The Amino Terminus of Polyomavirus Middle T Antigen Is Required for Transformation

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In polyomavirus-transformed cells, pp60^{c-src} is activated by association with polyomavirus middle T antigen. These complexes have a higher tyrosine kinase activity compared with that of unassociated pp60^{c-src}. Genetic analyses have revealed that the carboxy-terminal 15 amino acids of pp60^{c-src} and the amino-terminal half of middle T antigen are required for this association and consequent activation of the tyrosine kinase. To define in greater detail the borders of the domain in middle T antigen required for activation of pp60^{c-src}, we constructed a set of unidirectional amino-terminal deletion mutants of middle T antigen. Analysis of these mutants revealed that the first six amino acids of middle T antigen are required for it to activate the kinase activity of pp60^{c-src} and to transform Rat-1 fibroblasts. Analysis of a series of insertion and substitution mutants confirmed these observations and further revealed that mutations affecting the first four amino acids of middle T antigen reduced or abolished its capacity to activate the kinase activity of pp60^{c-src} and to transform Rat-1 cells in culture. Our results suggest that the first four amino acids of middle T antigen constitute part of a domain required for activation of the pp60^{c-src} tyrosyl kinase activity and for consequent cellular transformation.

Polyomavirus (Py)-mediated transformation results principally from the action of one of the Py early gene products, middle T antigen (39). Middle T antigen is both necessary and sufficient to transform established (34, 51) and, in some cases, embryonic cells (24, 46) in culture and to induce tumors in hamsters (1), chickens (24), and transgenic mice (2, 52). Middle T antigen is a phosphoprotein (17, 40, 41, 44), is composed of 421 amino acids, and has a measured molecular weight of either 56,000 or 58,000, depending on its state of phosphorylation (41). Phosphorylation occurs primarily on serine and threonine residues *in vivo* and to a much lesser extent on tyrosine (41). By contrast, when immunoprecipitates of Py-infected or -transformed cells are incubated *in vitro* with [γ -³²P]ATP, middle T antigen becomes phosphorylated principally on tyrosine residues (17, 40, 41). The capacity of middle T antigen to act as a substrate for phosphorylation *in vitro* is thought to be relevant to transformation, because there are no known transformation-competent mutants of middle T antigen that lack this capacity.

The protein kinase activity in immunoprecipitates is not intrinsic to middle T antigen (42, 43) but is due, at least in part, to its association with the cellular proto-oncogene product, pp60^{c-src} (5, 14, 15). The pp60^{c-src} molecules associated with middle T antigen differ from their noncomplexed counterparts by a phosphate-free tyrosine at residue 527 (9) and a higher tyrosine kinase specific activity as measured *in vitro* (5, 12). It has been proposed that middle T antigen may effect transformation by preventing phosphorylation of Tyr-527 (12), because mutants of pp60^{c-src} with a substitution of

phenylalanine for tyrosine at residue 527 readily transform NIH 3T3 cells, whereas wild type (wt) c-src does not or does so inefficiently (8, 23, 37). Transformation by middle T antigen may include additional effects, however, because the Tyr-527 substitution mutants of c-src do not transform as well as middle T antigen does (8), and because there are mutants of middle T antigen that activate the tyrosine kinase activity of pp60^{c-src} but fail to transform established rat cells (31, 36, 50).

Genetic analyses have revealed that the amino-terminal half of middle T antigen is required for its capacity to transform cells (3, 6, 10, 31, 49) and, by implication, for it to associate with and activate the kinase activity of pp60^{c-src}. Large deletions (33) and numerous point mutations (30, 38) in the carboxy-terminal half of the protein, except residues involved in anchoring the protein to the plasma membrane (7, 30, 48), do not significantly alter the transforming activity of middle T antigen or its capacity to activate the tyrosine kinase activity of pp60^{c-src}. By contrast, a truncated middle T antigen whose translation is initiated at the first internal methionine (Met-30) does not transform cells and does not enhance the tyrosyl kinase specific activity of pp60^{c-src} (49), implying that a region within the first 30 amino acids of middle T antigen is important for transformation. To define this region more precisely, we introduced deletion, insertion, and substitution mutations within sequences encoding the first 12 amino acids of middle T antigen and measured the capacity of these mutants to activate the tyrosine kinase activity of pp60^{c-src} and to transform Rat-1 cells. The results of these analyses revealed that the first four amino acids of middle T antigen are required for activation of the tyrosine kinase activity of pp60^{c-src} and for transformation. We suggest that these amino acids form a part of the domain in middle T antigen required for the activation of pp60^{c-src}.

