Transformation by polyoma virus is drastically reduced by substitution of phenylalanine for tyrosine at residue 315 of middle-sized tumor antigen

(tumor viruses/tyrosine kinase/protein phosphorylation/neoplastic transformation/in vitro mutagenesis)

GORDON CARMICHAEL*, BRIAN S. SCHAFFHAUSEN†, GAIL MANDEL‡, T. JAKE LIANG‡, AND THOMAS L. BENJAMIN‡

*Department of Microbiology, University of Connecticut Health Center, Farmington, CT 06032; †Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, MA 02111; and ‡Department of Pathology, Harvard Medical School, Boston, MA 02115

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ABSTRACT We used an oligonucleotide to introduce an A → T transversion at nucleotide position 1178 in polyoma virus DNA. The single effect of this mutation is to substitute phenylalanine for tyrosine at residue 315 of the middle-sized tumor (mT) protein (antigen). This site was previously identified as a major phosphate acceptor in the protein kinase reaction of immunocomplexes containing mT antigen. Reconstituted polyoma virus with the transversion, Py-1178-T, produces an altered mT protein that shows about 20% of the activity of wild-type mT antigens in the immunocomplex kinase assay. This residual activity appears to be directed primarily at another tyrosine at position 322 in the mT protein. The transforming ability of Py-1178-T is drastically reduced compared to wild-type virus. The efficiency of transformation by the mutant is <1% of that of wild type in focus assays and <0.1% in soft-agar growth assays. Cells identified in focus assays with Py-1178-T are generally less transformed in their phenotype than wild-type transformed cells.

The demonstration of a protein kinase activity associated with the product of the transforming gene of Rous sarcoma virus (1, 2) has been followed by reports of a similar activity in a number of different tumor virus systems. The tyrosine specificity of these tumor virus-related protein kinases was first shown for the polyoma virus middle-sized tumor (mT) antigen (3), a protein that is essential, though not sufficient, for transformation (4–7). Tyrosine-specific kinase activities also have been shown for the Abelson virus transforming protein (8) and in other virus systems (9, 10). In most instances the activity is measured in vitro by incubating immunocomplexes containing the viral protein with [γ-32P]ATP. The heavy chains of immunoglobulin often serve as the major phosphate acceptor in these reactions, although the viral gene product or products themselves also may be preferred substrates. In the case of Rous and Abelson viruses, the activity seems to be intrinsic to the viral proteins as shown by copurification and by the presence of tyrosine-specific protein kinase activity in the viral gene products cloned and expressed in Escherichia coli (11, 12).

In the case of polyoma virus, the source of the activity seen in immunocomplexes is unclear. The mT antigen appears to lack ATP-binding activity (13), and at least two laboratories have succeeded in expressing mT protein in E. coli without attendant kinase activity (unpublished data; K. Palme and W. Eckhart, personal communication). The possibility that mT antigen associates with cell-specified tyrosine kinase(s) has been supported recently by evidence showing the presence of c-src antigen in anti-polyoma-tumor (T) antigen immunocomplexes (14). In addition to serving as an acceptor for a tyrosine kinase activity measured in vitro, polyoma mT antigen is also a substrate for kinases in vivo with either or both serine and threonine being the major phosphoamino acids (15, 16).

Two considerations point to the importance of these phosphorylation reactions in the mechanism of transformation. First, there is strong genetic evidence in several viral systems linking kinase activities to transforming ability. Thus, in the case of polyoma virus, whether mT antigen turns out to possess an intrinsic kinase activity or to interact with a cellular enzyme(s), manifestation of the associated in vitro kinase activity and the ability to serve as a substrate in vivo appear to be necessary correlates of transforming ability. Second, protein phosphorylation provides a logical mechanism underlying the known pleiotropic action of tumor viruses. The intervention of tumor viral gene products in cellular pathways involving protein modification is a generally plausible way of explaining how single viral genes affect the multiple cellular changes that accompany transformation.

