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Characterization of *am*404, an Amber Mutation in the Simian Virus 40 T Antigen Gene

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We analyzed the biological activity of an amber mutation, *am*404, at map position 0.27 in the T antigen gene of simian virus 40. Immunoprecipitation of extracts from *am*404-infected cells demonstrated the presence of an amber protein fragment (*am* T antigen) of the expected molecular weight (67,000). Differential immunoprecipitation with monoclonal antibody demonstrated that *am* T antigen was missing the carboxy-terminal antigenic determinants. The amber mutant was shown to be defective for most of the functions associated with wild-type T antigen. The mutant did not replicate autonomously, but this defect could be complemented by a helper virus (D. R. Rawlins and N. Muzyczka, J. Virol. **36**:611–616, 1980). The mutant failed to transform nonpermissive rodent cells and did not relieve the host range restriction of adenovirus 2 in monkey cells. However, stimulation of host cell DNA, whose functional region domain has been mapped within that portion of the protein synthesized by the mutant, could be demonstrated in *am*404-infected cells. A number of unexpected observations were made. First, the *am* T antigen was produced in unusually large amounts in a simian virus 40-transformed monkey cell line (COS-1), but overproduction was not seen in nontransformed monkey cells regardless of whether or not a helper virus was present. This feature of the mutant was presumably the result of the inability of *am* T antigen to autoregulate, the level of wild-type T antigen in COS-1 cells, and the unusually short half-life of *am* T antigen in vivo. Pulse-chase experiments indicated that *am* T antigen had an intracellular half-life of approximately 10 min. In addition, although the *am* T antigen retained the major phosphorylation site found in simian virus 40 T antigen, it was not phosphorylated. Thus, phosphorylation of simian virus 40 T antigen is not required for the stimulation of host cell DNA synthesis. Finally, fusion of *am*404-infected monkey cells with *Escherichia coli* protoplasts containing appropriate procaryotic suppressor tRNAs showed that *am*404 is a suppressible nonsense mutation.

Although nonsense mutations have been very useful in the identification of essential procaryotic and lower eucaryotic genes, their use in mammalian genetics has been largely prohibited by the lack of appropriate suppressor cell lines. Indeed, only a limited number of nonsense mutations are currently available in mammalian genes (7, 10, 15, 24, 64). In a previous report, we described the construction of a nonsense mutation in the simian virus 40 (SV40) T antigen gene (64). We chose SV40 T antigen because it is a multifunctional, autoregulatory protein (94) which, therefore, might be useful for the selection of even weak suppressor cell lines by several different approaches.

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The SV40 mutant *am*404 has an amber mutation at map position 0.27 in the T antigen gene (64) which was constructed in vitro by the site-directed bisulfite mutagenesis technique of Shortle and Nathans (81). DNA sequencing indicated that a C-to-T transition had occurred at nucleotide 3206 (6) which converted glutamine residue 538 to an amber codon. Because this nucleotide was also part of a *Pst*I recognition site, *am*404 was missing the *Pst*I site at 0.27 map units. The mutant was incapable of autonomous replication in monkey cells, but it could be complemented by a helper virus containing a late deletion (64).

SV40 T antigen is a multifunctional protein which is involved in SV40 replication (85), control of its own transcription (2, 39, 67, 88), transformation (reviewed in reference 94), stimulation of host cell DNA synthesis (11, 23, 34,

TABLE 1. Mammalian cell cultures used

Cells	Animal source	Relevant phenotype	Reference/source
Primary rat embryo, Fisher	Rat embryo	Primary culture, nontransformed	M. A. Bioproducts
BALB/c 3T3	Mouse embryo	Continuous line, nontransformed	1/W. W. Brockman
CV-C	African green monkey kidney	Continuous line, nontransformed	68/C. Anderson
BSC-40	African green monkey kidney	Continuous line, nontransformed, adapted to 40°C	5/D. Nathans
COS-1	African green monkey kidney	Continuous line, transformed with SV40	25/Y. Gluzman
Clone 412	Mouse	Hybridoma, fusion of BALB/c 3T3 spleen cells with myeloma cells NS1	32/E. Gurney; cell distribution center, Salk Institute

35, 76, 91), adenovirus (Ad) host range restriction (21, 63), activation of host rRNA genes (23, 82, 83), and the induction of other host cell functions (48, 55, 61, 74). T antigen also undergoes several forms of covalent modification including various degrees of phosphorylation (17, 27, 29, 51, 72, 87, 96). Biochemical characterizations of T antigen have shown it to be an ATPase (92) which can bind to specific SV40 sequences (50, 66, 77, 90) as well as form complexes with host proteins (30, 44, 46).

A wide variety of genetic techniques have been used to investigate the role played by T antigen in these various functions, including the use of temperature-sensitive mutants (86), Ad-SV40 hybrids (37), nonconditional deletion mutants (42), and, more recently, microinjection of cloned restriction fragments (53). The results indicate that at least some of the functions of T antigen reside in specific and independent regions on the T antigen protein (12, 20, 23, 31, 53, 83). Each of these approaches, however, has inherent limitations in the amount of genetic and biochemical information it can provide. Furthermore, relatively little information is available about the behavior of mammalian nonsense mutants (7, 10, 15, 24, 64). It was, therefore, of interest to determine the physical and biological characteristics of the truncated *A* gene product of *am*404. In this report, we examine the size, stability, and phosphorylation of the *am*404 T antigen as well as some of its biological activities.

TABLE 2. Recombinant plasmids used[a]

Designation	Insert	Site of insertion	Reference or source
pDR404	SV40 *am*404	*Pst*I	64
pMS2	SV40 *dl*1007	*Bam*HI	R. J. Samulski and N. Muzyczka (unpublished)
pDR1	SV40	*Bam*HI	D. Rawlins and N. Muzyczka (unpublished)

[a] The plasmid vector in all cases was pBR322.

MATERIALS AND METHODS

Cells, viruses, bacteria, and plasmids. Tables 1, 2, and 3 summarize the cell lines, bacterial strains, and plasmids which were used in this work as well as the laboratories which generously donated them. The BSC-40 line of African green monkey kidney cells was used routinely for production of wild-type SV40 and SV40 DNA and for complementation experiments (54). All cell lines, except clone 412, were routinely maintained in minimal Eagle medium (MEM) supplemented with 10% fetal calf serum (FCS) under a 5% carbon dioxide atmosphere at 37°C. Clone 412 was grown in Dulbecco modified Eagle medium with high glucose (4.5 g/liter) and 10% FCS.

The wild-type SV40 was the small plaque strain 776 (93) and was grown in BSC-40 cells in MEM with 2% FCS. Mixed stocks of SV40 mutants *dl*1007 (76) and *am*404 (64) were produced from DNA transfections (49) of BSC-40 cells. *am*404 viral stocks were grown in COS-1 cells in MEM with 10% FCS. Ad type 2 (Ad2) was a gift from R. Bohenzky.

Wild-type SV40 titers were determined by plaquing on CV-C cells as previously described (54) and were expressed as PFU. The titers for the *am*404 virus stocks were estimated by the ability to induce SV40-specific late proteins in COS-1 cells as described by

TABLE 3. Bacterial strains used

E. coli strain	Suppressor (Su)	Source
c	Su^-	D. Duckworth
HB101	Su^+ (Glu tRNA Sup, SuII)	H. Smith
DP50SupF	Su^+ (Glu tRNA Sup, SuII and Tyr tRNA Sup, SuIII)	F. Blattner

Gluzman (25). Titers for *am*404 were expressed as fluorescent focus units.