MATERIALS AND METHODS

Cells. All cell lines were propagated on plastic tissue culture dishes in Dulbecco modified Eagle medium supple-

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FIG. 1. Procedure to generate amino-terminal deletion mutants of Py middle T antigen. pPymT(X) carries a cDNA encoding middle T antigen. This DNA was linearized with *Xho*I, and various lengths of DNA encoding the amino terminus of middle T antigen were removed from the ends of the linear DNA with *Bal* 31 exonuclease. Sequences required for transcription and translation were restored to these DNAs as described in the text.

mented with gentamicin (50 μ g/ml), fungizone (2.5 μ g/ml), and 10% fetal bovine serum and were maintained at 37°C in a humidified CO₂ incubator.

Construction of recombinant plasmids. All cloning procedures were performed as described by Maniatis et al. (29). Recombinant plasmids were isolated from *Escherichia coli* DH1 or DH5. Restriction enzymes and DNA-modifying enzymes were used according to the specifications of the manufacturers.

A series of deletion mutants was generated using pPymT(X) as a substrate (Fig. 1). The plasmid has an insert of Py middle T cDNA bounded by a unique *Xho*I site at nucleotide (nt) 154 (a former *Hph*I site), and an *Eco*RI site at nt 1560 (18). The plasmid was linearized by cleavage with *Xho*I and subjected to deletion mutagenesis by incubation for various periods with *Bal* 31 exonuclease. The recessed 3' ends were made blunt by incubation of the DNA with the Klenow fragment of *E. coli* DNA polymerase I (Klenow) in the

presence of all four deoxynucleotide triphosphates. The double-stranded oligodeoxynucleotide shown in Fig. 1 was then ligated to the plasmid DNA. The oligodeoxynucleotide contains an *Nco*I site embedded within a eucaryotic translation initiation consensus sequence (25–28) and has a *Hind*III recognition site. After ligation, the DNA was cleaved with *Hind*III to ensure that only one oligodeoxynucleotide was retained on the plasmid DNA. The plasmid DNA was then purified from an agarose gel, circularized by ligation, and used to transform *E. coli* DH1. Cleavage with *Hind*III would also remove the *Nco*I site from plasmids in which the oligonucleotide had been inserted in the wrong orientation with respect to the middle T antigen coding sequences. Ampicillin-resistant colonies were therefore screened for plasmids that had acquired a new *Nco*I site as well as the *Hind*III site, thereby confirming that the oligonucleotide was situated in the correct orientation, juxtaposing the initiator ATG to the deletion endpoint. The approximate size of the deletions was determined by polyacrylamide gel electrophoresis after cleavage of the DNA with *Hind*III and *Acc*I (nt 368). The sequence of plasmids bearing small deletions in middle T antigen coding sequences was determined by the method of Maxam and Gilbert (32). Those plasmids that were predicted to encode an in-frame, truncated middle T antigen, whose terminal methionine would be encoded by the ATG within the oligonucleotide, were processed further. These DNAs were cleaved with *Hind*III, dephosphorylated by incubation with calf intestinal alkaline phosphatase, ligated to the simian virus 40 (SV40) *Hind*III C fragment containing the SV40 early and late promoters, and used to transform *E. coli*. The orientation of the SV40 sequences in the recombinant plasmids was determined by cleavage of the DNA with *Bgl*II and analysis of the fragments by electrophoresis through agarose gels. Plasmids carrying the SV40 early promoter next to the middle T antigen-coding sequences were analyzed further. Three mutants (*dl*192, *dl*201, and *dl*207 [Table 1]) bearing short deletions in middle T antigen-coding sequences were isolated in this way. pmT165 was constructed in a similar manner, except that the oligonucleotide was inserted in the opposite orientation with respect to the middle T antigen-coding sequences. Consequently, pmT165 possesses a *Hind*III site but not an *Nco*I site.

Two deletion-substitution mutants were generated by enzymatically correcting the reading frame of two out-of-frame deletion mutants. *in*179 was constructed by altering the reading frame of an out-of-frame deletion mutant (*dl*179) at its novel *Nco*I site. Because the Py early region contains another *Nco*I site at nt 1211, *dl*179 DNA was cleaved partially with *Nco*I to linearize the plasmid. The cohesive ends of the linear DNA were rendered blunt by the Klenow fragment, and the oligonucleotide was ligated to them. The DNA was then cleaved with *Hind*III and *Eco*RI, and the middle T antigen-coding sequences were isolated and inserted between the *Eco*RI and *Hind*III sites of pML-2. The predicted sequence of this mutant was confirmed by DNA sequencing (Table 1). The SV40 early promoter-enhancer region was inserted in front of the *in*179-coding sequences as described above.

