The EMBO Journal Vol.1 No.4 pp.461 – 466, 1982

Mutant *din*-21, a variant of polyoma virus containing a mouse DNA sequence in the viral genome

DaMing Ding[1], M.D. Jones, Anne Leigh-Brown, and Beverly E. Griffin*

Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, UK

Communicated by B.E. Griffin
Received on 5 April 1982

An unusual non-defective mutant of polyoma virus with an anomalously large genome, designated *din*-21, has been isolated. The viral chromosome lacks 49 base pairs of the putative control region between the origin of replication and the initiation codon for the early proteins, the T-antigens. In their stead, 95 base pairs, with limited homology to the deleted sequence and apparently of mouse origin, have been inserted. The primary sequence of the insert DNA has been determined and some of the biological properties of the mutant examined. It transforms rat-1 cells slightly better than wild-type virus and grows slightly less well in lytically infected mouse cells. It does not interfere with the growth of wild-type polyoma virus. The properties of this mutant suggest that it is a natural isolate of mouse cells. The mutant was presumably generated by reciprocal recombination between polyoma DNA and mouse host DNA. This could be associated with the integration of a viral DNA sequence into the host chromosome during the viral replicative cycle.

Key words: viral mutant/transfection/lytic response/transformation/transcription

Introduction

The DNA tumour virus, polyoma, grows lytically in mouse cells. In the process of identifying regions within the genome that are not essential for the reproductive growth of this virus, we isolated an unusual variant from mouse cells with a genome greater in size than that found in most wild-type polyoma virus strains. Further investigation into the properties of this mutant showed it to differ from several previously described variants with anomalously large genomes. Whereas the latter appear to have either duplications of viral sequences from around the origin of replication (Fried and Griffin, 1977; Magnusson and Nilsson, 1977) or duplicated, rearranged "late region" sequences (Katinka *et al.*, 1980; Sekikawa and Levine, 1981), our mutant, designated *din*-21 (*d*eletion-*in*sertion), has mouse cellular DNA replacing a viral sequence in the putative control region between the origin of replication and the initiation codon for the viral early proteins (Soeda *et al.*, 1979). Somewhat surprisingly, this substitution has little apparent effect on the properties of the virus examined so far. The exchange of mouse sequence for viral sequence could have arisen by a reciprocal recombination event associated with viral replication. If so, one might expect variants similar to *din*-21 to arise frequently following infection of mouse cells with polyoma virus. They may only be observed, however, under conditions similar to those used in our experiments, in which most cells are not challenged by an infectious species. Studies by Türler (1977) on the polyoma virus replicative cycle suggested that unlike subsequent findings on SV40 (Rigby and Berg, 1978), integration into chromosomal DNA can occur during the productive infection of mouse cells by polyoma virus. Mutant *din*-21 could have arisen as a consequence of such integration.

Results

Polyoma virus mutant *din*-21 was isolated from a single plaque produced on whole mouse embryo cells (WME) following transfection with full-length linear molecules from wild-type A2 strain DNA. The latter were generated by cleavage of supercoiled viral DNA in the presence of ethidium bromide (Parker *et al.*, 1977) with a restriction endonuclease, *Hinf*I. About 30 plaques were examined, of which only one produced a mutant virus with the properties described here.

Analysis of the DNA from mutant *din*-21 with the restriction endonuclease *Hpa*II showed it to be ~1% larger than DNA of strain A2, the extra sequence residing in fragment *Hpa*II-5 (Figure 1). This fragment is of particular interest since it contains the viral origin of replication and the ATG initiation codon for the early proteins, the T-antigens (Griffin *et al.*, 1974; Soeda *et al.*, 1980a). DNA sequence analysis (data shown in Figure 2 and illustrated in Figure 3) showed *din*-21 DNA to have lost the viral sequence between nucleotides 48 and 98 and to have gained 95 bp from elsewhere. A computer search of the sequence of wild-type DNA failed to locate the insert sequence, or any substantial portion of it, at any other position within the viral DNA.

Analysis of the inserted sequence predicted it to contain recognition sequences for the known restriction endonucleases *Asu*I, *Hae*III, *Hgi*AI, *Eco*PI, *Ava*II, *Spn*I, *Eco*RII, *Kpn*I, and *Rsa*I. To substantiate in part this prediction, *din*-21 DNA was cleaved with *Kpn*I (two sites in wild-type DNA) and *Hgi*AI (seven sites in wild-type DNA) and the results were found in each case to confirm the prediction. That is, *Kpn*I produced three fragments of the size predicted, and *Hgi*AI eight fragments. Similarly, wild-type DNA fragment *Hae*III-14 (Griffin, 1977) was absent in the mutant and a new fragment ~100 bp long was found, as predicted. The data (some of which are shown in Figure 4) support the conclusion that the sequence alteration illustrated in Figure 3 is the major, and probably the sole, change in the mutant relative to wild-type DNA. Moreover, immunoprecipitation of viral antigens from 3T6 mouse cells infected either with the *din*-21 or with wild-type viruses showed no detectable differences (Figure 5).

