Expression of biologically active middle T antigen of polyoma virus from recombinant baculoviruses

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ABSTRACT

Two different recombinant baculoviruses have been generated for expressing the middle T antigen (MT) of polyoma virus in insect (Sf9) cells. One (pAcI-PyMT) produces moderate levels of MT and the other (pVL-PyMT) high levels. Indirect immunofluorescence and cellular fractionation studies with pAcI-PyMT infected Sf9 cells give results similar to those observed with wild type polyoma virus infected mouse cells, and show MT to be mainly associated with cytoplasmic membranes in the insect cell. In the latter, a sub-population of MT is phosphorylated in *in vitro* protein kinase assays. The yields of MT from pVL-PyMT infected cells are high enough to suggest that this protein can now be produced by this method in sufficient amounts for definitive biochemical and crystallographic analyses.

INTRODUCTION

Middle T-antigen (MT), the major oncogene of polyoma virus, is a membrane-associated protein (1,2). Alone, it can transform established rodent cell lines. In combination with other oncogenes, including an "immortalizing function" localized in a second polyoma virus oncogene the large T-antigen (LT), it can transform primary rodent cells and produce tumours in susceptible animals (as reviewed, 3). No separate function corresponding to MT has yet been identified in other members of the papovavirus group. Few, if any, enzymatic activities of this protein have been identified to date. However, numerous studies carried out to assess the biological rôle of MT in altering both the cellular pheno- and genotype have demonstrated interactions between the polyoma virus antigen and other cellular proteins, including pp60^{C-src} (in mammalian cells), p62^{C-yes} (in chick cells) and an 85kD phosphatidylinositol kinase (PI) activity (as reviewed, 4). Further protein studies have revealed associations between MT and other, as yet, unidentified cellular proteins (5,6). As well as these protein/protein interactions, the cellular location and membrane binding region of MT are thought to be important in its transforming properties; mutations that affect any of these either reduce or abolish the capability of MT to transform cells (7-9).

Several things are apparent from further studies on MT: First, small amounts only of the antigen are present in infected cells in its natural host (mouse) or in polyoma-virus induced rodent tumours. Secondly, it is not a single biochemical entity. At least two species

exist with different electrophoretic mobilities (10,11) and studies with monoclonal antibodies reveal several immunologically distinct populations (12-13). Finally, only a small proportion of MT interacts with proteins such as pp60^{C-src} and PI kinase; the biological rôle of the remainder of MT is unknown.

One of the major problems hampering studies on MT is the difficulty of obtaining abundant amounts of biologically active protein. Here we show that large quantities of soluble MT, as a non-fused gene product, can be generated from baculovirus vectors. Further, a portion of this antigen (but not all) is phosphorylated in infected insect cells in an *in vitro* protein kinase assay either in complex with homologues of mammalian protein kinases (14) or by an autocatalytic mechanism (11). Our data suggest that with regard to some of its properties MT generated from baculoviruses resembles its mammalian counterpart.

MATERIALS AND METHODS

The recombinant viruses expressing the polyoma middle T antigen (MT) were constructed using the Autographa californica nuclear polyhedrosis virus (AcNPV) cloning system and the corresponding bacterial transfer vectors, as described by Summers and coworkers (15,16).

Bacteria: Transfer vectors and recombinant plasmids were propagated in E. coli strain DHI (17). E. coli strain JM101 (18) was used in sequencing procedures. E. coli strain TG2 (F', recA⁻, T. Gibson, personal communication) was used for cloning in the Bluescribe (Stratagene) M13+ vector. The bacteria were cultivated and transformed as described by Maniatis et al. (19).

Plasmids: The transfer vectors pAc373 (20) and pVL941 (21; Summers, personal communication) with the polyhedrin promoter and BamHI cloning site were used to construct recombinant plasmids where polyoma MT was placed under the control of the polyhedrin promoter. Vector pAc373 contains a deletion between the -8 position (8 bases upstream from the polyhedrin ATG initiation codon) and the natural BamHI site at nucleotide +171 of the polyhedrin gene. An additional 9 bases (CGAGATCCG) were inadvertently introduced into the construct together with the BamHI linker, between nucleotides -8 and +171 (22). The transfer vector pAcI originated from the pAc373 vector by replacing the BamHI - KpnI fragment (position -8 to +640 of the polyhedrin gene) with the polylinker BamHI-BglII-EcoRI-PstI-KpnI (a gift from M. Jones). In the pVL941 vector all the upstream sequences and the first 35 coding nucleotides of the polyhedrin gene are retained, but the ATG codon has been changed by point mutation to ATT (21). The sequences of the transfer vectors in the region around the polyhedrin ATG, compared with those of wild type AcNPV, are shown in Fig. 1A.