To better understand the phosphorylation reactions in relation to the biological activities of polyoma virus, we have used site-specific mutagenesis to specifically alter regions of the mT protein. Introduction of a termination codon just upstream of the hydrophobic tail near the COOH terminus results in failure of the protein to associate with cellular membranes, failure to undergo phosphorylation both in vitro and in vivo, and failure to transform cells (6). Here we report results of introducing a single amino acid substitution in which the tyrosine at residue 315 is replaced by phenylalanine. We chose to alter this site based on the following considerations. (i) Previous attempts to map the site of in vitro phosphorylation indicated tyrosine-315 as a major acceptor (15). These experiments were based on a two-dimensional mapping procedure involving partial proteolysis in the second dimension. The tyrosines at positions 315 and 322—both downstream of the glutamic acid-rich region (residues 309–314) that gives rise to a labeled COOH-terminal peptide by V8 protease cleavage—were indicated as potential sites of phosphorylation. The mutant dl-1014, in which the tyrosine at 322 has been deleted, shows essentially normal levels of kinase activity as well as normal transforming ability (15, 17, 18). (ii) We have made antiserum to a nonapeptide with a sequence matching that of the tyrosine-315 region in mT antigen. This antiserum specifically precipitates the mT protein. Furthermore, as expected if tyrosine-315 were the major acceptor site, such immunocomplexes are virtually negative in the kinase reaction. Incubation of these immunocom-

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Abbreviations: T antigen (or protein), tumor antigen (or protein); mT antigen (protein), middle-sized T antigen (protein); kDa, kilodalton(s).
plexes with soluble peptide, however, results in recovery of mT protein kinase activity (19). Despite these findings, there is no direct evidence linking phosphorylation of this site in vivo to any biological activity of the virus. By constructing a mutant in which phenylalanine replaces tyrosine-315, we hoped to maximally preserve the character of the wild-type protein while preventing phosphorylation at this site. We have shown that this mutant retains a mT antigen-associated kinase activity at a level roughly 20% of that of the wild-type protein. The residual activity in the mutant appears to be directed primarily to tyrosine-322. Despite retention of this activity, the mutant is drastically reduced in its ability to transform cells.

MATERIALS AND METHODS

Materials. Enzymes for DNA work were from New England BioLabs; chymotrypsin, from Worthington; Staphylococcus aureus V8 protease was from Miles. The synthetic undecanucleotide 5'-d(G-G-A-G-T-T-C-A-T-G-C)-3' was from Collaborative Research (Waltham, MA). [α-32P]dNTPs for DNA sequence analysis were from Amersham. [35S]Methionine and [32P]ATP for protein kinase assays was from ICN.

Cells and Viruses. Virus stocks were prepared on primary baby mouse kidney cells (20). Plaque assays were on mouse UC1-B cells (21). Tests for transformation were with Fischer rat F-111 fibroblasts (22, 23).

Phage and Bacteria. Wild-type polyoma virus DNA [NG59RA (24)] was digested with Pst I and EcoRI. The 1,076-base-pair fragment from nucleotides 484–1560 on the viral genome was isolated and cloned into phase M13 mp8, with E. coli strain JM103 as the host. This recombinant was designated PR3.

In Vitro Mutagenesis and Virus Reconstruction. Efforts were made to prepare a matched virus pair differing only at position 1178. This was done by reconstructing virus in a three-way ligation in which two of the three pieces were derived from wild-type viral DNA and the third from phage replicative-form molecules (see below).