Enzymes. The restriction enzymes *Bam*HI and *Pst*I were purchased from Bethesda Research Laboratories. T4 DNA ligase was purchased from Bethesda Research Laboratories or Collaborative Research Laboratories. Pancreatic RNase A and lysozyme were obtained from Worthington Biochemical Corp. and Sigma Chemical Co., respectively.

DNA preparations. Wild-type SV40 DNA was isolated by the method of Hirt (33) as previously described (54). Plasmid DNA was also isolated as described previously (64). *am*404 DNA and *dl*1007 DNA were extracted from the recombinant plasmids pDR404 and pMS2, respectively, by restriction of the plasmids with the appropriate enzyme and separation of the SV40 and pBR322 sequences by RPC-5 chromatography (64). The viral DNA was then ligated to form monomer circles, and the form I DNA was isolated by centrifugation in cesium chloride-ethidium bromide gradients. All recombinant DNA experiments were done in compliance with the National Institutes of Health guidelines.

Antisera. Hamster anti-SV40 tumor (T antigen) sera were obtained from J. S. Cole III, Biological Carcinogenesis Branch, National Cancer Institute. Two lots of these sera, 72X-783 and 80X-3, were used in these studies. Antibody 412 (32) was prepared from the suspension growth medium in which the clone 412 cells had been grown. The protein in 1 liter of growth suspension medium was precipitated with 50% ammonium sulfate. The protein pellet was dissolved in 30 ml of distilled water, and the solution was dialyzed extensively against phosphate-buffered saline (PBS). The antibody was further concentrated with polyethylene glycol to a volume of 6 ml. Horse anti-SV40 neutralizing serum was purchased from Flow Laboratories. This antiserum showed no cross-reactivity with SV40 early proteins and was used in immunofluorescence assays for the SV40 capsid antigens. Rabbit anti-Ad2 fiber serum was a generous gift from C. W. Anderson.

Fluorescein-labeled antibodies (conjugates) were used for the indirect immunofluorescence technique. Goat anti-hamster immunoglobulin G (IgG) (heavy and light chains) was obtained from Cappel Laboratories, and rabbit anti-hamster IgG (whole molecule) was purchased from Miles Laboratories. No differences were noticed in the quality of staining achieved with these two antisera. Goat anti-mouse IgGs (heavy and light chains) were purchased from Cappel Laboratories and United States Biochemical Corp., respectively. Rabbit anti-horse IgG (heavy and light chains) was also obtained from United States Biochemical Corp. Goat anti-rabbit IgG was a gift from E. Siden. All conjugates were diluted 1:10 with PBS before use. Antisera were stored at −20°C.

Labeling and extraction of viral and cellular proteins. Proteins in virus-infected or noninfected cells were labeled in vivo with [^{35}S]methionine (1,200 to 1,400 Ci/mmol; Amersham Corp.) or $^{32}P_i$ (Amersham). At 30 to 35 h postinfection, the cells were washed once with methionine-free or phosphate-free MEM. The cells were then incubated in MEM with 75 to 125 μCi of [^{35}S]methionine per ml or with 100 to 1,000 μCi of $^{32}P_i$ per ml for 5 min at 37°C. Preliminary experiments (not shown) indicated that the optimum labeling time for the 67,000 (67K)-molecular-weight amber T peptide (*am* T antigen) was 5 to 15 min. The cells were washed three times with cold PBS, and 1 ml of protein extraction solution (10 mM Tris-hydrochloride [pH 8.0], 140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, 1.0% Nonidet P-40, 200 μg of phenylmethylsulfonyl fluoride per ml) was added to each 10-cm dish. The cells were scraped into a 15-ml centrifuge tube 5 min later and placed at 0°C for 20 min. The sample was then centrifuged at 2,500 rpm for 10 min at 4°C to remove cellular debris and cell nuclei. The supernatant was clarified by centrifugation at 100,000 × *g* for 30 min at 4°C. Bovine serum albumin (Sigma) was added to a final concentration of 100 μg/ml, and the extract was stored at −20°C.

Immunoprecipitation of T antigens. Radiolabeled proteins from virus-infected and noninfected cells were incubated with antisera for 16 to 24 h at 4°C. Antigen-antibody complexes were adsorbed to Formalin-treated protein A-bearing staphylococci (Bethesda Research Laboratories) as described previously by Kessler (38). The antibody precipitates were resuspended in protein sample buffer (62.5 mM Tris-hydrochloride [pH 6.8], 2% sodium dodecyl sulfate [SDS], 20% glycerol, 70 mM 2-mercaptoethanol, 0.001% bromphenol blue) and boiled for 5 min. The boiled samples were clarified by centrifugation, and the supernatant was removed for electrophoresis.

Immunoprecipitated proteins were fractionated by discontinuous SDS-polyacrylamide gel electrophoresis as described by Laemmli (41). Electrophoresis was at 75 to 100 V through a 4% stacking gel and a 7.5% resolving gel (15 by 19 by 0.15 cm). If the proteins were labeled with [^{35}S]methionine, the gel was fluorographed by immersing it in 1 M sodium salicylate (9). The positions of the protein bands were determined by autoradiography at −70°C against Dupont Cronex-4 or Kodak XAR-5 X-ray film with a Dupont HiPlus intensifying screen.

Pulse-chase analysis. Monolayers of COS-1 cells in 6-cm dishes were infected with *am*404 virus stock (1 ml per 6-cm dish) at a multiplicity of infection of 3. At 30 h postinfection, a pulse-chase schedule was begun in which the cells were labeled for 5 min (pulse period) with [^{35}S]methionine (75 μCi/ml) in methionine-free MEM, washed with MEM containing a 100-fold excess of unlabeled methionine, and incubated for various lengths of time (chase period) in MEM with unlabeled methionine. When the chase period was completed, protein extracts were prepared as described above. The total protein in each extract was determined by the procedure of Bradford (3), and equal amounts of protein were immunoprecipitated and analyzed by electrophoresis on a 7.5% SDS-polyacrylamide gel. Examination of the ^{35}S radioactivity in trichloroacetic acid precipitates of the protein extracts indicated that the specific activity of the total protein did not increase during the chase period. Densitometer tracings were made by scanning the film with a double-beam Joyce-Loebl microdensitometer. The turnover rate of *am* T antigen was determined as the rate of disappearance of the protein as represented by the area under its peak.

Indirect immunofluorescence. Viral proteins were detected in infected and in transformed cells by modifications of the immunofluorescent staining procedure of Pope and Rowe (62). Indirect immunofluorescence was used to demonstrate the presence of SV40 T

antigen, the carboxy terminus of SV40 T antigen, SV40 late proteins, and Ad2 fiber proteins in cells which had been infected with virus or transfected with DNA. Nuclear fluorescence was observed with a Zeiss fluorescence microscope. Randomly chosen fields were scored for fluorescent cells until a total of 100 cells had been counted. Cells were photographed with Kodak Ektachrome film (ASA400).

