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Construction and Expression of a Recombinant DNA Gene Encoding a Polyomavirus Middle-Size Tumor Antigen with the Carboxyl Terminus of the Vesicular Stomatitis Virus Glycoprotein G

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We constructed a molecular clone encoding the N-terminal 379 amino acids of the polyomavirus middle-size tumor antigen, followed by the C-terminal 60 amino acids of the vesicular stomatitis virus glycoprotein G. This hybrid gene contained the coding region for the C-terminal hydrophobic membrane-spanning domain of the G protein in place of the C-terminal hydrophobic domain of the middle-size tumor antigen. The hybrid gene was expressed in COS-1 cells under the control of the simian virus 40 late promoter. The hybrid protein was located in cell membranes and was associated with a tyrosine-specific protein kinase activity, as was the middle-size tumor antigen. Plasmids encoding the hybrid protein failed to transform mouse NIH 3T3 or rat F2408 cells.

The polyomavirus genome encodes three early proteins, the small, middle-size (MT), and large T antigens. The coding regions for these three proteins overlap, so that all three share an N-terminal region of 79 amino acids. The small and MT antigens share an additional 112 amino acids not found in the large T antigen. The three T antigens have unique C-terminal regions translated from different reading frames in the viral DNA (29).

The three proteins are at different locations in the cell: the large T antigen is found in the nucleus, the small T antigen is found in the cytoplasm, and the MT antigen is found in a cell membrane fraction (14, 27). The mechanisms by which the three proteins are directed to different intracellular locations are not understood.

The MT antigen is of interest because it is primarily responsible for cell transformation by polyomavirus. Recombinant DNA plasmids encoding only the MT antigen can transform established cell lines (32). Many mutations affecting the MT antigen reduce or abolish cell transformation (1, 5, 11, 15, 17–19, 31). Although the MT antigen alone can transform established cell lines, a portion of the large T antigen is required in addition for transforming primary cells (20).

The MT antigen is associated with a protein kinase activity (6, 24, 28). The protein kinase phosphorylates a tyrosine residue of the MT antigen in immunoprecipitates (6). With few exceptions (18, 24), mutations in the MT antigen that affect cell transformation also affect the protein kinase activity, suggesting that the activity may be involved in transformation (1, 6, 24, 28, 31). However, polyomavirus-transformed cells do not have elevated levels of phosphotyrosine in total cellular protein, as do cells transformed by retroviruses encoding tyrosine-specific protein kinases (26).

The MT antigen has an uninterrupted stretch of 22 hydrophobic and uncharged amino acids bounded by positively charged amino acids near its C-terminus (29). The size and location of this C-terminal hydrophobic region are similar to the size and location of regions involved with membrane association in other proteins. In particular, the vesicular stomatitis virus (VSV) glycoprotein G has an uninterrupted stretch of 20 hydrophobic and uncharged amino acids which are also bounded by positively charged amino acids and are located near the C-terminus of the protein (23). The hydrophobic region of the VSV G protein spans the cell membrane in which the G protein is located (23). Whether the hydrophobic region of the MT antigen also spans the membrane in which the MT antigen is located is not known. However, the hydrophobic region of the MT antigen appears to be important for membrane association, cell transformation, and protein kinase activity. MT antigens lacking the hydrophobic region fail to become associated with cell membranes, to transform cells, or to show the associated protein kinase activity (1, 31).

Because the hydrophobic regions of the MT antigen and the G protein are similar in size and location in the two proteins, we wondered whether the hydrophobic region of the G protein could substitute for the hydrophobic region of the MT antigen. Cloned genes encoding modified forms of the VSV G protein (21) and the influenza virus hemagglutinin (9) have been inserted into expression vectors and used to study the role of hydrophobic anchoring sequences in processing and secretion of these membrane-associated proteins. We constructed a molecular clone encoding the N-terminal 379 amino acids of the MT antigen, followed by the C-terminal 60 amino acids of the G protein. We introduced this clone into the simian virus 40 (SV40) expression vector, JC119 (30), and studied the protein expressed in COS-1 cells (10). In COS-1 cells, the hybrid protein was associated with cell membranes and with a tyrosine-specific protein kinase activity. However, the cloned DNA encoding the hybrid protein failed to transform mouse NIH 3T3 or rat F2408 cells.

MATERIALS AND METHODS

Cell cultures and DNA transfection. The polyomavirus large plaque virus strain WS, the nucleotide sequence of which has been determined, was used in these studies (8). Cells were grown in Dulbecco modified Eagle medium supplemented with 5 or 10% calf serum. For transient expression experiments, plasmid DNA was diluted to a concentration of 10 μg/ml in serum-free Dulbecco modified

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Eagle medium containing DEAE-dextran at a concentration of 400 μg/ml. Cell cultures in 6-cm dishes were incubated with DNA for 2 h at 37°C. The medium was replaced by Dulbecco modified Eagle medium containing 10% calf serum and chloroquine diphosphate at a concentration of 100 μM for 2 to 6 h. This medium was replaced with Dulbecco modified Eagle medium containing 10% calf serum. (Chloroquine treatment increased the amount of the MT antigen by about 50%.) For transformation experiments, ca. 10^5 rat F2408 or NIH 3T3 cells in 6-cm dishes were transfected with 1 to 5 μg of plasmid DNA by the calcium phosphate coprecipitation procedure, essentially as described by Wigler et al. (33), except that no carrier DNA was used and the cells were not replated after transfection.

