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Large-Scale Production of Polyoma Middle T Antigen by Using Genetically Engineered Tumors

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A recombinant plasmid containing a metallothionein promoter-polyoma middle T cDNA fusion was constructed and used to transfect NIH 3T3 cells. Transformed cells expressing middle T were injected into nude mice. Within 3 weeks, each mouse produced tumors containing middle T equivalent to that in 250 to 1,000 100-mm dishes of polyomavirus-infected cells. This middle T, partially purified by immunoaffinity chromatography, retained activity as measured by its ability to be phosphorylated in vitro. The combined approach of fusing strong promoters to genes of interest and utilizing nude mice to grow large quantities of cells expressing the gene provides a quick, inexpensive alternative to other expression systems.

Middle T antigen of polyomavirus is required for cell transformation (4, 5, 10, 13, 16, 20, 22, 30). It is a membrane-bound protein anchored by a stretch of hydrophobic amino acids at its C terminus (4, 12, 28). There are two forms of middle T, a predominant 56,000-molecular-weight (56K) species and a minor 58K species (23, 24) differing in their serine and threonine phosphorylation. The protein has an associated kinase activity (9, 25, 27, 29) which phosphorylates protein tyrosine residues (1, 9) and phosphatidylinositol [M. Whitman, D. Kaplan, B. Schaffhausen, L. Cantley, and T. M. Roberts, Nature (London), in press]. However, middle T appears to lack intrinsic kinase activity (24, 25, 28). $pp60^{c\text{-}src}$ is associated with middle T (1, 7, 8) and is apparently the source of the associated kinase activity.

Although genetic and radiochemical techniques have been quite useful for the analysis of middle T, progress has been hampered by the lack of sufficient protein for biochemical analysis. Walter and his colleagues purified limited amounts of middle T protein with an antibody to a synthetic peptide (32). However, the detailed analyses carried out for other purified viral proteins, such as $pp60^{v\text{-}src}$, have not been possible for middle T. In this study, a method is described for producing large quantities of middle T without the need for large-scale tissue culture.

A plasmid expressing middle T under the control of the mouse metallothionein promoter was constructed from pTR890 (25) and a plasmid containing the metallothionein gene and promoter, pMT (3). The salient features of pTR890 are diagrammed in Fig. 1. There is a unique *BclI* site immediately upstream from the initiation codon of the middle T gene. The middle T gene is a cDNA (31) rather than a genomic clone, and hence only middle T can be produced. Polyoma sequences extend from the initiation codon to a *Hin*dIII site 159 base pairs downstream from the middle T gene. This includes the polyadenylate addition site used in virus-transformed cells (14). The metallothionein promoter was excised from pMT as a *PstI-BglII* fragment. This fragment includes some 1,600 base pairs of upstream sequences and sequences encoding a portion of the untranslated leader of the metallothionein message as well as an active promoter. Insertion of this fragment into pTR890 by replacement of the *PstI-BclI* fragment by standard techniques (21) generated pTR960. In pTR960, the initiation codon for middle T is in almost exactly the same position relative to the promoter as the metallothionein initiation codon.

NIH 3T3 cells were cotransfected with pTR960 and pSVgpt by the calcium phosphate technique (6, 11). Cells were selected for their ability to grow in media containing mycophenolic acid (18). Selected colonies were then screened for expression of middle T by the in vitro kinase assay (26, 27). The level of expression of middle T in the different cell lines varied by approximately 10-fold. All clones expressing middle T were morphologically transformed, as assayed by their ability to form foci and grow in soft agar (data not shown).