Transformation of nonpermissive cells. BALB/c mouse 3T3 cells and primary rat embryo cells were used for transformation by SV40 virus or DNA. The cells were grown to 20% confluency in 10-cm dishes (ca. 3×10^6 cells per dish), and the growth medium was removed. For viral infections, 1 ml of virus stock (wild-type SV40 or *am*404) was added to the cells (multiplicity of infection, 1 to 10) and allowed to adsorb for 1.5 h. The cells were incubated in MEM-5 for 12 h and then trypsinized, diluted 10-fold, and plated onto 10-cm dishes.

DNA-mediated transformation utilized the calcium phosphate transfection procedure of Graham and van der Eb (28) as modified by Frost and Williams (22). Both virus-infected and DNA-transfected cells were fed with MEM-2 at 3- to 4-day intervals, and viral transformation was detected by the formation of densely staining foci (Giemsa stain). Transformed foci were subcloned (only one focus from each dish) and tested for the presence of SV40 T antigen by indirect immunofluorescence.

Ad2 fiber protein expression. Support of Ad2 infection of CV-C monkey cells by SV40 or *am*404 was demonstrated by indirect immunofluorescence as described previously by Zorn and Anderson (98). Subconfluent CV-C cells on cover slips (11 by 22 mm) were transfected with 1 μg of SV40 DNA, 1 μg of *am*404 DNA, or 1.8 μg of pDR404 DNA or mock transfected by the DEAE-dextran technique. The transfection solutions also contained Ad2 at a high multiplicity. At 30 to 36 h postinfection, the cells were fixed and stained, and the number of Ad2 fiber-positive cells was determined by counting the cells demonstrating positive nuclear fluorescence on each cover slip. For comparative purposes, the numbers were normalized to correct for various numbers of cells on the cover slips.

Stimulation of cellular DNA synthesis. A modification of the technique of Scott et al. (76) was used to assess the stimulation of cellular DNA synthesis by SV40 and *am*404. Briefly, BALB/c mouse 3T3 cells were uniformly labeled for three generations with [^{14}C]thymidine (10 μCi per dish), and confluent monolayer cells were growth arrested by incubation for 48 h in MEM supplemented with 2% FCS that had been heated to 70°C. The medium was then removed and the cells were infected. After infection, the conditioned medium was replaced, and the cells were labeled from 18 to 30 h postinfection with [^{3}H]thymidine (50 μCi per dish). The cellular DNA was extracted, and acid-precipitable ^{3}H counts per minute were measured and normalized to a constant number of ^{14}C counts per minute to correct for variations in the harvesting of cellular DNA.

Protoplast fusion. Bacterial protoplasts were formed by the lysozyme-EDTA treatment of Weiss (97), except that 50 mM Tris-hydrochloride (pH 8) was used throughout, and DNase was added to 20 μg/ml as described previously by Schaffner (71). Protoplasts were then diluted 1:1 with MEM. At 12 h postinfection, 50% confluent CV-C cells (6-cm dish) were washed once with PBS and overlaid with 10^4 protoplasts per CV-C cell. The protoplasts were centrifuged onto the mammalian cell layer (200 rpm, 3 min, 25°C), the supernatant was decanted, and 1 ml of 50% polyethylene glycol 1,000 in MEM was added to the dish for 1.5 min at 25°C. The polyethylene glycol was then removed, and the cells were rinsed three times with PBS at 25°C. The cells were then either incubated in MEM with 10% FCS and gentamicin (10 μg/ml) for 24 h and fixed for immunofluorescence or labeled with ^{32}P, for 24 h before harvesting.

RESULTS

Detection of *am*404 T antigen. The amber mutation in *am*404, at map position 0.27, was expected to truncate the coding region for the SV40 T antigen by 24% (6). Therefore, an *am* T antigen was expected that was approximately 76% of the wild-type protein. The molecular weight of T antigen seems to vary depending on the host cell line and the extraction procedure (86, 94), but in this investigation it was consistently 88 to 90K on SDS-polyacrylamide gels. Thus, the predicted molecular weight for *am* T antigen was 67 to 68K.

When *am*404 plus *dl*1007-infected BSC-40 cells were labeled with [^{35}S]methionine, an immunoprecipitable protein of 67K could be demonstrated (Fig. 1 lane c). The 67K protein was not found in immunoprecipitates from mock-infected cells (lane b), in immunoprecipitates from SV40-infected cells (lane d), or in immunoprecipitates of *am*404 plus *dl*1007-infected cells when normal hamster serum was used (not shown). In this particular experiment (Fig. 1, lane c), the amount of *am* T antigen compared with the amount of wild-type T antigen produced was comparable to the relative amounts of *am*404 and *dl*1007 DNA found in similarly infected cells (not shown). More frequently, however, there was decidedly less *am* T antigen than wild-type T antigen produced even when the ratio of mutant DNA to wild type was 1:1. No *am* T antigen was detected after *am*404 DNA transfection or *am*404 viral infection in the absence of helper (not shown).

The anti-T antigen serum used in Fig. 1 was a polyclonal antibody with heterogeneous binding activities directed against many antigenic determinants of SV40 T antigen. Consequently, it could not distinguish which portion of the SV40 T antigen was missing in the 67K *am* T peptide. To show that the *am* T antigen was specifically missing the C-terminal portion of SV40 T antigen, we used the monoclonal antibody produced by hybridoma clone 412 (32). The 412 antibody has been shown to be specific for the C-terminal antigenic determinants of T antigen coded by the nucleotide sequences between 0.28 and 0.17 map units on the SV40 genome (16). A sequen-

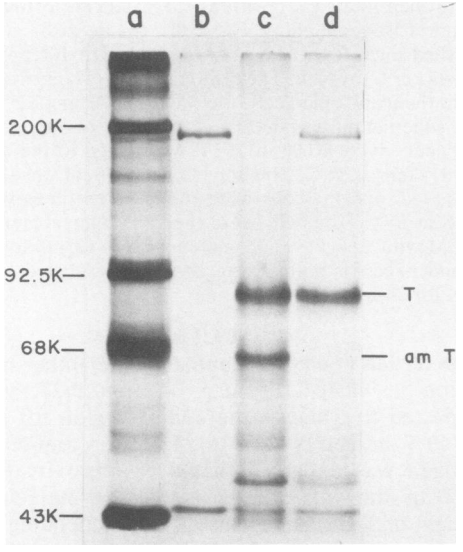

FIG. 1. Immunoprecipitation of *am*404 T antigen from BSC-40 monkey cells. [^{35}S]methionine-labeled immunoprecipitates from infected or noninfected BSC-40 cells were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis and fluorography. Anti-T antigen serum from tumor-bearing hamsters was used for the immunoprecipitations. Lane a, ^{14}C-labeled marker proteins (myosin, 200K; phosphorylase b, 92.5K; bovine serum albumin, 68K; ovalbumin, 43K); lane b, immunoprecipitate from mock-infected cells; lane c, immunoprecipitate from *am*404 plus *dl*1007-infected cells; lane d, immunoprecipitate from wild-type SV40-infected cells.

tial immunoprecipitation procedure was used to address this issue. When the protein extract from cells infected with *am*404 and *dl*1007 was precipitated with 412 antibody, only the full-length T antigen was precipitated (Fig. 2, lane b). When the supernatant of the 412 immunoprecipitation was precipitated with additional 412 antibody, again only the T antigen was precipitated (not shown). However, when the supernatant was precipitated with hamster anti-SV40 T antigen, both the 67K *am* T antigen and the remaining T antigen were precipitated (Fig. 2, lane c). These results suggest that the protein designated *am* T antigen lacks the carboxy-terminal portion of SV40 T antigen.