Another mutant of this type, *in*197, was constructed by fusing sequences encoding the first five amino acids of wt middle T antigen to another deletion mutant (*dl*197) lacking the first eight amino acids of middle T antigen as follows. DNA sequences encoding amino acid residues 8 through 421 were isolated by partial cleavage of *dl*197 DNA with *Nco*I, treatment of the recessed 3' ends with the Klenow fragment,

TABLE 1. Sequence and transforming capacity of middle T antigens

Plasmid	Nucleotide and amino acid sequence ^a													Transformation frequency (% of wt)						
pmT165(H)	AAGCTT CCACCATC ATG GAT AGA...													100						
dl166	AAGCTTACCATGGCACCATC ATG GAT AGA...													10						
wt	ATG GAT AGA GTT CTG AGC AGA GCT GAC AAA GAA AGG CTG CTA...													100						
	Met	Asp	Arg	Val	Leu	Ser	Arg	Ala	Asp	Lys	Glu	Arg	Leu	Leu...						
dl207	ATG GGG CTG CTA...													<0.1						
	Met											Gly	Leu	Leu...						
dl201	ATG GAA GAA AGG CTG CTA...													<0.1						
	Met											Glu	Glu	Arg	Leu	Leu...				
dl192	ATG GGA GCT GAC AAA GAA AGG CTG CTA...													<0.1						
	Met											Gly	Ala	Asp	Lys	Glu	Arg	Leu	Leu...	
	GCA TGG ^b																			
in179	ATG AGA GTT CTG AGC AGA GCT GAC AAA GAA AGG CTG CTA...													<0.1						
	Met	Arg	Val	Leu	Ser	Arg	Ala	Asp	Lys	Glu	Arg	Leu	Leu...							
in197	ATG GAT AGA GTT CTG ACA TGG GAC AAA GAA AGG CTG CTA...													30						
	Met	Asp	Arg	Val	Leu	Thr	Trp	Asp	Lys	Glu	Arg	Leu	Leu...							
bs176	ATG AAT AGA GTT CTG AGC AGA GCT GAC AAA GAA AGG CTG CTA...													62						
	Met	Asn	Arg	Val	Leu	Ser	Arg	Ala	Asp	Lys	Glu	Arg	Leu	Leu...						
bs182	ATG GAT AGA ATT CTG AGC AGA GCT GAC AAA GAA AGG CTG CTA...													76						
	Met	Asp	Arg	Ile	Leu	Ser	Arg	Ala	Asp	Lys	Glu	Arg	Leu	Leu...						
bs183	ATG GAT AGA GAT CTG AGC AGA GCT GAC AAA GAA AGG CTG CTA...													<0.1						
	Met	Asp	Arg	Asp	Leu	Ser	Arg	Ala	Asp	Lys	Glu	Arg	Leu	Leu...						
bs195	ATG GAT AGA GTT CTG AGC AGA GTT GAC AAA GAA AGG CTG CTA...													69						
	Met	Asp	Arg	Val	Leu	Ser	Arg	Val	Asp	Lys	Glu	Arg	Leu	Leu...						
bs197	ATG GAT AGA GTT CTG AGC AGA GCT AAC AAA GAA AGG CTG CTA...													81						
	Met	Asp	Arg	Val	Leu	Ser	Arg	Ala	Asn	Lys	Glu	Arg	Leu	Leu...						

^a Sequences derived from the oligonucleotide are underlined. The codon for the initiator methionine is in boldface print. The nucleotide changes and amino acid substitutions resulting from mutagenesis are italicized and in boldface print.

^b These sequences are inserted between the first and second codons displayed below them (in179).

and complete cleavage with *Bam*HI. Sequences encoding the first five amino acid residues of middle T antigen were isolated from pPyBR-1, which contains the Py early region from a *Bam*HI site at nt 4632 to an *Eco*RI site at nt 1560. pPyBR-1 DNA was cleaved to completion with *Dde*I (nt 185), the recessed 3' ends were made blunt by the Klenow fragment, and the Py sequences were isolated after cleavage with *Bam*HI. This DNA fragment was ligated to the *dl*197 DNA and used to transform *E. coli* DH1. Recombinant plasmids were identified by the loss of the *Dde*I site at the junction between the pPyBR-1 and *dl*197 DNA. The predicted sequence of this recombinant was confirmed by DNA sequencing (Table 1).

Single-base-pair substitution mutants were generated by the deletion loop mutagenesis procedure of Kalderon et al. (20). Briefly, a heteroduplex was formed between pm T165(H) DNA and *dl*207 DNA and treated with sodium bisulfite. Upon transformation of *E. coli*, both strands of the heteroduplex were represented in DNA isolated from single colonies. To resolve the different plasmids, approximately 100 colonies were pooled, and the DNA was extracted and used to transform *E. coli*. Plasmid DNA isolated from these colonies was the size of either the deletion mutant or the wt. Plasmids the size of the wt bearing single-base-pair transitions were detected by colony hybridization with two overlapping oligonucleotide probes (5'-ATGGATAGAGTTCTGA-3' and 5'-TGAGCAGAGCTGACA-3'), which spanned the region corresponding to the deletion in *dl*207. Mutants were distinguished from the wt based on the different thermostabilities of the hybrids formed (10). Putative mutant plasmid DNAs that hybridized with the probe at a reduced thermostability by comparison to the wt were further characterized by DNA sequencing. Five mutants bearing single point mutations were isolated in this way.