PR3 phage were propagated in JM103 cells and purified by precipitation with 4% polyethylene glycol in 0.5 M NaCl (25). Single-stranded DNA was prepared by extraction with phenol/chloroform and precipitation with ethanol. For mutagenesis 1 μg of single-stranded PR3 DNA was incubated in 20 μl with a 100-fold molar excess of 5'-phosphorylated oligonucleotide for 5 min at 37°C in 0.05 M Tris chloride, pH 8/0.05 M NaCl/0.02 M MgCl2/0.001 M diethiothreitol/1 mM each of the four dNTPs and ATP. The reaction was then put at 0°C, and 9 units of E. coli DNA polymerase I, large fragment, and 20 units of T4 DNA ligase were added. The sample was incubated 30 min at 0°C, followed by 4 hr at 22°C. It then was loaded onto a horizontal 1% agarose gel containing ethidium bromide (1 μg/ml) and electrophoresed 4 hr at 50 V. The band migrating at the position of closed circular DNA was excised, and the DNA was extracted with glass beads (26) and used to transform CaCl2-treated JM103 cells (27). Plaques were picked, and phage replicative-form molecules were prepared (28) and screened for the mutation by digestion with Rsa I. The desired base change of an A → T at position 1178 destroys a Rsa I site. One mutant was chosen, and the polyoma Pst I/EcoRI fragment was isolated. For construction of the wild-type partner, the Pst I/EcoRI fragment was isolated from PR3 replicative-form DNA derived from the same stock as used for mutagenesis. From NG59RA DNA we prepared a BamHI/Pst I fragment (spanning nucleotides 4632–490) and an EcoRI/BamHI fragment (spanning nucleotides 1560–4632). The two viral- and one phage-derived pieces were ligated together and used to infect primary mouse kidney cells by the calcium phosphate procedure (29). These reconstructed viruses were plaque-purified before subsequent analysis. By DNA sequence determination from the Ava I site at position 1016 to the EcoRI site at 1560, we found no difference other than that at position 1178; the reconstructed virus that we shall call wild type has an adenose (Py-1178-A) and the reconstructed mutant has a thymidine (Py-1178-T).

T-Antigen Analyses. Procedures for labeling cells, immunoprecipitation, and analysis of T antigens have been described (15, 17, 30–32). Partial proteolysis also was carried out as before (15, 30, 32). Briefly, T antigens were first resolved on cylindrical 10% NaDodSO4 gels. The cylinders were placed head-to-head on top of a 12.5% slab gel, and a digestion solution containing either chymotrypsin or V8 protease was layered on top of the gel. Electrophoresis was then carried out for 16 hr.

Detailed descriptions of chemical cleavage procedures will be presented elsewhere. For the CNBr digestion, T antigens were first resolved on 10% NaDodSO4 gels. The gels were fixed in 7.5% (vol/vol) acetic acid/5% (vol/vol) methanol at room temperature. Each gel was transferred to a beaker containing 5 ml of 7% acetic acid at 45°C. Digestion was carried out by the addition of 0.6 ml of a CNBr solution (2 g of CNBr per ml of acetonitrile) for 1 hr. The gel was then soaked in 0.125 M Tris, pH 6.8/0.1% NaDodSO4 for 30 min. The cylindrical gel was then placed on top of a 15% NaDodSO4 gel, and electrophoresis was carried out at 50 V for ~18 hr.

RESULTS

Tyrosine Codon (TAC) Is Replaced by a Phenylalanine Codon (TTC) in Py-1178-T. The oligonucleotide chosen to mutate polyoma DNA introduced an A → T transversion at nucleotide position 1178. In the reading frame for mT antigen, this resulted in the change of tyrosine-315 to a phenylalanine (see Fig. 1). In the overlapping large T-antigen reading frame, there was no change of protein sequence: GTA(Val) → GTT(Val).

Both wild-type (Py-1178-A) and mutant (Py-1178-T) viruses were reconstructed from DNA fragments derived from wild-type virus and viral DNA segments cloned into phage M13 as described. Sequence assay confirmed the presence of the A → T change and revealed no other difference. The two viruses were routinely propagated in baby mouse kidney cells and were found to grow to equally high titers.