In Fig. 2, clone 14-2 is compared with 3T3 cells infected with polyomavirus. Both cell lines produced equivalent amounts of middle T, as estimated by immunoprecipitation of [^{35}S]methionine-labeled protein (Fig. 2A). Incubation of immunoprecipitates with [γ-^{32}P]ATP (5, 9, 27) resulted in labeling of both the 56K and 58K forms of middle T from polyomavirus-infected cells (Fig. 2B, lane PYa). The 14-2 cells (Fig. 2B, lane MTa) showed an equivalent level of phosphorylation of the 56K species and a reduced level of 58K phosphoprotein, compared with the infected cells. This is a common finding in polyomavirus-transformed cells (23). Partial proteolytic mapping confirmed the identity of the 56K and 58K species from the infected 3T3 and 14-2 cells (data not shown). Treatment of 14-2 cells with 0.5 μM cadmium sulfate 7 h before labeling resulted in a threefold increase in middle T assayed by the in vitro kinase reaction (Fig. 2C). This is consistent with the results of Pavlakis and Hamer, who observed 1.3- to 2.5-fold increases in human growth hormone after cadmium treatment of mouse cells harboring a metallothionein-human growth hormone hybrid gene (19).

To grow tumors, BALB/c nude mice were injected subcutaneously on each flank with 5×10^6 14-2 cells. The 14-2 cells were scraped from tissue culture dishes, washed, and suspended in 0.2 ml of phosphate-buffered saline before injection. Small tumors were detected within 7 days. Tumor growth accelerated over the course of approximately 3

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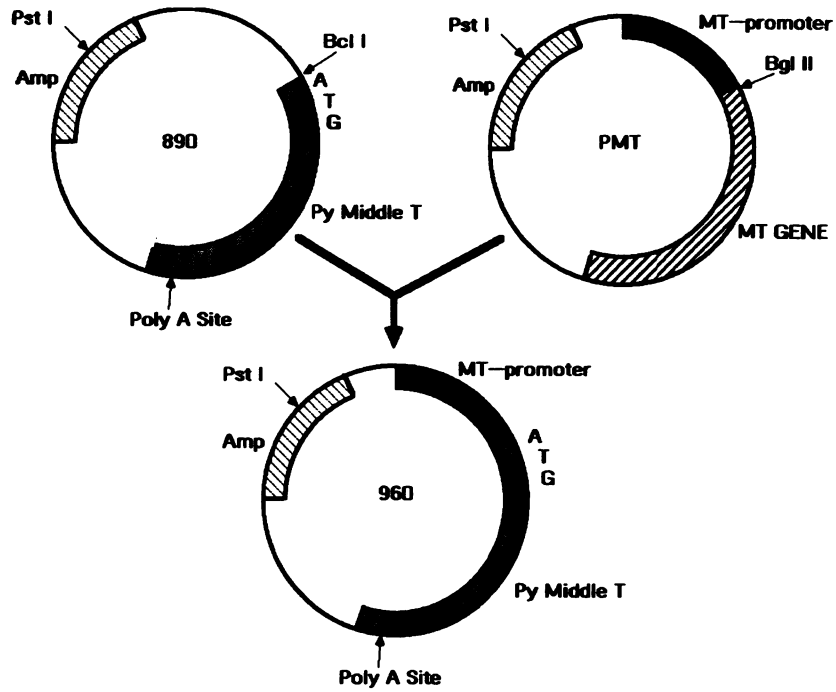

FIG. 1. Diagram of the construction of pTR960, a plasmid in which the polyoma middle T gene is expressed from a mouse metallothionein promoter. Details are as described in the text.