DNA transfections. CaPO₄ transfections of Rat-1 cells were conducted as described previously (16). DEAE-dextran-mediated DNA transfections of COS-1 cells (19) were also performed as described previously (35), except that the cells were subjected to a 2-min shock with 10% dimethylsulfoxide in phosphate-buffered saline immediately after removal of the DNA and washed twice with phosphate-buffered saline before incubation in growth medium. Cells were harvested 72 h posttransfection.

RNA analysis. RNA was isolated from cells by the method of Chirgwin et al. (11). The RNA pellet was dissolved in sterile distilled water, ethanol precipitated, and stored at -70°C until required. RNA concentration was determined by A₂₆₀. Py-specific RNA was detected by using 10 µg of total cellular RNA in an RNase protection assay.

Middle T antigen analysis. Radiolabeling of cells, T antigen immunoprecipitations, and in vitro kinase assays with the polyclonal antiserum were all performed as described previously (16). The EE hybridoma cell line was a generous gift of Gernot Walter, Department of Pathology, University of California, San Diego, La Jolla. The antibodies produced by this line recognize a peptide, Glu-Glu-Glu-Glu-Tyr-Met-Pro-Met-Glu, corresponding in sequence to an internal region of middle T antigen. The MTC antiserum was a gift of Rob Harvey, Genzyme Corp., Boston, Mass., and recognizes a peptide corresponding to the six carboxy-terminal amino acids of middle T antigen, Lys-Arg-Ser-Arg-His-Phe. Immunoprecipitations with both anti-peptide antibodies were conducted as follows. Cell lysates were prepared as described previously (16) and incubated with 5 µl of cell supernatant for 1 h at 4°C, and antigen-antibody complexes were collected by the addition of 20 µl of 50% protein A-Sepharose beads (Pharmacia Fine Chemicals, Piscataway, N.J.) for 1 h. The beads were pelleted, washed twice with lysis buffer (16)

and once with phosphate-buffered saline, and incubated with sample buffer at 85°C for 5 min before loading on the gel.

RESULTS

Experimental strategy. We wished to generate and characterize amino-terminal mutated middle T antigens to define the domain of the protein required for activation of the pp60^{c-src} tyrosyl kinase activity and consequent transformation. To accomplish this, we deleted various lengths of DNA from an *Xho*I site situated 20 base pairs upstream of the middle T antigen-coding sequences in pPymT(X) (Fig. 1). In so doing, sequences required for the initiation of translation and transcription and sequences encoding the amino terminus of middle T antigen were deleted from the plasmid. Sequences required for transcription initiation were provided to appropriate mutants by the SV40 early promoter-enhancer region. This promoter was chosen because it is a generally strong promoter (18) and because a fragment bearing it carries a *Hind*III recognition site situated only 7 base pairs upstream of the translation start codon for the SV40 small and large T antigens. Therefore, nearly all sequences close to the translation start site that might be important for efficient translation initiation or mRNA stability in SV40 ought to be retained in the hybrid mRNA. Replacement of the translation initiation signal was accomplished by ligation of a synthetic double-stranded oligodeoxyribonucleotide to the deletion endpoint (Fig. 1). The oligonucleotide contains an *Nco*I recognition site embedded within the eucaryotic translation initiation signal (5'-AC CATGG-3'), which has been shown to be optimum for ribosome binding and initiation of translation of preproinsulin mRNA (25-28). When situated upstream and out of frame with a reporter gene translation start site, the oligonucleotide was able to reduce synthesis of the reporter gene product by a factor of 10-fold (data not shown), as would be expected if the synthetic translation initiation signal were functional (27). We inferred from this result that the synthetic AUG is likely a strong translation initiation signal *in vivo*. Consequently, we employed the oligonucleotide as a translation initiation signal with mutant DNAs from which the natural ATG at nt 173 had been deleted.

Primary structure of mutant DNAs. Deletion of sequences encoding the amino-terminal amino acids of middle T antigen was achieved as described above. Plasmids that had sustained short deletions of middle T antigen-coding sequences with the oligonucleotide positioned in the correct orientation were characterized by DNA sequencing. Of 11 different plasmids sequenced, 3 were predicted to yield truncated middle T antigens upon transcription and translation (Table 1). The other eight mutants were predicted to encode nonsense polypeptides and were therefore of little immediate interest.

Using available out-of-frame deletion mutants, we constructed two plasmids that should encode middle T antigens in which two foreign amino acids replaced residues of wt middle T antigen. *in179* encodes a protein with a substitution of two amino acids, Ala and Trp, for the second residue, Asp. *in 197* encodes a middle T antigen with a substitution of two different amino acids, Thr and Trp, for the wt amino acids, Ser, Arg, and Ala, at residues 6 through 8 (Table 1).

