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Expression of polyoma early gene products in *E. coli*

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Abstract

The three products of the early region of polyoma virus have been cloned for expression in E. coli using the Tac promoter. Although the identical promoter and ribosome binding site are used in each final construction, the observed level of protein expression is different for each protein. While plasmids expressing wild type T antigens as well as a plasmid expressing the truncated Py-1387T middle T antigen lacking the membrane-anchoring sequence give rise to synthesis of proteins readily detectible by ^{35}S-methionine labeling and immunoprecipitation, only small T and the middle T of Py-1387T are made in amounts sufficient for ready detection in total cell protein. Unlike middle T expressed in animal cells, middle T produced in E. coli is not detectibly phosphorylated. Further, the E. coli protein lacks tyrosine kinase activity.

INTRODUCTION

The early region of polyoma virus encodes three proteins: large T, middle T and small T antigens (1). Large T antigen is essential for viral DNA replication and autoregulates its transcription (2,3). It is required for transformation by the virus (4) and is involved in integration of viral DNA into the host chromosomes (5). It also is required for the DNA mediated transformation of primary cells, perhaps functioning in 'immortalization' (6). Middle T and small T are important in the maintenance of transformation and in tumorigenicity by the virus. Middle T is required in transformation. Mutants in which middle T, but not small T, are altered are affected in transformation (7-10). cDNA coding for middle T alone can transform some established cell lines (11), although the degree of transformation clearly depends upon the cell (12). A precise role for small T has not been defined. However, its presence is required for tumor formation and for the transformation of certain cell types (13). Small t also appears to be involved in virion assembly (14).

Of the three polyoma T antigens, middle T has been the protein best studied. It is associated with membrane fractions and is found on the inner

surface of the plasma membrane (15,16). Membrane association is required for transformation (9). Mutants of middle T unable to insert in the membrane do not transform. If γ^{32}P-ATP is added to T antigen immunoprecipitates, middle T is phosphorylated on tyrosine residues, primarily at 315 (17) and to a lesser extent, 322 (18,19,54) and other sites (54,55). Mutants lacking tyr 315 are defective in their ability to transform under some circumstances (19,56); hence, phosphorylation on tyrosine 315 appears to be important for transformation. Mutants lacking tyr 322 transform normally (8). Middle T can be resolved into two subfractions which differ dramatically in their in vitro kinase activity. The high specific activity 58K form is phosphorylated in vivo at serine and/or threonine residues different from those phosphorylated in the 56K form, leading to the suggestion that second-site modification may affect the tyrosine kinase activity (17). Interestingly, both the tyrosine kinase activity and the 58K-specific in vivo phosphorylation seem to require that middle T become membrane associated (9). One important issue to be resolved is whether middle T itself has tyrosine kinase activity. Middle T lacks ATP binding activity (16) and the bulk protein can be distinguished from the kinase-active fraction by a number of different criteria (20). These facts are consistent with the notion that middle T is not a kinase. Recently, Courtneidge and Smith reported that middle T can associate with $pp60^{c\text{-}src}$, which possesses a tyrosine kinase activity (21).

Although studies based on genetics and isotopic labelling techniques have been quite informative, detailed biochemical analysis of the early gene products has been hampered by the lack of material. There have been no good sources of the early proteins comparable to the SV80 cells or the D2 SV40-adenovirus hybrids used to produce SV40 large T (22,23). The work described here represents an approach to the solution of this problem by the molecular cloning and expression of these proteins in E. coli. This approach has been successful for SV40 small T antigen (24,25). Polyoma genomic DNA encoding small T has been cloned, and a protein related to small T, but with additional amino acids has been expressed (26,27). We report here the expression of all three polyoma early gene products. The emphasis of our work has been on the middle T antigen because of its obvious importance to transformation. The results described here indicate that middle T expressed in E. coli lacks tyrosine kinase activity and is not modified at serine and/or threonine residues.

MATERIALS AND METHODS

Materials

Chymotrypsin was obtained from Worthington. ^{35}S-methionine and $^{32}PO_4$ were obtained from New England Nuclear; γ^{32}P-ATP was obtained from ICN.

Cells and Viruses

Baby mouse kidney cells, prepared as described by Winocour (28), were used to prepare virus stocks and infected to provide control T antigens. Wild type virus, NG59RA (29), and the deletion strain, dl 45 (30), were used to prepare T antigen controls.

Bacterial Strains

E. coli LG90 (31), MM294 (32) and Deg44 (33) were cultured as previously reported (32).

Plasmid Construction

The 9 plasmids mentioned in the text were constructed from fragments of 10 other plasmids constructed as a part of this project and not yet described in print. All of the technical details of plasmid construction (buffers, enzymes, DNA preparation, etc.) and the general cloning steps were exactly as previously described (25,31,34,35). Since the actual details of construction are quite lengthy to describe and are of interest to a very limited audience, we will content ourselves here to giving exact descriptions of the 9 plasmids mentioned in the body of the paper. Details of construction will be published elsewhere.

