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N-Terminal Amino Acid Sequences of the Polyoma Middle-Size T Antigen Are Important for Protein Kinase Activity and Cell Transformation

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We constructed deletion mutations which removed N-terminal coding sequences of various lengths from a cloned polyoma middle-size T antigen (MT antigen) gene. We introduced the MT antigen genes into a simian virus 40 expression vector so that they were expressed at high levels under the control of the simian virus 40 late promoter in COS-1 cells. The deletion mutant genes synthesized truncated MT antigens whose size was consistent with the deletion of either 70 or 106 amino acids from N termini, owing to initiation of translation at internal methionine codons in the MT antigen-coding region. The truncated MT antigens were found in cell membrane fractions but failed to show MT antigen-associated protein kinase activity. The cloned deletion mutant DNAs failed to transform rat F2408 or mouse NIH 3T3 cells. Therefore, N-terminal amino acid sequences of the polyoma MT antigen, as well as C-terminal sequences, are important for protein kinase activity and cell transformation.

The middle-size tumor antigen of polyoma virus (MT antigen) causes neoplastic transformation of established cell lines (14). The MT antigen consists of 421 amino acids encoded in the early region of the polyoma genome. The MT antigen shares 79 amino acids at its N terminus with the large (LT) and small (ST) antigens. The MT and ST antigens share an additional 112 amino acids not found in the LT antigen. The C-terminal regions of the three proteins (4 amino acids for ST, 230 for MT, and 701 for LT) are unique.

The MT antigen is located in a cell membrane fraction (6, 10). The MT antigen is associated with protein kinase activity in immunoprecipitates (3, 9, 11). The protein kinase activity phosphorylates tyrosine residues in the MT antigen in immunoprecipitates (3) and soluble extracts after partial purification (16).

There is little information concerning the involvement of N-terminal amino acid sequences in the functions of the MT antigen. Katinka and Yaniv (7) reported that two deletions of polyoma DNA extending into the N-terminal coding region for the three T antigens retained a small fraction of their transforming activity. They suggested that truncated MT antigens with decreased transforming activity might be produced by the deletion mutants through initiation at internal methionine residues. However, it was difficult to interpret these results because it was not possible to characterize the putative MT antigens produced after DNA infection and because the effects of mutations on the individual T antigens could not be separated. In addition, the deletions removed the normal signals for transcription and translation, including the TATA box, the major cap sites, and the normal initiation codon, making it difficult to predict the structure of the new mRNAs that would be required for synthesizing modified T antigens.

The availability of a molecular clone encoding only the MT antigen made it possible to study the effects of mutations on the MT antigen in the absence of LT and ST antigens (14). We used this cloned DNA to isolate and characterize deletion mutations in the N-terminal region of the MT antigen. We introduced the mutated DNA into a simian virus 40 (SV40) expression vector so that the MT antigens were expressed under the control of the SV40 late promoter and synthesized at high levels after infection of COS-1 cells. Deletion mutants produced truncated MT antigens whose size was consistent with initiation at internal methionine residues. The truncated MT antigens were localized in cell membrane fractions but failed to show the MT antigen-associated protein kinase activity. The cloned DNAs failed to transform rat F2408 or mouse NIH 3T3 cells.

MATERIALS AND METHODS

Cell culture and biochemical analysis. Cultures of mouse NIH 3T3, rat F2408, and monkey COS-1 cells were maintained in Dulbecco modified Eagle medium (DME) supplemented with 5 or 10% calf serum. Infection of COS-1 cells for transient expression was carried out by adding 5 μg of DNA in 0.5 ml Dulbecco modified Eagle medium containing 400 μg of DEAE-dextran per ml to cultures containing ca. 10^6 cells per 6-cm dish. Infection of NIH 3T3 or F2408 cells for transformation was carried out by adding 1 to 5 μg of plasmid DNA to cultures containing 10^5 cells by using the calcium phosphate coprecipitation procedure of Wigler et al. (17).

Analysis of MT antigen synthesis by radiolabeling, immunoprecipitation, and polyacrylamide gel electrophoresis was performed as described previously (5). Protein kinase assays were also performed as described previously (3).