To assess the contribution of individual residues at the amino terminus of middle T antigen to its capacity to transform cells, we generated single-base-pair mutations in pmT165(H) DNA by deletion-loop mutagenesis (20). A total of six different mutants were isolated, and five of these were

predicted to encode middle T antigens with single amino acid substitutions (Table 1). The substitutions include the following amino acid changes: Asp to Asn at the second residue, Val to either Ile or Asp at the fourth residue, Ala to Val at the eighth residue, and Asp to Asn at the ninth residue. The other mutant bore a silent mutation that did not alter the amino acid sequence of middle T antigen.

Transforming capacity of mutants. The various mutants were tested for their capacity to transform Rat-1 cells by the focus assay. As expected, about 10³ foci per µg of DNA per plate of 3 × 10⁵ transfected cells were observed 3 weeks after transfection with pmT165(H) DNA, the wt control (33, 34). By contrast, several of the plasmid DNAs failed to induce a single focus on the cell monolayer, even after 6 weeks of incubation (Table 1). These included the plasmids encoding truncated middle T antigens, *dl207*, *dl201*, and *dl192* as well as *in179*, which encodes a middle T antigen with a replacement of Ala and Trp for the second residue Asp, and *bs183*, which encodes a single amino acid change of Asp for Val at the fourth residue.

The other plasmids induced foci on the Rat-1 monolayers at frequencies ranging from 30 to 80% of that of the wt control DNA (Table 1). These included the following plasmids: *in197*, which encodes a substitution of Thr and Trp for Ser, Arg, and Ala at residues 6 through 8; *bs176*, which encodes a substitution of Asn for Asp at residue 2; and *bs182*, *bs195*, and *bs197*, which encode conservative amino acid substitutions at residues 4, 8, and 9, respectively. The focus assays were performed at least three times for each DNA, with the same result.

Analysis of mutant middle T antigens. To determine whether the nontransforming mutant DNAs were capable of being expressed to yield stable middle T antigen, these DNAs were used to transiently transfect COS-1 cells. The transfected cells were labeled with [³⁵S]methionine, and the middle T antigens were immunoprecipitated from lysates of these cells with an antiserum (MTC) that recognizes the six carboxy-terminal residues of the protein (Fig. 2A). Middle T antigen was efficiently immunoprecipitated from lysates of COS-1 cells transfected either with wt DNA (lane 2) or with the mutant DNAs (lanes 3 through 12) but not from mock-transfected cells (lane 1). Similar results were obtained when a rat polyclonal antiserum or a mouse monoclonal antibody (E-E) that recognizes an internal region of middle T antigen was used to immunoprecipitate the middle T antigens (data not shown).

Immune complex protein kinase assays were conducted on the mutant middle T antigens with the MTC antiserum to determine whether they were associated with protein kinase activity (Fig. 2B). The middle T antigens that were transformation competent were associated with a kinase activity that phosphorylated enolase *in vitro*. These included the wt middle T antigen (lane 2) as well as the middle T antigens encoded by the transformation-competent mutants *in197*, *bs176*, *bs182*, *bs195*, and *bs197* (lanes 7, 8, 9, 11, and 12, respectively). In general, the middle T antigens that failed to transform Rat-1 cells were not associated with protein kinase activity. These included the amino-terminal deletion mutants *dl207*, *dl201*, and *dl192* (lanes 3 through 5, respectively) as well as the base substitution mutant *bs183* (lane 10). The transformation-defective mutant *in179* encoded a middle T antigen that was associated with a much reduced but measurable amount of protein kinase activity (lane 6).

Rat-1 cells were also transfected with the mutant DNAs together with pSV2neo DNA (45), and the cells were subjected to selection in G418-supplemented medium. The

FIG. 2. Analysis of mutant middle T antigens expressed in COS-1 cells. COS-1 cells were transfected with no DNA (lane 1), wt DNA (lane 2), or the indicated mutant DNAs. The cells were radiolabeled with [^{35} S]methionine at 72 h posttransfection, and the middle T antigens were immunoprecipitated with the MTC antiserum. The washed immunoprecipitates were either incubated with sample buffer and loaded directly on a gel (A) or used to conduct immune complex protein kinase assays (B). The positions of middle T antigen and enolase are indicated by arrows.