1) pTR508 consists of the large Rl, Hind III backbone fragment of pLG400 (31) a 95 bp Rl, Pvu II fragment containing the UV5 lac promoter taken from pGL101 (36), a Hind III linker, and a 280 bp fragment of polyoma extending from the Hae III site at bp 105 to the Hinf I site at bp 385. The fragments are joined in the following manner: The Rl site of the lac promoter is joined to the backbone Rl site. The linker is attached to the Pvu II end of the promoter fragment. The Hae III end of the polyoma fragment is joined to the linker and the Hinf I end of the polyoma fragment (rendered flush with DNA polymerase) is joined to the Hind III end of the backbone (similarly rendered flush).

2) pTR509 is simply a deletion of the Hind III linker and adjacent 65 bp of polyoma sequence of pTR508.

3) pTR814 consists of 3 sequences: The large Rl, Hind III backbone of pBR322 (37), the RI, Acc I fragment containing lac promoter and amino terminal sequences of polyoma from pTR509 and a fragment of the polyoma cDNA for middle T (11) extending from the Acc I site at bp 368 to the Hind

III site at bp 1656. These fragments can be assembled in only one way with proper joining of like sticky ends.

4) pTR841 consists of 3 fragments: the large Rl, Pst I backbone of pBR322, the Rl Ava I fragment of pTR814 containing the lac promoter, ribosome binding site and coding sequence for the first 151 amino acids common to τ and t, and a Ava I, Pst I fragment of cDNA coding for the C terminus of small T taken from pEl.1 (38). (Note that the Pst of the last fragment is a result of the cDNA cloning and is not a normal polyoma restriction site). Once again these three fragments can be assembled into a plasmid in only one way.

5) pTR850 consists of 4 sequences: (1) a Bam cut pBR322 backbone piece into which are inserted (2) the Bam I to Acc I fragment of pTR841 containing the amino terminus of the tet resistance element plus the lac promoter and common amino terminal region of the polyoma early region coding sequence (3) the Acc I to Ava I fragment of large T cDNA from pE2.1 (38) and (4) a fragment of viral DNA extending from the Ava I site at bp 1016 to the Bam I site at bp 4632.

6,7, 8) pTR1070, pTR885 and pPT are identical to pTR509, pTR814, and pTR850, respectively, except that the Rl Hpa II fragment of the lac promoter has been replaced by an Rl Cla I fragment of pEA108 (39).

9) pTR900 consists of 3 fragments: (1) The Rl Pvu II backbone of pBR322 containing the ampicillin resistance element and replication origin (2) the same Rl to Cla I trp promoter fragment used above and (3) the fragment of pTR841 extending from the Hpa II site in the lac promoter to the Hae III at bp 837 in the polyoma sequence.

T Antigen Analysis

The procedures for labeling animal cells and for extraction of T antigens have all been described previously (17,40,41). For the preparation of T antigens in bacteria, cells were grown overnight in Luria broth (42). An inoculum (0.2 ml) was then transferred to 5 ml of M9 (42) containing ampicillin (20 μg/ml) and permitted to grow for 2 hrs ($OD_{600} \approx 0.5$). Cells were harvested at 10,000 rpm in a J21 Beckman Centrifuge for 5 min, and resuspended in 50 μl of medium. After 5 min at 37°C, ^{35}S-Methionine (20 μC/sample) and 5 mM [final concentration] isopropyl-β-D-thiogalactoside (IPTG) was then added for the specified period, usually 2 min. The cells were then frozen in a dry ice/ethanol bath. 0.5 ml of T antigen extraction buffer (0.137M NaCl, 0.02M Tris-HCl, pH 7, 0.001M $MgCl_2$, 0.001M $CaCl_2$ 10% v/v glycerol, 1% v/v NP40) was then added, and the cells were sonicated

until broken in a Raytheon cavity sonicator. Anti-T ascites (10 µl/sample) and Protein A-Sepharose (Pharmacia) [40 µl of 50% (v/v)] were added and the sample agitated for 20 min at 4°C. Immunoprecipitates were collected by centrifugation in a Sorval GLC-1 centrifuge at 2000 g. The samples were then washed using phosphate-buffered saline and 0.5M LiCl in the usual manner (17). The T antigens were analyzed on SDS 10% acrylamide gels (43).

Partial proteolysis was also carried out as previously described (17,40,41). T antigens were first resolved on an SDS 10% acrylamide gel. The cylinders were then placed head to head on top of a 12.5% acrylamide slab gel and overlayed with digestion solution [0.125M Tris-HCl, pH 6.8, 0.01M EDTA, 20% glycerol (v/v), 50 µg/ml bovine serum albumin, 50 µg/ml chymotrypsin]. Electrophoresis was carried out until a bromphenol blue marker reached the bottom of the gel (≈16 hrs at 50 v).