To study the biological properties of the mutant, rat-1 cells were infected with equal quantities (plaque forming units) of either wild-type or mutant *din*-21 viruses. Visible foci were scored after 3 weeks on eight dishes infected with each virus, four maintained at 37°C and four at 32°C. Whereas there was an ~4-fold difference of foci at the two temperatures, the ratio of foci produced by *din*-21 virus relative to A2 virus was 3:1 at each temperature. These data suggest that *din*-21

[1]On leave from Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.

*To whom reprint requests should be sent.

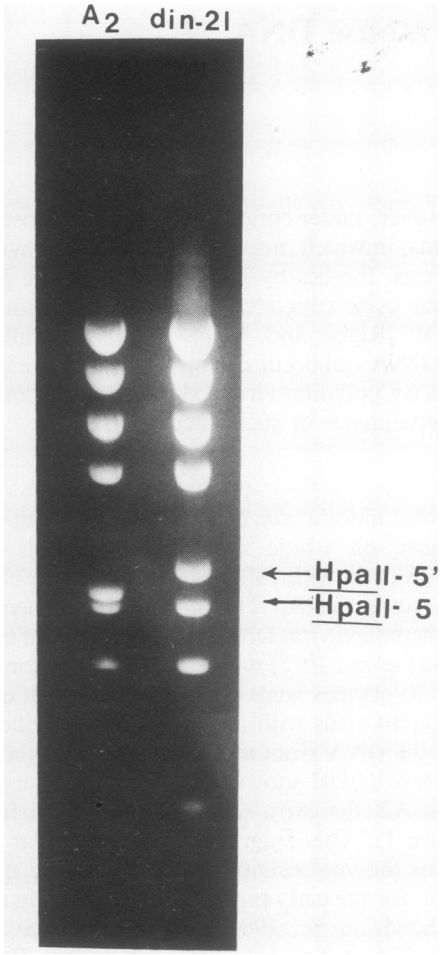

Fig. 1. DNA from the mutant *din*-21 was cleaved with the restriction endonuclease *Hpa*II and fragments separated by electrophoresis on 1.4% agarose gels in the presence of ethidium bromide (Griffin *et al.*, 1974). Wild-type strain A2 DNA was used as a control. Eight fragments were produced on cleavage of *din*-21 DNA, seven of them with sizes indistinguishable from wild-type DNA. One mutant fragment, *Hpa*II-5', was larger than the corresponding wild-type fragment, *Hpa*II-5.