Construction of recombinant DNAs. Recombinant DNA plasmids were grown in *Escherichia coli* C600. The plasmids were purified by density gradient centrifugation in cesium chloride containing ethidium bromide by standard methods. Restriction enzymes, DNA modifying enzymes, and synthetic DNA linkers were obtained commercially and were used according to the instructions of the manufacturers. The pMT/G1 hybrid gene was prepared as follows. We chose the *Sst*I site in polyomavirus DNA at nucleotide 1,388 as the site for insertion of G protein DNA. We first subcloned a fragment of plasmid pPyMT1 between the *Ava*I (nucleotide 1,031) and *Eco*RI (nucleotide 1,575) sites into pBR322. The *Sst*I site at nucleotide 1,388 is the only *Sst*I site in this fragment of polyomavirus DNA. We digested the plasmid DNA with *Sst*I and removed the single-stranded 3′ end, using the Klenow fragment of DNA polymerase I in the presence of all four deoxynucleoside triphosphates. We added synthetic *Hin*dIII linkers, 10 bases long, to the digested DNA, producing a *Hin*dIII site in place of the former *Sst*I site. We then isolated the *Ava*I to *Eco*RI fragment containing the *Hin*dIII linker and substituted it for the corresponding fragment in a pPyMT1 plasmid which was modified by digestion with *Eco*RI and ligation to remove the polyomavirus sequences distal to the *Eco*RI site. The resulting plasmid had a single *Hin*dIII site and a single *Eco*RI site which could be used to remove the coding region for the C-terminus of the MT antigen and replace it with a *Hin*dIII to *Eco*RI fragment of VSV DNA encoding the C-terminus of the G protein. Plasmid pGR125 (22) encodes the VSV G protein and has an *Eco*RI site just beyond the G protein termination codon. We digested pGR125 DNA with *Taq*I, replaced one base of the 5′ overhang using Klenow DNA polymerase and dGTP, and removed the remaining single-stranded residue with S1 nuclease. We then added *Hin*dIII linkers, digested the DNA with *Eco*RI and *Hin*dIII, and replaced the *Eco*RI to *Hin*dIII fragment of polyomavirus DNA described above with the *Eco*RI to *Hin*dIII fragment of VSV DNA. Finally, we restored polyomavirus DNA sequences distal to the *Eco*RI site, which was deleted earlier, creating a plasmid identical to pPyMT1, with the exception of the isolated fragment of VSV DNA. We verified the DNA sequence in the junction region by nucleotide sequencing and characterized the hybrid plasmid, pMT/G1, by restriction enzyme digestion.

The pMTSVL1 and pMT/GSVL1 plasmids were prepared as follows. We digested plasmids pPyMT1 and pMT/G1 with *Nar*I, which cleaves polyomavirus DNA at nucleotide 99 and cleaves the vector DNA at several sites. We repaired the two-base 5′ overhang remaining after *Nar*I digestion by synthesis with the Klenow fragment of *E. coli* DNA polymerase I in the presence of all four deoxynucleoside triphosphates. We ligated synthetic *Xho*I linkers to the resulting blunt-ended fragments and purified the fragments by gel filtration chromatography with Sephadex G50. We digested the purified fragments with *Xho*I and *Bam*HI and isolated the 4.5-kilobase fragment of polyomavirus (nucleotides 99 to 4,657) by agarose gel electrophoresis. We then digested the expression vector, pSVGL3, which consists of sequences encoding the VSV G protein and the expression vector, pJC119, with *Xho*I (at the 5′ end of the expressed gene) and *Bam*HI (at the 3′ end of the expressed gene) to liberate the 7-kilobase expression vector. We purified the expression vector DNA by agarose gel electrophoresis and ligated the *Xho*I-*Bam*HI polyomavirus DNA fragments from pPyMT1 and pMT/G1 to it. The resulting plasmids are characterized by restriction enzyme digestion.

Protein analysis. The radiolabeling of cell cultures, preparation of cell extracts, immunoprecipitation, and polyacrylamide gel electrophoresis were performed as described previously (13). Protein kinase assays were performed as described previously (6).

Immunofluorescence microscopy. Approximately 5×10^3 COS-1 cells were seeded in a 50-μl drop on epoxy-gridded glass microscope slides (Carlson Scientific, Peotone, Ill.) and transfected as described above. At 24 h after infection, the cells were rinsed with Tris-buffered saline (TBS) chilled to 4°C and incubated for 10 min with fluorescein-conjugated wheat germ agglutinin (P-L Biochemicals, Inc., Milwaukee, Wis.) diluted 1:500 in TBS. The cells were rinsed and fixed for 10 min at room temperature in 3.7% formaldehyde. The cells were then permeabilized with 0.5% Nonidet P-40 in TBS for 2 min at room temperature. The slides were incubated with rat antitumor ascites fluid (diluted 1:50 in TBS and absorbed against methanol-fixed COS-1 cells) for 20 min at room temperature. The slides were rinsed thoroughly and stained with rhodamine-conjugated rabbit anti-rat serum (Cappel Laboratories, Downingtown, Pa.) diluted 1:200 in TBS. Photomicroscopy was carried out with a Nikon microscope and Tri-X film at ISO 400.

RESULTS

Construction of an MT antigen-VSV G protein hybrid gene. We used a cDNA clone of the polyomavirus MT antigen, pPyMT1 (32), and a cDNA clone of the VSV G protein, pGR125 (22), as starting materials. The pPyMT1 plasmid contains the entire polyomavirus genome, with the exception of the intron of the MT antigen. We replaced a C-terminal portion of the MT antigen coding region with a C-terminal portion of the G protein coding region as described above (Fig. 1). Briefly, we cleaved the polyomavirus DNA with *Sst*I at nucleotide 1,388 (numbering system of Friedmann et al., [8]), 41 nucleotides upstream of the MT hydrophobic region, and cleaved the VSV DNA with *Taq*I 35 nucleotides upstream of the G protein hydrophobic region. We removed one nucleotide at the site of *Taq*I cleavage to adjust the reading frame and added *Hin*dIII linkers to both DNAs. We isolated the fragment encoding the C-terminus of the G protein by cleaving the modified VSV DNA with *Hin*dIII and *Eco*RI and inserted this fragment into the polyomavirus DNA cleaved with *Hin*dIII and *Eco*RI. We analyzed the nucleotide sequence of the hybrid gene plasmid, pMT/G1, in the junction region to confirm the correctness of the reading frame. Figure 2 shows the nucleotide sequence in the junction region and shows how the coding regions were produced.

