Truncated Forms of the Polyomavirus Middle T Antigen Can Substitute for the Small T Antigen in Lytic Infection

DENNIS TEMPLETON,^{1,2†} SUZANNE SIMON,¹ AND WALTER ECKHART^{1*}

Molecular Biology and Virology Laboratory, The Salk Institute, San Diego, California 92138,¹ and Department of Biology, University of California, San Diego, La Jolla, California 92093²

Received 29 April 1985/Accepted 30 September 1985

Cloned polyomavirus genomes encoding the small T antigen or truncated forms of the middle T antigen facilitated the growth of genomes encoding only the large T antigen in mouse 3T6 cells. We conclude that an N-terminal domain of the middle T antigen, in the appropriate cellular location, can substitute for the small T antigen during lytic infection.

The polyomavirus genome encodes three proteins (T antigens) involved in lytic infection and cell transformation. The large T antigen (LT) is required for viral DNA replication. The small and middle T antigens (ST and MT) are required for lytic infection in certain cells: host range transforming (*hr-t*) mutants, which synthesize LT but not ST and MT, grow poorly in these cells (1-3, 6, 11). We used cloned viral genomes encoding individual T antigens to test whether ST or MT can supply the *hr-t* function required for the growth of genomes encoding only LT.

Cloned polyoma genomes lacking the intervening sequences for each of the T antigens (10) were excised from their plasmids and circularized with DNA ligase. These genomes, encoding individual T antigens, are referred to as LTV, MTV, and STV. As expected, none of the DNAs produced visible plaques on mouse 3T6 cells, which are nonpermissive or semipermissive for the growth of *hr-t* mutants, whereas LTV alone produced small plaques on a polyoma-transformed cell line, Py6, which is fully permissive (1, 4). We transfected cultures of 3T6 cells with LTV, MTV, and STV DNAs singly and in pairs, subcultured the cells, and examined the cultures for cytopathic effect (CPE). The singly transfected cultures showed little or no CPE after 4 weeks. Cultures transfected with LTV-plus-STV DNAs showed complete CPE after 3 weeks, suggesting that ST can facilitate the growth of genomes encoding LT. This is consistent with the observation that a mutation in the 3' splice acceptor site for MT in RNA blocks the production of MT mRNA and protein but does not render the mutant virus nonviable (5). Four of six cultures transfected with LTV and MTV behaved like cultures transfected with LTV alone, suggesting that MT cannot facilitate the growth of LTV genomes. However, in two of six cultures, CPE progressed to completion after 4 weeks. Lysates of the cultures in which CPE was complete showed rapid CPE in a second cycle of infection, suggesting that the cultures contained infectious virus. The infectivity of LTV-plus-STV and LTV-plus-MTV lysates after complete CPE was comparable to that of wild-type virus having a titer of 10^8 PFU/ml when the lysates were used undiluted for infection of 3T6 cells (see below). However, the infectivity as measured by plaque assay after serial dilution was 10^6 PFU/ml or less. This behavior is what

would be expected if the lysates contained complementing defective particles.

To study the nature of the virus yield, we analyzed the T antigens produced in 3T6 cells infected with the lysates of cycle 1 DNA transfections by LTV alone, LTV plus STV, and LTV plus MTV, in which CPE was complete (Fig. 1). Control infections by wild-type virus and a premature termination mutant, MOP 1033 (7), produced normal LT and ST. The mutant produced a truncated form of MT, as described previously (7). LTV infections produced LT, but no MT or ST, and reduced amounts of the major capsid

FIG. 1. T antigens in cells infected by the yields of LTV, MTV, and STV DNA infections. Subconfluent cultures of mouse 3T6 cells were infected with stocks of wild-type polyoma (WT) or the premature termination mutant, MOP 1033 (MOP), at a multiplicity of 10 PFU per cell. Other cultures were infected with undiluted lysates of cultures infected with LTV, MTV, and STV DNA in the combinations indicated. The cultures were radiolabeled with [³⁵S]methionine 24 to 27 h postinfection, and cell extracts were analyzed by immunoprecipitation with polyoma antiserum, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Arrowheads indicate truncated forms of MT.

* Corresponding author.

† Present address: Department of Pathology, New England Deaconess Hospital, Boston, MA 02215.

FIG. 2. MTV and LTV genomes. The MTV and LTV genomes, 5,233 and 4,910 bp, respectively. The cutting sites for *Bam*HI (B), *Eco*RI (R), and *Ava*I (A), coding regions for MT and LT, and deleted intervening sequences of 62 and 385 bp are shown.