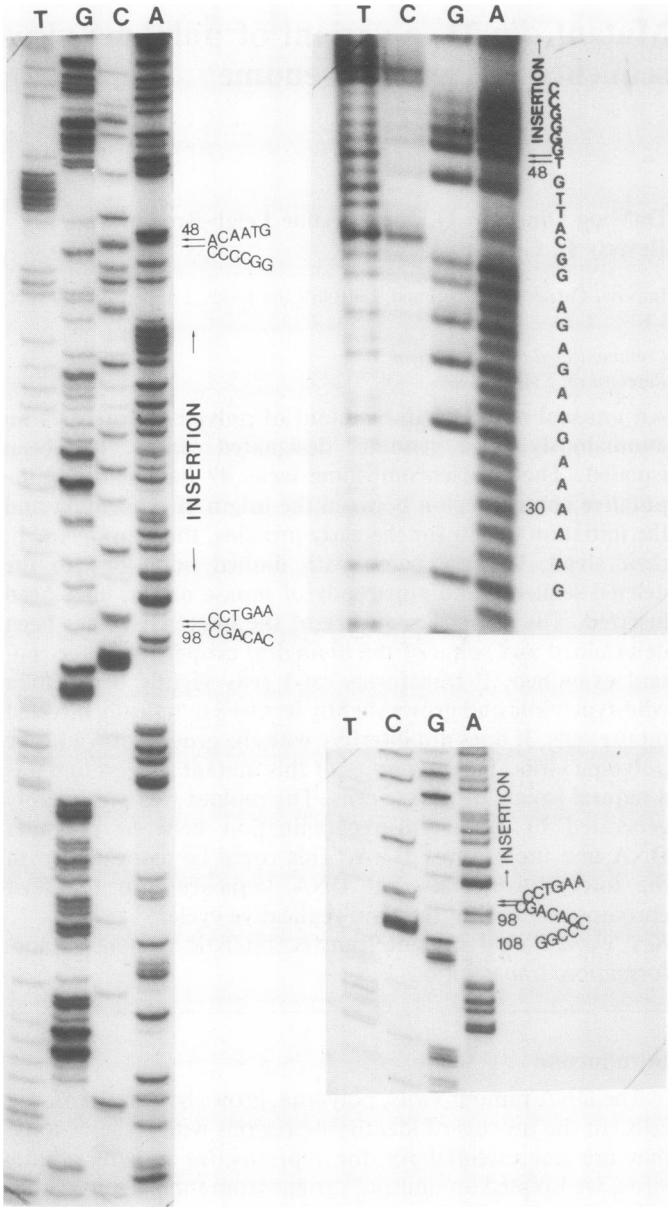

Fig. 2. DNA sequence analysis of the region in mutant *din*-21 showing sequence alterations and joins to wild-type DNA. **Left**, sequence from the "late region" strand of polyoma virus indicative of the insertion that replaces viral nucleotides between positions 48 and 98. This region of the mutant DNA was cloned in the vector M13 (Messing *et al.*, 1981) and sequenced by the dideoxy method of Sanger *et al.* (1977). **Right, upper panel**: a Maxam-Gilbert (1977) sequencing gel of "early region" strand DNA showing the altered sequences around viral nucleotide 48; **lower panel**, a second dideoxy sequencing gel which illustrates more clearly the altered sequences around viral nucleotide 98. Nucleotide numbering is taken from Soeda *et al.* (1980a).

may be a slightly better transforming virus than the wild-type strain, and it is not temperature sensitive. Similar experiments carried out with the deletion mutant *dl*-8 which appears to be superior to wild-type virus in transformation studies (Griffin and Maddock, 1979), showed ratios of *din*-21:*dl*-8 to be 1:2 at 37°C and 1:5 at 32°C.

In simple competition experiments, 3T6 mouse cells were infected with a mixture containing equal amounts of wild-type and *din*-21 mutant viruses. Supercoiled DNA was isolated after 60 h. As a control, similar competition experiments were carried out using mixtures of wild-type and mutant *dl*-8 viruses, the latter known to produce only small amounts of DNA (Griffin and Maddock, 1979). Restriction enzyme analyses of the isolated DNAs (data not shown) suggest that no detectable recombination occurred between the viruses and that neither mutant had an appreciable effect on the replication of wild-type virus, or *vice versa*.

To determine the origin of the novel sequence in *din*-21, hybridisation experiments were carried out on secondary WME cells, using as probes similar amounts of either radioactively labelled wild-type or *din*-21 DNAs. As controls, hybridisations were also carried out on DNA from monkey cells (CV1) and salmon sperm, using the same probes; SV40 DNA was also used as a probe in experiments with WME cells and salmon sperm. The relevant results are shown in Figure 6. The data suggest that, although detectable hybridisation occurs between mouse embryo DNA and DNAs from both wild-type and *din*-21 viruses, there is >4-fold difference in the levels seen between the two species, the greater level of hybridisation being observed with *din*-21. To confirm these results, and also to question the origin of the polyoma virus-like DNA in mouse embryo cells, high mol. wt. DNA made from WME cells, from organs of nude mice, and from baby hamster kidney (BHK) cells were cleaved with

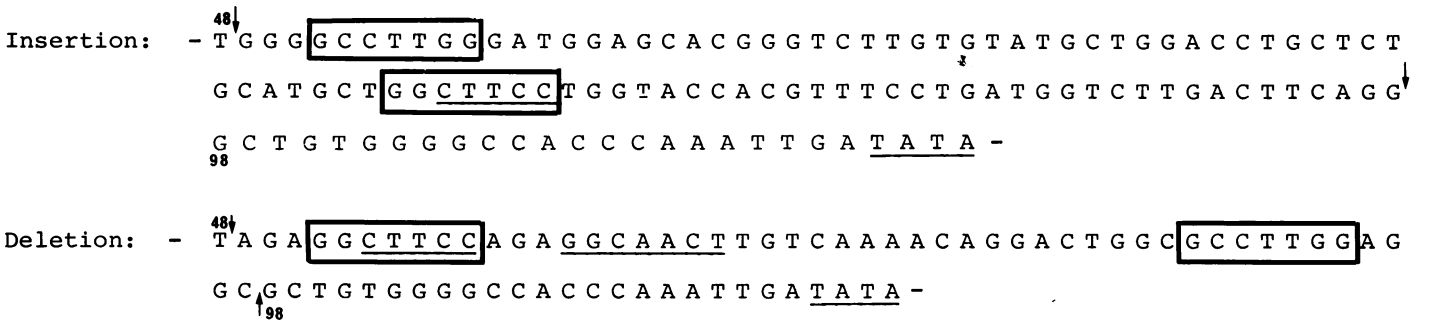

Fig. 3. Sequence alterations found in mutant *din*-21. Sites of deletion and insertion are indicated by arrows. Wild-type nucleotides 49–97 (Soeda *et al.*, 1980a), inclusive, are deleted in the mutant and replaced by 95 bp of apparent mouse origin. Two regions of perfect homology (boxes) between deleted and inserted sequences, with conserved polarity but different locations, can be observed. Signals playing possible roles in transcription of early virus genes are underlined (see text).

