

Mechanism of interferon action: Simian virus 40-specific early polypeptides synthesized in untreated and interferon-treated monkey kidney cells

(tumor antigens/antiviral agents/translational control)

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ABSTRACT The effect of interferon treatment on proteins synthesized in simian virus 40 (SV40)-infected cells in the presence of cytosine arabinoside was investigated. The following results were obtained: (i) In addition to previously described large tumor (T) antigen (94 kilodaltons) and small tumor (t) antigen (19 kilodaltons), a 62-kilodalton polypeptide was immunoprecipitated by SV40 anti-T antiserum from extracts of infected CV-1 and BSC-1 monkey kidney cells and transformed SV3T3 mouse cells. The 94-, 62-, and 19-kilodalton polypeptides were not precipitated with normal serum from extracts of infected cells, and they were not present in extracts of uninfected cells. (ii) The *de novo* synthesis of the 94-, 62-, and 19-kilodalton tumor antigens was inhibited in CV-1 and BSC-1 cells treated with interferon before infection; total cellular protein synthesis was not significantly affected by interferon treatment. The relative interferon sensitivity of the three polypeptides in lytically infected monkey cells was comparable; by contrast, interferon did not affect their synthesis in transformed mouse cells. (iii) The 62-kilodalton polypeptide was detected in monkey cells infected with the following strains of SV40: tsA30 at both 33°C and 41°C; wt 708, the parent of tsA30; dl 884; and wt 830, the parent of dl 884. The amount of the 62-kilodalton species relative to T antigen was significantly greater in tsA30-infected cells as compared to cells infected with other SV40 strains. (iv) T, t, and 62-kilodalton polypeptides were readily labeled with [³⁵S]methionine during a 10-min pulse; in a subsequent chase, the ³⁵S-labeled 94-kilodalton T antigen was apparently converted to 89- and 84-kilodalton polypeptides but not to either the 62-kilodalton polypeptide species or t antigen. (v) Partial peptide maps suggest that the 62-kilodalton polypeptide and T antigen are closely related. (vi) In addition to the above described 62-kilodalton polypeptide, a 54-kilodalton polypeptide was also detected. However, the 54-kilodalton species appears to be of cellular origin because it was immunoprecipitated with both normal and anti-T antiserum from uninfected and lytically infected cells and from virally transformed cells.

Interferon inhibits the replication of a wide range of animal viruses, in most cases at the level of accumulation or translation, or both, of virus-specific mRNAs (1, 2). Treatment of monkey kidney cells with interferon before infection by wild-type simian virus 40 (SV40) strains results in a reduction in the accumulation of virus-specific early RNA (3–6); the synthesis of SV40-specific early proteins is also reduced in SV40-infected monkey cells (3–5) but not in SV40-transformed mouse cells (7, 8). The primary mechanism of the interferon-mediated inhibition of viral genome expression in SV40-infected permissive cells is not yet resolved (5–9, 10).

The two major early polypeptides detected in extracts prepared from SV40 lytically infected and transformed cells are large tumor (T) antigen with an apparent size of 90–100 kilodaltons (kDal) and small tumor (t) antigen with an apparent

size of 17–20 kDal (11–13). These polypeptides are specified by two distinct 19S early mRNA species with different splicing patterns (14, 15). Several polypeptides in addition to T and t antigens can also be detected after electrophoretic analysis of immunoprecipitates derived from SV40 lytically infected and transformed cells; these additional polypeptides have been termed middle T antigens and have apparent sizes of 45–63 kDal (16–22). The origin of these polypeptides is unclear; some appear to be cell-coded (19–22) whereas others may be virus-coded (16, 18). A class of virus-coded middle T antigens has also been described in polyoma-infected mouse cells (23, 24).

As part of our studies of the molecular mechanism of interferon action, we have investigated the mechanism of interferon-mediated inhibition of SV40 replication with the aim of understanding the relative contribution of the inhibition of RNA accumulation and the inhibition of protein accumulation to the overall reduction in SV40 virus yields. The present studies were undertaken to quantitate the effect of interferon on the *de novo* synthesis of the different classes of SV40 early polypeptides, T, t, and middle T antigens, present at early times after virus infection. Most of our results were obtained by using lytic infection of CV-1 African green monkey kidney cells with the temperature-sensitive mutant tsA30 (25) at 41°C in the presence of cytosine arabinoside. The reasons for choosing this system were: only early viral functions are studied (26); there are no complicating effects of autoregulation of the A gene at the nonpermissive temperature (27); and higher levels of early viral RNA and protein are synthesized with tsA as compared to wild-type SV40 strains (27, 28).