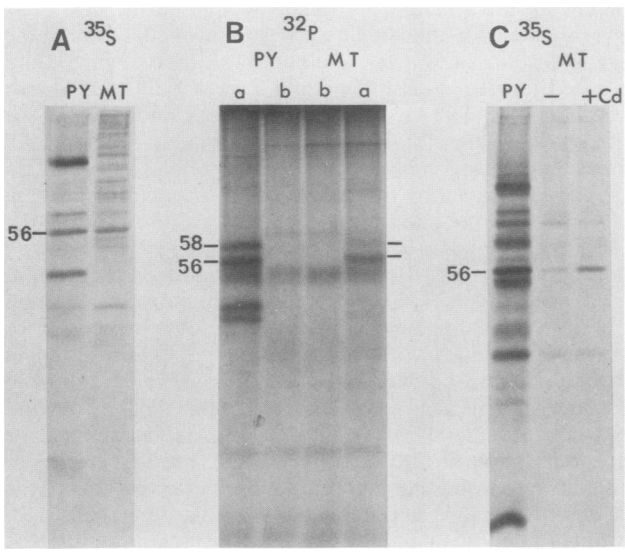

FIG. 2. Middle T antigen production by 14-2, a 3T3 cell line isolated after transfection with pTR960. (A) Immunoprecipitation of middle T antigen (56K) from wild-type infected 3T3 cells (PY) or 14-2 cells which express middle T from the metallothionein promoter (MT). Equal numbers of cells (5×10^5) were labeled with [^{35}S]methionine, and the T antigens were extracted, immunoprecipitated, and resolved in a 10% sodium dodecyl sulfate-acrylamide gel (15). (B) The phosphorylation of middle T antigens from infected 3T3 cells (PYa), 14-2 cells (MTa), or control NIH 3T3 cells (b) was compared in the in vitro kinase reaction by adding [γ-^{32}P]ATP to washed immunoprecipitates. Both the 56K and 58K forms of middle T are shown. (C) Comparison of immunoprecipitated middle T labeled with [^{35}S]methionine in 14-2 cells treated either in the presence (+) or in the absence (−) of 0.5 μM cadmium sulfate. Panels A and C are independent experiments and cannot be directly compared.

weeks, whereupon tumors were harvested. The average tumor size was 6 to 10 g; thus, a maximum of 20 g of tumor tissue was produced per mouse.

To estimate the amount of middle T in the tumors, in vitro kinase assays were performed. The amount of middle T

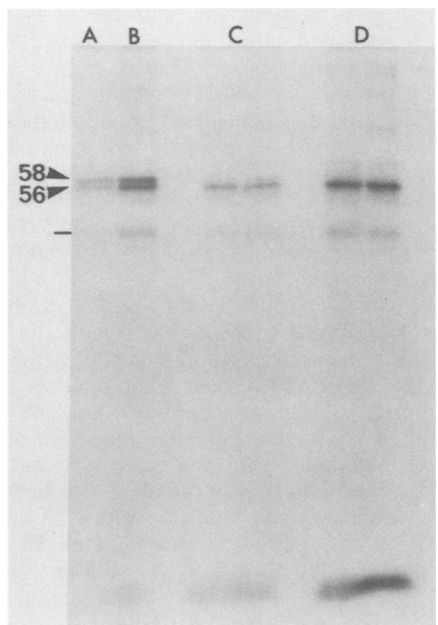

FIG. 3. Assay of middle T antigen from a tumor. Wild-type-polyomavirus-infected 3T6 cells (lane A, 1.25×10^6 cells; lane B, 5.0×10^6 cells) and duplicate samples of increasing amounts of tumor tissue (lane C, 10 mg; lane D, 40 mg) were extracted, and the T antigens were assayed by the in vitro kinase reaction. The 56K middle T antigen and the 58K form are indicated by arrowheads.

labeling was directly proportional to the amount of infected cell extract (A and B) or tumor cell extract (C and D) used (Fig. 3). Calculations from this and other experiments show that one tumor provides levels of middle T antigen equivalent to 250 to 1,000 100-mm dishes of infected cells. The tumor cells, like the cells used to derive the tumor, contained less of the 58K form of middle T.

The identity of middle T protein in the tumors was confirmed by *Staphylococcus aureus* V8 proteolytic mapping. The 24K band which originates from the 56K form of middle T and the 18K overlapping C-terminal fragment common to both the 56K and 58K forms (23) are clearly visible in digests of both tumor and infected cell middle T (Fig. 4). However, as expected from Fig. 3, the 24K' fragment which originates from the 58K species is only observed at low levels in the tumor material.

Middle T was partially purified from the tumors with an immunoaffinity column. The affinity column employed the anti-middle T monoclonal antibody pAB815 (2). DEAE-cellulose–reactive blue 2 column-purified immunoglobulin G was coupled to Affigel 10 (Bio-Rad Laboratories) according to the instructions of the manufacturer. The pAB815 affinity gel bound the middle T protein efficiently and could be used directly in in vitro kinase assays.