RESULTS

Plasmid Constructions

The format for construction of plasmids which direct synthesis of T antigens has been described in detail in our previous work on expressing eukaryotic genes in *E. coli*. Briefly stated, the procedure consists of four basic steps:

i) A hybrid gene was constructed whose amino terminus was derived from a restriction fragment of viral DNA bearing those polyoma sequences coding for the initiator ATG and the next 70 amino acids common to all three T antigens (as well as an additional 60 base pairs of sequences upstream of the ATG), and whose carboxyl terminus was a large fragment of the lac Z gene. The lac Z fragment used codes for an enzymatically active ß-galactosidase but does not have a translational start. Hence translation of ß-galactosidase is dependent upon ribosomes initiating protein synthesis at the ATG of the polyoma T antigens (see Fig. 1A). The plasmid bearing this construct (pTR508) was also engineered so that there was a unique Hind III site some 60 base pairs upstream from the polyoma initiator codon.

ii) A functional ribosome binding site was constructed for the polyoma lac Z gene fusion. To do this, pTR508 DNA was first opened at the Hind III site and resected for varying times with a combination of Exonuclease III and S1 nuclease (Fig. 1B), and then a fragment bearing the promoter and ribosome binding site of the lac Z gene was ligated into place. The resulting plasmids were used to transform lac Z^- *E. coli* and the clones were screened on indicator plates for ß-galactosidase production. Of the

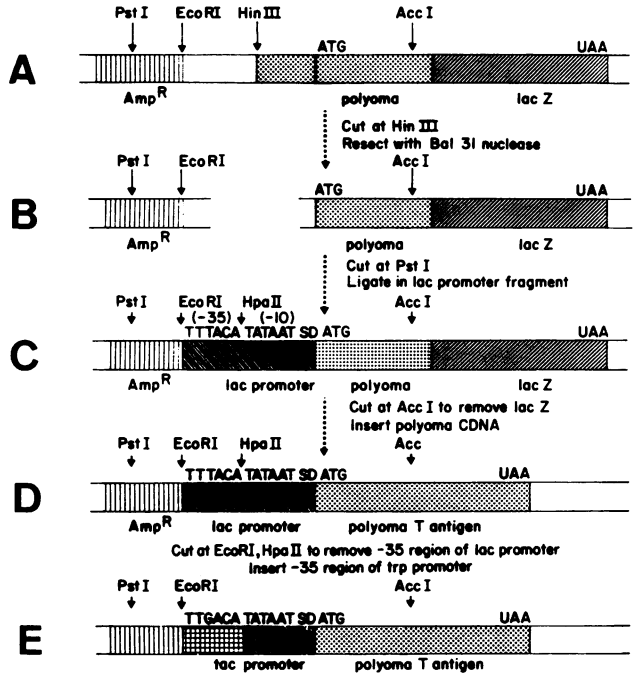


Figure 1. Schematic representation of plasmid construction. (A) A portion of pTR508 containing the polyoma-lac Z fusion gene used for assaying ribosome binding sites; (B) Exonuclease mediated removal of polyoma sequences upstream from the initiator ATG in preparation for the insertion of bacterial promoter and ribosome binding site. (C) A portion of pTR509 showing the resulting construction with functioning bacterial ribosome binding site and promoter. (D) A portion of a plasmid in which lac Z sequences have been removed and replaced with the appropriate polyoma cDNA giving an intact T antigen gene capable of being expressed in E. coli. (E) A portion of a plasmid expressing T antigen from a tac promoter. Stippled areas represent polyoma sequences, light cross-hatching represents the lac promoter, and sequences represent trp promoter sequences. In each case the plasmid backbone is pBR322. For pTR900, the coding sequences are those for small T. For pTR885, the coding sequences are those for middle T and for pPT, the coding sequences are those for large T.

many potential ribosome binding site configurations resulting from the exonuclease resection, some are more functional than others. The best ribosome binding sites lead to levels of β-galactosidase production which yield highly distinguishable red colonies on the indicator plates used. Colonies expressing high levels of β-galactosidase were picked and plasmid DNA from them was sequenced in the ribosome binding site region. A clone with separation of 8 base pairs between the Shine-Dalgarno sequence and

polyoma ATG (pTR509) gave the highest β-galactosidase levels and was chosen for further study (Fig. 1C).

iii) The lac Z sequences in pTR509 were replaced with cDNA for each of the three polyoma T antigens. This cDNA was isolated from plasmids constructed by R. Treisman in R. Kamen's laboratory (11,38). The actual separation of polyoma sequences from lac Z sequences was accomplished using the Acc I site near the 3' end of the polyoma DNA in pTR509. The result of this process (a rather long and involved set of subclonings described in the Materials and Methods section) were three plasmids: pTR810 (middle T), pTR840 (small T) and pTR850 (large T) (Fig. 1D).