The hybrid gene was predicted to encode 379 amino acids of the N-terminal portion of the MT antigen, 4 amino acids derived from the *Hin*dIII linker, and 60 amino acids of the C-

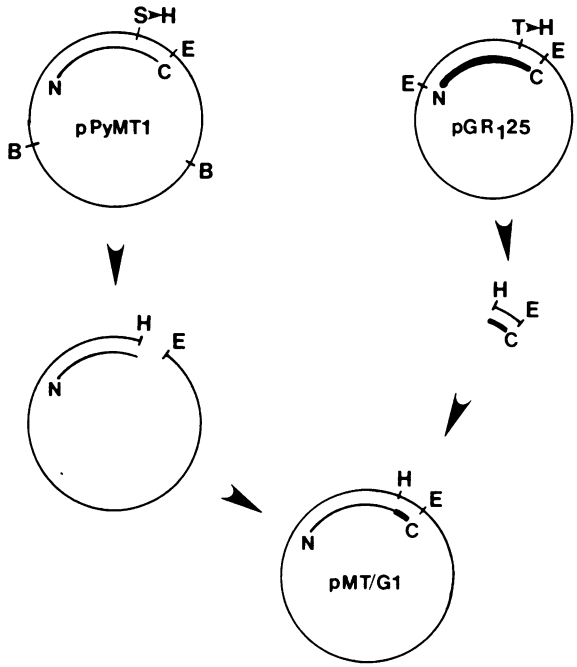

FIG. 1. Origin of coding regions for the MT-G hybrid protein. The coding region for the MT-G fusion protein was constructed by replacing the C-terminal coding region of the MT antigen in plasmid pPyMT1 by the C-terminal coding region of the VSV G protein which was derived from plasmid pGR125. The coding regions for the MT antigen and the G protein are represented by arcs within the respective plasmids. Synthetic *Hin*dIII linkers were inserted at one *Sst*I site in polyomavirus DNA and a *Taq*I site in VSV DNA upstream of the hydrophobic regions of the proteins. The small *Hin*dIII to *Eco*RI fragment from pGR125 was ligated to the large fragment from pPyMT1 to form plasmid pMT/G1 encoding the hybrid protein. Details are given in the text. Abbreviations: S, *Sst*I; B, *Bam*HI; H, *Hin*dIII; T, *Taq*I; E, *Eco*RI.

terminal portion of the G protein. Figure 3 shows the predicted C-terminal amino acid sequences of the MT antigen and the MT-G fusion protein, including the hydrophobic regions.

Expression of the MT-G fusion protein. We were unable to detect either the MT antigen or the MT-G fusion protein in NIH 3T3 cells after transfection with pPyMT1 or pMT/G1 DNA. Therefore, we used SV40 expression vector pJC119 (30) which has been used to express the VSV G protein (21) and the nucleocapsid protein N of VSV (30) in COS-1 cells. This vector utilizes the late promoter of SV40 for the expression of genes inserted at an *Xho*I site proximal to the initiation codon of the SV40 major capsid protein, VP1. Plasmid pJC119 replicates in COS-1 cells because of the presence of the SV40 large T antigen in these cells (10). The replication of the plasmid and the use of the SV40 late promoter favor a high level of expression of genes inserted into the vector.

We inserted DNA containing the complete coding sequences for the MT antigen or the MT-G fusion protein into pJC119 at the *Xho*I site, as described above. We used a DNA fragment including sequences between the *Nar*I site of polyomavirus (nucleotide 99), which is proximal to the MT initiation codon at nucleotide 188, and the *Bam*HI site (nucleotide 4,657) in the polyomavirus late region. In these constructions, the first initiation codon in the message is that of the MT antigen; therefore, neither the MT antigen nor the MT-G fusion protein produced by the expression vector should contain amino acids contributed by SV40.

Figure 4 shows immunoprecipitates of the MT antigen and the MT-G fusion proteins made in COS-1 cells analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Both proteins were immunoprecipitated with rat antitumor serum. The MT antigen had a greater electrophoretic mobility than the MT-G fusion protein (Fig. 4, lanes 6 and 7), which is consistent with the prediction that the MT-G fusion protein should be 22 amino acids larger than the MT antigen. Antiserum directed against a synthetic peptide identical to the C-terminus of the MT antigen precipitated the MT antigen but failed to precipitate the G protein or the MT-G fusion protein, as would be expected if the MT-G fusion protein had a different C-terminus (Fig. 4, lanes 8 through 10). The antipeptide serum also failed to precipitate a truncated MT protein produced by a nonsense mutant of the MT antigen, MOP 1033 (Fig. 4, lane 3), as reported previously (31). Therefore, the results of the immunoprecipitation analysis support the predicted structure of the MT-G fusion protein.

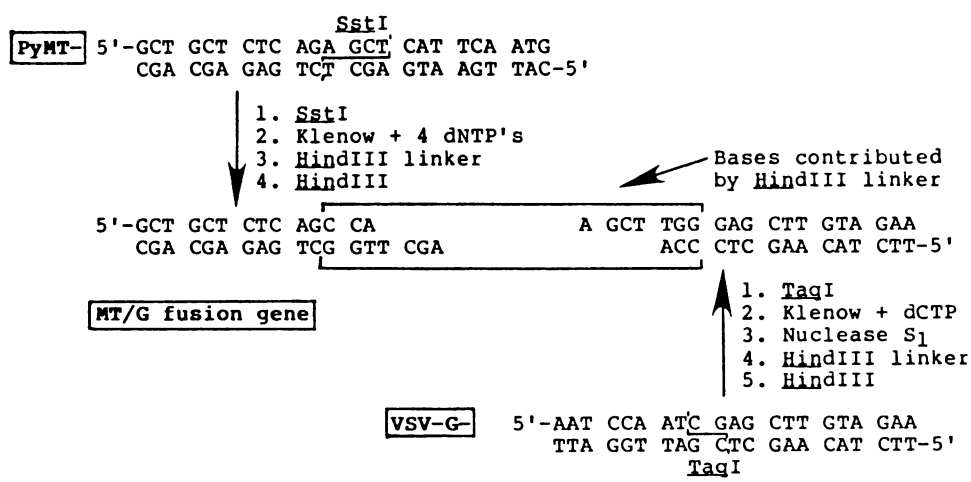

FIG. 2. Joining of the MT antigen and G protein coding regions. The nucleotide sequence of polyomavirus DNA around the *Sst*I site upstream of the hydrophobic region of the MT antigen is shown at the top of the figure. The sequence of VSV DNA around the *Taq*I site is shown at the bottom. The steps in constructing the fragments to be joined are numbered. The nucleotide sequence of the MT-G fusion protein at the junction is shown in the middle of the figure.