Plasmid pPyMTE, containing the gene for polyoma virus middle T antigen, is derived

from the BamHI -EcoRI fragment of the polyoma genome (map position 58 to 100 units, nucleotides 4631 to 1560; ref 23) inserted into the pAT153 plasmid; the intron of the MT gene (61 nucleotides long) was removed by site specific mutagenesis (24). The insertion containing the gene for MT antigen starts at the BstXI site at nucleotide 165 and ends 60 nucleotides before the EcoRI site. In order to construct recombinant viruses for expressing polyoma virus MT in insect cells, the intronless MT gene (equivalent to cDNA) was removed from the pPyMTE plasmid (24) and recloned into transfer vectors pAcI and pVL941 (15,16,21). To generate the plasmid pAcI-PyMT (Fig. 1B), pPyMTE was linearized with BstXI and the 3'-protruding ends were removed by the exonucleolytic activity of T4 DNA-polymerase. The linearized plasmid was then digested with EcoRI and the 1300 base pair fragment containing the complete intronless MT gene subcloned in the Bluescribe vector to insert a BamHI cloning site in front of the MT initiation ATG codon. The MT gene was removed from the Bluescribe plasmid with BamHI and EcoRI, and ligated with the pAcI transfer vector which had been completely cleaved with BamHI and partially cleaved with EcoRI. Competent bacteria were transformed with the ligation mixture and the plasmid pAcI-PyMT was selected. The recombinant plasmid pVL-PyMT (Fig. 1C) was obtained by replacing the BamHI-KpnI fragment of the pVL941 transfer vector with the BamHI-KpnI fragment of the plasmid pAcI-PyMT. Detailed construction maps are available on request.

Plasmid DNAs were isolated using the NaOH-SDS procedure of Birnboim and Doly (25), followed by CsCl-ethidium bromide gradient centrifugation. The recombinant plasmids which were destined for cotransfection of insect cells were treated with proteinase K as described by Summers and Smith (16).

Sequencing of the recombinant vector: In order to verify the DNA sequence of recombinant plasmid pAcI-PyMT at the junction of the polyhedrin promoter and the MT gene, it was cleaved with EcoRV (-98bp. upstream from the polyhedrin initiator codon) and PstI (+311bp. downstream from the initiator codon of the MT gene). The fragment was subcloned in the SmaI digested vector M13mp9, and sequenced using the dideoxy chain termination method of Sanger et al. (26) and the 17-mer M13 universal oligonucleotide (New England BioLabs) as a sequencing primer.

Cells and viruses

Spodoptera frugiperda (Sf9) cells were propagated as monolayer cultures at 27°C using TNM-FH medium, supplemented with 10% foetal bovine serum as described by Hink (27). Stocks of wild-type virus AcNPV were prepared as follows: A Falcon flask (175cm²) was seeded with 20ml of a suspension containing 2×10^7 cells; the suspension was prepared by scraping off the cells from a culture that had just reached confluence. After a one hour incubation, by which time the cells were attached, medium was removed and the cells were infected with 4ml of virus inoculum at about 1×10^8 plaque forming units/ml. To allow absorption of the virus, the cells were incubated for 60 - 90 min with occasional gentle

rocking of the flask. The fluid was then replaced with 30ml fresh complete medium. The extracellular virus was harvested after four days of incubation at 27°C and the virus suspension was clarified by centrifugation at 1000 x g for 10 min.

The virus titre was determined by plaque assay: Log phase Sf9 cells were seeded into Nunc tissue culture dishes (60 x 15mm) using 2×10^6 cells per plate, in TNM-FH medium without foetal bovine serum. After the cells were attached, the medium was removed and 0.5ml of dilute virus was added. The plates were incubated for 60 - 90 min with occasional rocking to achieve absorption. The liquid was then removed and the layer of cells overlaid with 4ml 1.5% agarose (Sea Plaque, low melting) in complete medium at 37°C. The dishes were placed on trays and sealed in plastic bags together with dampened paper towels to provide humidity and incubated at 27°C. The plaques were well formed after about five days.

Stocks of recombinant viruses were prepared by picking a single pure plaque (see below) and transferring it into a 25cm² flask which had been pre-seeded with 1×10^6 Sf9 cells. The virus was harvested after five days of incubation at 27°C.