The pattern of Coomassie blue-stained protein eluted from the affinity reagent with 3.5 M KSCN (pH 6.8) is shown in Fig. 5. The middle T band was not observed in the lanes of material eluted from absorbent lacking pAB815 monoclonal antibody or in material bound to the affinity reagent from control liver extracts (data not shown). The identity of the band labeled middle T was confirmed by mapping the eluted protein with V8 protease. The silver-stained mapping gel of middle T from the tumor (Fig. 5B) shows the same 24K and 18K fragments as the ^{32}P-labeled peptides of middle T extracted from infected cells and labeled in vitro (Fig. 5C).

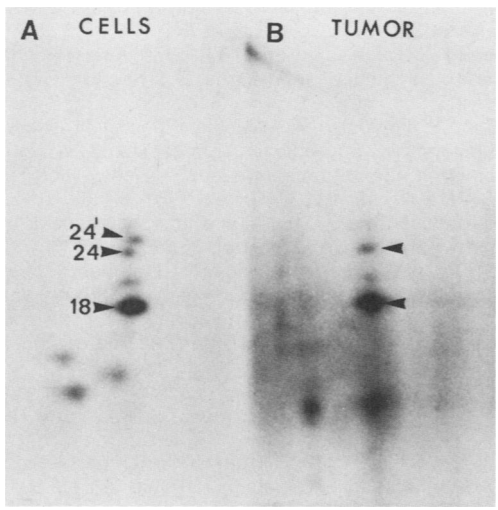

FIG. 4. *S. aureus* V8 protease mapping of middle T extracted from cells infected in vitro or extracted from tumors. Middle T was extracted from polyomavirus-infected 3T6 cells (A) or from tumors (B), immunoprecipitated, and labeled in vitro with [γ-^{32}P]ATP. Each sample was electrophoresed on cylindrical 10% acrylamide gels, and the cylinders were placed on top of a 12.5% sodium dodecyl sulfate-acrylamide slab gel. Partial digestion with V8 protease was carried out as described previously (23). The 24K fragment derived from the 56K middle T, the 24K' fragment derived from the 58K form of middle T, and the 18K fragment common to both forms are indicated by the arrowheads.

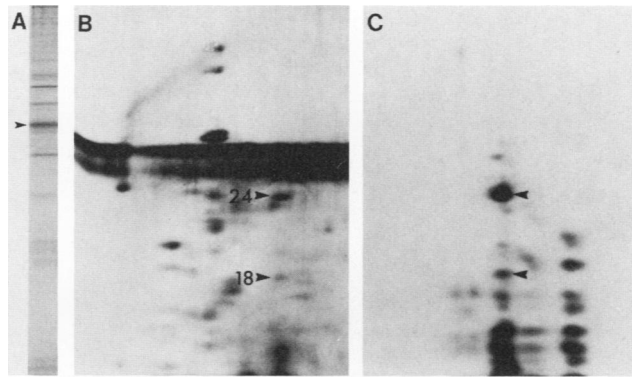

FIG. 5. (A) Affinity purification of middle T. Tumor extract (10 ml; 250 mg/ml) was incubated overnight at 4°C with 0.3 ml of Affigel 10 to which pAB815 had been coupled. The middle T was batch eluted as described in the text and electrophoresed on a 10% sodium dodecyl sulfate-acrylamide gel. The gel was stained with Coomassie blue. The position of the middle T band is indicated by the arrowhead. (B) and (C) *S. aureus* V8 mapping of affinity-purified middle T. Panel B is the silver-stained mapping gel of middle T from tumors, and panel C is the autoradiograph of ^{32}P-labeled middle T from infected cells digested on the same gel. Affinity-purified middle T was electrophoresed on a cylindrical 10% sodium dodecyl sulfate-acrylamide gel. Middle T immunoprecipitated from polyomavirus-infected 3T6 cells and labeled in vitro with [γ-^{32}P]ATP was run on a second cylindrical gel. The two cylinders were placed on top of a 12.5% sodium dodecyl sulfate-acrylamide slab gel. Partial digestion with V8 protease was carried out as described previously (23). The gel was silver stained (17), dried, and autoradiographed. Panels B and C represent the left- and right-hand sides of the same slab gel. The 24K and 18K fragments characteristic of polyoma middle T are shown by the arrowheads.