majority of drug-resistant colonies on plates transfected with transforming DNAs (Table 1) were morphologically transformed, indicating that most colonies that expressed the neomycin resistance gene also expressed the cotransfected middle T gene. By contrast, colonies derived by cotransfection with plasmids that failed to induce foci on Rat-1 monolayers appeared morphologically normal (data not shown). Approximately 100 colonies were pooled from each transfection, and steady-state levels of middle T antigen in the cell populations were determined by [^{35} S]methionine labeling followed by extraction and immunoprecipitation of the middle T antigen with a rat polyclonal antiserum. The results of immunoprecipitations with nondenaturing conditions were difficult to interpret because of relatively small amounts of middle T antigen in the Rat-1 cells and the presence of cellular proteins that coprecipitated with it (data not shown). The lysates were therefore denatured before immunoprecipitation of the middle T antigens, because we observe fewer nonspecific contaminating proteins in immunoprecipitates of middle T antigen when this is done (16). The results of one such experiment are shown in Fig. 3A. Middle T antigen was observed in immunoprecipitates of lysates of cells transfected with either the wt DNA (lane 2) or mutant DNA (lanes 3 through 12). However, the amount of middle T antigen immunoprecipitated from these Rat-1 cell lysates varied considerably. For example, very little truncated middle T antigen was immunoprecipitated from lysates of cells trans-

FIG. 3. Analysis of mutant middle T antigens expressed in Rat-1 cell populations. Immunoprecipitations of middle T antigen were conducted with the polyclonal antiserum by using lysates of Rat-1 cell populations expressing wt DNA (lane 1), no Py DNA (lane 2), or the indicated mutant DNAs. (A) [^{35} S]methionine-labeled middle T antigen was denatured and immunoprecipitated with the polyclonal antiserum. (B) Middle T antigen was immunoprecipitated under nondenaturing conditions and used to conduct immune-complex protein kinase assays. The positions of phosphorylated middle T antigen are indicated by arrows.

fected with the deletion mutants *dl207*, *dl201*, and *dl192* compared with the amount immunoprecipitated from wt cell lysates (compare lane 2 with lanes 3 through 5). The middle T antigens encoded by *in179*, *in197*, and *bs183* (lanes 6, 7, and 10, respectively) were immunoprecipitated better than the truncated middle T antigens but were poorly immunoprecipitated relative to wt middle T antigen. Although each middle T antigen that failed to transform the Rat-1 cells was also poorly immunoprecipitated by the polyclonal antiserum, the converse was not true: the *in197*-encoded middle T antigen was poorly immunoprecipitated by the polyclonal antiserum (lane 7), although *in197* retained 30% of wt transforming activity (Table 1). Finally, each middle T antigen encoded by single-amino-acid substitution mutants was precipitated efficiently by the polyclonal antiserum (lanes 8, 9, 11, and 12) with the exception of *bs183*, a nontransforming mutant (lane 10).

RNAse protection experiments revealed no significant difference in the amount of Py-specific mRNA between the wt and mutant middle T antigen-expressing Rat-1 cell populations (data not shown). These middle T mRNAs were all translated efficiently in two different *in vitro* translation systems to yield middle T antigens of the expected size, and these *in vitro*-synthesized proteins were all stable when added to fresh extracts of Rat-1 cells (data not shown). Pulse-chase experiments conducted with the cell populations expressing the various mutants revealed no significant difference in the half-lives of the wt- and mutant-encoded middle T antigens *in vivo* (data not shown). When immunoprecipitations of the various middle T antigens expressed in COS-1 cells were also conducted under denaturing conditions, the truncated middle T antigens were also poorly

immunoprecipitated relative to wt middle T antigen (data not shown). These data suggest that several of the mutant middle T antigens are poorly immunoprecipitated by the polyclonal antiserum when denaturing conditions are employed. Therefore, the various middle T antigens were likely present in the transfected Rat-1 cells, as they were in the transfected COS-1 cells.

We also carried out immunocomplex protein kinase assays of lysates of Rat-1 cells transfected with either wt or mutant DNA. Middle T antigen from the control wt-transfected population was readily phosphorylated in the reaction with the polyclonal antiserum (lane 1), as were a 60-kilodalton protein, probably pp60^{c-src}, and an 80-kilodalton protein, which may represent the recently described phosphatidylinositol kinase (13, 21). The other major phosphorylated protein, migrating slightly faster than middle T antigen, was unrelated to middle T antigen-mediated transformation because it was also observed in the lane corresponding to cells expressing only the *neo* gene. Phosphorylated middle T antigens were also observed in lysates from cells that expressed the mutant plasmids that induced focal growth on Rat-1 monolayers: *in197*, *bs176*, *bs182*, *bs195*, and *bs197* (lanes 7 through 9 and 11 and 12, respectively). By contrast, no phosphorylated middle T antigens were observed in lanes corresponding to cell populations that expressed the plasmids that failed to transform Rat-1 cells: *dl192*, *dl201*, *dl207*, *in179*, and *bs183* (compare lanes 3 through 6 and lane 10 with lane 1). This was true even after prolonged exposure of the autoradiogram. Interestingly, the *in197*- and wt-encoded middle T antigens were associated with essentially equivalent kinase activities (Fig. 3A, compare lane 2 with lane 7), although far less ³⁵S-labeled middle T antigen was immunoprecipitated from Rat-1 cells transfected with *in197* DNA than from cells transfected with wt DNA (Fig. 3B, compare lane 7 with lane 2). A likely explanation for this is that the immunoprecipitations of [³⁵S]methionine-labeled middle T antigen were conducted under denaturing conditions, whereas the kinase assays were done under nondenaturing conditions.