Cell fractionation was performed as follows. Radiolabeled cultures in 3-cm dishes were rinsed with Tris-buffered saline and incubated in 1 ml of hypotonic buffer (10 mM Tris [pH 7.5], 1 mM EDTA, 1% aprotinin) for 5 min at 4°C. The cells were scraped into a Dounce homogenizer and homogenized by 20 strokes with an A pestle. Nuclei and unbroken cells were removed by centrifugation at 100 × *g*, and the supernatants were centrifuged in microfuge tubes in a Beckman JA21 rotor at 20,000 rpm for 1 h (40,000 × *g*). The high-speed

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supernatants were supplemented with 1% Nonidet P-40–1% deoxycholate–0.1% sodium dodecyl sulfate, and the pellets were extracted with 1 ml of the same buffer. The extracts were cleared by centrifugation at 12,000 × *g* before being used for immunoprecipitation.

Expression vector construction. The expression vector JC119 (12) consists of a portion of a late-region deletion mutant of SV40 cloned into a plasmid. The deletion removes most of the coding region of the major capsid protein VP1, including the initiation codon. There is a unique *Xho*I site in the SV40 late region in the vector, inserted in such a way that fragments cloned into this site will be translated from the first ATG codon, using the initiation, splicing, and termination signals for VP1 messages. We prepared a fragment of the polyoma genome from the molecular clone pPyMT1, which encodes only the MT antigen in its early region (14). We digested plasmid pPyMT1 with *Nar*I, which cleaves polyoma sequences at nucleotide 99 (the numbering system of Friedmann et al. [4] was used). We reparied the two-base 5′ overhang left after *Nar*I digestion by synthesis with the Klenow fragment of *Escherichia coli* DNA polymerase I. We ligated synthetic *Xho*I linkers to the resulting blunt-ended fragments and separated the fragments from the unligated linkers by gel filtration with Sephadex G-50. We then digested the fragments with *Xho*I and *Bam*HI and isolated the 4.5-kilobase fragment (nucleotides 99 to 4,657 of the polyoma) by agarose gel electrophoresis. We inserted the polyoma sequences into a modified form of the pJC119 expression vector between an *Xho*I site (at the 5′ end of the inserted MT gene) and a *Bam*HI site (at the 3′ end of the inserted gene). In this orientation, translation is initiated at the authentic MT antigen initiation codon (nucleotides 188 to 190) under the control of the SV40 late promoter. This plasmid was designated pMTSVL1.

Preparation of deletion mutants. The expression vector plasmid containing MT antigen-coding sequences was digested with *Xho*I, extracted with phenol, and precipitated with ethanol. Of the digested plasmid DNA, 5 μg was treated with 2 U of BAL 31 nuclease for various lengths of time at 22°C. Synthetic *Xho*I linkers were attached to the BAL 31-treated fragments, and the fragments were digested with *Xho*I and *Bam*HI. The digested fragments were separated by agarose gel electrophoresis, and fragments migrating slightly faster than fragments not treated with BAL 31 nuclease were isolated. These fragments, presumably deleted in polyoma sequences, were inserted into the expression vector between the *Xho*I and *Bam*HI sites. Individual isolates were radiolabeled and characterized by restriction enzyme digestion with *Xho*I and *Ava*I.

RESULTS

Construction of N-terminal deletion mutants. We introduced cloned polyoma DNA encoding the MT antigen into a derivative of the SV40 expression vector pJC119 (12) as described above. The resulting plasmid, pMTSVL1, expressed the MT antigen at high levels in COS-1 cells under the control of the SV40 late promoter. The cloned DNA had an *Xho*I cleavage site at polyoma nucleotide 99. This site is 89 nucleotides upstream from the T antigen initiation codon at nucleotide 188. We digested pMTSVL1 DNA with *Xho*I, followed by BAL 31 exonuclease to generate deletions as described above. We attached synthetic *Xho*I linkers to the exonuclease-treated fragments, ligated the mixture to recircularize the plasmids, and released the deleted polyoma fragments by digestion with *Xho*I and *Bam*HI. We recloned the fragments into the expression vector and screened potential deletion mutants by restriction enzyme digestion with *Xho*I and *Ava*I and by expression of truncated MT antigens in COS-1 cells.

The properties of three N-terminal deletion mutants are described below.