The partially purified middle T eluted from the affinity gel with 3.5 M KSCN was phosphorylated in the in vitro kinase reaction (Fig. 6). Since no immunoprecipitation was performed, some additional bands besides middle T were also labeled. Only 20% or less of the activity bound to the column was eluted in active form. Experiments are under way to determine whether this represents denaturation of the activity or dissociation of the middle T–c-*src* complex.

The work described here presents a combined approach to obtaining substantial quantities of middle T antigen. To make a cell expressing middle T at high levels, we utilized promoter fusion. The metallothionein promoter was chosen for its efficiency and inducibility. 14-2 cells expressed middle T antigen at a level comparable with that of a productive polyomavirus infection. This is significantly better than the level ordinarily seen in transformed mouse cells. Constructions with other promoters, such as the Harvey sarcoma virus long terminal repeats, have not resulted in significantly better expression. It is possible that levels of middle T are limited by the ability of the cells to tolerate the protein. The value of the observed threefold inducibility of the promoter is more questionable. The cells are morphologically transformed in the absence of cadmium; therefore, induction is not of value in studying transformation. Induction is clearly useful in increasing protein production in tissue culture. However, limited experiments to demonstrate cadmium, zinc, or selenium induction in tumors have not yet proven successful.

To avoid mass cell culture, cells expressing middle T were grown as tumors in nude mice. The tumors are easily manipulated and do not develop excessively invasive vascular systems or necrotic areas. Frozen tumor tissue stored at

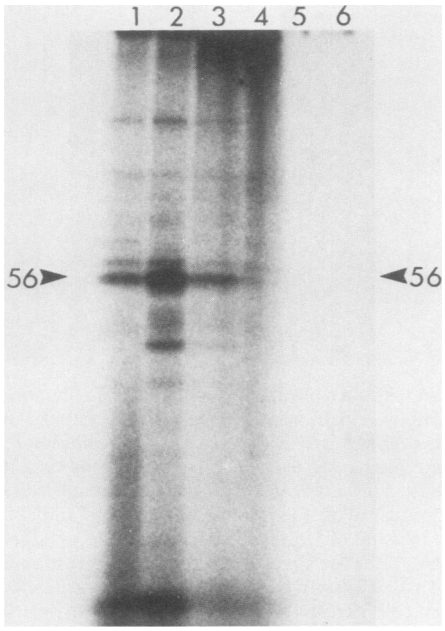

FIG. 6. Elution of kinase active middle T from the affinity gel. Tumor extract and affinity gel were incubated together. Washed affinity gel was loaded on top of a G10 column. The column was eluted with 3.5 M KSCN, and fractions were collected beginning with the void volume. The concentration of $MgCl_2$ was increased to 5 mM in the eluant, and the middle T was labeled with [γ-^{32}P]ATP. The proteins were then precipitated with trichloroacetic acid and electrophoresed on a 10% sodium dodecyl sulfate-acrylamide gel. The position of 56K middle T is indicated by an arrowhead. Minor bands represent contaminating protein of the sort seen in Fig. 5.

−70°C retained kinase activity for periods of greater than 6 months. Tumors from a single animal give the equivalent of 250 to 1,000 100-mm tissue culture dishes of polyomavirus-infected cells. We were able to obtain approximately 10 μg of partially purified middle T protein from a gram of tumor, as estimated from Coomassie blue-stained gels. This is a dramatic step forward in developing preparations for detailed biochemical analysis, as hundreds of grams of tumor tissue are available for purification. Although middle T has been produced with *Escherichia coli* (25) and simian virus 40 (33) expression systems, the tumors are the best source of middle T which is active in the in vitro kinase reaction.

Nude mouse tumors may be a general system to overproduce products from other transfected genes expressed in middle T-transformed NIH 3T3 cells. The middle T-expressing tumors from nude mice also express a level of the coselected gene (bacterial xanthine-guanine phosphoribosyltransferase) equivalent to that in 250 to 1,000 100-mm tissue culture dishes of 14-2 cells (data not shown). As there was no selection for this enzyme during tumor production, we anticipate that other unselected genes can be similarly overexpressed.

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