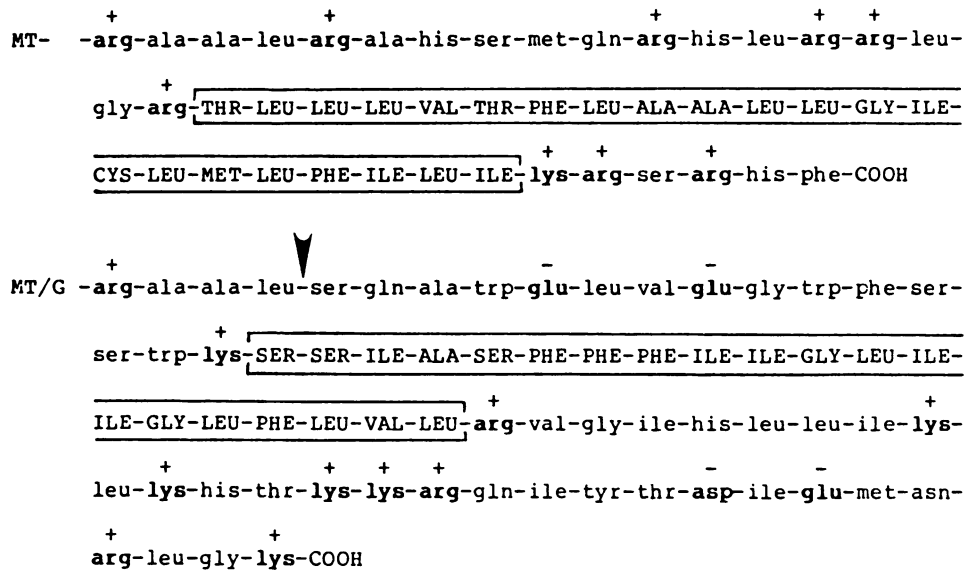

FIG. 3. Amino acid sequences of the C-terminal regions of the MT antigen and the MT-G hybrid protein. The predicted amino acid sequences for the C-termini of both proteins are shown. The hydrophobic regions are boxed. The junction between the MT antigen amino acid 379 and the linker and VSV sequences in the MT-G protein is indicated by an arrowhead.

Subcellular localization of the MT-G fusion protein. To determine whether the MT-G fusion protein became associated with cell membrane fractions, as did the authentic MT antigen, we transfected COS-1 cells with pMTSVL1 or pMT/GSVL1 DNA. We radiolabeled the cultures and lysed the cells by homogenization in hypotonic buffer containing EDTA as described above. We separated the extracts into particulate or soluble fractions by centrifugation. Both the immunoprecipitable MT-G fusion protein and the authentic MT antigen were found exclusively in the 40,000 × *g* pellet (Fig. 5). (Although the amount of the MT-G protein appeared to be less than the amount of the MT antigen in Fig. 5, the levels of the two proteins were similar in other experiments.)

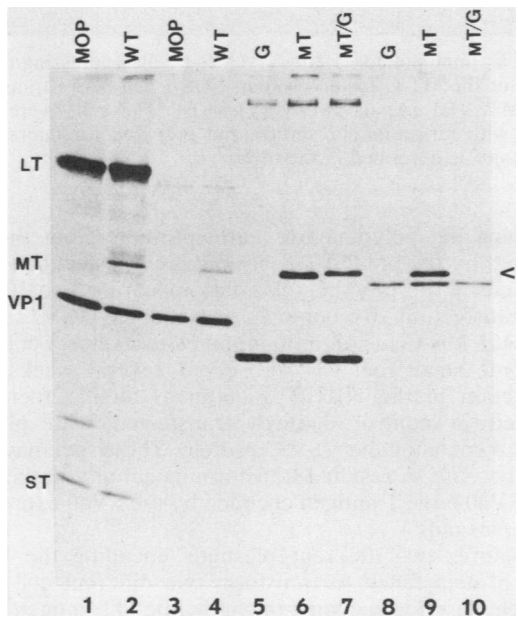

FIG. 4. Immunoprecipitation of the MT-G fusion protein expressed in COS-1 cells. COS-1 cells were transfected with plasmid pSVGL3 expressing the G protein (lanes 5 and 8), with pMTSVL1 expressing the MT antigen (lanes 6 and 9), and with pMT/GSVL1 expressing the MT-G hybrid protein (lanes 7 and 10). Mouse 3T6 cells were infected with wild-type polyomavirus (lanes 2 and 4) or with the MOP 1033 mutant, which causes premature termination of translation of the MT antigen (lanes 1 and 3). Radiolabeled cell extracts were precipitated with rat antitumor serum (lanes 1, 2, 5, 6, and 7) or with serum directed against a synthetic peptide corresponding to the six C-terminal amino acids of the MT antigen (lanes 3, 4, 8, 9, and 10). The positions of the MT antigen and the MT-G fusion protein, which migrates slightly slower, are indicated by an arrowhead at the right.

