding region for the viral small (22K) and middle (55K) T-antigens intact (8,34,35).

To probe further into the effects of sequence alterations on transformation and as part of a broader study to define the minimal viral coding sequence required for transformation, we have introduced specific deletions into a recombinant plasmid (pA_2) that carries a fragment of polyoma virus DNA which is known to code for full-sized small and middle T-antigens. After recloning, use of such viral DNA sequences has allowed us to analyse the role of the C-terminus of middle T-antigen in transformation. This protein contains some interesting chemical features, including a very hydrophobic sequence (12,34). The pA_2 plasmid was linearised at the EcoR I site (nucleotide number 1560) which is sixty base pairs (bp) beyond the termination codon of middle T antigen (21). Nucleotides were then sequentially removed from the plasmid and viral DNA by treatment with the exonuclease BAL 31 (see Fig. 1). A number of mutants carrying a deletion either between the viral EcoR I site and the Acc I site at 1500 bp (p46, p37 and p56) or beyond either the Acc I site (p42, p52 and p7), the Hpa II site at 1488 bp (p46, p12 and p40), or the Sst I site at 1373 bp (p2, p4, p5 and p11) were constructed (see Figs. 1 and 2). Most of the recombinants which lacked sequences between the EcoR I and Ava II sites were then sequenced. A knowledge of the precise join between viral and plasmid sequences allowed the prediction of the amino acid sequences of the virus-related proteins encoded within the recombinant plasmid to be made (Fig. 3). Depending on the sequence joins, the middle T antigen-related protein contained different amounts of viral and plasmid derived sequences. The plasmid recombinants pA_2, p43, p56, and p37 contain viral sequences that extend beyond the termination codon for middle T-antigen and with respect to functions associated with this protein, should behave identically. They should vary only in their respective coding sequences for large T antigen-related proteins. The recombinants p42, p52, p7, p46, p12, p40, p2, p4, p5 and p11, on the other hand, all have varying amounts of viral

deletions which affect the C-terminus of middle T antigen and some of them remove a putative polyadenylation signal at 1476-1482 bp (see Fig. 3) in the viral DNA (21). Since the latter deletions also remove the termination codon for middle T-antigen (at 1498-1500 bp), translational termination must depend upon the presence of a suitable codon within the plasmid DNA. The various altered viral middle T-antigens can be predicted to acquire between two (p12) and seventy-eight (p46) amino acids from the plasmid sequence. It cannot be ruled out that these additional amino acids have some influence on the expression or function of middle T-antigen. For example, it would appear likely that the conformation of the viral middle T-antigen may have been altered in the recombinants p7 and p46. Both of these species produced proteins only marginally different in mobility from the wild type species (see Fig. 5), in apparent conflict with prediction (Table I). The size deduced for the wild type middle T-antigen (55K) from its mobility on SDS gels is not consistent, however, with its size as predicted from the DNA sequence (16,21). Studies on mlt mutants suggest that the mobility of this protein is very sensitive to sequence alterations (16). The simplest explanation of our data therefore is that in both p7 and p46, mobilities reflect conformational differences rather than size alterations. None of the recombinant plasmids would be expected to have altered the sequence coding for the viral small T-antigen.

The sole cellular property studied in our experiments was transformation and the results of dense focus assays are given in Table I. In general, once the sequence coding for middle T-antigen had been reached by exonuclease digestion, the transformation frequency was found to be diminished. In another assay, cell lines established from dense foci were tested for their ability to grow in soft agar; in this assay, lines from p42 and p46 grew less well than other cell lines. If these observed differences are significant, it is possible that acquired plasmid-coded sequences may in some cases perturb the function of middle T-antigen (see below). It is noteworthy that p42, which exhibits a reduced efficiency of transformation in both assays, contains eight additional amino acids as well as all those found

in the wild type middle T-antigen.