EXPERIMENTAL PROCEDURE

Cell Cultures. The CV-1 and BSC-1 lines of monkey kidney cells, obtained from John Carbon (University of California, Santa Barbara) and Edward Penhoet (University of California, Berkeley), respectively, were grown in Eagle's minimal essential medium (GIBCO) containing 7% fetal calf serum (Irvine Scientific). The transformed cell line SV3T3-47.3, obtained from Howard Green (Massachusetts Institute of Technology, Cambridge), was grown in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal calf serum. All media contained penicillin (100 units/ml) and streptomycin (100 µg/ml).

Virus Stocks. Temperature-sensitive mutant tsA30 (25) and its wild-type parent, wt 708 (VA45-54), were obtained from Peter Tegtmeyer (State University of New York at Stony Brook); deletion mutant dl 884 (29) and its wild-type parent, wt 830 (SV-S), were obtained from Paul Berg (Stanford University Medical Center).

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Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen; t antigen, small tumor antigen; kDal, kilodalton(s).

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Interferon Preparation and Assay. Monkey interferon induced by Newcastle disease virus was prepared and assayed with vesicular stomatitis virus as described (30).

Virus Infections and Radioactive Labeling. Confluent monolayer cultures in 75-cm² flasks (Corning) or 21-cm² dishes (Lux) were infected at multiplicities of 5–10 plaque-forming units/cell. Infected cultures were incubated in Eagle's medium containing 5% fetal calf serum and 30 μ g of cytosine arabinoside per ml; temperatures were as indicated under the respective figure legends. Cells were radioactively labeled 34 hr after infection with 25 μ Ci of [³⁵S]methionine per ml (New England Nuclear; 670–930 Ci/mmol, 1 Ci = 3.7 \times 10¹⁰ becquerels) in methionine-free Eagle's medium; the length of labeling and chase are indicated in *Results*. In pulse-chase experiments, the labeling medium was replaced with Eagle's medium containing unlabeled methionine and 5% fetal calf serum.

Preparation and Analysis of Cell Extracts. All extraction and fractionation procedures were done at 4°C. Cells were rinsed with phosphate-buffered saline and disrupted with extraction buffer [20 mM Tris-HCl, pH 9.0/137 mM NaCl/1 mM MgCl₂/1 mM CaCl₂/50 μ g of phenylmethylsulfonyl fluoride per ml/10% (vol/vol) glycerol/1% (vol/vol) Nonidet P-40], 1.2 ml per flask and 0.4 ml per dish. After 10 min at 4°C the disrupted cells were centrifuged at 500 \times g for 10 min. The supernatant fraction was then centrifuged for 30 min at 25,000 \times g; the resulting extracts were stored at –80°C.

Proteins were immunoprecipitated from extracts with either normal serum or immune serum from hamsters bearing SV40-induced tumors (provided by Jack Gruber, National Cancer Institute, Bethesda, MD) by the indirect *Staphylococcus aureus* protein A procedure (31). Extract (100 μ l) was incubated with normal serum (9 μ l) for 2 hr at 4°C; heat-inactivated formalin-fixed *S. aureus* (100 μ l, 10% wt/vol; provided by Duane Sears, University of California, Santa Barbara) was then added and incubation was continued for 30 min at 4°C followed by centrifugation. The supernatant fraction was removed and incubated with either hamster anti-T antiserum or normal serum (7 μ l) for 24 hr at 4°C, then mixed with *S. aureus* (100 μ l) and further incubated for 30 min at 4°C. The immunoprecipitate was collected by centrifugation, washed three times at 4°C with 0.1 M Tris-HCl, pH 7.4/0.15 M NaCl, suspended in 40 μ l of electrophoresis sample buffer, boiled for 10 min, centrifuged, and analyzed by using a discontinuous Tris/glycine-buffered NaDodSO₄/polyacrylamide gel electrophoresis system essentially as described (32). Slab gels with a 12.5% acrylamide running layer were routinely used. Fluorography was performed as described (33). Apparent molecular weight values were estimated (34) by the relative mobility of reovirion polypeptides λ (150,000), μ_{1C} (75,000), σ_2 (45,500), and σ_3 (41,500); formyltetrahydrofolate synthetase (60,000 monomer); carbonic anhydrase (31,000); and myoglobin (17,200).