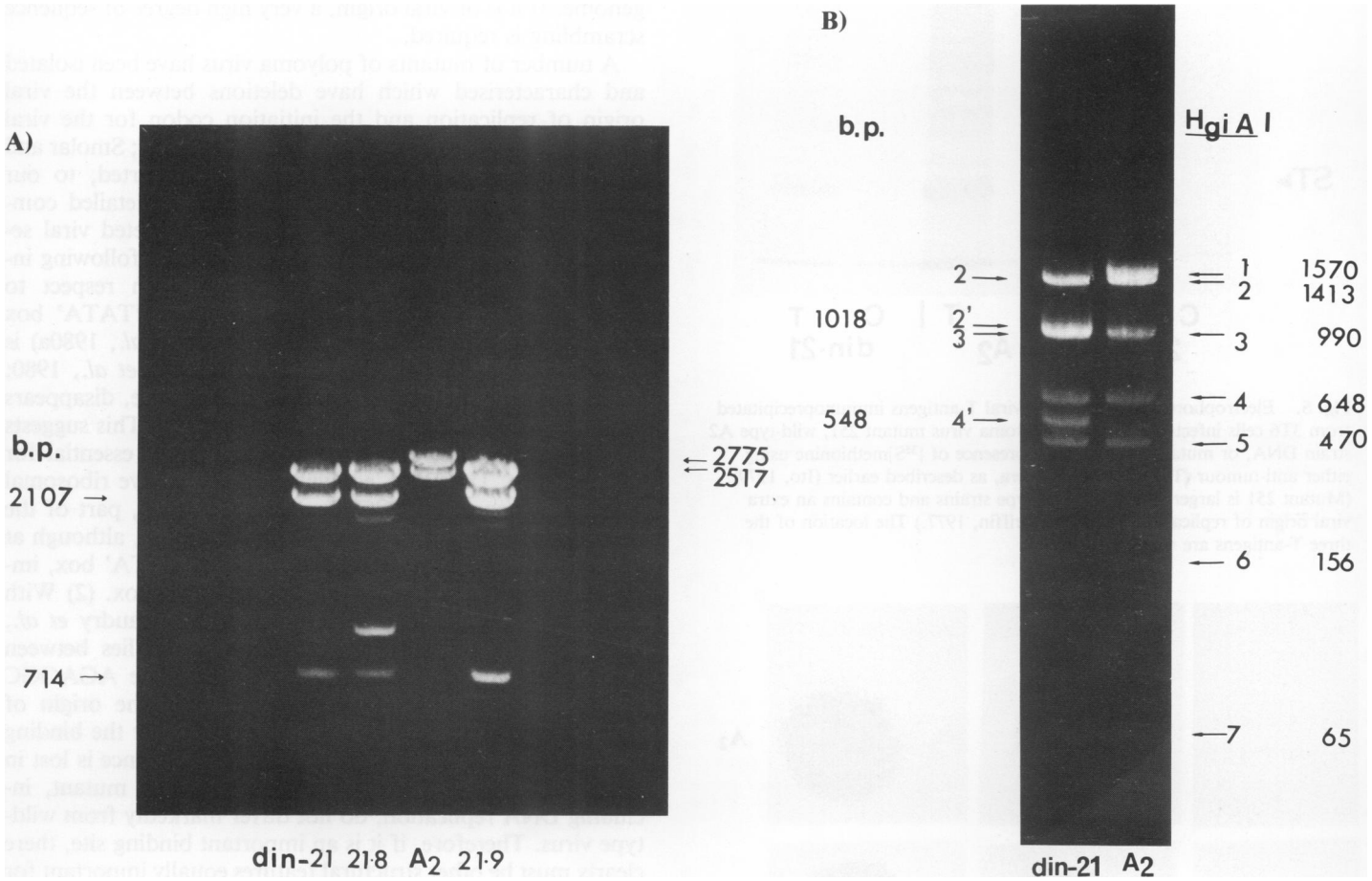

Fig. 4. Comparison of restriction enzyme digests of polyoma virus wild-type A2 strain and mutant *din*-21 DNAs. **A** *Kpn*I digest which shows the absence of the largest wild-type fragment (2775 bp) in *din*-21 DNA and the presence of two new bands with predicted sizes of 2107 and 714 bp. Mutant 21.8 is discussed in Figure 6; 21–9 is another plaque isolate of *din*-21. **B** *Hgi*AI digests which show that the largest wild-type fragment (1570 bp) is absent in *din*-21 DNA and two new bands (labelled 2′ and 4′, with predicted sizes 1018 and 548 bp, respectively) are present.

the restriction enzymes *Alu*I and *Hae*III respectively, and equal quantities of each DNA species were separated by electrophoresis on agarose gels. Control wells contained A2 strain DNA cleaved with the same two enzymes. Employing well-documented procedures (Southern, 1975), hybridisations were carried out with radioactively labelled probes made from A2 strain, *din*-21, and SV40 DNAs. Analysis of the results by autoradiography (data not shown) allowed the conclusion that, under conditions where there was negligible hybridisation of either WME, nude mouse, or BHK DNAs with A2 DNA, there was strong hybridisation between the DNA of *din*-21 and both WME and nude mouse DNAs. (The SV40 experiment was negative throughout.)