Overproduction of *am* T antigen in COS-1 cells. During an *am*404 plus *dl*1007 infection of BSC-40 cells, there is an increase in the copy number of both amber and wild-type T antigen genes as a result of viral DNA replication (64). Because SV40 T antigen has the capacity to regulate its own transcription (2, 39), the expression of *am* T antigen in the presence of a limited amount of wild-type T antigen was investigated. COS-1 cells have been shown to produce a low constitutive level of T antigen from the single integrated SV40 genome per cell (25). For this reason, COS-1 cells were infected with *am*404 virus, and the resulting protein extracts were examined after immunoprecipitation with polyclonal hamster anti-T antigen sera. The extract in Fig. 3, lane a, was from uninfected cells, and only the wild-type T antigen was detected. The immunoprecipitate in Fig. 3, lane b, was from cells infected with *am*404 virus. Clearly, there was a much higher rate of synthesis of the 67K *am* T protein in COS-1 cells than in cells coinfected with *am*404 and *dl*1007 (Fig. 1). Differential immunoprecipitation with monoclonal antibody 412 was used to confirm that this was *am* T antigen (not shown). Immunoprecipitations after comparable infections of BSC-40 cells or CV-C cells with the same *am*404 viral stock did not

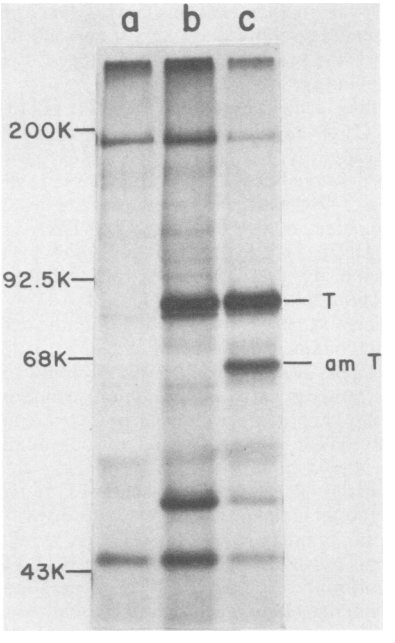

FIG. 2. Differential immunoprecipitation of *am*404 T antigen and wild-type T antigen from BSC-40 cells. [^{35}S]methionine-labeled immunoprecipitates from infected or noninfected BSC-40 cells were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis and fluorography. The extract from the noninfected cells (lane a) was immunoprecipitated with anti-T antigen serum from tumor-bearing hamsters. The extract from the cells infected with *am*404 plus *dl*1007 (lane b) was immunoprecipitated with monoclonal antibody 412. The supernatant from the monoclonal antibody precipitation was then precipitated with the polyclonal anti-T antigen serum from tumor-bearing hamsters (lane c). Molecular weight markers (not shown) are as described in the legend to Fig. 1.

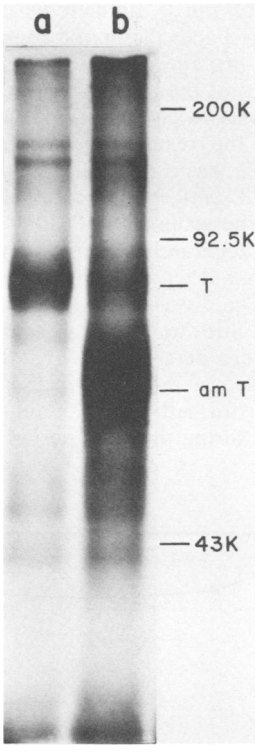

FIG. 3. Immunoprecipitation of *am*404 T antigen from COS-1 cells. [^{35}S]methionine-labeled immunoprecipitates from *am*404-infected or noninfected COS-1 cells were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis and fluorography. Anti-T antigen serum from tumor-bearing hamsters was used for the immunoprecipitations. Lane a, Noninfected COS-1 cells; lane b, *am*404-infected COS-1 cells. The molecular weight markers (not shown) are as described in the legend to Fig. 1.

detect any truncated protein or full-length T antigen (not shown). We estimated that there was at least a 50-fold-higher rate of synthesis of *am* T antigen in COS-1 cells than that seen in mixed *dl*1007 plus *am*404 infections of BSC-40 cells (Fig. 1).

Phosphorylation of *am* T antigen. One of the major phosphorylation sites on the SV40 T antigen has been shown to be near the amino-terminal end of the protein (70, 72, 75, 95, 96), and therefore it was expected that *am* T antigen would be phosphorylated. Experiments designed to determine the level of phosphorylation of *am* T antigen were carried out with *am*404-infected COS-1 cells. In an experiment exactly paralleling the one illustrated in Fig. 3, COS-1 cells were infected with *am*404 virus stock and labeled with $^{32}P_i$, and the resulting protein extracts were immunoprecipitated and analyzed in an SDS-polyacrylamide gel (Fig. 4). Again, lane a was the immunoprecipitate from uninfected cells and lane b contained the immunoprecipitate from *am*404-infected cells. Although in Fig. 3 a large amount of ^{35}S-labeled protein was found at the position of *am* T antigen in extracts from infected cells, little or no difference was apparent in the ^{32}P-labeled immunoprecipitates from either infected or uninfected cells shown in Fig. 4. We concluded that the *am*404 T antigen is not phosphorylated in vivo.

In vivo stability of *am* T antigen. The selective degradation of abnormal proteins, including nonsense peptides, has been well documented in both procaryotic and eucaryotic cells (26). The fact that *am* T antigen was difficult to isolate after extended pulse periods or under conditions in which the amber mutant was not amplified prompted a pulse-chase analysis of the protein to assess its stability in vivo. The apparent over-

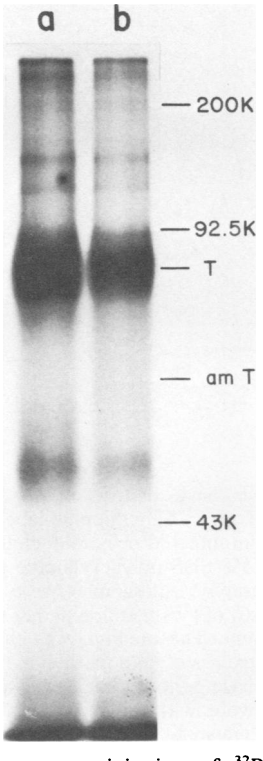

FIG. 4. Immunoprecipitation of ^{32}P-labeled proteins from *am*404-infected COS-1 cells. ^{32}P-labeled immunoprecipitates from infected or noninfected COS-1 cells were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis and autoradiography. Immunoprecipitations were with anti-T antigen serum from tumor-bearing hamsters. Lane a, Noninfected cells; lane b, *am*404 virus-infected cells. Molecular weight markers (not shown) are as described in the legend to Fig. 1.