Peptide Mapping. Peptide map analysis of the 94- and 62-kDal proteins was performed with *S. aureus* V8 protease (Miles) and the partial proteolysis, polyacrylamide gel analysis technique of Cleveland *et al.* (35) as modified (36) for recovery of the protease digestion products from the gel slices.

RESULTS

SV40 Tumor Antigens in CV-1 Cells Infected by Temperature-Sensitive Mutant tsA30. Fig. 1 shows the polypeptides detected in immunoprecipitates of CV-1 cells infected with SV40 mutant tsA30 at both the restrictive temperature (41°C) and the permissive temperature (33°C). T antigen (94 kDal) and t antigen (19 kDal) were readily detected at both temperatures (tracks D and E). In addition to the large and small an-

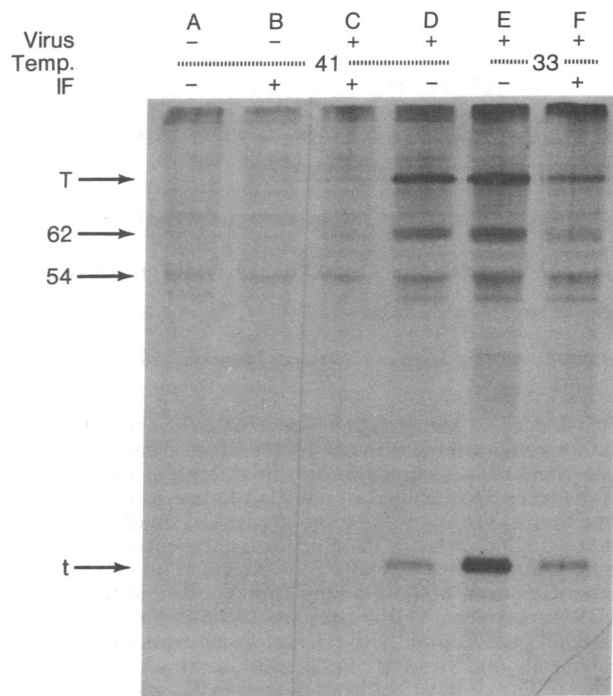


FIG. 1. Effect of interferon (IF) treatment and culture temperature (°C) on patterns of proteins precipitated with SV40 anti-T immune serum from tsA30-infected (+) and uninfected (-) CV-1 cells. Interferon treatment was with 100 units/ml at 37°C for 24 hr before infection; cultures were infected and labeled at either 41°C or 33°C in the presence of cytosine arabinoside. See text for details.

tigens, a polypeptide migrating near 62 kDal was also prominent in extracts prepared from tsA30-infected CV-1 cells at both 41°C (track D) and 33°C (track E). The 62-kDal species was detected in all of more than 50 independently prepared extracts prepared from tsA30-infected CV-1 cells, both in the presence and absence of cytosine arabinoside, and immunoprecipitated with different lots of anti-T antiserum, although some variation in levels relative to T antigen was observed between extracts. The 62-kDal polypeptide was also detected in extracts prepared from tsA30-infected monkey BSC-1 cells at a level comparable to that observed in tsA30-infected CV-1 cells (result not shown). The 94-, 62-, and 19-kDal polypeptides were not present in extracts prepared from uninfected cells (tracks A and B) or from infected cells when treated with normal serum (result not shown). Other prominent polypeptides of sizes near 54-kDal were routinely observed, but because these polypeptides were present both in virus-infected and uninfected cells treated with either immune or normal serum, they are probably of cellular rather than viral origin.

The synthesis of the 62-kDal polypeptide, as well as T and t antigens, was significantly reduced by treatment with interferon (100 units/ml) before infection (tracks C and F). Some cellular proteins (e.g., those in the 50- to 56-kDal range, which were routinely precipitated with immune sera) were not significantly affected by interferon (track B). Furthermore, there was no significant effect of interferon on the synthesis of total cellular proteins analyzed before immunoprecipitation.