Discussion

As part of a general probe into essential and non-essential regions within the polyoma virus genome, we obtained a very interesting mutant, *din*-21, which we postulate could have arisen by recombination between mouse cellular and polyoma

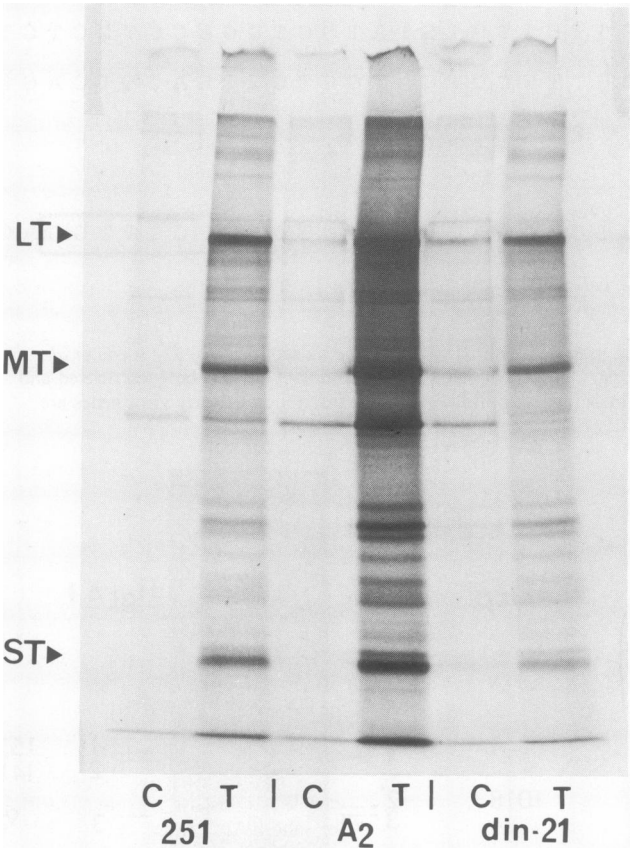

Fig. 5. Electrophoretic separation of viral T-antigens immunoprecipitated from 3T6 cells infected either with polyoma virus mutant 251, wild-type A2 strain DNA, or mutant *din*-21, in the presence of [^{35}S]methionine using either anti-tumour (T) or control (C) sera, as described earlier (Ito, 1979). (Mutant 251 is larger than most wild-type strains and contains an extra viral origin of replication, Fried and Griffin, 1977.) The location of the three T-antigens are shown.

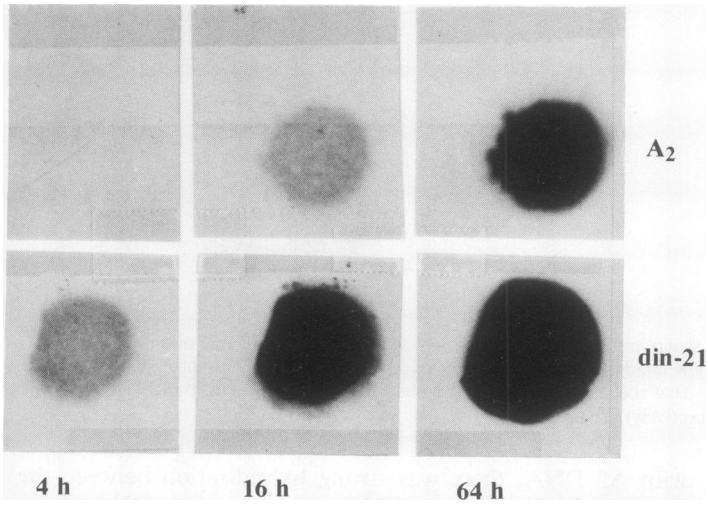

Fig. 6. Nitrocellulose discs containing DNA hybridised against equal amounts of either [^{32}P]nick-translated A2 strain or *din*-21 DNAs. Discs were autoradiographed for the times indicated in order to allow comparative hybridisations to be observed. In comparable experiments against either CV1 or salmon sperm DNAs, no hybridisation with either probe was evident even after exposure for 64 h. Similarly, [^{32}P]nick translated SV40 DNA used as a probe against WME or salmon sperm DNA showed no detectable hybridisation after 64 h.

virus DNAs during the productive replication cycle of the virus.

In our experiments aimed at generating deletion mutants (see Griffin and Maddock, 1979), WME cells were transfected with full-length linear polyoma virus molecules generated by cleavage of supercoiled DNA with the restriction endonuclease, *Hinf*I, followed by S1 nuclease. We assume that the mutant *din*-21 described here, whose lesion is shown in Figure 3, originated either from an uncleaved minor population of circular DNA in the linear population or from a recircularised species, since *din*-21 DNA appears to contain all the *Hinf*I sites present in wild-type DNA; the *Hinf*I recognition sequence in wild-type DNA nearest to the lesion in *din*-21 is ~200 bp away and appears to be retained in the mutant. The novel sequence found in *din*-21 shows no extensive homology to the viral sequence lost nor to any of the rest of the viral genome. If it is of viral origin, a very high degree of sequence scrambling is required.