iv) Finally, the lac promoter was converted to the 5-10-fold more efficient tac promoter. This was done, as described in our earlier work on SV40 t antigen expression (24), by making use of the Hpa II site on the lac promoter to cut away the inefficient structural element situated 35 base pairs upstream of the startpoint of transcription and replace it with a similar but more efficient element from the trp promoter. Again, this involved multiple constructions (described in Materials and Methods) which resulted in the following four final products: pTR900 (small T), pTR885 (middle T), and pPT (large T) (Fig. 1E).

Expression of T Antigen-β-galactosidase Fusion Protein and the Three Polyoma T Antigens

As described in the preceding section, a plasmid (pTR509) directing synthesis of a fusion protein consisting of the 71 N-terminal amino acids of the domain common to the three polyoma early proteins fused to β-galactosidase was constructed first. The expression of this protein is shown in the left hand panel of Fig. 2. Bacterial cells were labeled with ^{35}S-methionine for 8 min at the time of induction with IPTG. Proteins solubilized by sonication in T antigen extraction buffer were immunoprecipitated with anti-T ascites fluid. Lane B shows the fusion protein, while lane A shows an immunoprecipitate from cells lacking the plasmid for the fusion protein and lane C shows the labeling of the protein in uninduced cells. The band corresponding to the fusion protein is shown in lane B, but it also can be detected in uninduced cells. This shows that the promoter is not completely shut off in these cells in the absence of inducer. The fact that this fusion protein was produced in levels comparable to that of fully induced wild type β-galactosidase (data not shown) indicates that the promoter and ribosome binding site in the present plasmid used for subsequent expression of T antigens function efficiently.

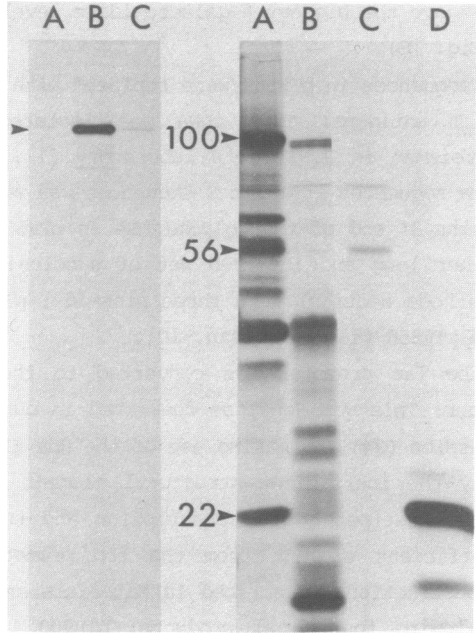


Figure 2. Left Panel: E. coli were incubated for 8 min at 37° in the presence of 50 μC ^{35}S-methionine. For lanes A and B, 5 mM isopropyl-β-D-thiogalactosidase was also present. At the end of the labeling, the fusion protein was extracted, immunoprecipitated, and resolved on a 10% acrylamide SDS gel as described in Materials and Methods. (A) Control cells; (B,C) cells containing the plasmid (1070) expressing the fusion protein. The arrowhead indicates the position of the fusion protein.

Right Panel: Baby mouse kidney cells infected with wild-type polyoma virus were pulse-labeled for 2 min at 30 min after induction with 5 mM IPTG. The T antigens were extracted, immunoprecipitated and resolved on a 10% acrylamide SDS gel. The positions of the large T (100K), middle T (56K) and small T (22K) are shown by the arrowheads. Lane A: T antigens from BMK cells; lane B: T antigens from E. coli containing the plasmid (pPT) expressing large T; lane C: from E. coli containing the plasmid expressing middle T (pTR885); lane D: from E. coli containing the plasmid expressing small T (pTR900).