Results of immune complex protein kinase assays with the MTC antiserum were similar to those observed with the polyclonal antiserum (data not shown). Middle T antigens that were transformation competent were associated with a protein kinase activity that phosphorylated enolase, whereas those that were defective for transformation were not associated with a protein kinase activity.

DISCUSSION

The experiments described in this report were initiated to map the boundaries of the domain in Py middle T antigen required for it to activate the kinase activity of pp60^{c-src} and for cellular transformation. Because much genetic evidence (6, 10, 31, 49) links this domain to the region encoded by the first exon of the middle T gene, we began by constructing DNAs that lack sequences encoding the extreme amino terminus of middle T antigen. These DNAs proved uniformly defective in their capacity to induce foci on a Rat-1 monolayer, suggesting that amino acids close to the amino terminus are required for transformation. To investigate the requirement for specific amino acids in this region for transformation, we generated a number of mutant DNAs that encoded amino acid substitutions within this region and tested the capacity of these DNAs to transform Rat-1 cells. DNAs predicted to encode nonconservative amino acid

changes of the first four residues of middle T antigen were completely defective in the focus assay, and their encoded proteins did not associate with a protein kinase activity in vitro. These included plasmid DNAs that encode the following amino acid changes: deletion of amino acid residues 2 through 6, 2 through 9, and 2 through 11; substitution of two amino acids, Ala and Trp, for the second residue, Asp; and substitution of Asp for the fourth residue, Val. Each of these DNAs harbors a mutation that affects either the second, fourth, or both residues of middle T antigen, suggesting that these residues are important to transformation. Interestingly, not all mutations that affected these amino acids rendered middle T antigen transformation defective. Rather, plasmid DNAs encoding the substitution of an amino acid of similar structure to the one it replaced retained transforming activity, even though the substituted amino acid had a different charge. For example, *bs176* encodes a change of the second residue from Asp to Asn, an amino acid similar in structure but different in charge, and *bs182* encodes a relatively conservative change of the fourth amino acid, Val, to Ile. Both of these plasmids (*bs176* and *bs182*) were transformation competent, and their encoded proteins were associated with tyrosine kinase activity in vitro. Therefore, functional middle T antigen was not completely intolerant of amino acid substitutions at its amino terminus. The data suggest that only those mutations predicted to effect a structural change at the amino terminus of middle T antigen rendered the protein transformation defective.

Each plasmid bearing a mutation that affected amino acids proximal to the fifth residue retained transforming activity. This group includes two single-amino-acid substitution mutants, *bs195* and *bs197*, which encode conservative changes to residues 8 and 9, respectively, as well as *in197*, which encodes a more drastic replacement of residues 6 through 8 (Ser, Arg, and Ala) by two unrelated amino acids (Thr and Trp). Taken together, the data suggest that the first four residues of middle T antigen constitute part of a domain important for transformation, whereas amino acids 6 through 9 do not.

The nontransforming DNAs generally encoded middle T antigens that did not associate with a protein kinase activity in vitro. Their lack of associated kinase activity likely accounts, at least in part, for the inability of these mutant middle T antigens to transform Rat-1 cells. This conclusion is based on the observation that no transformation-competent mutant of middle T antigen has ever been reported that fails to associate with a protein kinase activity in vitro. One nontransforming mutant, *in179*, encoded a middle T antigen that associated with a detectable kinase activity when the mutant was expressed in COS-1 cells but not when it was expressed in Rat-1 cells. This discrepancy may be due to the larger amounts of middle T antigen made in COS-1 cells compared with the Rat-1 cell population. However, it is also possible that a species-specific difference in pp60^{c-src} accounts for this observation. The mutation in *in179* may have a greater effect on the capacity of middle T antigen to associate with rat pp60^{c-src} than it does on its capacity to associate with simian pp60^{c-src}.

We noted that less middle T antigen was immunoprecipitated with the polyclonal antiserum from Rat-1 cell populations expressing the nontransforming mutants than from wt-expressing cells. We think that this observation is artifactual and is due to the inability of the mutant middle T antigens to renature properly after denaturation. This inference is supported by several observations. First, the Rat-1 cells transfected with the mutant DNAs contained as much

Py-specific mRNA as did cells transfected with wt DNA. Second, these mRNAs were efficiently translated *in vitro*. Third, the mutant middle T antigens were stable *in vitro* and *in vivo*. These data suggest that the various middle T antigens were synthesized and were stable in the Rat-1 cells that expressed them.