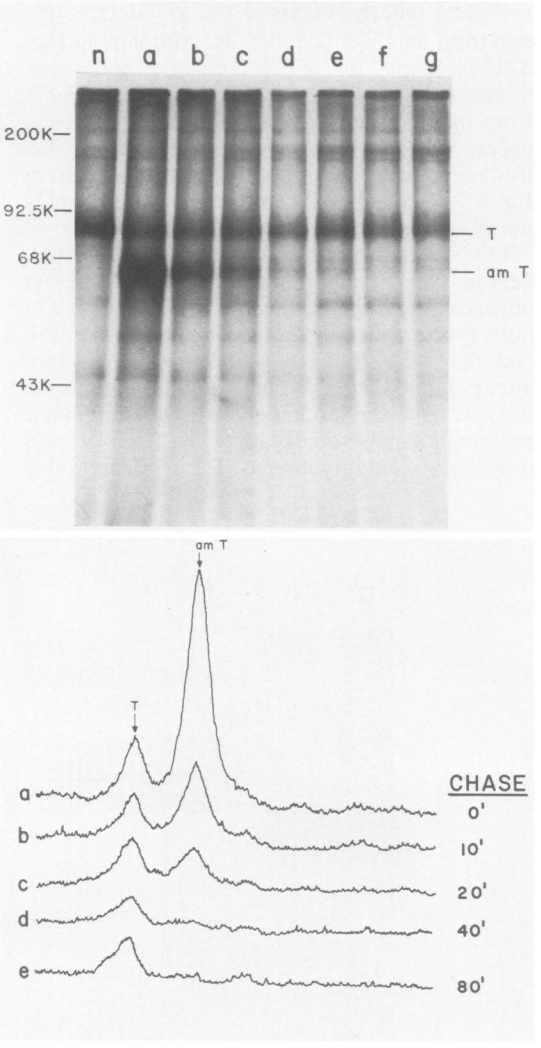

FIG. 5. Pulse-chase analysis of *am* T antigen in COS-1 cells. Top, [^{35}S]methionine-labeled immunoprecipitates from infected or noninfected COS-1 cells analyzed by 7.5% SDS-polyacrylamide electrophoresis and fluorography. All samples were labeled for 5 min with 100 μCi of [^{35}S]methionine per ml in methionine-free medium. The medium was then removed, and a 100-fold excess of cold methionine medium was added for the chase periods indicated below. Immunoprecipitations were with anti-T antigen serum from tumor-bearing hamsters. Lane a, No chase; lane b, 10-min chase; lane c, 20-min chase; lane d, 40-min chase; lane e, 80-min chase; lane f, 160-min chase; lane g, 320-min chase. Lane n is the immunoprecipitate from noninfected cells (no chase period). The molecular weight markers (not shown) are as described in the legend to Fig. 1. Bottom, Shorter exposure of the immunoprecipitates seen in the top panel traced with a densitometer. Tracings a to e correspond to the lanes seen in the top panel.

expression of *am* T antigen in *am*404 virus-infected COS-1 cells facilitated consistent recovery of the protein and measurement of the turnover rate of the amber peptide. The pulse-chase analysis was performed as described above, and the results are shown in Fig. 5. Clearly, there was a rapid disappearance of the labeled *am* T antigen, indicating a very short half-life for the protein.

To more accurately determine the turnover rate for the *am* T antigen, a light exposure of the gel seen in Fig. 5 was scanned with a microdensitometer. Values for the areas under each protein peak were determined (Fig. 6). Wild-type SV40 T antigen, as expected from investigations of SV40-transformed cells (17), did not turn over appreciably during the time period used in this

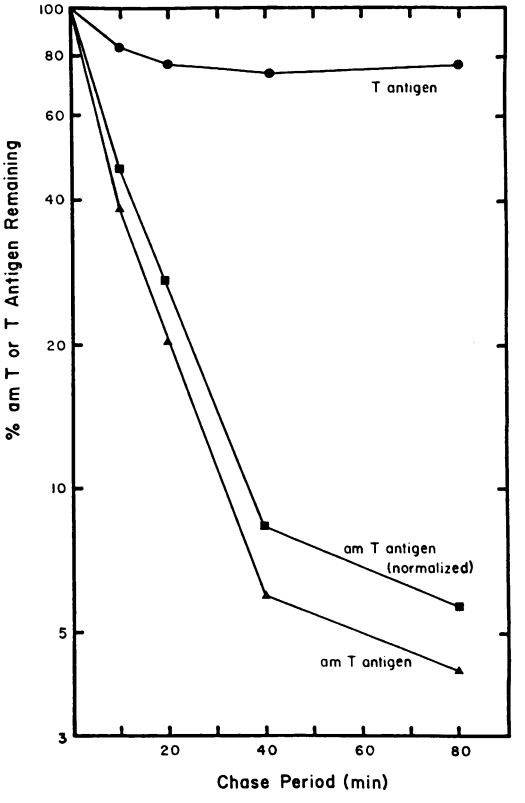

FIG. 6. Turnover of *am* T antigen in COS-1 cells. The disappearance of [^{35}S]methionine-labeled *am* T antigen or wild-type SV40 T antigen during the chase period of the pulse-chase analysis is shown as the percentage of labeled *am* T antigen or T antigen remaining at the specified time points. The values at the time points were determined from the areas under the protein peaks of the densitometer tracing shown in Fig. 5. Symbols: ●, wild-type SV40 T antigen; ▲, *am* T antigen; ■, *am* T antigen values normalized to a constant value for wild-type SV40 T antigen.

TABLE 4. Transformation of nonpermissive cells by pDR404 DNA

Cells[a]	Transforming agent	Amount per dish	No. of foci per dish	T antigen-positive subcloned foci (%)[b]
BALB/c mouse 3T3	SV40	5×10^7 PFU	>60	100 (5/5)
	None[c]		<2	0 (0/5)
	pDR404 DNA	10 μg	<2	0 (0/3)
	pDR404 DNA	5 μg	<2	0 (0/3)
	pDR404 DNA	1 μg	<2	0 (0/3)
	pMS2 DNA	10 μg	7	80 (4/5)
	pMS2 DNA	5 μg	4	100 (2/2)
	pMS2 DNA	1 μg	<2	NT[d]
Primary rat embryo	SV40	5×10^7 PFU	176	100 (5/5)
	None[c]		2	0 (0/2)
	pDR404 DNA	20 μg	2	0 (0/2)
	pDR404 DNA	10 μg	2	0 (0/2)
	pDR404 DNA	5 μg	0	NT
	pMS2 DNA	20 μg	22	100 (5/5)
	pMS2 DNA	10 μg	6	NT

[a] Cells were infected or transfected when at a density of 3×10^6 cells per 10-cm dish.
[b] Percentage of foci which were positive for SV40 T antigen by indirect immunofluorescence; numbers in parentheses indicate the number of T-positive clones/total number of clones tested.
[c] Mock-transfecting solutions contained 50 μg of carrier herring sperm DNA per ml.
[d] NT, Not tested.

experiment. If the turnover of T antigen is assumed to be negligible, then the variation in the amount of wild-type T antigen at each chase time can be used as an internal control for variations in the total protein used for each time point. When each *am* T antigen value was normalized against its respective T antigen value, the half-life of the amber peptide was found to be approximately 10 min.