Cotransfection: Recombinant baculoviruses with the MT gene under control of the polyhedrin promoter were prepared by allelic exchange between wild type AcNPV DNA and the DNA of recombinant plasmids pAcI-PyMT or pVL-PyMT. Total DNA from AcNPV infected Sf9 cells was used as a source of viral DNA sequences for cotransfection. It was prepared from about 2×10^6 infected cells (60 - 72 hrs post-infection, p.i.), lysed with 5ml lysis buffer (0.3M Tris, pH 7.5, 0.01M Mg-acetate, 1% Nonidet P40) for 5-10 min on ice, with occasional vortexing. The nuclei were collected by low-speed centrifugation (1000 x g, 5 min). The pellet was washed in cold phosphate buffered saline (PBS). The nuclei were then incubated with proteinase K (200µg) in 4.5ml TE buffer (10mM Tris, pH 8.0, 1mM EDTA) for 1 hr at 50°C. After addition of 10% sarcosyl (0.5ml), the incubation was continued for 3 hrs. DNA was deproteinated by extraction with an equal volume of phenol and chloroform-isoamyl alcohol, precipitated with ethanol and dissolved in TE buffer.

Transfection was performed as follows: 2.5×10^6 Sf9 cells were transferred into a 25cm² flask. After cell attachment, the medium was replaced with 2ml of TNM-FH medium supplemented with 10% foetal bovine serum. 1ml of transfection mixture [2µg DNA from AcNPV-infected cells, 4µg pAcI-PyMT or pVL-PyMT plasmid DNA, 950µl HEBS buffer, pH 7.05 (0.137 M NaCl, 6mM D (+) glucose, 5mM KCl, 0.7 mM Na₂HPO₄, 20mM HEPES) and 50µl 2.5 M CaCl₂] was added and the cells were incubated at 27°C for 4 hrs. The transfection mixture was then replaced with fresh complete medium. After 5 - 6 days of incubation at 27°C, the medium was clarified by low speed centrifugation (1000 x g, 10 min) and plated at different dilutions on fresh cell monolayers for plaque screening.

Screening of recombinant plaques: Plaques containing the desired recombinant virus were detected by combining visual screening with either plaque hybridization (16) or plaque

immunoassay. For visual screening, the cell layer was illuminated using the room fluorescent light as the only light source and observed at low magnification on an inverted microscope. Plaque immunoassay was performed in the following manner: The agarose and the plate were marked for orientation using a Pasteur pipette for the agarose and a fine-point pen for the plate. The agarose layer was carefully transferred to a larger dish and stored at 4°C. The cells remaining on the original dish were fixed with acetone-methanol (1 : 1, v/v) for 3 min and dried. To prevent nonspecific antibody binding, the fixed cells were saturated by treatment with MPT solution (5% solution of commercial dry milk in PBS containing 0.05% Tween 20). Dishes were gently shaken for 1 hr at room temperature. Milk solution was poured off and the cells were washed 3 times with PBS by shaking for a minimum of 3 x 20 min. MT16 monoclonal rat antibody tissue culture fluid (1ml) (10), diluted with MPT solution (4ml), was added to the washed cells. After shaking (1hr), the cells were again washed 3 times with PBS, as above, then incubated with a solution of rabbit anti-rat antibody-peroxidase conjugate (DAKOPATTS, diluted 1 : 1000) in MPT solution. Plaques were washed (3x) with PBS, then stained with a freshly prepared mixture of DAB (3,3'-diaminobenzidine tetrahydrochloride) (0.4mg/ml) in phosphate-citrate buffer, pH 5.0, containing 0.01% H₂O₂. The staining was examined by microscopy. As soon as the plaques turned brown (usually 1 - 2 min), the peroxidase reaction was stopped by rinsing with water.

Protein blot immunoassay: Sf9 cells were harvested at different times p.i. and resuspended in protein sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 62.5mM Tris-HCl, 0.01% bromophenol blue, pH 6.8). After boiling (5 min), the proteins were resolved on 10% SDS-PAGE as described by Laemmli (28). The proteins were transferred by electroelution onto a nitrocellulose membrane filter, the filter washed (10 min) in PBS containing 0.05% Tween 20, then incubated with MPT solution (1 hr) with gentle shaking. The filter was cut into strips corresponding to different time points and these were incubated in plastic bags with the MT16 monoclonal antibody (as above) for 1 hr. The strips were washed 3 times for 20 min with PBS - 0.05% Tween 20, then incubated with the monoclonal antibody OX 12 (anti rat K light chain antibody, Sera-lab) that had been diluted 500 times with MPT solution. After washing (as above), strips were incubated (1 hr) with rabbit anti-mouse antibody - peroxidase conjugate (DAKOPATTS, 1000 times diluted in MPT), then washed and stained with DAB as described above.