We also examined the transfected cells by immunofluorescence microscopy 24 h after infection. We stained the cells with fluorescein-conjugated wheat germ agglutinin before permeabilization to show plasma membranes and then with rat antitumor serum and rhodamine-conjugated anti-rat serum to show the MT antigen and the MT-G fusion protein. The fluorescence patterns of COS-1 cells expressing the MT-G fusion protein were indistinguishable from those of cells expressing the MT antigen (Fig. 6). In both cases, the stained cells had a clear nuclear ring and a perinuclear reticular staining, which is characteristic of the endoplasmic reticulum. Therefore, the cellular distribution of the MT-G fusion protein was, to the limits of sensitivity of this analysis, identical to the distribution of the MT antigen.

MT-G fusion protein is associated with a tyrosine-specific protein kinase activity. We transfected COS-1 cells with pMTSVL1 DNA and assayed immunoprecipitates of cell extracts for protein kinase activity capable of phosphorylating the MT antigen or the MT-G fusion protein, as described above. Figure 7A shows that both the MT antigen and the MT-G fusion protein were phosphorylated in the assay. We eluted the phosphorylated proteins from the gel, hydrolyzed them in acid, and separated the phosphoamino acids by two-dimensional, thin-layer electrophoresis to identify the phosphorylated residues (12). Both the MT-G fusion protein and the MT antigen contained only phosphotyrosine (Fig. 7B). The mobilities of the phosphorylated MT antigen and MT-G fusion protein were similar; the size difference in the non-phosphorylated proteins (Fig. 4) was not apparent in the phosphorylated proteins. The reason for this is not clear. It would be of interest to compare the sites of phosphorylation in the two proteins to determine whether differences in

phosphorylation might affect the relative mobilities of the proteins.

Plasmids encoding the MT-G fusion protein do not transform mouse or rat cells. We tested the ability of plasmids encoding the MT antigen or the MT-G fusion protein to transform mouse NIH 3T3 or rat F2408 cells by the calcium phosphate transfection procedure (33). (COS-1 cells cannot be used for this assay because they are already transformed.) Initially, we tested plasmid pPyMT1, which encodes the MT antigen, and plasmid pMT/G1, which encodes the MT-G fusion protein. In these plasmids, both proteins are under the control of the early promoter of polyomavirus. Table 1 shows the results of a typical experiment. The plasmid encoding the MT antigen produced foci 10 to 14 days after transfection, confirming the results reported previously (32). No foci were observed in cultures transfected with the plasmid encoding the MT-G fusion protein, even when the cultures were incubated for up to 8 weeks. We conclude that the efficiency of transformation by plasmid pMT/G1 was at least 50-fold lower than the efficiency of transformation by pPyMT1.

We also tested transformation by the plasmids encoding the MT antigen or the MT-G fusion protein under the control of the SV40 late promoter. The plasmid encoding the MT antigen produced foci in both cell types (Table 1). The frequency of transformation was approximately the same as the frequency of transformation by the pPyMT1 plasmid

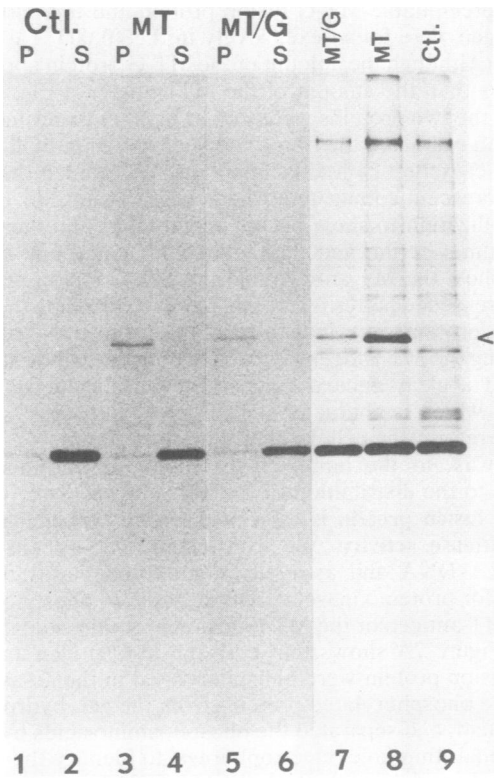

FIG. 5. Fractionation of cells expressing the MT antigen or the MT-G fusion protein. COS-1 cells were transfected with pSVGL3 (Ctl.), pMTSVL1 (MT), or pMT/GSVL1 (MT/G). Radiolabeled cell extracts were precipitated directly with rat antitumor serum (lanes 7, 8, and 9) or were fractionated into particulate (P) and soluble (S) fractions by centrifugation at 40,000 × *g* before precipitation. The locations of the MT antigen and the MT-G protein are indicated by the arrowhead at the right.

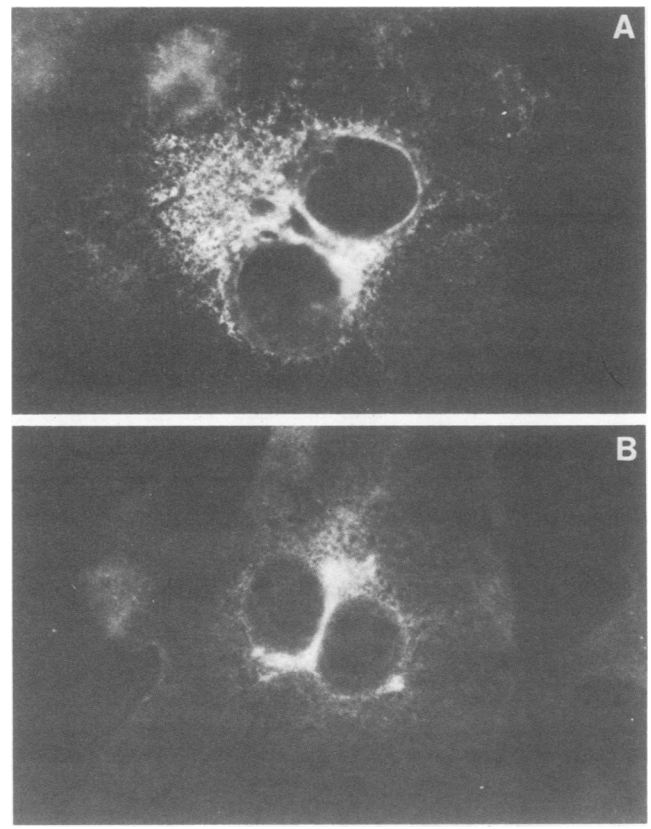

FIG. 6. Immunofluorescence of COS-1 cells expressing the MT antigen or the MT-G fusion protein. COS-1 cells were transfected with pMTSVL1 (A) or pMT/GSVL1 (B). The cells were fixed, treated with rat antitumor serum, and prepared for fluorescence microscopy as described in the text.