A number of mutants of polyoma virus have been isolated and characterised which have deletions between the viral origin of replication and the initiation codon for the viral T-antigens (Wells *et al.*, 1979; Bendig *et al.*, 1980; Smolar and Griffin, 1981), but none have yet been reported, to our knowledge, with an overall net insertion. A detailed comparison of the insertion sequence and the deleted viral sequence in *din*-21 (Figure 3) allows some of the following interesting observations to be made: (1) With respect to transcription, whereas the Goldstein-Hogness 'TATA' box control signal (Kamen *et al.*, 1980;. Soeda *et al.*, 1980a) is outside the lesion, the 'CAAT' site, (Benoist *et al.*, 1980; Efstradiatis *et al.*, 1980), GGCAAC in this case, disappears and is not replaced by a comparable sequence. This suggests that in mouse cells, at least, this signal is not essential for transcription. The DNA equivalent of a putative ribosomal binding sequence, CTTCC (Soeda *et al.*, 1979), part of the deleted sequence, reappears within the insertion, although at a slightly different location relative to the 'TATA' box, implying a need for conservation of this signal box. (2) With regard to replication, it has been reported (Gaudry *et al.*, 1981) that the integrity of the sequence that lies between nucleotides 48–65, including two of the three AGAGGC hexamers found in wild-type virus between the origin of replication and the 'TATA' box, is required for the binding of large T-antigen to the viral DNA. This sequence is lost in *din*-21 and yet the observed properties of the mutant, including DNA replication, do not differ markedly from wild-type virus. Therefore, if it is an important binding site, there clearly must be other structural features equally important for replication. (3) Two heptanucleotide sequences of similar composition, GCCTTGG and GCTTCC, are found near the ends of the viral deleted sequence (see Figure 3). They also appear in the inserted sequence and account for the chief homology between the deleted and inserted sequences, but are reversed in location. Therefore, no simple model of homologous recombination between cellular and viral DNAs, at least at these sites, would appear to account for the deletion-insertion in *din*-21. (4) Finally, in *din*-21, the triplet CTT appears near the termini of both the inserted and deleted sequences flanked at the ends by purine-rich sequences. In fact, six of the 93 triplets present in the inserted sequence are CTT; the same ratio of ~1 CTT in 15 triplets is found in the viral deletion. These results are compatible with the notion that the virus may have acquired from its host (see below)

structurally similar elements, and possibly even biologically similar ones.

Since mutant *din*-21 differs little from wild-type virus in its known biological properties and was produced by transfection of mouse cells with linear viral DNA, the question of the origin of the novel DNA sequence in the mutant seems highly relevant. Comparison between the latter and the sequence of wild-type A2 strain DNA (Soeda *et al.*, 1980a) showed negligible homology, suggesting a non-viral source. On the other hand, hybridisation against total DNA derived from either WME cells, or organs of an adult nude mouse, gave positive results. (The nude mouse experiments were carried out to distinguish between a genuine hybridisation to host cellular DNA and the possibility of a viral contaminant in WME cells). In three separate experiments, the level of hybridisation with cellular mouse DNA was shown to be significantly greater with *din*-21 than with wild-type viral DNA. Some of the data are shown in Figure 6. The fact that some hybridisation was also seen with A2 strain DNA is perhaps not surprising; we have previously argued that polyoma virus might contain sequences derived from its natural host (Soeda *et al.*, 1980b).

The data appear to support the conclusion that the insertion sequence in mutant *din*-21 is derived from host cellular DNA, possibly following integration during the lytic cycle (Türler, 1977). Whether there is a functional relationship between the cellular and viral sequences remains to be determined.

Materials and methods

Materials

The restriction endonuclease *Eco*RI was purchased from Boehringer Corp. and *Hinf*I, *Kpn*I, and *Hgi*AI from New England BioLabs. *Hpa*II and *Hae*III were prepared by standard procedures. [^{32}P]Deoxyribonucleotides (high specific activity), and [^{35}S]methionine were purchased from The Radiochemical Centre (Amersham, UK) and dideoxynucleoside triphosphates from CalBioChem. Virus stocks were made from WME cells and viral DNA from established 3T6 mouse cell lines (Griffin and Fried, 1976). Transformation studies were carried out on rat-1 cells (Freeman *et al.*, 1973, recloned by J. Wyke) using standard procedures. Viral protein analyses were performed using anti-tumour antiserum, as described by Ito (1979).