FIG. 3. Restriction enzyme digestion of LTV and MTV viral genomes. (A) Viral DNA was extracted from 3T6 cells infected with LTV-plus-MTV2 virus, digested, and analyzed by gel electrophoresis. Lanes 1 to 3, MTV marker; lanes 4 to 6, LTV marker; lanes 7 to 9, LT-plus-MT2 DNA digested with *Bam*HI (lanes 1, 4, and 7), *Bam*HI plus *Eco*RI (lanes 2, 5, and 8), and *Bam*HI plus *Ava*I (lanes 3, 6, and 9). (B) Viral DNA extracted from purified virions of LTV-plus-MTV2 virus digested and analyzed as in panel A. Lanes 1 to 3, LT-plus-MT2 DNA; lanes 4 to 5, LTV marker; lane 6, MTV marker digested with *Bam*HI (lanes 1, 4, and 6), *Bam*HI plus *Eco*RI (lane 2), and *Bam*HI plus *Ava*I (lanes 3 and 5). The deleted fragments of 3,620 and 2,151 bp (Table 1) are indicated.

protein, VP1, as expected. LTV-plus-STV infections produced LT and ST and normal amounts of VP1. (VP1 is precipitated nonspecifically by the antitumor serum and is present in variable amounts in immunoprecipitates; however, it gives a rough indication of the amount of VP1 in the cells.) The two independent stocks of LTV-plus-MTV virus (designated MT1 and MT2) in which CPE was complete produced LT and normal amounts of VP1 but no full-length MT. Instead, each stock produced a smaller protein of unique size, apparent M_r about 35,000 for MT1 and 56,000 for MT2.

We were unable to precipitate these new proteins with antiserum directed against a C-terminal peptide sequence of authentic MT. This result suggested that the new proteins were variant forms of MT lacking C-terminal amino acid sequences. We confirmed this by partial proteolysis of the ^{35}S -radiolabeled proteins, using *Staphylococcus aureus* V8 protease. We compared the digestion products of the authentic MT, the new proteins, and two N-terminal fragments of MT produced by premature termination mutants. The new proteins had the same 26,000-molecular-weight N-terminal digestion product as authentic MT but different C-terminal digestion products. We also studied the intracellular location of the new proteins by separating radiolabeled cell lysates into cytoplasmic and membrane fractions by centrifugation at $40,000 \times g$ before immunoprecipitation. The authentic MT was found in the membrane fraction, whereas the new proteins were found in the cytoplasmic fraction, as were the truncated MT proteins, lacking C-terminal amino acids, produced by the premature termination mutants.

To see whether the new proteins were produced by altered MT genomes in the LTV-plus-MTV virus stocks, we analyzed the viral DNAs from infected cells by restriction enzyme digestion. We used *Bam*HI, *Eco*RI, and *Ava*I, singly or in combination, to study the N- and C-terminal T antigen coding regions. The structures of the parental LTV and MTV genomes and the cutting sites of each of the enzymes are shown in Fig. 2. The sizes of the fragments observed after digestion of viral DNAs extracted from cells infected with two independently isolated LTV-plus-MTV virus stocks are shown in Table 1. (One of the analyses is shown in Fig. 3A.) The fragments expected for the LTV genome were present in all the digests. The additional bands observed were consistent with the presence of deleted MTV genomes: digestion with *Bam*HI alone gave a single additional band, smaller than the full-length MTV genome; digestion with *Bam*HI plus *Eco*RI showed that the *Eco*RI

TABLE 1. Restriction enzyme digestion products of genomes in LTV-plus-MTV virus stocks^a

DNA digested	Source of fragments (genome)	Fragments produced (bp) by digestion with:		
		<i>Bam</i> HI	<i>Bam</i> HI + <i>Eco</i> RI	<i>Bam</i> HI + <i>Ava</i> I
pLT	LT	4,910	3,082, 1,828	3,626, 1,284
pMT	MT	5,233	3,082, 2,151	3,626, 1,310, 297
LT + MT1	LT	4,910	3,082, 1,828	3,626, 1,284
	MT1	(4,360)	(4,360)	(2,800), 1,310, 297
LT + MT2	LT	4,910	3,082, 1,828	3,626, 1,284
	MT2	(3,620)	(3,620)	(2,151), 1,310, 297

^a DNA from plasmids pLT and pMT and DNAs from 3T6 cells infected with two independently derived LTV-plus-MTV virus stocks were digested with the restriction enzymes shown, and the digestion products were analyzed by polyacrylamide gel electrophoresis. DNA fragments with deletions are shown in parentheses.