containing the polyomavirus early promoter, but the foci produced by the pMTSVL1 plasmid grew faster than those produced by the pPyMT1 plasmid, appearing 7 to 10 days after transfection. By contrast, plasmid pMT/GSVL1, encoding the MT-G fusion protein, failed to induce foci (Table 1). (Some small foci were observed several weeks after transfection in the NIH 3T3 cultures. Similar foci were observed in control cultures transfected with plasmid pSVGL3 encoding the VSV G protein. These foci may have arisen because of residual transforming activity in the truncated SV40 large T antigen encoded by the SV40 expression vector plasmids.)

Therefore, two different plasmids encoding the MT-G fusion protein failed to transform two different cell lines, although plasmids encoding the authentic MT antigen transformed the cell lines under similar conditions.

DISCUSSION

We replaced the C-terminal hydrophobic domain of the polyomavirus MT antigen with the C-terminal hydrophobic domain of the VSV G protein. The MT-G fusion protein was expressed in COS-1 cells under the control of the SV40 late promoter. The MT antigen and the MT-G fusion proteins had similar subcellular locations in cell membranes, as judged by immunofluorescence microscopy and fractionation of cell extracts. The MT-G fusion protein was associated in immunoprecipitates with a protein kinase activity which phosphorylated a tyrosine residue of the MT-G fusion protein.

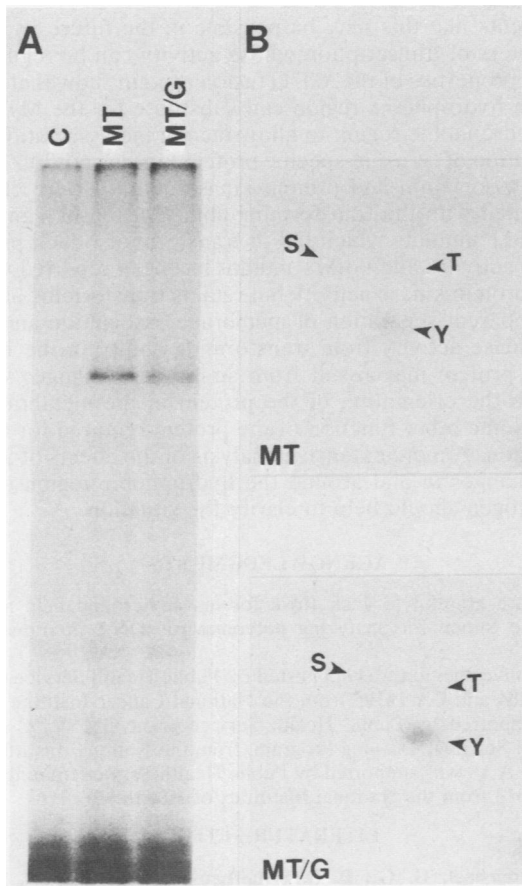

FIG. 7. Tyrosine protein kinase activity associated with the MT-G fusion protein. COS-1 cells were transfected with pSVGL3 (C), pMTSVL1 (MT), or pMT/GSVL1 (MT/G) DNA. Cell extracts were immunoprecipitated and assayed for protein kinase activity as described in the text. (A) Autoradiogram of the gel used to separate the phosphorylated proteins. (B) Phosphoamino acids of the MT antigen and the MT-G fusion protein analyzed by two-dimensional thin-layer electrophoresis. Abbreviations: S, serine; T, threonine; Y, tyrosine.

Although the MT antigen and the MT-G fusion protein were similar in these respects, plasmids encoding the MT antigen transformed mouse NIH 3T3 and rat F2408 cells, whereas similar plasmids encoding the MT-G fusion protein failed to do so. These results raise several questions about the role of the C-terminal hydrophobic domain of the MT antigen in membrane localization, associated protein kinase activity, and cell transformation.

How are the MT antigen and the MT-G fusion proteins associated with cell membranes? In the case of the VSV G protein, the C-terminal hydrophobic region serves as a membrane-spanning domain. The G protein is inserted into the membrane by an N-terminal "signal sequence" which subsequently is cleaved, leaving the G protein spanning the membrane with its N-terminus on one side and its C-terminus on the other. The main function of the C-terminal hydrophobic region appears to be to anchor the protein in the membrane. The MT antigen and the MT-G fusion proteins have no N-terminal signal sequences. Perhaps some feature of the N-terminal portion of the MT antigen contained in the MT-G fusion protein, such as an internal signal sequence (16) or a helical hairpin (7), is involved in the association of the protein with the membrane. In the case of the MT antigen, it is not known whether the C-terminal hydrophobic region acts as a membrane-spanning domain, as it does in the G protein. Our observations show that the MT antigen and the MT-G fusion protein are associated with cell membranes, but subtle changes in membrane association easily could have escaped detection by our assays. Because C-terminal sequences affect the localization of the VSV G protein (21), it would be of interest to make a more detailed comparison of the localization of the MT antigen and the MT-G fusion protein.