We noted that, although significantly less [³⁵S]methionine-labeled middle T antigen was immunoprecipitated from Rat-1 cells expressing *in197* than from wt-expressing cells, the *in197* middle T antigen was associated with a kinase activity comparable to that of wt middle T antigen. It is likely that this discrepancy is due to the fact that the kinase assays were conducted under nondenaturing conditions, whereas the immunoprecipitations of Rat-1 cell lysates were done under denaturing conditions. When the immunoprecipitations and kinase assays were both conducted under nondenaturing conditions, no significant difference in the amount of middle T antigen or its associated kinase activity was observed between cells expressing *in197* DNA and cells expressing wt DNA. Taken together, the data strongly suggest that all of the mutant DNAs were efficiently expressed in Rat-1 cells, but that some of the mutant middle T antigens were precipitated poorly by the polyclonal antiserum when denaturing conditions were employed. Although this result was unforeseen, there is a precedent in the literature for a similar observation. Taylor et al. have reported that several different preparations of a rabbit polyclonal serum which efficiently immunoprecipitate the wt *v-fps* gene product fail to immunoprecipitate a mutant version of the protein (47). Moreover, like the middle T antigens described here, a monoclonal antibody raised against an epitope of *v-fps* unaffected by the mutation, efficiently immunoprecipitated both wt *v-fps* and the mutant *v-fps*. This observation suggests that most of the antibodies in the anti-*fps* polyclonal antisera recognize a single epitope and that mutations that affect the structure of the epitope prevent efficient immunoprecipitation by that antiserum. It is therefore possible that the majority of antibodies in the rat polyclonal antiserum recognize an epitope at the amino terminus and that mutations that affect the structure of this region impair the capacity of middle T antigen to renature and to be immunoprecipitated by the antiserum.

Unfortunately, we were unable to directly demonstrate the presence of middle T antigen (including wt middle T antigen) in lysates of the Rat-1 cell populations by immunoprecipitation with the EE antibody or with the polyclonal antibody under nondenaturing conditions because of the relatively low amounts of middle T antigen in the Rat-1 cell populations. Therefore, it remains formally possible that the mutant mRNAs were translated efficiently in COS-1 cells but poorly in Rat-1 cells. We consider this unlikely however, because two of the middle T antigens that were poorly immunoprecipitated by the polyclonal antiserum (encoded by *bs183* and *in197*) are translated from mRNAs whose base changes are situated at least 10 nucleotides downstream from the translation start site and are therefore unlikely to affect the efficiency of translation initiation. Moreover, other mRNAs whose base changes were situated closer to the translational start site (at nt 176 and nt 182) encoded middle T antigens that were precipitated well by the polyclonal antiserum. The important difference between these pairs of mutants is likely that the former encode middle T antigens with structural alterations at their amino termini, whereas the latter encode middle T antigens with a structure similar to that of the wt. Again, this suggests that the inability of these mutants to transform Rat-1 cells is due to structural

alterations of their amino termini rather than to their inability to be translated in Rat-1 cells.

Amino-terminal deletion mutants of middle T antigen have also been described by other groups. Katinka and Yaniv (22) reported that deletion of N-terminal sequences of middle T antigen reduced but did not abolish transformation. However, no attempt was made to replace signals required for transcription and translation initiation, making it difficult to predict the structure of the mutated middle T antigens or the mRNAs that encoded them. Templeton and Eckhart (49) described a truncated middle T antigen (*d113*) whose translation is initiated from the first internal methionine (Met-30). The *d113*-encoded middle T antigen fails to transform rat cells and fails to activate the kinase activity of pp60^{c-src}, suggesting that the first 30 residues of middle T antigen are important for its transforming activity. Data presented here confirm this result and define more precisely the amino acids at the amino terminus that are important for transformation. Like DNA from the various mutants described here, *d113* DNA was efficiently expressed in COS-1 cells to yield as much middle T antigen as wt DNA when the analysis was performed with the MTC antiserum. However, Templeton and Eckhart did not report on the analysis of *d113*-encoded middle T antigen in the rat cells in which transformation was assayed. We do not know, therefore, whether *d113*-encoded middle T antigen is also poorly precipitated by polyclonal serum when expressed in rat cells.

Because many other mutations in the amino-terminal half of middle T antigen have also been found to reduce the transforming potential of middle T antigen (6, 10, 31; Cook and Hassell, unpublished data), we suggest that the first four amino acids of middle T antigen participate with other noncontiguous amino acids in the amino-terminal half of the molecule to form a structural domain that interacts with pp60^{c-src}. Alternatively, mutations affecting the amino terminus may change the conformation of the protein, thereby preventing a distant region of middle T antigen from contacting pp60^{c-src}. Whatever the explanation, an intact amino terminus of Py middle T antigens is apparently required for its capacity to activate the tyrosine kinase activity of pp60^{c-src} and to transform cells.

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