FIG. 4. T antigens in cells infected by complementing defective genomes. Subconfluent cultures of mouse 3T6 cells were infected with wild-type polyoma (WT) or the premature termination mutant, *am1294*, at multiplicities of 10 to 20 PFU per cell. Other cultures were infected with undiluted lysates of cultures infected with LTV DNA alone or LTV-plus-MTV DNA carrying the *am1294* mutation. The truncated form of the MT produced by the *am1294* mutant is indicated by the arrowhead.

site was missing from each of the deleted genomes; digestion with *Bam*HI plus *Ava*I showed that the smaller *Bam*HI to *Ava*I fragment was intact and that both *Ava*I sites were present, implying that the deletions began after the *Ava*I site at nucleotide 1031. Therefore, the LTV-plus-MTV virus stocks contained normal LTV genomes plus MTV genomes which had normal N-terminal coding regions including information for at least the first 261 amino acids of MT, but had deletions in the coding regions for the C-terminal portions of MT. The origin of these deleted genomes is uncertain; however, they probably arose during DNA replication in the infected cells, rather than being present in the original cloned plasmid DNA used to prepare the MTV genomes.

The MT2 genome had a deletion of about 1,613 base pairs (bp), making it 30% shorter than the wild-type MT genome (Table 1). It was interesting and unexpected that genomes having such large deletions could be assembled into virus particles. Therefore, we purified virus particles from lysates of cells infected with LTV plus MTV2 by equilibrium centrifugation in CsCl (the virion bands were diffuse, as would be expected if they contained particles having different densities), extracted the viral DNA, and digested it with *Bam*HI, *Eco*RI, and *Ava*I, as described above. The digestion products are shown in Fig. 3B. The digestion products predicted for LT and the deleted MT2 genomes were present in all three digests. We conclude that the MT2 genomes, despite having large deletions, are assembled into virus particles.

The results of these experiments suggested that truncated

forms of MT, lacking C-terminal amino acid sequences, could substitute for ST to provide the *hr-t* function for growth of LTV in 3T6 cells. To verify this, we performed a reconstruction experiment, using LTV DNA and MTV DNA containing a mutation causing premature termination of translation at amino acid 348 of MT (*aml294*) (8). Mixed infection of 3T6 cells by the two DNAs resulted in complete CPE, and a second round of infection, using this lysate, produced rapid CPE and the expected T antigens (Fig. 4).

We conclude that truncated forms of MT can substitute for ST during lytic infection of mouse 3T6 cells. The results emphasize the importance of intracellular location in determining whether the functions of the T antigens are correctly expressed. The N-terminal domain of MT includes 191 of 195 amino acids of ST. Amino terminal sequences of MT are required for transformation (9). Therefore, the N-terminal domain of MT may function in transformation like ST in lytic infection, but in a different cellular location.

This investigation was supported by Public Health Service grants CA-13884 and CA-14195, awarded by the National Cancer Institute. D.T. was supported by Public Health Service grant GM-07198 of the Medical Scientist Training Program, National Institutes of Health.

LITERATURE CITED

1. Benjamin, T. L. 1970. Host range mutants of polyoma virus. *Proc. Natl. Acad. Sci. USA* **67**:394-399.
2. Benjamin, T. L. 1982. The *hr-t* gene of polyoma virus. *Biochim. Biophys. Acta* **695**:69-95.
3. Garcea, R. L., and T. L. Benjamin. 1983. Host range transforming gene of polyoma virus plays a role in virus assembly. *Proc. Natl. Acad. Sci. USA* **80**:3613-3617.
4. Goldman, E., and T. L. Benjamin. 1975. Analysis of host range of non-transforming polyoma virus mutants. *Virology* **66**:372-384.
5. Liang, T. J., G. G. Carmichael, and T. L. Benjamin. 1984. A polyoma mutant that encodes small T antigen but not middle T antigen demonstrates uncoupling of cell surface and cytoskeletal changes associated with cell transformation. *Mol. Cell. Biol.* **4**:2774-2783.
6. Staneloni, R., M. Fluck, and T. L. Benjamin. 1977. Host range selection of transformation-defective *hr-t* mutants of polyoma virus. *Virology* **77**:598-609.
7. Templeton, D., and W. Eckhart. 1982. Mutation causing premature termination of the polyoma virus medium T antigen blocks cell transformation. *J. Virol.* **41**:1014-1024.
8. Templeton, D., and W. Eckhart. 1984. Characterization of viable mutants of polyomavirus cold sensitive for maintenance of cell transformation. *J. Virol.* **49**:799-805.
9. Templeton, D., and W. Eckhart. 1984. N-terminal amino acid sequences of the polyoma middle-size T antigen are important for protein kinase activity and cell transformation. *Mol. Cell. Biol.* **4**:817-821.
10. Treisman, R., U. Novak, J. Favaloro, and R. Kamen. 1981. Transformation of rat cells by an altered polyoma virus genome expressing only the middle T protein. *Nature (London)* **292**:595-600.
11. Türlér, H., and C. Salomon. 1985. Small and middle T antigens contribute to lytic and abortive polyomavirus infection. *J. Virol.* **53**:579-586.