By immunofluorescence microscopy, we found that the MT antigen and the MT-G fusion protein had intracellular distributions characteristic of the endoplasmic reticulum. This result is in agreement with observations on the location of the MT antigen obtained by immunoelectron microscopy (S. Dilworth, personal communication). However, the MT antigen was previously shown to be present in membrane fractions having the buoyant density of the plasma membrane (14, 25; unpublished data). The reason for the difference in the apparent location of the MT antigen is not clear. One possible explanation is that the MT antigen—if it is present in the endoplasmic reticulum—is present in a fraction which has a lower density than the majority, e.g., the smooth endoplasmic reticulum or a region of the rough endoplasmic reticulum that is devoid of ribosomes. Another possibility is that the locations of the proteins are changed in cells producing large amounts of protein, such as COS-1 cells transfected with the SV40 expression vector.

What is the relationship between the C-terminal hydrophobic region and the tyrosine-specific protein kinase activity associated with the MT antigen and the MT-G fusion protein? The C-terminal hydrophobic region seems to be necessary for the appearance of the activity associated with the MT antigen, because proteins lacking the hydrophobic region, owing to premature termination of translation, do not show the activity (1, 31; unpublished data). However, the amino acid sequence of the region can vary because the G protein hydrophobic region can substitute for the MT antigen hydrophobic region in allowing the activity to be expressed. It seems likely that a hydrophobic region is required for membrane association, and membrane association in turn is required for appearance of the protein kinase activity.

TABLE 1. Transforming activity of plasmids encoding the MT antigen and the MT-G fusion protein

Plasmid	No. of transformed foci[a]	
	NIH 3T3 cells	F2408 cells
pAT153[b]	0	0
pSVGL3[c]	9[d]	0
pPyMT1	59	42
pMT/G1	0	0
pMTSVL1	114	49
pMT/GSVL1	5[d]	0

[a] Mouse NIH 3T3 or rat F2408 cells were seeded at a density of 10^5 cells per 6-cm plate and transfected with 2.5 μg of the indicated plasmid DNA as described in the text. The numbers of foci are the total number on three plates.

[b] Plasmid pAT153 is the parental plasmid of pPyMT1 and pMT/G1.

[c] Plasmid pSVGL3 encodes the VSV G protein.

[d] Small foci appearing after prolonged incubation (see text).

Prematurely terminated MT antigens lacking the hydrophobic region do not show protein kinase activity, but proteolytic fragments, derived from membrane-associated MT antigens, do show activity, even though they no longer have the hydrophobic region (1). This suggests that the protein kinase activity is acquired by the membrane-associated MT antigen. One possibility is that the protein kinase activity is intrinsic to the MT antigen, and membrane association is required to activate it. Alternatively, the protein kinase could be a membrane-associated cellular enzyme which binds to the MT antigen in the membrane. Two cellular tyrosine kinases are known to be membrane associated, the cellular homolog of the Rous sarcoma virus transforming protein, $pp60^{c\text{-}src}$ (3), and the epidermal growth factor receptor protein kinase (2). The $pp60^{c\text{-}src}$ protein kinase is found in association with the MT antigen (4). The presence of the hydrophobic region is not in itself sufficient for the appearance of the protein kinase activity because some *hr-t* mutants with intact hydrophobic regions lack kinase activity (6, 24) and because MT antigens with intact hydrophobic regions, but lacking 70 or 106 amino acids at their N-termini, become associated with cell membranes but do not show protein kinase activity (unpublished data).

Why do plasmids encoding the MT-G protein fail to cause cell transformation? The MT antigen and the MT-G fusion protein differ in several respects. The hydrophobic region of the MT antigen is 22 amino acids long, whereas the hydrophobic region of the MT-G fusion protein is 20 amino acids long. Both regions allow membrane association, and it seems unlikely that the difference in length itself would drastically alter the function of the protein. The MT antigen has 6 amino acids at its C-terminus distal to the hydrophobic region, whereas the MT-G fusion protein has 29 amino acids. An altered MT antigen with a deletion of 2 amino acids at its C-terminus, followed by an additional 11 amino acids encoded by a plasmid vector, retains 8% of its normal transforming activity (19); therefore, some flexibility in the length of the sequence distal to the hydrophobic region can be tolerated. The MT antigen and the MT-G fusion protein also differ in the amino acid sequences immediately surrounding the hydrophobic regions (see Fig. 3). Two lines of evidence suggest that these amino acids are important for the function of the MT antigen in transformation. Removal of the amino acids bordering the MT antigen on the C-terminal side by deletion reduces transforming activity (19). A mutant of polyomavirus which is cold sensitive for transformation has an amino acid change two residues upstream of the hydrophobic region (D. Templeton and W. Eckhart, J. Virol., in press). Therefore, it is possible that plasmids encoding the MT-G protein fail to transform cells because the protein does not have the appropriate "anchor" residues bordering the hydrophobic region. This possibility could be examined by attempting to restore transforming activity to the MT-G encoding plasmids by making site-specific alterations in the amino acid residues surrounding the hydrophobic region.

Another possibility is that the MT-G fusion protein is not expressed at sufficiently high levels to cause transformation. We isolated cells containing the MT-G encoding plasmid which show normal growth properties, but we were unable to measure the amounts of the MT-G protein expressed in them. Therefore, we cannot rule out quantitative differences as the explanation for the lack of transformation. In any case, it is not clear how much of the authentic MT antigen is required for transformation because the relationship between the level of expression of the MT antigen and transformed cell growth properties has not been measured. Measurements like this may be possible in the future by using promoters of transcription whose activity can be regulated.

The properties of the MT-G fusion protein show that the G protein hydrophobic region can substitute for the MT antigen hydrophobic region to allow membrane association and acquisition of tyrosine-specific protein kinase activity. Membrane association and protein kinase activity were closely correlated with the transforming ability of the MT antigen. Most MT mutants which fail to transform also lack protein kinase activity, and no MT mutant has been reported which lacks protein kinase activity but retains transforming ability. The apparent separation of membrane association and protein kinase activity from transforming ability in the MT-G fusion protein may result from amino acid changes which perturb the orientation of the protein in the membrane or affect some other function of the protein required for transformation. A more extensive analysis of the effects of amino acid changes in and around the hydrophobic region of the MT antigen should help to clarify the situation.