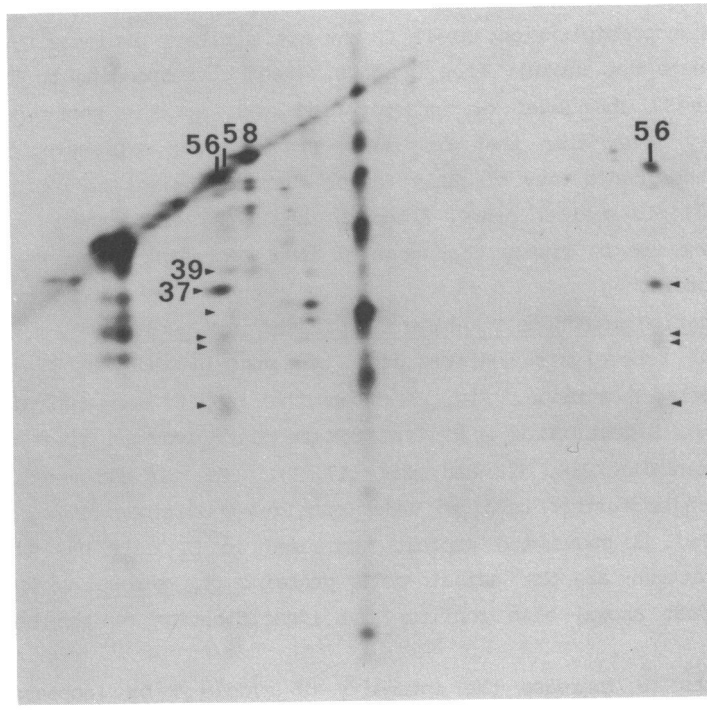
The right hand panel compares T antigens from animal cells with those from E. coli producing each of the three primary viral products using identical promoters and ribosome binding sites. Panel A shows the usual T antigen pattern observed in baby mouse kidney cells: 100K large T, 56K middle T, and 22K small T antigen, as well as the 63K and 36K non-viral T antigens. Lanes B, C, and D show large T, middle T and small T antigens labeled in a 2 min pulse after 30 min of induction. The clone expressing small T (lane D) clearly gives the most satisfactory results. When aliquots

of total protein labelled with ^{35}S-methionine are run directly on SDS gels without immunoprecipitation, small T, but not middle T or large T, can be observed (data not shown). Lane C shows a band corresponding to the wild type middle T. The level of incorporation even in this short pulse is considerably lower than that for small T. The clone expressing large T (lane B) shows bands very slightly faster than the animal cell protein as well as multiple smaller bands. Although these smaller fragments have not been studied, it is likely that most of them represent fragments of the large T protein.

Characterization of Middle T Antigens Expressed in E. Coli

Partial proteolysis confirms that the protein made in E. coli is indeed middle T antigen. Fig. 3 shows the results of analysis with chymotrypsin. Digestion of wild type 56K middle T antigen gives rise to major fragments of 37K, 31K and ≈26K (17,41). The 31K fragment labeled with ^{35}S can be further resolved under some gel conditions (17). The top panel of Fig. 3 shows the protein expressed in E. coli has the same fragment pattern as the animal cell protein. S. aureus V8 protease digestion (not shown) also confirms the identification of the bacterial middle T.

Attempts to increase the intensity of middle T by increasing the length of the labeling time to 20 min were unsuccessful. This result as well as those from pulse-chase experiments suggest that the middle T produced in E. coli is not stable. Middle T is a membrane protein (15,16) and membrane associated proteins have previously been shown to present problems in E. coli (44). To test this possibility, the mutant Py-1387T has been used. Py-1387T induces a truncated middle T which is found in the soluble fraction of animal cells (9). The mutant protein lacks the 'hydrophobic tail' at the C terminus of wild type middle T required for membrane attachment. The coding sequence for Py-1387T middle T was inserted into pTR885 so that the resulting plasmid, pTR925, differs by only a single base pair change from the parent pTR885. Fig. 4 compares the level of mutant protein production directed by pTR925, compared to that of pTR885 for the wild type protein. It is clear that the Py-1387T construction yields much more labeled protein than does the wild type. The identity of the mutant protein was confirmed by partial protease mapping. Py-1387T middle T is known to have truncated 37K and 31K fragments ≈5K shorter than the wild type fragments (9); the bottom of Fig. 3 shows the expected pattern of the mutant bacterial protein. As is the case for small T, a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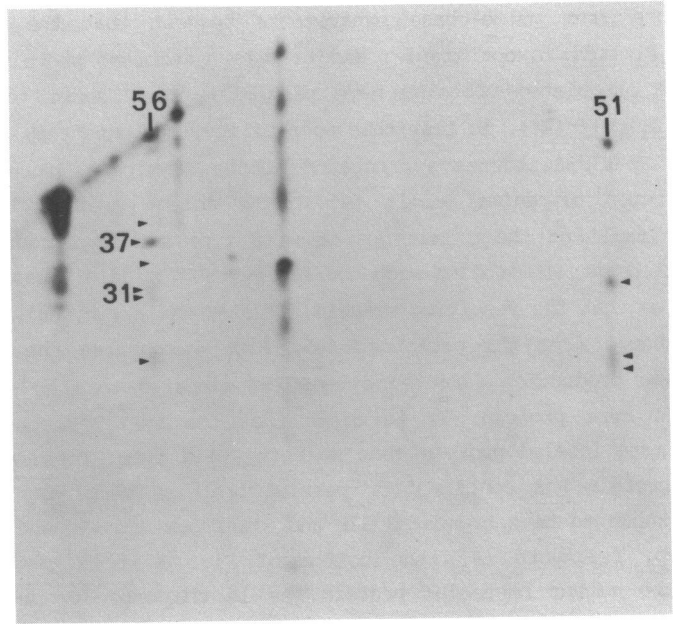
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labeled band corresponding to the mutant protein can be observed in patterns of total cell protein (not shown).