N-terminal coding regions of the deletion mutants. We analyzed the nucleotide sequences of the deletion mutants in the regions in which the N-terminal MT antigen-coding sequences were inserted into the expression vector. Figure 1 shows the N-terminal coding regions of the deletion mutants. The deletions removed different amounts of N-terminal sequences. The polyoma sequences in mutant *dl*3 began at nucleotide 488. (The initiation codon in the wild-type virus begins at nucleotide 188.) The first methionine codon in the *dl*3 mutant was an in-frame codon at nucleotides 506 to 508. The sequences in mutant *dl*5 began at nucleotide 419. There were two out-of-frame methionine codons, at nucleotides 438 to 440 and 444 to 446, before the first in-frame methionine codon at nucleotides 506 to 508. The sequences in mutant *dl*13 began at nucleotide 365. The first methionine condon in mutant *dl*13 was an in-frame condon at nucleotides 398 to 400. If translation were initiated from the first in-frame methionine codon in each case, mutant *dl*13 would synthesize an MT antigen of 351 amino acids, shortened by 70 amino acids compared with the wild-type protein. Mutants *dl*3 and *dl*5 would synthesize MT antigens of 315 amino acids, shortened by 106 amino acids compared with the wild-type protein.

Truncated MT antigens expressed by deletion mutants. We transfected COS-1 cells with expression vector plasmid DNAs containing the N-terminal deletion mutations and analyzed the MT antigens synthesized in the infected cells by immunoprecipitation and polyacrylamide gel electrophoresis (Fig. 2). All three mutants produced truncated MT antigens. Mutant *dl*13 produced an MT antigen of apparent M_r 48,000 (compared with an apparent M_r of 59,000 for the wild-type MT antigen in this gel system). Mutants *dl*3 and *dl*5 produced MT antigens of apparent M_r 44,000. The sizes of the truncated MT antigens were consistent with initiation of translation at the first in-frame methionine codon in each case (The predicted apparent M_r of an MT antigen initiated at nucleotides 398 to 400 is 49,000 in this gel system; the apparent M_r of an MT antigen initiated at nucleotides 506 to 508 is 44,000. The only other in-frame methionine codons in this part of the early region are at nucleotides 275 to 277 and 650 to 652.). Therefore, the sizes of the truncated MT antigens and the fact that mutants *dl*3 and *dl*5 produced MT antigens of the same apparent size, although the mutants had

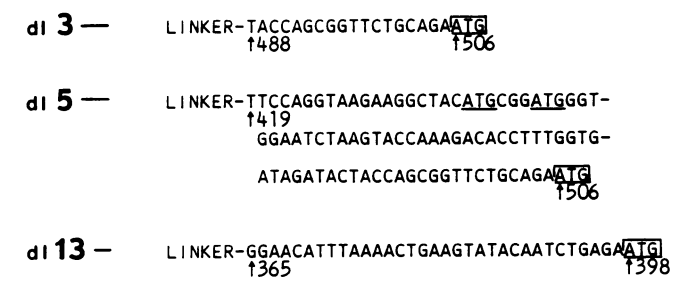

FIG. 1. Nucleotide sequences of N-terminal deletion mutants. The nucleotide sequence between the linker and the first in-frame initiator codon is shown. Out-of-frame methionine codons in mutant *dl*5 are underlined.

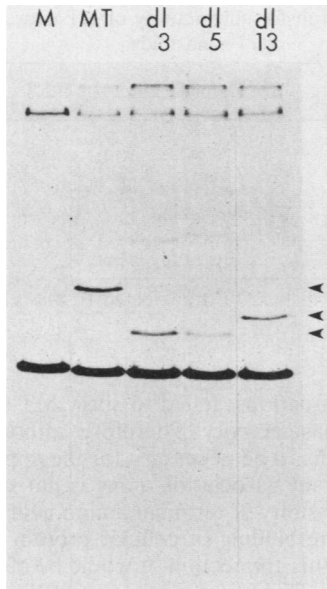

FIG. 2. MT antigens synthesized by N-terminal deletion mutants. Extracts of COS-1 cells transfected with expression vector DNAs encoding wild-type or deletion mutant MT antigens were immunoprecipitated and analyzed by polyacrylamide gel electrophoresis. Lanes: M, mock infected; MT, wild type; *dl*3, *dl*5, and *dl*13, deletion mutants. The positions of the MT antigens are indicated by arrows on the right.

different N-terminal coding regions, argued strongly that the truncated MT antigens were produced by initiation at internal methionine codons.

We verified that the truncated MT antigens produced by N-terminal deletion mutants had intact C termini by precipitating extracts of mutant-infected cells with antiserum directed against a synthetic peptide corresponding to the six C-terminal amino acids of the MT antigen (15). Both classes of truncated MT antigens were precipitated efficiently by the C-terminal specific antiserum (Fig. 3).