Mutant Py-1178-T Shows a Normal Pattern of 35S-Labeled T Antigens but an Altered In Vitro Kinase Activity for mT Antigen. The typical pattern of [35S]methionine-labeled wild-type T antigens—large T antigen [100 kilodaltons (kDa)], mT (Py-1178-A)/Py-1178-T: Phe for Tyr at Position 315 of Middle T

![Fig. 1. Oligonucleotide mutagenesis substituting thymidine for adenine at position 1178. The undecanucleotide shown was used to prime DNA synthesis by using a wild-type viral DNA segment cloned into bacteriophage M13 as template. The A → T transversion gives rise to a phenylalanine (TTC) instead of a tyrosine (TAC) at position 315 of the mT protein.](image-url)
antigen (56 kDa), and small T antigen (22 kDa) as well as the nonviral species (63 and 36 kDa)—is shown in Fig. 2. Py-1178-T induced the same pattern as wild type, including the nonviral products previously shown to be lacking in immunoprecipitates of hr-t mutant-infected cells (30–33).

Incubation of wild-type immunoprecipitates with [γ-32P]ATP resulted in the labeling of mT antigen. Fig. 2 shows that Py-1178-T mT antigen was much less active in the in vitro reaction. Densitometry of gels such as those of Fig. 2 (32P/32P ratio) showed that the mT antigen of Py-1178-T is about 20% as active as that of the wild-type virus containing tyrosine instead of phenylalanine at residue 315.

Although they are not well resolved in Fig. 2, two species of mT antigen, the 56- and 58-kDa forms, are phosphorylated in the in vitro reaction (15, 17). Each form gave rise to a characteristic pair of chymotryptic fragments on partial digestion. The 39- and 33-kDa fragments derived from the 58-kDa species and the 37- and 31-kDa fragments derived from the 56-kDa species were observed for both wild-type parent and Py-1178-T, and the relative amounts of the fragments were the same for both viruses (see Fig. 3). Therefore, Py-1178-T is not impaired in the ability to produce both mT-antigen forms. It previously has been shown that the two forms differ in their patterns of in vivo phosphorylation at serine or threonine residues, or at both (15). As expected from the in vitro kinase results, 56- and 58-kDa patterns of in vivo phosphorylation were normal for Py-1178-T (not shown).

Partial proteolysis with S. aureus V8 protease showed a difference between wild type and Py-1178-T. Digestion of wild-type mT antigen labeled in the in vitro reaction gave rise to two overlapping COOH-terminal fragments of 24- and 18-kDa. The stretch of six glutamic acid residues from 309 to 314 is the likely site for the V8 protease cleavage giving rise to the 18-kDa fragment (15). Digestion of Py-1178-T mT antigen gave rise to little of the 24-kDa fragment and a preponderance of the 18-kDa fragment (Fig. 3). The substitution of phenylalanine for tyrosine at residue 315 apparently increased the probability of cleavage within the adjacent glutamic acid-rich cluster.

CNBr cleavage of Py-1178-T mT antigen labeled in vitro showed loss of labeling at residue 315. The detailed analysis of CNBr mapping will be presented elsewhere. In the wild-type mT-antigen digestion (Fig. 4), fragment A (actually a doublet) contained amino acids 284 to 316–318 of mT antigen. That fragment was unlabeled in the Py-1178-T pattern. On the other hand, fragment B contained only downstream sequences (316–318 to 371 or 384), including the phosphorylation site at tyrosine-322. As expected, the B fragment was not affected by the A → T mutation at 1178. Fragments D and E represent upstream fragments, from residues 254 and 225, respectively, to 316 or 318. In the mutant, the labeling of these fragments compared to the labeling of the B fragment was drastically reduced. The presence of any labeling of these fragments in Py-1178-T is likely due to minor upstream phosphorylations. Fragments C/C' and F/F' of wild type are unresolved fragments containing both Tyr-315 and Tyr-322. Their intensities relative to B were also reduced with the mutant, but the effect was less dramatic than for D and E because the fragments containing the 322 site are normal.