Mutant production

Viable deletion mutants of polyoma virus were isolated using a modification of the protocol previously described (Griffin and Maddock, 1979): wild-type A2 strain viral DNA was cleaved with the restriction endonuclease *Hinf*I in the presence of ethidium bromide and subsequently treated with S1 nuclease to produce "full-length" linear DNA, which was separated from other DNA species by electrophoresis in 1.0% agarose gels. DNA from the appropriate region of the gel was excised and isolated by electrophoretic elution into dialysis bags. In a typical experiment, polyoma DNA (25 μg) in 0.006 M Tris (pH 7.5), 0.006 M $MgCl_2$, 0.001 M DDT (50 μl) was treated with an equal volume of 1% ethidium bromide, enzyme (3 μl) added and the solution digested for 1.5 h at 37°C. Under these conditions, >50% of the DNA migrated together with full-length linear DNA as determined using as a control polyoma virus DNA cleaved with *Eco*RI. WME cells were transfected with linear viral DNA using the DEAE-dextran technique (McCutchan and Pagano, 1968). Thereafter, the procedure followed was essentially that described by Griffin and Maddock (1979).

Plaque analysis

In order to analyse a large number of plaques easily, a DNA minipreparation method was used: a single plaque was removed under sterile conditions with an Agar pipette into Dulbecco's modified Eagle's media (E4) (1 ml) containing 1% foetal calf serum (FCS). The mixture was freeze-thawed (3 times) and half of it used to overlay a plate (50 mm) of 3T6 cells. After 1.5 h at 37°C, media with 5% FCS were added and cells left 48–60 h, at which time media were removed and viral DNA was separated from cellular DNA by the Hirt procedure (1967). The total mixture (500 μl) was put into an Eppendorf tube, spun at highest speed for 20 min, and the supernatant removed to another Eppendorf tube and extracted (twice) with phenol. Two volumes of ethanol were added to the aqueous extract. The tube was cooled in dry ice-ethanol, spun at highest speed, and the pellet dried. About one-tenth of redissolved material was cleaved with restriction endonucleases by standard procedures. DNA fragments were separated by electrophoresis on agarose gels, denatured, transferred to nitrocellulose filters using the Southern procedure (1975), and probed with ^{32}P-labelled nick-translated polyoma virus DNA (Rigby *et al.*, 1977). Mutant *din*-21 was selected for further study on the basis of its anomalous *Hpa*II restriction digest pattern.

The procedure described above was used routinely for the analysis of material from plaques, as well as to assay purity of original virus stocks. DNAs made from 10 individual plaques derived from the original *din*-21 viral isolate were cleaved with *Hinf*I, separated, and analysed. The results are shown in Figure 7.

DNA sequencing

Primary sequence data were obtained from *din*-21 *Hpa*II fragment 5′ cloned in the *Acc*I site of M13 (Messing *et al.*, 1981) using the dideoxy method of Sanger *et al.* (1977), or from terminally labelled *Hpa*II-5′ using the method of Maxam and Gilbert (1977).

Fig. 7. Analysis of material from 10 plaques isolated following replaquing of the original mutant viral stock, using a protocol outlined in Materials and methods. Except for DNA obtained from plaque 8 (21–8), all the others yielded materials that were indistinguishable. (The slow migrating band in channel 10 was a partial digestion product.) DNA from plaque 8 was found to be a mixture of at least two mutants (see Figure 4a) and was not further analysed. Virus stock made from plaque-5 (see channel 5) was used in all studies of the *din*-21 mutant.

Competition replication experiments

Equal mixtures (plaque forming units) of either *din*-21 and wild-type A2 strain viruses, or *dl*-8 and A2 viruses, at low multiplicities of infection (5 p.f.u./cell), were used to infect WME cells under standard conditions (Griffin and Fried, 1976). Ten plates of cells for each of the two experiments were harvested 60 h post-infection. As controls, analogous experiments were carried out using each virus (wild-type or mutants *dl*-8 and *din*-21) separately. Viral DNA was extracted (Hirt, 1967) and supercoiled DNA isolated in CsCl-ethidium bromide gradients. DNA was analysed following cleavage with the *Hpa*II restriction endonuclease.

Hybridisation experiments

(a) Dishes of confluent WME or CV1 cells were used to prepare total chromosomal DNA. For each experiment, cells from a 50-mm dish were washed with phosphate buffered saline (PBS) scraped off, and collected by low speed centrifugation. They were lysed, and the DNA denatured, fragmented, and made single-stranded by an alkaline procedure previously described (Griffin *et al.*, 1981). Neutralised solutions of non-viscous material were collected by filtration through nitrocellulose discs. The latter were dried, baked overnight at 80°C, and hybridised at 68°C in 1 x Denhart's solution – 6 x SSC (Southern, 1975), using as probe either [^{32}P]nick-translated A2, *din*-21 or SV40 (control) DNAs. As a further set of controls, similar experiments were carried out on comparable amounts of salmon sperm DNA using the same probes. Discs were washed exhaustively with 0.3 x SSC – 0.1% SDS, one of the washes being carried out at 68°C, and then autoradiographed.