The main conclusion allowed by our data is that any perturbation of the C-terminus of middle T-antigen has a concomitant effect upon transformation. Further, our results show that the DNA sequence of polyoma virus which lies between the termination codon of middle T-antigen (1498-1500 bp) and the EcoR I site (1560 bp), while coding for a part of large T-antigen, is not required for the initiation or maintenance of cellular transformation, nor does the removal of this portion of the genome have a measurable effect upon the frequency of transformation.

If the termination codon for middle T-antigen is removed, either alone or together with a few additional codons, the efficiency of transformation drops dramatically. The smallest deletion which gave rise to a non-transforming species is exemplified by the recombinant p12 in which the DNA sequence suggests that the terminal fourteen amino acids should have been deleted from the wild type middle T-antigen and only two plasmid coded amino acids added. p12 did not transform Rat-1 cells in any of our experiments. The middle T-antigen of polyoma virus was originally detected by Ito et al. in association with the host cell plasma membrane (36). From the DNA sequence, middle T-antigen can be predicted to have a C-terminus characteristic of membrane bound proteins (see ref. 37, and references therein). In polyoma virus middle T-antigen, a stretch of twenty-two hydrophobic amino acids is preceded and followed by polar (basic) amino acids. We have postulated that this protein might be attached to the membrane by its hydrophobic region and positioned or stabilised by the basic amino acids immediately adjacent to this region (12). One explanation for the dramatic effect upon transformation that is observed when sequences that code for the C-terminal region of middle T-antigen are removed may lie in the fact that they alter the structure of the hydrophobic region of middle T-antigen (38) and lead to a reduced interaction with the cellular membrane. From our data, the break between the point at which transformation is still observed and when it is no longer detectable, occurs between the removal of six (p46) and eight (p12) of the hydrophobic amino acids.

It would appear from our data that not only the removal of sequences coding for middle T-antigen reduce the efficiency of transformation, but the addition of plasmid-coded amino acids to the viral antigen are also significant in this regard. The simplest explanation for the variation in transformation efficiency among recombinants p42, p52, p7 and p46 (see Table I and Fig. 3) lies in the assumption that sequences added to the C-terminus of middle T-antigen may change its conformation in such a way as to alter its function(s) in transformation. This assumption best fits the efficiencies observed with these recombinants and explains why differences in transformation observed among them do not correlate directly with loss of viral amino acid sequences.

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REFERENCES

1. Ito, Y. and Spurr, N. (1980) Cold Spring Harb. Symp. Quant. Biol. 44, 149-157.
2. Francke, B. and Eckhart, W. (1973) Virol., 55, 127-135.
3. Cogen, B. (1978) Virol., 85, 222-230.
4. Basilico, C., Gattoni, S., Zouzias, D. and Della Valle, G. (1979) Cell, 17, 645-659.
5. Fried, M. (1965) Proc. Natl. Acad. Sci. 53, 486-491.
6. Lania, L., Gandini-Attardi, D., Griffiths, M., Cooke, B., de Cicco, D. and Fried, M. (1980) Virol. 101, 217-232.
7. Israel, M.A., Simmons, D.T., Hourihan, S.L., Rowe, W.P. and Martin, M.A. (1979) Proc. Natl. Acad. Sci., 76, 3713-3716.
8. Novak, U., Dilworth, S.M. and Griffin, B.E. (1980) Proc. Natl. Acad. Sci. USA, 77, 3278-3282.
9. Hassell, J.A., Topp, W.C., Rifkin, D.B. and Moreau, P.E. (1980) Proc. Natl. Acad. Sci., 77, 3978-3982.
10. Bastin, M., Borgaux-Ramoisy, D. and Borgaux, P. (1980) J. Gen. Virol. 50, 179-184.
11. Moore, J.L., Chowdhury, K., Martin, M.A. and Israel, M.A. (1980) Proc. Natl. Acad. Sci. 77, 1336-1340.
12. Novak, U. and Griffin, B.E. (1980) Cell Biology 1980-1981 (ed. Schweiger, H.G.), Springer-Verlag, pp. 448-456.
13. Della Valle, G., Fenton, R.G. and Basilico, C. (1981) Cell 23, 347-355.
14. Smolar, N. and Griffin, B.E. (1980) J. Virol., in press.