Effect of Interferon Dose on Synthesis of SV40 Tumor Antigens in tsA30-Infected CV-1 Cells. The effect of increasing doses of interferon, ranging from 0.3 to 100 units/ml, on the synthesis of SV40 tumor antigens was examined. As shown in Fig. 2, there was a dose-dependent reduction at early times after infection of newly synthesized T and t antigens and the 62-kDal polypeptide. To estimate the relative sensitivities of the tumor antigens to reduction by interferon, the fluoro-

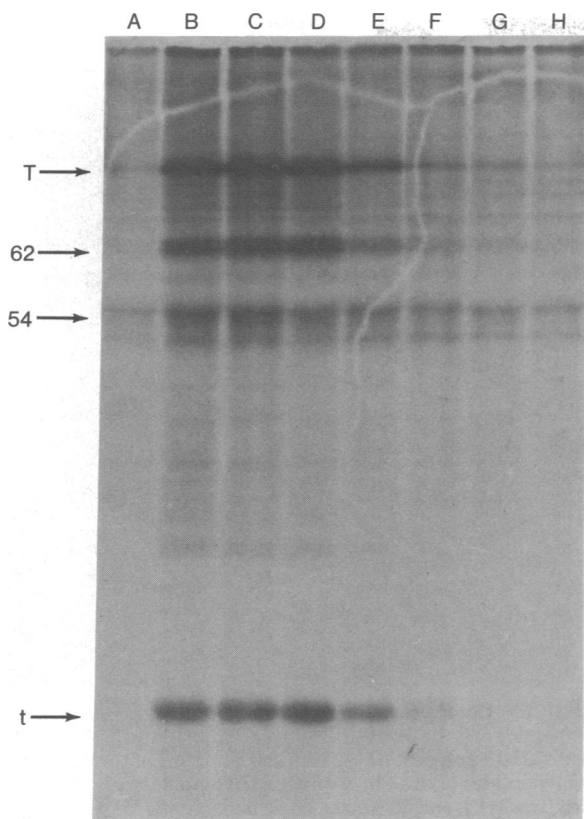


FIG. 2. Effect of interferon dose on the synthesis of proteins in tsA30-infected CV-1 cells. Treatment with 0 (track B), 0.3 (track C), 1 (track D), 3 (track E), 10 (track F), 30 (track G), and 100 (track H) units of interferon per ml was at 37°C for 24 hr before infection. Track A, uninfected cells not treated with interferon. Cultures were labeled for 1 hr from 34 to 35 hr after infection. Equal amounts of radioactively labeled extract (1.40×10^6 cpm) were precipitated with anti-T antiserum.

grams shown in Fig. 2 were scanned with a densitometer and the peak areas were integrated and compared. The 94-, 62-, and 19-kDal antigens possessed a comparable sensitivity to interferon; an interferon dose of about 3 units/ml was required to reduce the amount of radioactivity in each of the tumor antigens by 50%. With the maximal dose of interferon tested (100 units/ml), from less than 1-fold to about a 3-fold change was observed in the amount of the 54-kDal cellular protein immunoprecipitated (Figs. 1 and 2).

The effect of interferon in SV40-infected CV-1 cells on middle T antigens has not been previously reported. A further characterization of the 62-kDal polypeptide precipitated selectively by anti-T antiserum was therefore undertaken both to establish its relationship to middle T antigens recently described by others (16–22) and as a preliminary to defining its origin and the basis of its interferon sensitivity.

Effect of SV40 Strain and Cell Type on Nature of T Antigens Observed in Untreated and Interferon-Treated Cells. To determine the effect of virus strain and cell type on the synthesis of the 62-kDal polypeptides observed in tsA30-infected CV-1 cells, we examined monkey cells lytically infected by different wild-type or mutant strains of SV40 and SV40-transformed mouse 3T3 cells.