Labelling of proteins: Sf9 cells (10⁶ cells per well) were transferred to a six-well dish and infected with virus (multiplicity of 10/cell). At different time intervals, the infected cells were incubated for 1 hr in methionine-free Grace's medium (GIBCO) to deplete the intracellular pool of methionine. The methionine-free medium was replaced with 2ml fresh methionine-free Grace's medium containing 100μCi [³⁵S]-methionine (1000 Ci/mmol). After 3 hrs incubation, cells were washed with cold PBS and proteins from lysed cells

analysed either directly by SDS PAGE (28), or by immunoprecipitation with MT16 antibody followed by SDS PAGE, then detected by autoradiography.

Immunoprecipitation of MT: About 10^6 labelled cells were washed with PBS and resuspended in 0.1 ml of lysis buffer (150mM NaCl, 5mM EDTA, 50mM Tris, pH 7.4, 0.05% Nonidet P40, containing Aprotinin (SIGMA), 0.3TIU/ml, and the proteolytic inhibitor, PMSF, 2 μ g/ml). To remove non-specific binding, a half volume of a 20% (v/v) suspension of fixed *Staphylococcus aureus* Cowan I strain (SAC) were added to the lysate at 0°C and after 30 min, the mixture was centrifuged for 5 min. (Supernatant could be stored in liquid nitrogen for subsequent use.) Supernatant (30 μ l) was incubated on ice for 1 hr with MT16 monoclonal antibody (10 μ l) and 400 μ l NET buffer (150mM NaCl, 5mM EDTA, 50mM Tris, pH 7.4, 0.05% NP40) in 0.2 % gelatin (SIGMA). SAC (25 μ l) treated with OX 12 monoclonal antibody (1 μ l) was added. After 30 min, the SAC immunoprecipitates were diluted with NET buffer (10ml), sedimented by centrifugation and washed 2 times by resuspending in RIPA buffer (NET buffer supplemented with 1% deoxycholic acid, 1% Triton X100, and 0.1% SDS). SAC sediment was then resuspended in protein sample buffer and boiled for 5 min. After centrifugation the supernatant was analysed by SDS-PAGE and autoradiography.

Immunofluorescence: All operations were carried out at room temperature. Infected cells were washed with PBS and fixed with acetone-methanol 1 : 1 (v/v). Nonspecific antibody-binding sites were blocked by incubation with PBS containing 0.25% gelatin and 0.25% bovine serum albumin (BSA) (30 min). The cells were incubated with MT16 antibody (30 min) then with fluorescein-conjugated rabbit anti-rat IgG (DAKOPATTS) (30 min) diluted 50 times with PBS. After each incubation the cells were extensively washed in PBS, then mounted in 90% glycerol-10% PBS containing 1mg/ml p-phenylenediamine. Cells expressing MT were visualized by fluorescence microscopy.

Subcellular fractionation: Cytoplasm and cytoplasmic membranes were isolated essentially as described by Courtneidge et al. (29). Briefly, the cells were suspended in PBS, centrifuged (1000 x g, 5 min), washed once with PBS, and resuspended in hypotonic solution (20mM HEPES, 50mM KCl, 1mM MgCl₂, pH 7.4) at ca. 2×10^7 cells/ml, then incubated 5 - 10 min on ice. After cell breakage by Dounce homogenization (15 strokes in a tight-fitting pestle), the nuclei and unbroken cells were removed by centrifugation (1000 x g) for 5 min. The homogenate was then fractionated by centrifugation (100 000 x g, 30 min) to separate cytoplasm (supernatant) from membranes (pellet). For immunoprecipitation, the supernatant was diluted with 1 volume of 2x lysis buffer (see above) prior to incubation with the antibody. Membranes were resuspended in lysis buffer.

In vitro protein kinase assay: Lysates of whole cells, or of membranes, were immunoprecipitated as described above. The SAC precipitates were washed 3 times with TBS buffer (150mM NaCl, 50mM Tris-HCl, pH 7.4, 0.05% NP40). Each pellet was

FIGURE 1:**A. Sequences of transfer vectors pAc373, pAcI and pVL941, compared with wild type baculovirus AcNPV**

The 5'-proximal leader sequence and part of the polyhedrin coding sequences of AcNPV are presented (15,16). Deletions in homologous regions of transfer vectors are marked. Insertions and a point mutation in the ATG codon of pVL941 (21) vector are given in lower case letters.