Py-1178-T Transforms Rat Fibroblasts Weakly and with Reduced Efficiency. Fig. 5 (Left) shows the results of a focus assay on F-111 rat fibroblasts. Py-1178-T showed a drastic

Fig. 2. Baby mouse kidney cells were infected with either Py-1178-T (lanes B and D) or wild-type (lanes A and C) virus. [35S]Methionine-labeled T antigens (lanes A and B) were labeled in vivo, whereas the mT antigens (lanes C and D) were labeled in vitro with [γ-32P]ATP. The T antigens were resolved by electrophoresis on discontinuous buffer NaDodSO4/10% acrylamide gels.

Fig. 3. (Upper) Partial chymotryptic digestion of wild-type (WT) and Py-1178-T mT antigens labeled in vitro with [γ-32P]ATP. The arrowheads indicate the major chymotryptic fragments, including the 39/33-kDa pair derived from the 58-kDa form of mT antigen and the 37/31-kDa pair derived from the 56-kDa form. (Lower) Partial digestion with S. aureus V8 protease. Both maps use a 12.5% acrylamide/NaDodSO4 gel for the second dimension.

Fig. 4. CNBr digestion of wild-type (WT) and Py-1178-T mT antigens labeled in vitro with [γ-32P]ATP. The CNBr fragments are resolved in the second dimension on a 15% acrylamide/NaDodSO4 gel. The arrowheads indicate the position of the major fragments. Fragments labeled A, D, and E contain tyrosine residue 315 but not residue 322. Fragment B contains tyrosine-322 but not tyrosine-315. Peptides marked C/C' and F/F' include pairs of fragments including both sites.
reduction in the efficiency of transformation compared to its wild-type control. In repeated experiments, the efficiency of transformation by the mutant (focus-forming units per plaque-forming unit) was lower by a factor of 200-1,000. Moreover, the foci appeared later in the mutant-infected cultures and were smaller in size. The mutant also was defective in transformation at 33°C. Cells within the typical mutant focus appeared less transformed than their wild-type counterparts (Fig. 5 Right). They grew less densely and were more adherent, while maintaining a more bipolar and less refractile appearance compared to wild-type transformed cells. When transformation was scored by the soft-agar growth assay, the mutant appeared to be even more drastically affected, consistent with its qualitatively and quantitatively weaker behavior in focus assays. Mutant foci were picked, grown, and shown to contain mT antigen with altered kinase activity typical of the mutant: the mT antigen had a low level of kinase activity, which showed the CNBr pattern of Py-1178-T.

**DISCUSSION**

We constructed and compared two strains of polyoma virus shown to differ by an A → T transversion at nucleotide position 1178 in the viral DNA. The sole effect of this mutation is to substitute phenylalanine (Py-1178-T) for tyrosine (Py-1178-A) at position 315 of the mT protein. The mutant Py-1178-T produces normal levels of all T antigens by [35S]-methionine labeling and immunoprecipitation. In the *in vitro* kinase reaction, however, the mutant mT protein shows only 20% of the activity of wild type. Proteolytic and chemical cleavage maps comparing the mutant and wild-type mT antigens labeled *in vitro* show a lack of phosphorylation in the mutant at position 315 but significant phosphorylation at tyrosine-322. Thus, these results confirm the assignment of tyrosine-315 as the major site of phosphorylation in the immunocomplex kinase reaction (15, 17). Phosphorylation of tyrosine-315 cannot be required for the action of the serine/threonine kinases, which recognize mT antigen *in vivo* because Py-1178-T shows a normal pattern of *in vitro* phosphorylation. Phosphorylation of tyrosine-322, which occurs in the wild-type as well as the mutant mT protein, appears not to be essential for high levels of *in vitro* kinase activity or for transforming ability (18). Py-1178-T is reduced both quantitatively and qualitatively in its transforming ability when tested on a rat embryo fibroblast cell line. Thus, the retention of tyrosine at position 315 is deemed to be essential for maximal transforming ability. The most likely explanation for these results is that tyrosine-315 is utilized as a phosphorylation site *in vivo*. Phosphorylation of mT antigen on tyrosine *in vivo* has been reported by one laboratory (16). However, it cannot be ruled out that tyrosine at this position is required in some other manner (conformational, hydrogen-bond donor, sulfation, etc.) for which phenylalanine is inadequate. It remains unclear to what extent the ability to be phosphorylated at position 322 is essential for the residual transforming ability of Py-1178-T. The mutant does induce small foci at a low frequency compared to wild type (0.1–0.5%), and small colonies in soft agar can sometimes be detected at a low frequency (<0.1%). This limited transforming potential depends in some manner on the altered mT antigen because Py-1387-T, the “tail-less” mutant that totally lacks kinase activity, fails to induce morphologically discernible foci on F-111 cells (6). Our results with Py-1178-T are consistent with previous reports that deletions removing tyrosine-315 result in decreases in both *in vitro* kinase activity and transformation (34–36).