Fig. 5 compares the stability of mutant or wild type middle T to that of small T. Cells were labeled for 2 min and the T antigens extracted from aliquots of the culture (lanes B, E, H). An excess of cold methionine was then added to the remainder, and the cells incubated for an additional 5 min (lanes C, F, I) or 20 min (lanes D, G, J). The chase is not completely successful since the amount of label in small T increases slightly during the 5 min chase (lane H vs. I). Nevertheless, the wild type middle T is largely degraded in the 5 min chase and is barely detectable after 20 min. By contrast, the Py-1387T 51K middle T is easily detected after a 20 min chase. Densitometry (data not shown) reveals that the half life of middle T is less than two minutes while that of small T and the 51K 1387 middle T antigens are greater than 15 minutes. This difference in half lives is sufficient to explain almost all the difference in incorporation seen in Fig. 4.

The Middle T Expressed in Bacteria is Inactive in Tyrosine Phosphorylation In Vivo and In Vitro

Fig. 6 shows an *in vitro* kinase assay for the bacterial protein. Animal cell (A) or bacterial (B) middle T were immunoprecipitated using anti-T ascites and γ^{32}P-ATP added to washed immunoprecipitates in the usual manner. No labeling is observed in the immunoprecipitate from lane B. Experiments were carried out to test the possibility that the bacterial extract contained an inhibitor of kinase activity. In lane C, wild type animal cell extract and bacterial extract were mixed in equal proportions and the T antigens immunoprecipitated. Clearly, there is no difficulty identifying kinase-active middle T, arguing against the presence of some potent inhibitor or phosphatase. Since we have previously shown that animal cell immunoprecipitates are contaminated with other kinases (41), it seems

Figure 3. Top: T antigens extracted from baby mouse kidney cells (left) or *E. coli* expressing wild type middle T (right) labeled with ^{35}S-methionine were resolved on 10% cylindrical SDS gels and then digested in the second dimension 12.5% SDS slab gel using chymotrypsin. The 37K fragment as well as the 31K doublet and 25K spot derived from the 56K form of middle T are indicated by the arrowheads; the 39K spot derived from the 58K form is also marked. On the right side the 37K, 31K and 25K spots derived from the bacterial middle T are also marked with arrowheads.

Bottom: Comparison of wild type T antigens from baby mouse kidney cells to the mutant middle T or Py-1387T middle T expressed in *E. coli*. As shown for the mutant virus in animal cells (9), the 37K and 31K chymotryptic fragments are shortened by approximately 6K in the mutant.

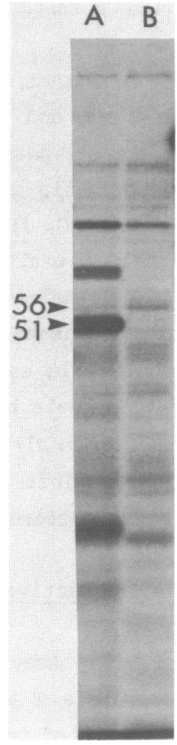

Figure 4. T antigens from E. coli expressing Py-1387T middle T (A) or wild type middle T (B). The bacteria were labeled for 8 min with ^{35}S-methionine. The 56K arrowhead indicates the position of the wild type middle T; the 51K arrowhead indicates the position of the truncated middle T of Py-1387T. 10% SDS gel.

probable that the difference in the general background between E. coli and animal cell immunoprecipitates stems from the lack of kinase activity in bacteria. In a variation of the experiment shown in lane C, extracts from dl 45-infected cells which contain a shortened middle T active in the kinase reaction were mixed with the wild type protein made in bacteria. No kinase activity towards the wild type protein was observed, although the dl 45 middle T was phosphorylated in the usual manner (data not shown). These experiments suggest that middle T differs from the pp60$^{v\text{-}src}$, which retains its kinase activity upon expression in bacteria (45).