15. Griffin, B.E., Ito, Y., Novak, U., Spurr, N., Dilworth, S.M., Smolar, N., Pollack, R., Smith, K. and Rifkin, D. (1980) Cold Spring Harb. Symp. Quant. Biol. 44, 271-282.
16. Ito, Y., Spurr, N. and Griffin, B.E. (1980) J. Virol. 35, 219-232.
17. Magnusson, G. and Berg, P. (1979) J. Virol. 32, 523-529.
18. Magnusson, G., Nilsson, N.G., Dilworth, S.M. and Smolar, N., submitted for publication.
19. Benjamin, T.L. (1970) Proc. Natl. Acad. Sci. 67, 394-399.
20. Feunteun, J., Sompayrac, L., Fluck, M. and Benjamin, T.L. (1976) Proc. Natl. Acad. Sci. 73, 4169-4173.
21. Soeda, E., Arrand, J.R., Smolar, N., Walsh, J.E. & Griffin, B.E. (1980) Nature, 283, 445-453.
22. Kamen, R., Favaloro, J., Parker, J., Treisman, R., Lania, L. Fried, M. and Mellor, A. (1980) Cold Spring Harb. Symp. Quant. Biol. 44, 63-75.
23. Griffin, B.E. and Fried, M. (1976) Methods in Cancer Research XII (ed. Busch, H.) Academic Press, pp. 49-86.
24. Sutcliffe, J.G. (1978) Nuc. Acids. Res. 5, 2721-2728.
25. Birnboim, H.C. and Doly, J. (1979) Nuc. Acids. Res. 7, 1513-1523.
26. Maxam, A.M. & Gilbert, W. (1977) Proc. Natl. Acad. Sci. 74, 560-564.
27. Freeman, A.E., Gilden, R.V., Vernon, M.L., Wolford, R.G., Hugunin, P.E. & Huebner, R.J. (1973) Proc. Natl. Acad. Sci. 70, 2415-2419.
28. Van der Eb, A.J. and Graham, F.L. Methods in Enzymology (ed. L. Grossman and K. Moldave, Academic Press) Vol. 65 826-838.
29. Ito, Y. (1979) Virology, 98, 261-266.
30. Gray, H.B., Ostrander, D.A., Hodnett, J.L., Legerski, L.J. & Robberson, D.L. (1975) Nuc. Acids. Res. 2, 1459-1492.
31. Sutcliffe, J.G. (1978) Proc. Natl. Acad. Sci. 75, 3737-3741.
32. Griffin, B.E., Soeda, E., Barrell, B.G. and Staden, R. (1980) In DNA Tumor Viruses (ed. J. Tooze) Cold Spring Harbor Laboratory, p. 888.
33. Griffin, B.E., Fried, M. and Cowie, A. (1974) Proc. Natl. Acad. Sci. 71, 2077-2081.
34. Soeda, E., Arrand, J.R. & Griffin, B.E. (1979) Nuc. Acids. Res. 7, 839-857.
35. Friedmann, T., Esty, A., LaPorte, P. & Deininger, P. (1979) Cell, 17, 715-724.
36. Ito, Y., Brocklehurst, J.R. & Dulbecco, R. (1977) Proc. Natl. Acad. Sci. 74, 4666-4670.
37. Rose, J.K., Welch, W.J., Sefton, B.M., Esch, F.S. & Ling, N.C. (1980) Proc. Natl. Acad. Sci. 77, 3884-3888.
38. Soeda, E., Arrand, J.R., Smolar, N. and Griffin, B.E. (1979) Cell, 17, 357-370.