Transformation of nonpermissive cells by *am*404. The ability of *am*404 to transform rodent cells was assayed after infection with *am*404 virus or after calcium phosphate (22, 28) transfection with pDR404 plasmid DNA. Positive transformation of both cell types was detected as the formation of dense foci of cells. The DNA transfection experiments (Table 4) indicated that pDR404 was incapable of transforming nonpermissive cells. Extensive attempts to transform rodent cells with *am*404 virus were equally unsuccessful. No BALB/c mouse 3T3 clones and only five transformed rat primary cell clones were found after *am*404 infection. Because the *am*404 viral stocks were contaminated (0.5%) with wild-type revertants (see below), it was not surprising to obtain a few SV40-transformed clones after infection by the mutant virus. Three of the rat primary clones were tested for T antigen expression, and all three were positive for wild-type T antigen by indirect immunofluorescence with monoclonal 412 antibody. One of these is shown in Fig. 7. Two of these lines were subsequently shown to contain at least one copy of the wild-type T antigen gene by Southern blotting experiments (i.e., at least one integrated SV40 copy contained an intact *Pst*I site at map position 0.27).

Finally, rodent (rat primary or BALB/c mouse 713) and monkey (CV-C) cells were infected with *am*404 virus and examined for T antigen immunofluorescence with hamster anti-T antigen or 412 antibody at 24 and 48 h postinfection. When this was done, no significant immunofluorescence was seen in either the nucleus or the cytoplasm of *am*404-infected cells. The appearance of these cells was indistinguishable from that of comparable cells stained with normal serum (Fig. 7C). Thus, neither BALB/c mouse 3T3 cells nor primary rat embryo cells appeared to be susceptible to transformation by the nonsense mutant. It should be noted, however, that the possibility that *am*404 can transform or immortalize rodent cells at a low frequency has not been completely excluded.

Support of productive Ad2 infection in monkey cells. The productive infection of monkey cells by Ad2 virus can be enhanced by coinfection of the cells with SV40 (21, 63). Because this function has been mapped to a region of wild-type T antigen that should be absent in *am* T antigen (12, 20, 31), *am*404 was tested to determine whether or not it could stimulate Ad2 infection. Measurement of Ad2 fiber protein expression has been used to demonstrate the SV40 stimulation effect on Ad2 virus infection of monkey cells (92), and the Ad-enhancing ability of *am*404 was assayed in this manner.

CV-C cells were transfected with SV40,

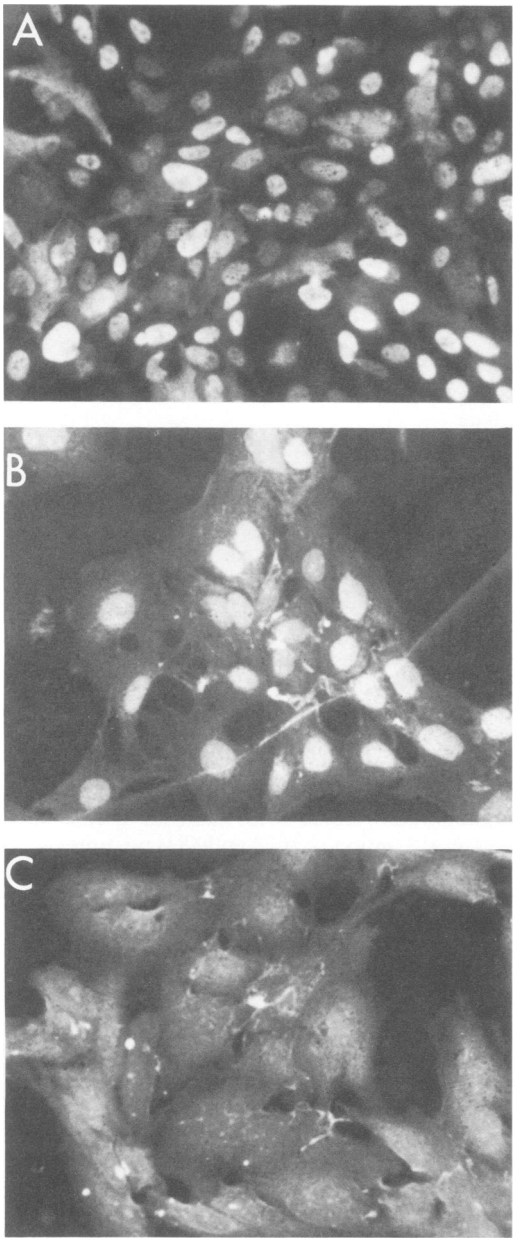

FIG. 7. Immunofluorescence in transformed primary rat embryo cells. T antigen in *am*RE-3, a subcloned cell line resulting from transformation with *am*404 virus stock, is shown with the following primary antisera: (A) anti-T antigen serum from tumor-bearing hamsters; (B) monoclonal antibody 412; (C) normal mouse serum. The fluorescein-labeled second antisera were the following: (A) rabbit anti-hamster immunoglobulin (×200 magnification); (B and C) rabbit anti-mouse immunoglobulin (×400 magnification).

TABLE 5. Support of productive Ad infection in CV-C monkey cells by *am*404 or pDR404 DNA transfection

DNA	DNA per dish (μg)	Ad2	No. of cells positive by immunofluorescence for Ad2 fiber protein per cover slip[a]
None	0	−	0
	0	+	14
SV40	1.0	−	0
	1.0	+	497
*am*404	1.0	−	0
	1.0	+	28
pDR404	1.8	−	0
	1.8	+	15

[a] 5×10^4 cells per cover slip.

*am*404, or pDR404 DNA with Ad2 (for infection) in the transfecting solutions. At 36 h postinfection, the cells were stained for Ad2 fiber protein by immunofluorescence. Cells exhibiting nuclear fluorescence were counted, and the counts were normalized to correct for variations in the numbers of cells in each experiment.

The results (Table 5) indicated that SV40 DNA was at least 17- to 33-fold more efficient in stimulating Ad2 fiber protein expression than *am*404 or pDR404 DNA. We concluded that *am*404 does not enhance the productive infection of Ad2 in CV-C cells.

Stimulation of host cell DNA synthesis by *am*404. The stimulation of host cell DNA synthesis by *am*404 (Table 6) was detected as the increased incorporation of [^{3}H]thymidine into newly synthesized cellular DNA after infection of growth-arrested BALB/c mouse 3T3 cells with *am*404 virus (see above). A preliminary

TABLE 6. Stimulation of host cell DNA synthesis by *am*404 virus

Virus[a]	Wild-type SV40 (PFU)	*am*404 (FFU)[b]	[^{3}H]DNA (cpm)
None[c]			9,406
SV40	2.5×10^7		582,815
SV40	2.5×10^5		36,463
SV40	2.5×10^2		2,331
*am*404	7.5×10^4	1.5×10^7	390,295

[a] 2×10^5 BALB/c mouse 3T3 cells were infected in each experiment.

[b] FFU, Fluorescent focus units.

[c] Frozen and thawed medium from mock-infected COS-1 cells was added during the infection period.

TABLE 7. Phenotypic suppression of *am*404 by protoplast fusion with Su^+ *E. coli*

Virus[a]	*E. coli* strain	% T antigen-positive cells[b]	Replication[c]
SV40		70	+
*am*404		<0.1	–
*am*404	c (Su^-)	<0.2	–
*am*404	HB101 (Su^+)	1	NT[d]
*am*404	DP50SupF (Su^+)	5	+
None	pDR1[e]	3	NT

[a] Multiplicity of infection was 1.