B. Schematic diagram showing the nucleotide sequence flanking the polyoma virus MT gene in the recombinant plasmid pAcI-PvMT

The polyhedrin leader sequence (-8 to -30), the intronless MT gene (+1 to +1266) and the junction between polyoma virus and AcNPV sequence, as determined by DNA sequencing analysis, are indicated. Compared with wild type polyoma virus, the 3'-terminal seven nucleotides preceding the initiation ATG codon are replaced by 18 nucleotides, of which the two cytosines between the BamHI site and the ATG come from the Bluescribe polylinker, and the other 16 nucleotides, containing the BamHI site, from the pAc373 vector. Translation initiation and termination codons of the MT gene are underlined.

C. Schematic diagram of the nucleotide sequences flanking the MT gene in the recombinant plasmid pVL-PvMT

The polyhedrin leader sequence (-1 to -30), polyhedrin and polyoma virus MT coding sequence, and the junction between polyoma virus and AcNPV sequence, are indicated. The mutated initiation codon of polyhedrin translation and the translation initiation and termination codons of the MT gene are underlined.

FIGURE 2:

Autoradiograms of proteins synthesized in insect cells infected with the recombinant virus AcI-PvMT(A) or with wild type AcNPV (B)

Sf9 cells were labelled with [³⁵S]- methionine for 3 hrs at the indicated times (0 - 60 hrs) p.i. Total lysates of labelled cells were resolved on SDS-PAGE as described in Materials and Methods. Locations of middle T-antigen (MT), an unidentified 25kD ($\triangle$) species and the polyhedrin protein (P) are indicated.

resuspended in 20mM Tris-acetate, pH 7.4, 5mM magnesium acetate (20 μ l). γ [³²P]-ATP (5 μ Ci, 5000Ci/mmol, Amersham) was added and the mixture was incubated at 30°C (15 min). The immunocomplexes were washed with ice-cold NET and RIPA buffers (see above), resuspended in protein sample buffer and incubated for 15 min at room temperature. The SAC was removed by centrifugation, the supernatant boiled 5 min and proteins separated on SDS-PAGE. Dry gels were autoradiographed for 1 - 3 days.

RESULTS AND DISCUSSION

Construction of the recombinant plasmids pAcI-PvMT and pVL -PvMT

The plasmids were constructed as described in Materials and Methods. Their composition, as determined by DNA sequence analysis, is given in part in Figs. 1B and C, respectively.

Transfer of the polyoma virus MT gene into the baculovirus genome

Sf9 cells were cotransfected with DNAs from AcNPV-infected cells and either pAcI-

PyMT or pVL-PyMT plasmids as described in Materials and Methods. Assuming that the total DNA of infected cells contains about 25% viral DNA (16), plasmid DNA was in approximately 50-fold excess over DNA of wild-type virus. About 0.5% of the total number of plaques obtained under these conditions were found to be polyhedra-minus, and most (if not all) of them hybridized with a polyoma virus MT DNA probe in a plaque-hybridization assay. The presence of polyoma virus MT was confirmed by a plaque immunoassay with the monoclonal antibody, MT16 (10). MT-positive plaques were picked and the viruses from several of these further plaque-purified to homogeneity and used to prepare viral stocks.

Production of polyoma virus MT by insect cells infected with AcI-PyMT baculovirus

In order to compare the efficiency of expression of polyoma virus MT with that of the baculovirus polyhedrin protein, Sf9 cells were infected with either wild type AcNPV or recombinant AcI-PyMT virus and pulse-labelled with [^{35}S] - methionine at various times post-infection. Radiolabelled proteins were analysed by SDS-PAGE (Fig. 2A and B). In wild type infected cells the polyhedrin (32kD) was detectable by 18 hrs. p.i. and its synthesis increased during the subsequent 42 hrs. [The polyhedrin band was observed to be contaminated with another viral p.rotein of similar (33kD) electrophoretic mobility whose synthesis did not appear to increase in the late phase of infection, that is, after 24 hrs p.i. This is best observed in Fig. 2A]. In the late stage of infection (60 hrs) polyhedrin was the predominant species on Coomassie blue-stained gels (not shown) as well as on autoradiograms of ^{35}S -labelled proteins (Fig. 2B). In lysates of AcI-PyMT-infected cells, polyhedrin was not seen. Rather, a new protein ca. 55kD in size (designated MT), which was absent in wild type-infected cells, appeared on the autoradiograms (Fig. 2A). Synthesis of this protein (which could not be positively identified on Coomassie blue-stained gels due to interference of cellular proteins with similar electrophoretic mobilities) reached a maximum at 36 - 42 hrs p.i. The novel protein (compare Fig. 2A with 2B) of size anticipated for polyoma virus middle T-antigen was assumed to be MT.