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LITERATURE CITED

1. **Carmichael, G. G., B. S. Schaffhausen, D. I. Dorsky, D. B. Oliver, and T. L. Benjamin.** 1982. Carboxy terminus of polyoma middle-sized tumor antigen is required for attachment to membranes, associated protein kinase activities, and cell transformation. Proc. Natl. Acad. Sci. U.S.A. **79**:3579–3583.
2. **Cohen, S., G. Carpenter, and L. R. King.** 1980. Epidermal growth factor receptor-protein kinase interactions. J. Biol. Chem. **255**:4834–4842.
3. **Courtneidge, S. A., A. D. Levinson, and J. M. Bishop.** 1980. The protein encoded by the transforming gene of avian sarcoma virus ($pp60^{src}$) and a homologous protein in normal cells ($pp60^{proto\text{-}src}$) are associated with the plasma membrane. Proc. Natl. Acad. Sci. U.S.A. **77**:3783–3787.
4. **Courtneidge, S. A., and A. E. Smith.** 1983. Polyoma virus transforming protein associates with the product of the c-src cellular gene. Nature (London) **303**:435–439.
5. **Ding, D., S. M. Dilworth, and B. E. Griffin.** 1982. *mlt* mutants of polyoma virus. J. Virol. **44**:1080–1083.
6. **Eckhart, W., M. A. Hutchinson, and T. Hunter.** 1979. An activity phosphorylating tyrosine in polyoma T antigen immunoprecipitates. Cell **18**:925–933.
7. **Engelman, D. H., and T. A. Steitz.** 1981. The spontaneous insertion of proteins into and across membranes: the helical hairpin hypothesis. Cell **23**:411–422.
8. **Friedmann, T., A. Esty, P. LaPorte, and P. Deininger.** 1979. The nucleotide sequence and genome organization of the polyoma early region: extensive nucleotide and amino acid sequence homology with SV40. Cell **17**:715–724.
9. **Gething, M. J., and J. Sambrook** 1982. Construction of influenza hemagglutinin genes that code for intracellular and secreted forms of the protein. Nature (London) **300**:598–603.
10. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
11. **Griffin, B. E., and C. Maddock.** 1979. New classes of viable deletion mutants in the early region of polyoma virus. J. Virol. **31**:645–656.
12. **Hunter, T., and B. M. Sefton,** 1980. The transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc.

Natl. Acad. Sci. U.S.A. **77**:1311–1315.
13. **Hutchinson, M. A., T. Hunter, and W. Eckhart.** 1978. Characterization of T antigens in polyoma-infected and transformed cells. Cell **15**:65–77.
14. **Ito, Y., J. Brocklehurst, and R. Dulbecco.** 1977. Virus-specific proteins in the plasma membrane of cells lytically-infected or transformed by polyoma virus. Proc. Natl. Acad. Sci. U.S.A. **74**:4666–4670.
15. **Ito, Y., N. Spurr, and B. E. Griffin.** 1980. Middle T antigen as primary inducer of full expression of the phenotype of transformation by polyoma virus. J. Virol. **35**:219–232.
16. **Lingappa, V. R., J. R. Lingappa, and G. Blobel.** 1979. Chicken ovalbumin contains an internal signal sequence. Nature (London) **281**:117–121.
17. **Magnusson, G., and P. Berg.** 1979. Construction and analysis of viable deletion mutants of polyoma virus. J. Virol. **32**:523–529.
18. **Nilsson, S. V., C. Tyndall, and G. Magnusson.** 1983. Deletion mapping of a short polyoma virus middle T antigen segment important for transformation. J. Virol. **46**:284–287.
19. **Novak, U., and B. E. Griffin.** 1981. Requirement for the C-terminal region of middle T antigen in cellular transformation by polyoma virus. Nucleic Acids Res. **9**:2055–2073.
20. **Rassoulzadegan, M., A. Cowie, A. Carr, N. Glaichenhaus, R. Kamen, and F. Cuzin.** 1982. The role of individual polyoma virus early proteins in oncogenic transformation. Nature (London) **300**:713–718.
21. **Rose, J. K., and J. E. Bergmann.** 1983. Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. Cell **34**:513–524.
22. **Rose, J. K., and C. J. Gallione.** 1981. Nucleotide sequences of the mRNA's encoding the vesicular stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. J. Virol. **39**:519–528.
23. **Rose, J. K., W. J. Welch, B. M. Sefton, F. S. Esch, and N. C. Ling.** 1980. Vesicular stomatitis virus glycoprotein is anchored in the viral membrane by a hydrophobic domain near the COOH terminus. Proc. Natl. Acad. Sci. U.S.A. **77**:3884–3888.
24. **Schaffhausen, B. S., and T. L. Benjamin.** 1979. Phosphorylation of polyoma T antigens. Cell **18**:935–946.
25. **Schaffhausen, B. S., H. Dorai, G. Arakere, and T. L. Benjamin.** 1982. Polyoma virus middle T antigen: relationship to cell membranes and apparent lack of ATP-binding activity. Mol. Cell. Biol. **2**:1187–1198.
26. **Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart.** 1980. Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. Cell **20**:807–816.
27. **Silver, J., B. Schaffhausen, and T. L. Benjamin.** 1978. Tumor antigens induced by nontransforming mutants of polyoma virus. Cell **15**:485–496.
28. **Smith, A. E., R. Smith, E. Griffin, and M. Fried.** 1979. Protein kinase activity associated with polyoma virus middle T antigen in vitro. Cell **18**:915–924.
29. **Soeda, E., J. R. Arrand, N. Smolar, J. E. Walsh, and B. E. Griffin.** 1980. Coding potential and regulatory signals of the polyoma virus genome. Nature (London) **283**:445–453.
30. **Sprague, J., J. H. Condra, H. Arnheiter, and R. A. Lazzarini.** 1983. Expression of a recombinant DNA gene coding for the vesicular stomatitis virus nucleocapsid protein. J. Virol. **45**:773–781.
31. **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.
32. **Triesman, 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.
33. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **76**:1373–1376.