Truncated MT antigens lack MT antigen-associated protein kinase activity. MT antigens which lack the C-terminal hydrophobic region because of premature termination of translation fail to show the tyrosine-specific protein kinase activity associated with the wild-type MT antigen (1.13, unpublished data). We tested the truncated MT antigens produced by plasmids pdl3 and pdl13 for the presence of protein kinase activity. Figure 3 shows the results of in vitro phosphorylation of MT antigens in immunoprecipitates of extracts of infected COS-1 cells. The wild-type MT antigen synthesized by pMTSVL1 was phosphorylated; the truncated MT antigens were not. Therefore, N-terminal sequences of the MT antigen, in addition to C-terminal sequences, are required for expression of the protein kinase activity.

Truncated MT antigens in cell membrane fractions. The wild-type MT antigen is located in a membrane fraction of lytically infected or transformed cells (6, 10). We tested whether the N-terminal amino acids missing from the truncated MT antigens of the deletion mutants were necessary for correct localization by studying the intracellular location of the truncated MT antigens. We infected COS-1 cells with plasmids pdl3 and pdl13 and fractionated radiolabeled cell extracts into soluble and particulate fractions by centrifugation as described above. Figure 4 shows the results of immunoprecipitation of each of the fractions. The wild-type MT antigen synthesized by pMTSVL1 and the truncated MT antigens synthesized by pdl3 and pdl13 all were located in the particulate membrane fraction and not in the soluble cytoplasmic fraction. By contrast, truncated MT antigens lacking the C-terminal hydrophobic region are located in the cytoplasmic fraction (1, 13; unpublished data). We conclude that the first 106 amino acids of the MT antigen are not required for localization of the protein in a cell membrane fraction.

Plasmids encoding truncated MT antigens fail to transform. We tested the transforming ability of plasmids encoding the truncated MT antigens with rat F2408 and mouse NIH 3T3 cells. Table 1 shows the results of an experiment in which rat F2408 cells were transfected with wild-type pPyMT1 and pMTSVL1 plasmids and with plasmids encoding the MT antigens having N-terminal deletions. Transformation was assayed by focus formation. Plasmids encoding wild-type MT antigens produced 30 to 40 foci per 10^5 transfected cells. By contrast, the plasmids encoding truncated MT antigens failed to produce foci. Therefore, the transforming efficiency of the plasmids encoding the truncated MT antigens was less than 1% that of the wild type. We obtained similar results with mouse NIH 3T3 cells, with the exception that very small foci appeared on all plates infected with plasmids derived from the vector pJC119 and on plates infected with pJC119 alone. These small foci may have arisen because of expression of the N-terminal 95% fragment of the SV40 LT antigen encoded by the vector.

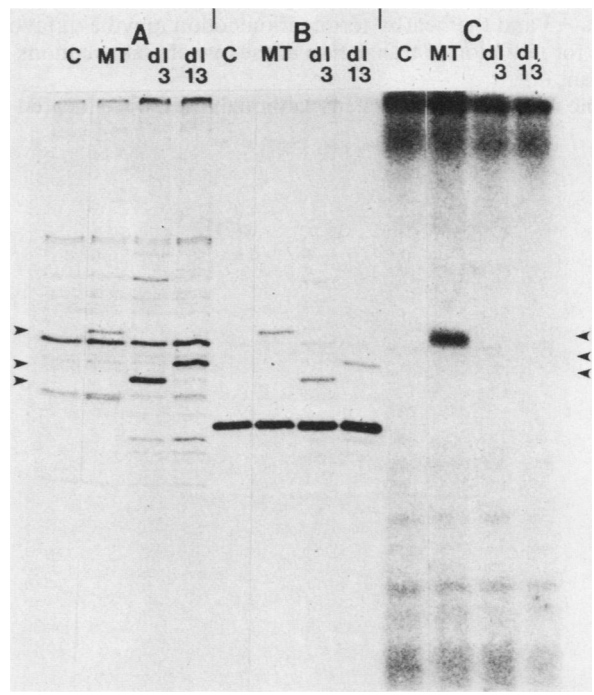

FIG. 3. MT antigens and associated protein kinase activities. Extracts of COS-1 cells transfected with expression vector DNAs encoding wild-type or deletion mutant MT antigens were immunoprecipitated with anti-C-terminal peptide serum (A) or rat antitumor serum (B and C). The immunoprecipitates were analyzed directly (A and B) or after incubation for protein kinase assay (C). The positions of the T antigen are indicated by arrows. Lanes: C, mock infected; MT, wild type; *dl*3 and *dl*13, deletion mutants.