To prove the identity of the 55kD protein, lysates of AcI-PyMT-infected cells were immunoprecipitated with the MT-specific monoclonal antibody, MT16, and the immunoprecipitates resolved on SDS-PAGE. The results are shown in Fig. 3A. Thus, it can be seen that the 55kD protein was immunoreactive and its appearance followed the same time course seen in Fig. 2A; it had the same electrophoretic mobility as polyoma virus MT immunoprecipitated from lysates of mouse cells infected with wild type virus (see panel labelled Py). Figure 3A also allows a comparison of yields of MT obtained in mouse and insect infected cells to be made since materials from the same number of cells were loaded in each track. Western immunoblots of proteins from uninfected, or from AcNPV-or AcI-PyMT-infected cells, 24 hrs p.i., showed that whereas no immunoreactive protein was found in the lysates of uninfected or AcNPV-infected cells (Fig. 3B, panels a and b), or in a

FIGURE 3

A. Time course of production of MT antigen in insect cells infected with recombinant AcI-PvMT virus

Cells were labelled with [35 S]- methionine at the indicated times p.i. Immunoprecipitates of cell lysates with the middle T-antigen specific monoclonal antibody, MT 16, were separated on 12% SDS-PAGE. A comparable number of 3T6 mouse cells infected with the wild type A2 strain of polyoma virus was assayed for the rate of MT production 28 hrs p.i., using the same procedure.

B. Western immunoblot of insect cell proteins

a, Uninfected Sf9 cells; b, cells infected with wild type AcNPV virus; and c, cells infected with the recombinant AcI-PvMT virus. The blots (tracks 2 and 3) were incubated with the rat monoclonal antibody MT16, followed by incubation with monoclonal mouse anti-rat antibody (OX 12) and with rabbit anti-mouse antibody-peroxidase conjugate, respectively. In track 1 MT specific antibody was omitted. M: molecular weight size markers given in kD. The position of the antigen (MT) and an unidentified 25kD protein ($\blacktriangleleft$) are indicated.

FIGURE 4

A. Cellular location of MT by indirect immunofluorescence in a heterogeneously infected population of cells. In this field can be observed some uninfected cells as well as cells at various stages of MT production. The asymmetric punctate assembly of high levels of MT around the nuclear membrane is particularly apparent in some cells.

B. Immunoprecipitation of MT from cellular fractions.

Immunoreactive proteins from cytoplasmic (track 1) and membrane (track 2) fractions of Sf9 cells infected with the recombinant virus AcI-PyMT and immunoprecipitated with the MT specific monoclonal antibody, MT16, are shown. M: molecular weight size markers.

control using non-immune serum (not shown), lysates of AcI-PyMT-infected cells contained two major immunoreactive proteins, one 55kD in size and the other about 25kD. The 25kD protein was always observed on Western immunoblots and a band of similar size is apparent in the time course (and immunoprecipitation) studies with AcI-PyMT- (Fig. 2) but not apparently with Ac-NPV-infected cells (Fig. 6). The 25kD species was initially presumed to be a major degradation product of MT since its amount relative to the 55kD protein varied among experiments, as did those of other (minor) products. We cannot exclude the possibility, however, that in the AcI-PyMT construction an alternative (internal) initiation codon might be used to produce the observed polypeptide, and, in this regard, MT contains a number of candidate ATG triplets. Luckow and Summers (21) have similarly noted smaller species related to β -galactosidase and β -glucuronidase gene products in some of their constructions.

Intracellular location of MT and its association with cellular protein kinase in insect cells

In its natural host (mouse cells), polyoma virus MT is associated with most cytoplasmic membranes, but mainly with the endoplasmic reticulum (2). A small proportion of MT forms complexes with the membrane-bound tyrosine kinase, pp60^{C-src}, and is apparently phosphorylated by its activity (30,31). Since there is a correlation between tumourigenicity and the interaction between pp60^{C-src} and MT, it seemed desirable to verify the cellular location of MT and whether an insect-cell homologue capable of phosphorylating MT exists. First, the location of MT in an infected Sf9 cell population was studied by immunofluorescence of intact cells. The data obtained regarding sub-cellular localisation (Fig. 4A) were indistinguishable from those reported previously for MT in mouse infected cells using this technique (2). That is, MT was found only in the cytoplasm of cells in dense, frequently asymmetric, arrangements around the nucleus, consistent with cytoplasmic membrane association. Subcellular fractionation was also carried out and the localization of MT in cells examined by immunoprecipitation. In these studies (Fig. 4B), most of the MT (>95%) was found in association with cytoplasmic membranes, as is also observed in mouse cells.