(b) High mol. wt. DNAs from WME or BHK cells and from the liver, spleen, and kidney of an adult nude mouse were cleaved with either *Hae*III or *Alu*I. Aliquots containing equal amounts of digested DNA fragments (~20–40 μg/well) were separated by electrophoresis in 0.1% agarose slab gels. Triplicates of the separated DNA fragments were transferred to nitrocellulose (Southern, 1975) and hybridised using as probes either [^{32}P]nick-translated wild-type A2 polyoma virus, *din*-21, or SV40 DNAs. Filters were washed as above and autoradiographed.

Acknowledgements

One of us (DD) gratefully acknowledges support received under the exchange scheme between the Royal Society (London) and the Chinese Academy of Sciences. We thank S.M. Dilworth for help with the protein analysis and useful discussion, and Dr. J. Wyke for recloned rat-1 cells.

References

Bendig,M.M., Thomas,T., and Folk,W.R. (1980) *Cell,* **20**, 401-409.
Benoist,C., O'Hare,K., Breathnach,R., and Chambon,P. (1980) *Nucleic Acids Res.,* **8**, 127-142.
Efstradiatis,A., Posakony,J.W., Maniatis,T., Lawn,R.M., O'Connell,C., Spritz,R.A., De Riel,J.K., Forget,B.G., Weissman,S.M., Slightom, J.L., Blechl,A.E., Smithies,O., Baralle,F.E., Shoulders,C.C., and Proudfoot,N.J. (1980) *Cell,* **21**, 653-668.
Freeman,A.E., Gilden,R.V., Vernon,M.L., Wolford,R.G., Huganin,P.E., and Huebner,R.J. (1973) *Proc. Natl. Acad. Sci. USA,* **70**, 2415-2419.
Fried,M., and Griffin,B.E. (1977) in Klein,G., and Weinhouse,S. (eds.), *Advances in Cancer Research,* **24**, Academic Press, NY, pp. 67-113.
Gaudry,P., Tyndale,C., Kamen,R., and Cuzin,F. (1981) *Nucleic Acids Res.,* **9**, 5697-5710.
Griffin,B.E., Fried,M., and Cowie,A. (1974) *Proc. Natl. Acad. Sci. USA,* **71**, 2077-2081.
Griffin,B.E., and Fried,M. (1976) in Busch,H. (ed.), *Methods in Cancer Research* XII, Academic Press, NY, pp. 49-86.
Griffin,B.E. (1977) *J. Mol. Biol.,* **117**, 447-471.
Griffin,B.E., and Maddock,C. (1979) *J. Virol.,* **31**, 645-656.
Griffin,B.E., Björck,E., Bjürsell,E., and Lindahl,T. (1981) *J. Virol.,* **40**, 11-19.
Hirt,B.J. (1967) *J. Mol. Biol.,* **26**, 365-369.
Ito,Y. (1979) *Virology,* **98**, 261-266.
Kamen,R., Favaloro,J., Parker,J., Treisman,R., Lania,L., Fried,M., and Mellor,A. (1980) *Cold Spring Harbor Symp. Quant. Biol.,* **44**, 63-75.
Katinka,M., Yaniv,M., Vasseur,M., and Blangy,D. (1980) *Cell,* **20**, 393-399.
McCutchan,J.H., and Pagano,J.S. (1968) *J. Natl. Cancer Inst.,* **41**, 351-357.
Magnusson,G., and Nilsson,M.-G. (1977) *J. Virol.,* **22**, 646-653.
Maxam,A.M., and Gilbert,W. (1977) *Proc. Natl. Acad. Sci. USA,* **74**, 560-564.
Messing,J., Crea,R., and Seeburg,P.H. (1981) *Nucleic Acids Res.,* **8**, 309-321.
Parker,R.C., Watson,R.M., and Vinograd,J. (1977) *J. Proc. Natl. Acad. Sci., USA,* **74**, 851-855.
Rigby,P.W.J., and Berg,P. (1978) *J. Virol.,* **28**, 475-489.
Rigby,P.W.J., Dieckmann,M., Rhodes,C. and Berg,P. (1977) *J. Mol. Biol.,* **113**, 237-252.
Sanger,F., Nicklen,S., and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA,* **74**, 5463-5467.
Sekikawa,K., and Levine,A.J. (1981) *Proc. Natl. Acad. Sci. USA,* **78**, 1100-1104.
Smolar,N., and Griffin,B.E. (1981) *J. Virol.,* **38**, 958-967.
Soeda,E., Arrand,J.R., Smolar,N., and Griffin,B.E. (1979) *Cell,* **17**, 357-370.
Soeda,E., Arrand,J.R., Smolar,N., Walsh,J.E., and Griffin,B.E. (1980a) *Nature,* **28**, 445-453.
Soeda,E., Maruyama,T., Arrand,J.R., and Griffin,B.E. (1980b), *Nature,* **285**, 165-167.
Southern,E.M. (1975) *J. Mol. Biol.,* **98**, 503-515.
Türler,H. (1977) *J. Virol.,* **23**, 272-285.
Wells,R.D., Hutchinson,M.A., and Eckhart,W. (1979) *J. Virol.,* **32**, 517-522.