[b] Determined with polyclonal anti-T antigen antibody.

[c] Replication was assessed by isolating Hirt (33) supernatant DNA from in vivo ^{32}P-labeled cells. The DNA was fractionated in a 1.4% agarose gel with appropriate markers.

[d] NT, Not tested.

[e] pDR1 is *E. coli* HB101 carrying the wild-type SV40-pBR322 recombinant plasmid pDR1. SV40 is inserted in the *Bam*HI site of pBR322.

time course (not shown) was done to determine the optimum labeling time.

Before growth arrest the cellular DNA had been uniformly labeled with [^{14}C]thymidine, and variations in the harvesting of the cellular DNA were corrected by normalization to a constant number of ^{14}C counts per minute. The results (Table 6) indicated that *am*404 had retained the capacity to stimulate host cell DNA synthesis at a level comparable to that of wild-type SV40.

As mentioned above, the *am*404 viral stock which was grown in COS-1 cells contained a significant amount of wild-type virus (Table 6). Presumably, this was due to the fact that wild-type revertants had a replicative advantage over the mutant during lytic growth in COS-1 cells (data not shown). To demonstrate that the stimulation of host cell DNA synthesis seen with *am*404 was not due to the contaminating wild-type virus, we did reconstruction experiments with various dilutions of wild-type SV40. The results (Table 6) indicated that the 0.5% wild-type contaminant in the *am*404 stock was not responsible for the stimulation.

Phenotypic suppression of amber mutant. To determine whether the nonsense mutation at map position 0.27 was the only defect in *am*404, we devised a method for phenotypically suppressing the nonsense mutant (see above) which is similar to a method previously used by Capecchi et al. (7). Protoplasts were made from *Escherichia coli* c, which was suppressor negative (Su^-), or from the suppressor-positive strains HB101 (SuII) and DP50SupF (SuII, SuIII). The protoplasts were then fused with CV-C cells (10^4 protoplasts per cell) which had been infected with *am*404 virus. We found (Table 7) that only CV-C cells which had been fused with Su^+ protoplasts were positive for T antigen immunofluorescence. Cells which had been fused with *E. coli* c (Su^-) protoplasts or infected with virus alone showed no evidence of T antigen expression. When protein extracts from *am*404-infected cells that had been fused with Su^+ protoplasts were immunoprecipitated with hamster α T antiserum, the presence of full-length T antigen could be demonstrated (not shown). In addition, when CV-C cells which had been fused with *E. coli* c or DP50SupF protoplasts were labeled in vivo with $^{32}P_i$, SV40 replication was seen only in those cells which had been fused with the Su^+ protoplasts (Table 7). Thus, the mutant could be transiently suppressed by the addition of the appropriate charged suppressor tRNA to the mammalian cytoplasm.

DISCUSSION

The gene *A* product of *am*404 was expected to be a truncated form of the SV40 T antigen with a molecular weight of 67 to 68K. A protein of this size (67K) was identified by immunoprecipitation of extracts from *am*404 plus *dl*1007-infected BSC-40 cells with anti-SV40 T antigen serum. Differential immunoprecipitation with monoclonal 412 antibody indicated that the C-terminal portion of T antigen was missing in *am* T antigen. In vivo phenotypic suppression of *am*404 by protoplast fusion with Su^+ bacteria indicated that the amber mutation was the only defect in *am*404.

Additional evidence that *am*404 is a point mutation is the fact that *am*404 reverts to the wild-type phenotype at a frequency of approximately 10^{-5}. Furthermore, restriction enzyme analysis of *am*404 revertants indicates that they invariably have regained the *Pst*I site at map position 0.27 (not shown). Thus, genetically, *am*404 appears to be a conventional nonsense mutant.

Both the 90K SV40 T antigen and the 67K *am* T antigen obviously migrate more slowly on SDS-polyacrylamide gels than would be predicted from their calculated sizes of 81 and 61.5K, respectively. We have no satisfactory explanation for the apparent molecular weight discrepancy.

A number of laboratories have mapped some of the functional regions of SV40 T antigen by the use of defined restriction fragments (23, 53), deletion mutants (8, 12, 13, 19, 42, 57–60, 70, 76, 79, 83), and point mutants (14, 43, 64, 80, 84) of the *A* gene as well as Ad-SV40 hybrids (20, 31). In addition, the phosphorylation sites of T antigen have also been mapped (40, 72, 73, 75, 95, 96). A partial summary of these results is shown in Fig. 8. Based on the position of the *am*404

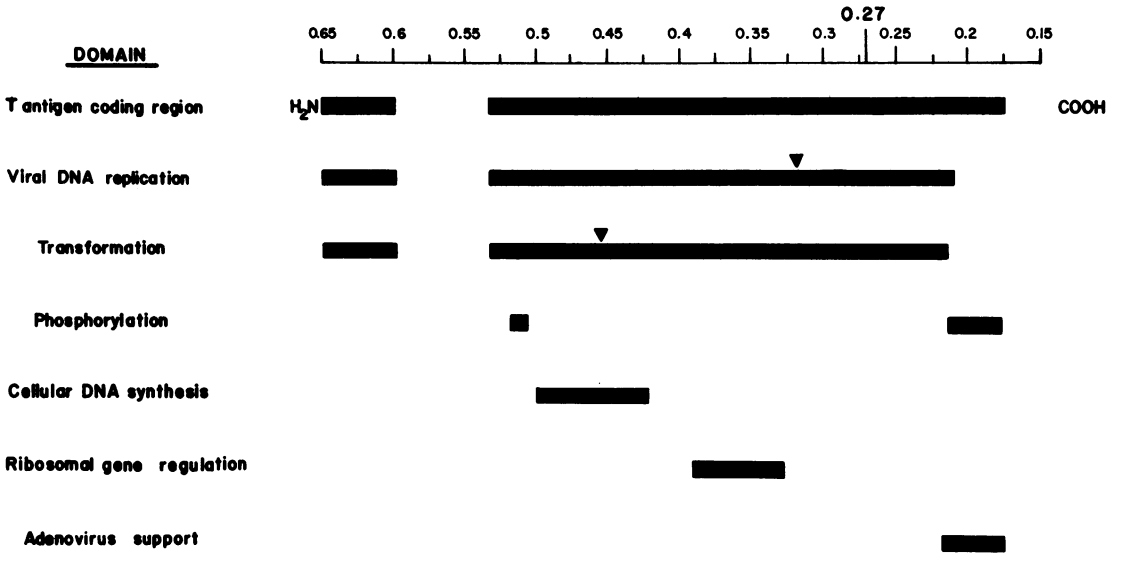

FIG. 8. Partial map of the functional regions of SV40 T antigen. The bars indicate the regions of T antigen which appear to be required for each function as determined by microinjection or transfection of restriction fragments or the study of deletion mutants, temperature-sensitive mutants, and Ad-SV40 hybrids. The information presented was taken from the following references: viral DNA replication and transformation (12–14, 19, 43, 58, 60, 76, 80, 84), phosphorylation (72), cellular DNA synthesis (83), ribosomal gene regulation (83), and Ad support (12, 20). The nonessential coding regions for viral DNA replication and transformation were deduced from the positions of deletions *dl*1140, *dl*1066, *dl*1263, *dl*1265, *tsA*1499, and *dl*2194. The arrows above the transformation and viral DNA replication lines indicate the positions of the point mutations SVR9D and *tsA*1642, respectively.

mutation (Fig. 8), it was expected that *am*404 (i) would not transform, (ii) would not replicate autonomously, and (iii) would not help Ad2 infections but that it (iv) would stimulate host cell DNA synthesis and (v) would be phosphorylated. All of these predictions were correct except for the last one. Although *am* T antigen could be detected in ^{35}S-labeled infected cell extracts, no evidence was found that it was phosphorylated, even in COS-1 cells in which it was overproduced. This is surprising because of the reported phosphorylation of the gene *A* product of SV40 *dl*1001 (70). SV40 *dl*1001 has an out-of-phase deletion in its early region (0.43 to 0.32 map units) which results in a 33K truncated T antigen which is phosphorylated. Thus, the lack of phosphorylation of *am* T antigen cannot be attributed to the loss of a C-terminal amino acid(s) which is an essential signal for phosphorylation.