The phosphorylation of the recombinant MT antigen was examined by the *in vitro* kinase assay (32). When immunocomplexes were prepared from the membranes of AcI-PyMT-infected Sf9 cells and incubated with γ -³²P]-ATP, MT antigen was found to be phosphorylated (Fig. 5, lane 1) as is MT from membranes of wild type polyoma virus infected mouse cells (lane 3). The intensity of the ³²P-signal in insect cells was not, however, significantly higher than in mouse cells under comparable conditions, in spite of the much higher MT content in the former (see Fig. 3A). Nevertheless, it may be inferred that insect cells contain a protein kinase which is capable of phosphorylating middle T antigen, although we do not yet know whether the site of phosphorylation is the same as that observed in mouse cells. Alternatively, the possibility of autophosphorylation of MT, as

FIGURE 5:

In vitro phosphorylation of MT by cellular protein kinases

Membrane lysates were precipitated with the MT16 antibody, washed, incubated with [32 P]-ATP and resolved on SDS-PAGE.

The tracks contain: 1, insect cells infected with AcI-PyMT virus; 2, insect cells infected with wild type AcNPV; 3, mouse cells infected with polyoma virus strain A2; 4, uninfected mouse cells. The location of the 55kD middle T-antigen (M) is indicated.

suggested elsewhere (11), cannot be excluded from these data. The extent of phosphorylation of MT *in vitro* did not increase, however, in proportion to its overexpression in insect cells. It would thus appear that only a small fraction of MT is capable of being phosphorylated. Although the nature of this sub-population has not yet been identified, similar observations have been described by Dilworth (11) with wild type polyoma virus infected mouse cells and by Schaffhausen *et al.* (33) with MT generated from an adenovirus expression system.

Abundant synthesis of MT antigen in insect cells using VL-PyMT recombinant baculovirus

Insect cells infected with the AcI-PyMT recombinant baculovirus produced MT in amounts that were substantially higher than those normally found in polyoma virus-infected mouse cells. However, even in this case densitometer scans indicated that MT in Sf9 cells accounted for only about 4 - 6% of the total labelled protein, whereas the polyhedrin content of baculovirus wild-type infected cells is nearer 50% (data not shown). Apparently, therefore, the construction based on the pAcI transfer vector was not capable of utilizing the full synthetic capacity of the baculovirus cloning system. Luckow and Summers (34) investigating signals important for expression of foreign genes also observed lower levels of non-fused gene products in constructions where genes were placed within the polyhedrin promoter region so as to be "upstream" of the normal polyhedrin initiation codon. They showed, however, that the latter, in its normal position in the gene, could be mutated to allow "downstream" ATG codons to be read with high efficiency (21). We therefore tested the producing capacity of the recombinant virus obtained with the pVL941 plasmid transfer

FIGURE 6:

Expression of polyoma virus MT in Sf9 cells infected with the VL-PyMT recombinant baculovirus

Sf9 cells were infected with wild-type AcNPV or with recombinant viruses. At 24 or 48 hrs p.i. (as indicated) the cells were labelled with [³⁵S]-methionine, the lysates were resolved by SDS-PAGE and proteins visualised by autoradiography.

Tracks 1 and 6, wild type AcNPV; 2a and 2b: two independent isolates of recombinant AcI-PyMT virus; 3, recombinant VL-PyMT virus; 4 and 5, five- and ten-fold dilutions, respectively, of the lysate shown in track 3. The positions of MT and polyhedrin (P) are indicated.

vector (21), which contains the whole intact polyhedrin leader sequence together with the first 35 polyhedrin coding nucleotides (with a single mutation in the ATG codon to inactivate it) (see Fig. 1A). Using this transfer vector, the recombinant virus VL-PyMT (Fig. 1C) was constructed. With this virus, as shown in Fig. 6, the efficiency of synthesis of MT protein was very high, accounting for about 50 - 60% of all the labelled proteins obtained, and is comparable with that of polyhedrin produced in wild type-infected cells. This fully confirms the prediction of Summers who designed the pVL941 vector for high expression (21). In a densitometry scan of autoradiograms of [³⁵S]- methionine labelled proteins resolved on SDS-PAGE, the peak of MT was found to be even higher than that of polyhedrin (not shown). This may be a genuine result, but, on the other hand, the possibility exists that under the cell lysis conditions routinely used (highly denaturing protein sample buffer), polyhedrin was not quantitatively released from occlusion bodies. In this regard, it is remarkable that in spite of the highly condensed state of MT in the late phase of the infection cycle, no large inclusion bodies were formed. Instead, a number of small granula associated with cytoplasmic membrane were observed (as is apparent in Fig.