We have been concerned with proving that the level of protein isolated from the bacteria is significantly above the detection limit of the protein kinase activity. Middle T produced by in vitro translation of RNA, for

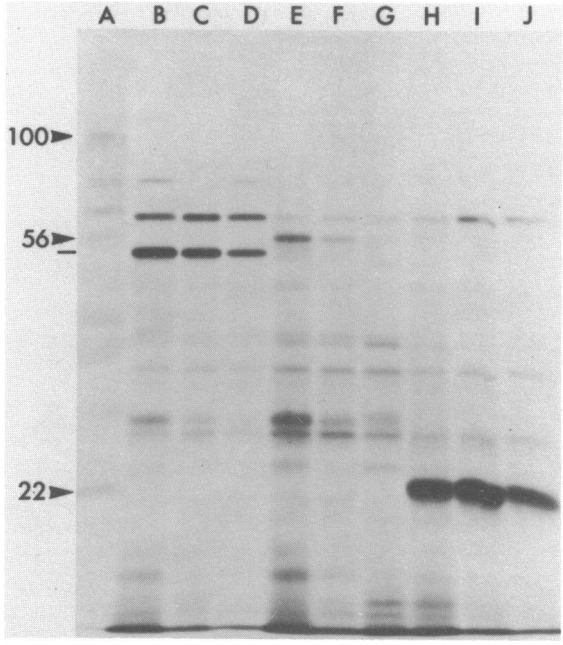

Figure 5. T antigens from wild type polyoma infected baby mouse kidney cells (lane A), from E. coli expressing Py-1387T middle T (B-D), expressing wild type middle T (E-G) or expressing small T (H-J) were labeled with ^{35}S-methionine. The kidney cells were labeled for 90 min. The bacterial cells were labeled for 2 min. Lanes B, E, and H were harvested at the end of the pulse. Cold methionine to a concentration of 10 mM was added to the remaining bacterial samples. C, F, and I were harvested after a 5 min chase. D, G, and J were harvested after a 20 min chase. The arrowheads indicate the position of large T (100K), middle T (56K) and small T (22K) from wild type polyoma. The bar indicates the position of the 51K middle T or Py-1387T. 10% SDS gel.

example, also appears to lack kinase activity (B. Schaffhausen, unpublished), but estimates of the amount of material synthesized suggests that it is below the limit of detection. To estimate the amount of middle T in our immunoprecipitates, parallel cultures expressing middle T or the β-galactosidase fusion protein were labeled with ^{35}S-methionine and the proteins immunoprecipitated. The relative amounts of middle T and fusion were determined directly by electrophoresis. The β-galactosidase activity of the fusion protein was then determined. From the amount of activity and the known specific activity of β-galactosidase, the amount of protein represented by the ^{35}S was then calculated. The amount of middle T in animal cells is known from continuous labeling experiments with ^{35}S-

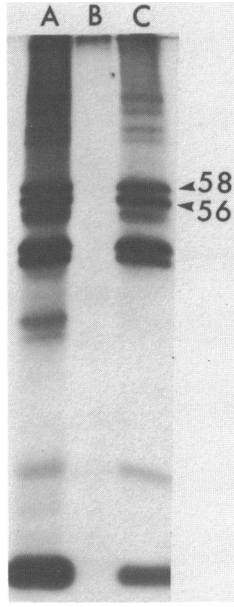

Figure 6. T antigens were extracted from wild type infected baby mouse kidney cells or from E. coli expressing wild type middle T. The T antigens were precipitated directly (A and B) or after mixing equal volumes of bacterial and animal cell extract (the total extract volume was the same in each case)(C). The immunoprecipitates were incubated with 50 μC γ^{32}P-ATP in 0.02M Tris-HCl, pH 7.5, 0.005M $MgCl_2$ for 20 min. After washing the T antigens were resolved on a 10% SDS gel.

methionine. The amount of middle T present in the bacterial cultures is ten thousand fold above that needed to detect kinase activity.

Wang and Baltimore have used a different approach in demonstrating activity of the Abelson transforming protein expressed in bacteria (47). Comparison of the labeling of bacterial proteins in the presence and absence of a plasmid expressing the Abelson product shows the appearance of new bands which contain phosphotyrosine. Using their protocols, the control E. coli cells used here can be labeled with $^{32}PO_4$ to give rise to a phosphoprotein pattern quite similar to theirs (Fig. 7). However, the expression of middle T in the bacteria (+ lane) does not lead to any obvious alteration in the pattern of cellular phosphoproteins.

DISCUSSION

Our goal has been to express the three polyoma early gene products in E. coli in order to produce these proteins for purification and analysis as

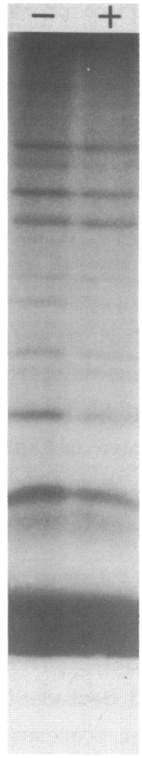

Figure 7. Control cells (-) or E. coli expressing wild type middle T (+) were labeled with $^{32}PO_4$ using the procedure of Wang and Baltimore (47). Cell pellets were washed twice in an ice cold buffer containing 0.05M Tris-HCl, pH 7.5, 0.01M $MgCl_2$, 0.10M NaCl. The cells were resuspended in the same buffer at a density of 3 x 10^9 cells/ml. To 45 µl of cells, 5 µl of $^{32}PO_4$ solution (300 µC/ml) was added. The cells were incubated at 37°C for 15 min. At the end of the labeling period, 100 µl of hot SDS buffer (5% SDS [W/V], 5% 2-mercaptoethanol [V/V], 0.12M Tris-HCl, pH 6.8, 20% [V/V] glycerol, 0.03% bromphenol blue) was added and the samples boiled for 15 min. Aliquots of the total protein were run at the 10% SDS gel.