We can imagine two alternative explanations for the lack of phosphorylation of *am* T antigen. First, it is possible that the tertiary structure of the *am*404 peptide is significantly different from shorter (33K) and longer (wild-type) T antigens and that this tertiary structure precludes phosphorylation. In this regard, Walter and Flory (96) have shown that the large T antigen of *tsA* mutants at restrictive temperature is phosphorylated to a lessor extent than wild-type T. This occurs despite the fact that the positions of *tsA* mutations map outside of the regions of T antigen which contain phosphate acceptor residues (43).

Second, it is possible that the short half-life of the *am*404 peptide does not allow sufficient time for phosphorylation to occur. The time required to phosphorylate serine and threonine acceptor residues which are contained within *am* T antigen is not known. However, it is worth noting that Scheidtmann et al. (72) have reported that the phosphorylation of the Thr 701 residue of T antigen required about 5 h. Conversely, it is tempting to speculate that the absence of phosphorylation itself leads to the rapid degradation of *am* T antigen by cellular proteases. Finally, it is possible that *am* T antigen is not phosphorylated because it is not transported to the necessary subcellular location.

Phosphorylation has been shown to be a mechanism for modifying the biological activity of proteins (69). At least eight amino acid residues of T antigen (both serine and threonine), clustered at two locations, can be phoshorylated (72), and in principle, this could lead to the modification of any of the several biological

functions associated with T antigen. Several investigators have reported a correlation between the level of phosphorylation and the DNA binding activity or the aggregation state of T antigen (18, 29, 47, 52, 56). No clear positive relationship, however, has been established between the phosphorylated state of T antigen and any of its in vivo functions. In addition, Shaw and Tegtmeyer (78) found that, in vitro, dephosphorylated T antigen bound to SV40 DNA as well as phosphorylated T antigen. It was, therefore, of particular interest to determine if the non-phosphorylated *am* T antigen retained any biological activity. Earlier studies had demonstrated that DNA fragments of the SV40 early region, found entirely within the *am* T antigen coding region (Fig. 7), could stimulate host cell DNA synthesis (23, 53, 83). The ability of *am*404 to stimulate host cell DNA synthesis indicated that at least one in vivo function of T antigen does not require phosphorylation of the protein. Furthermore, it demonstrated that the instability of *am* T antigen does not preclude functional activity in vivo.

It was quite apparent that much more of the 67K protein could be found in *am*404-infected COS-1 cells than in the *am*404 plus *dl*1007-infected BSC-40 cells. When the protein-to-viral DNA ratios of infected BSC-40 cells and infected COS-1 cells were compared, it was evident that there was a tremendous overproduction of the protein in the infected COS-1 cells. Indirectly, this overproduction suggests that the 67K *am* T protein does not have the autoregulatory capability that has been demonstrated for SV40 T antigen (67, 89). In the BSC-40 cells infected with both *am*404 and *dl*1007, there is an amplification of both viral DNAs (64). Because the wild-type T antigen is present in sufficient amounts, the expression of both the *am*404 and the *dl*1007 early genes is repressed. However, in the infected COS-1 cells, as the gene copy number of *am* T antigen increases, there is no concomitant increase in the number of genes which express wild-type T antigen. Therefore, it appears that in infected COS-1 cells, *am*404 can effectively saturate and then overcome the regulatory capacity of the endogenous T antigen.

Although *am* T antigen was eventually isolated and identified as described above, some of the difficulties encountered are worthy of discussion. The *am* T antigen could not be immunoprecipitated from cells transfected with *am*404 DNA. Furthermore, when BSC-40, CV-C, and COS-1 cells were infected with the same *am*404 viral stock, *am* T antigen could be immunoprecipitated only from the COS-1 cells. Finally, immunofluorescence for *am* T antigen was rarely positive when performed on BSC-40 or CV-C cells transfected with *am*404 DNA or infected with *am*404 virus. In part, these results can be attributed to the low copy number of the *am*404 genome in these experiments. Only the COS-1 cells are permissive for *am*404 DNA replication and would therefore lead to increased gene dosage. The difficulty in isolating *am* T antigen by immunoprecipitation can also be attributed to the short half-life of the protein. Pulse-chase analysis of *am* T antigen in COS-1 cells demonstrated a turnover time (half-life) for the protein of less than 10 min. The extreme nature of this result was emphasized even more by comparison of the turnover times of *am* T antigen and SV40 T antigen. The turnover time for the wild-type T antigen could not be calculated in this experiment because the endogenous T antigen produced in the COS-1 cells did not decay appreciably during the chase periods used. This was in agreement with the report of Edwards et al. (17) that the half-life of T antigen in transformed cell lines was greater than 24 h. Reddy et al. (65) have also reported a considerable variation in the stability of truncated T antigens. Although actual turnover times were not reported, it was clear that an N-terminal 33K peptide fragment was much less stable than an N-terminal 12K fragment. Our measurement of the turnover of the 67K *am* T antigen supports their conclusion that the stability of truncated T antigens is not a simple function of their size. Finally, if the formation of dimers or tetramers has a stabilizing effect on the protein, as has been suggested (4), *am* T antigen may be more labile because of its inability to participate in these aggregates. Examination of immunoprecipitates obtained with monoclonal antibody 412 indicated that *am* T antigen did not appear to form oligomeric structures with T antigen (Fig. 2). If *am* T antigen were complexed with T antigen, immunoprecipitation with antibody 412 would have precipitated both proteins, and this did not occur.

Although the preferential and accelerated degradation of abnormal proteins in procaryotic and eucaryotic cells has been well documented (26), the mechanism by which mammalian cells accomplish this has not been determined. In light of the overproduction of *am* T antigen under some conditions, *am*404 may be useful for the study of protein degradation in mammalian cells.

Finally, *am*404 represents one of only a handful of mammalian nonsense mutations which are currently available for study (7, 10, 15, 24, 64). The use of conditionally lethal nonsense mutations has been virtually impossible because of the lack of appropriate suppressor tissue culture lines. Recently, Temple et al. (89) and Laski et al. (45) have constructed suppressor tRNA genes which appear to be expressed in mammalian cells. This has led to the isolation of a

mammalian suppressor cell line (36). Because the autonomous replication of *am*404 and transformation by *am*404 are easily assayable characteristics whose expression would require suppression of the amber mutation, *am*404 may prove to be an excellent probe for identifying and isolating additional mammalian suppressor genes.

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