4A). These were readily soluble in denaturing media, yielding, on Coomassie blue-stained SDS-PAGE, a prominent, but rather diffuse band (about 55kD in size) with no perceptible degradation products (not shown). No insoluble residue has ever been observed.

Conclusions

For biochemical studies, and particularly for X-ray crystallographic analysis, large amounts of purified MT are essential. Also, for defining the rôle of MT in cellular transformation, a readily available source of reasonably pure MT is desirable. To these ends, a number of routes have been investigated for MT production. For example, polyoma virus transformed cells expressing MT can be grown as tumours in nude mice. Kaplan et al (35) obtained about 10 μ g of partially purified MT per gram of tumour, a result which suggested that this was a superior alternative to production either in bacterial (36) or SV40 (37) expression systems. (Moreover, MT produced in *E. coli* was not phosphorylated.) However, apart from the obvious disadvantages of using tumours as source material, the yield of MT per cell did not greatly exceed that found in lytically infected cells (35). This is also our experience. 10- to 40-fold higher expression of MT has been obtained using recombinant adenoviruses where MT is expressed off an adenovirus promoter in human host cells (38,39). Two problems were encountered in the latter studies, one being the failure of the cells to heed 3'- processing signals and the other the observation of aberrant splicing events.

As recent studies show, the baculovirus *Autographa californica* nuclear polyhedrosis system has proved a powerful method for the expression and modification of mammalian proteins in insect cells (16,20,21,34,41-44). For example, with regard to the latter, Lanford (45) has shown with SV40 that insect cells are capable of phosphorylating, palmitylating, glycosylating and oligomerising the T-antigens. Further, for large scale work, these cells can be grown simply and inexpensively in suspension culture at room temperature. The potential advantages inherent in this system led us to investigate the possibility that baculovirus recombinants might prove a preferred route for MT production. This investigation shows that full-length immunoreactive MT can be produced in insect cells upon infection with recombinant baculoviruses where genomes contain the (intronless) polyoma virus MT gene in place of the polyhedrin gene. Using a recombinant virus of moderate production, the cellular location of the MT antigen in insect cells was determined and found to resemble that in mouse cells. Furthermore, a small but definite portion of MT became phosphorylated *in vitro*, perhaps by a protein kinase of insect cells (14).

Using the transfer vector, pVL941, specifically designed for high production in insect cells (21), a recombinant virus (VL-PyMT) was constructed that mimicked in its production of MT the polyhedrin-producing capacity of wild type virus. Although no direct comparisons have been made, the data shown (Fig. 6) suggest that quantities of MT produced from this recombinant virus exceed even those reported elsewhere with the adenovirus expression system (33,38,39). Since the overproduced MT antigen did not appear

subject to intracellular proteolytic cleavage and could be completely solubilized by denaturing solvents without degradation, the VL-PyMT virus may be used for production of large amounts of MT. Our preliminary data show that MT produced in the baculovirus system interacts with a number of mammalian cellular proteins which, however, have yet to be identified.

Our data and those of others (33,35,39) point to the desirability of identifying the fraction of MT involved in complex formations with cellular proteins *in vivo*. Although several of the proteins that interact with MT have been identified, notably c-src and the 85kD PI kinase, mutant studies have shown that these do not fully account for all the effects on cells exerted by MT. Intuitively, it seems reasonable to hypothesise that many cellular proteins must be directly involved in the events required for cellular transformation under the influence of MT. In addition, fractions of MT that act independently of such interactions may exist. These may be required for the lytic cycle of the virus, as suggested by studies using a recombinant DNA-generated polyoma virus whose sole early function consists of the large T-antigen; this virus has been shown to grow poorly in mouse cells (C. Streuli, personal communication). It is obvious from our experiments that the baculovirus system provides ample amounts of MT for investigations aimed at assaying enzymatic activities and other biological functions. It still remains, however, to be seen whether the MT so-produced is a completely accurate model for the protein made *in vivo* following viral infection.

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