well as to answer some specific questions about middle T antigen. All three of the wild type T antigens as well as the mutant middle T of Py-1387T have been expressed in E. coli. As a by-product of the cloning procedure, a fusion protein expressing the 71 N-terminal amino acids common to the three T antigens connected to β-galactosidase has also been constructed. The fusion protein, small T and Py-1387T middle T are present at 0.5-1.0 mg/liter E. coli. With the clones presently available, the fusion protein, small T and the Py-1387T middle T are being purified. Indeed we have

already used partially purified fusion protein and small T antigen to raise high affinity, high titer rabbit antisera. These reagents, previously unavailable in the polyoma field, have been of great use in our studies on the virus (Pallas, Kaplan, and Roberts, unpublished). Monoclonal antibody production is in progress. The purified proteins are also being used to test biological activities. In the case of SV40 small T, microinjection of the protein produced in bacteria, has been shown to cause the loss of actin cables (24). It will be interesting to see if the polyoma protein gives similar results. Also, SV40 small T has been shown to associate with cellular proteins (46). The purified protein has been used as an affinity reagent for the identification and purification of associating cellular proteins (C. Murphy, I. Bikel, and D. Livingston, personal communication). Similar experiments are being carried out with the polyoma proteins.

The clones expressing wild type middle T and large T, although useful for some analytical purposes, do not provide a rich source (less than 10 mg/liter) of starting material for further purification. Considerable effort has been expended to obtain increased levels of middle T. Originally, the middle T was expressed using a lac promoter; to improve expression a tac promoter was substituted. Part of the problem with middle T is clearly related to its rapid degradation in the E. coli. Lon^- cells, which have an altered ATP-dependent protease system (48), have been used to try to prevent degradation of middle T. The lon^- cells give limited success perhaps because they retain partial lon-activity. To try to improve the stability of the message coding for the viral proteins, RNase-deficient mutants have also been employed; so far, they have also proven to be of limited use.

The instability of middle T antigen is especially worth noting. Middle T is associated with membranes (15,16). Bacterial fractionation experiments (D. Pallas and T. Roberts, unpublished observations) show that the middle T in E. coli is also associated with membrane fractions. The middle T of the mutant Py-1387T is soluble in animal cells because it lacks the hydrophobic sequence at the C-terminus (9). This protein is much more stable in the bacterium than the wild type middle T. Therefore, the instability of the wild type protein seems to be directly connected to its association with membranes. Whether the protease which degrades the middle T is itself membrane bound, or whether the protease only recognizes a membrane-bound substrate is not clear.

The middle T expressed in E. coli is not labeled with $^{32}PO_4$. It is

worth noting that large T is also a phosphoprotein (41) which undergoes modification from a rapidly migrating form to a slower form (49). As shown in Fig. 2, the large T from E. coli also migrates more rapidly than its animal cell counterpart. It seems likely that large T also is not modified in bacteria in the same way as in animal cells. This is not unexpected. As Fig. 7 shows, E. coli appears to have relatively little protein phosphorylation.

The middle T antigen in bacteria lacks tyrosine kinase activity. The lack of middle T tyrosine kinase activity differs from that of other tumor virus products [$pp60^{v-src}$ (45), Abelson protein (47)] expressed in E. coli. There are at least three possible explanations for this difference. The trivial explanation that the plasmid has picked up an adventitious mutation can be ruled out because middle T reconstructed from the plasmid DNA transforms cells and has kinase activity. The second explanation is that the protein is incorrectly folded or modified. There are examples of such problems for certain secreted proteins. Platelet-derived growth factor is a specific instance (50). However, eukaryotic proteins which function intracellularly generally retain their activity when expressed in E. coli (45,47,51-53). It remains possible that middle T requires secondary serine/threonine phosphorylations to be active in the tyrosine kinase reaction. We favor the third possibility - that middle T is not itself a protein kinase. Previously we have shown that middle T lacks ATP-binding activity and that the kinase-active fraction of middle T can be resolved from the major fraction of the protein (16). Recently, Courtneidge and Smith (21) have shown that $pp60^{c-src}$, which is known to have tyrosine kinase activity, is associated with middle T from animal cells. Such an association would account for middle T kinase activity. The present results with middle T from E. coli are entirely consistent with this idea.

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