DISCUSSION

We introduced deletions into the N-terminal coding region of the polyoma MT antigen expressed under the control of an SV40 late promoter. This system has several advantages for studying mutational alterations. The mutant proteins can be expressed at sufficiently high levels to be assayed biochemically in transfected cells. The plasmids express MT antigens, but not LT or ST antigens, so the effects of mutations on the MT antigen can be isolated from effects on the other two proteins. Finally, the MT antigens are expressed under the control of the SV40 promoter in the vector, so changes in polyoma early transcriptional control regions do not affect expression of the proteins.

The sizes of the truncated MT antigens were consistent with initiation of translation at internal methionine codons in the mutant MT antigen-coding regions. For two of the mutants, the first methionine codon downstream from the end of the deletion was in frame. One mutant, dl5, had two out-of-frame methionine codons preceding the first in-frame methionine codon. As a general rule, translation is initiated at the first methionine codon in eucaryotic mRNA (8). However, sequences flanking initiation condons are important in determining which codons are used for initiation (8). In particular, the binding of oligonucleotides to wheat germ ribosomes is enhanced by purines located three nucleotides before, or four nucleotides after, the A residue in the initiation codon (8). The two out-of-frame methionine codons in *dl5* had pyrimidine at position −3, whereas the in-frame codon had purine at position −3. In addition, the out-of-frame methionine codons (nucleotides 438 to 440 and 444 to 446) were followed closely by a termination codon, TAA, at nucleotides 456 to 458. The pyrimidine residues at positions −3 and the nearby termination codon may be unfavorable for initiation of translation at the out-of-frame codons in mutant *dl5*.

The truncated MT antigens, although they were located in cell membrane fractions, failed to show MT antigen-associated protein kinase activity. Therefore, although membrane association seems to be necessary for the appearance of the activity, membrane association alone is not sufficient for its appearance. Possibly, N-terminal amino acid sequences are important for the binding of cellular protein kinases to the MT antigen. In this connection, it would be of interest to test the ability of $pp60^{c-src}$ to associate with the truncated MT antigens of these deletion mutants, since $pp60^{c-src}$ has been shown to associate with the wild-type MT antigen (2).

Plasmids encoding the truncated MT antigens failed to transform rat F2408 or mouse 3T3 cells. There could be many reasons for this, including failure of the proteins to be expressed at appropriate levels or toxicity of the altered proteins for cells expressing them, in addition to possible failure of the proteins to function properly in transformation. To examine further the relationship between the MT antigen-associated protein kinase and transformation, it would be desirable to study other deletion mutations affecting the N-terminal amino acid sequences of the protein. The requirement for initiation of translation at internal methionine residues severely limits the kinds of deletion mutations that can be studied by the methods described here. Therefore, we are modifying the system to allow the selection of mutations which will initiate translation immediately at the end point of any deletion.

TABLE 1. Transforming activity of MT expression vector plasmids

Plasmid	No. of foci[a]
pAT153	<1
pPyMT1	119
pMTSVL1	88
pdl3	<1
pdl5	<1
pdl13	<1

[a] Total of three dishes, 10^5 transfected F2408 cells per dish.

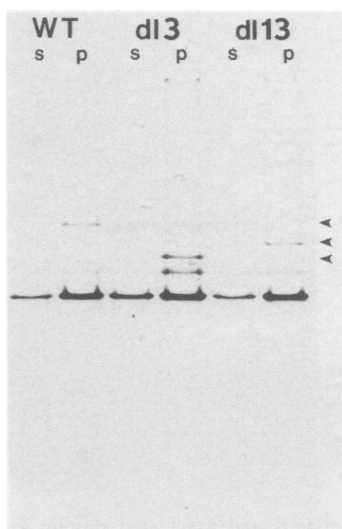

FIG. 4. Cellular location of deletion mutant MT antigens. COS-1 cells were transfected with wild-type or deletion mutant MT antigen expression vector plasmids, radiolabeled with [^{35}S]methionine, extracted by hypotonic swelling and Dounce homogenization, and separated into soluble (s) and particulate (p) fractions by centrifugation at 40,000 × *g*. Lanes: WT, wild type; *dl*3 and *dl*13, deletion mutants. The positions of the MT antigens are indicated by arrows.

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