Mutants of *src* that delete or alter sites of phosphorylation have recently been described (37, 38). Alterations of either the serine-17 or tyrosine-416 regions have no drastic effects on *in vitro* transformation or on kinase activity when measured with exogenous substrate. However, in at least one mutant (NYT10-1; ref. 38), deletion of the “autophosphorylation” site at tyrosine-416 does have an effect in inducing foci more slowly and being weaker in tumor induction. Although this mutation produces a superficially similar phenotype to that of Py-1178-T, the two virus systems are difficult to compare directly in view of the known enzymatic activity of src antigen and the uncertainty surrounding mT antigen.

We presently favor the view that to be effective in transformation, polyoma mT antigen must interact with at least two cellular kinases. One such enzyme would be the serine/threonine kinase responsible for inducing the 58-kDa form of mT antigen, which is particularly active in the *in vitro* kinase reaction (15, 17). This “58-kDa kinase” is presumably membrane-bound because the soluble “tail-less” form of mT antigen induced by Py-1387-T fails to be phosphorylated at the 58-kDa site and fails to acquire associated tyrosine kinase activity (6). The mT antigen of *hr-t* mutant NG-59, with a
two-amino-acid change from wild type, has no tyrosine kinase activity in vitro and also fails to be phosphorylated by the 58-kDa kinase in vivo (15, 17, 39). Phosphorylation of mT antigen on serine or threonine residues could result in acquisition of a tyrosine kinase activity by mT antigen itself or, more likely, facilitates association of mT antigen with a cellular kinase. This second enzyme, with tyrosine specificity, is deemed essential to interact with the mT protein in the 315–322 region of the molecule. The c-src protein recently shown to interact with mT antigen in immunocomplexes is a candidate for the latter activity (14).

Recently, Oostra et al. (40) have described mutations at and around tyrosine-315 of polyoma virus mT antigen. Their results differ from ours in two respects. First, they report that their phenylalanine-containing mutant has a normal level of kinase activity, although they have not identified the phosphorylation site(s). Second, they report that the mutant retains transforming ability. However, their protocols differ from ours in several important ways. In the study by Oostra et al., transformants of Rat-1 cells are first isolated after plasmid transfection and then tested for kinase activity. Our experiments are carried out after de novo viral infections of F-111 cells without selection for transformation. The initial selection of transformants may affect the observed level of mT antigen kinase activity. Rat-1 cells may be easier to transform than F-111 cells; mT antigen cDNA can transform Rat-1 cells (4), whereas in F-111 cells, middle T antigen cDNA induces only a partial transformation (unpublished observations). Transformation by DNA transfection may be more difficult to quantitate because the response is often nonlinear with DNA concentration (4). At the same time, transfections may give rise to higher copy numbers of integrated viral DNA than is usually found after virus infection. Either of these factors, together with intrinsic differences in the cell lines used, could account for the discrepancies. To rule out possible differences other than at codon 315 of mT between Py-1178-T and the plasmid (PAS-131) used by Oostra et al. (40), we have reconstructed and characterized a virus starting with PAS-131 (provided by Alan Smith). This virus behaves in a manner indistinguishable from Py-1178-T with respect to its mT-associated kinase activity and decreased transforming ability.

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