As shown in Fig. 3 *Left*, a prominent 62-kDal polypeptide was precipitated with anti-T antiserum from extracts of CV-1 cells infected with four different strains of SV40: wt 708, the parent strain of tsA30; tsA30; dl 884; and wt 830, the parent strain of dl 884. The synthesis of the 62-kDal polypeptide in CV-1 cells infected with the above strains of SV40 was sensitive

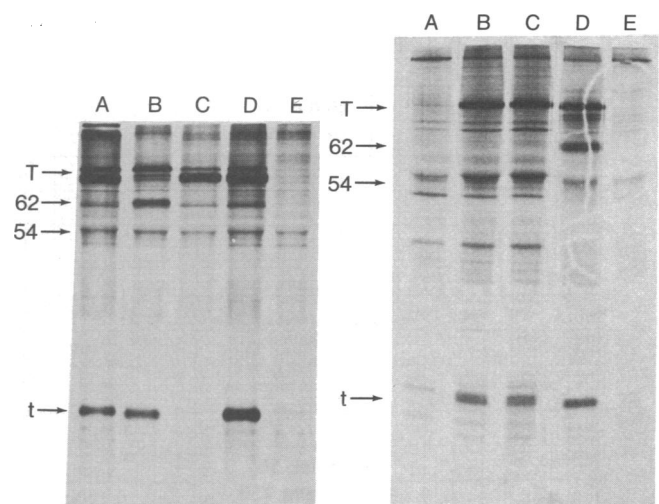


FIG. 3. Effect of SV40 strain and cell type on the nature of T antigens immunoprecipitated. (*Left*) Comparison of proteins immunoprecipitated with anti-T antiserum from extracts of CV-1 cells lytically infected with different strains of SV40. CV-1 cells were infected and labeled with [³⁵S]methionine for 1 hr from 34 to 35 hr after infection in the presence of cytosine arabinoside. Track A, wt 708, 41°C; track B, tsA30, 41°C; track C, dl 884, 37°C; track D, wt 830, 37°C; track E, uninfected, 37°C. (*Right*) Effect of interferon on proteins immunoprecipitated from SV40-transformed 3T3 cells. Transformed mouse cells, SV3T3-47.3, were treated for 24 hr with 300 units of interferon per ml and then labeled for 22 hr with [³⁵S]methionine. Proteins were precipitated with either normal or SV40 anti-T immune serum. Track A, untreated SV3T3, normal serum; track B, untreated SV3T3, anti-T antiserum; track C, interferon-treated SV3T3, anti-T antiserum; track D, untreated, tsA30-infected CV-1, anti-T antiserum; track E, untreated, uninfected CV-1, anti-T antiserum.

to interferon (unpublished observation). Relative to the amount of T antigen, the amount of 62-kDal polypeptide observed was greatest in cells infected with the tsA mutant.

In SV40-transformed mouse 3T3 cells, T and t polypeptides were readily detected; in addition, a 62-kDal species was also observed but at much lower levels relative to T antigen than in lytically infected monkey cells (Fig. 3 *Right*). The T, t, and 62-kDal polypeptides present in extracts of SV3T3 cells were immunoprecipitated with immune but not normal serum; however, the synthesis of these polypeptides in SV3T3 cells was not reduced by interferon. Additional polypeptides of about 54 kDal were detected with both immune and normal sera.

Stability of Tumor Antigens in Cells Infected by tsA30. Because T antigen is both overproduced and more rapidly turned over in infections at 41°C by the group A mutants as compared to wild-type virus (37), it was possible that the 62-kDal polypeptides observed during a 1- or 2-hr labeling period (Figs. 1–3) were post-translational cleavage products of 94-kDal T antigen. To test this hypothesis, CV-1 cells were infected with tsA30 at 41°C and at 34 hr after infection were pulse labeled for 10 min with [³⁵S]methionine and then either harvested immediately or chased for 110 min. The densitometer profiles of gel fluorograms of the SV40 anti-T immunoprecipitates are shown in Fig. 4. T, t, and 62-kDal polypeptides were readily detected after the 10-min labeling period. The amount of ³⁵S associated with the 62-kDal species before and after the chase was comparable, but T and t were relatively unstable. The radioactivity of the 94-kDal species decreased more than 50% during the chase and appeared to quantitatively accumulate in proteins with sizes of 84 and 89 kDal, polypeptides previously reported to be post-translational cleavage products of T antigen (28, 38, 39). Indeed, after a 6-hr chase essentially all of the ra-

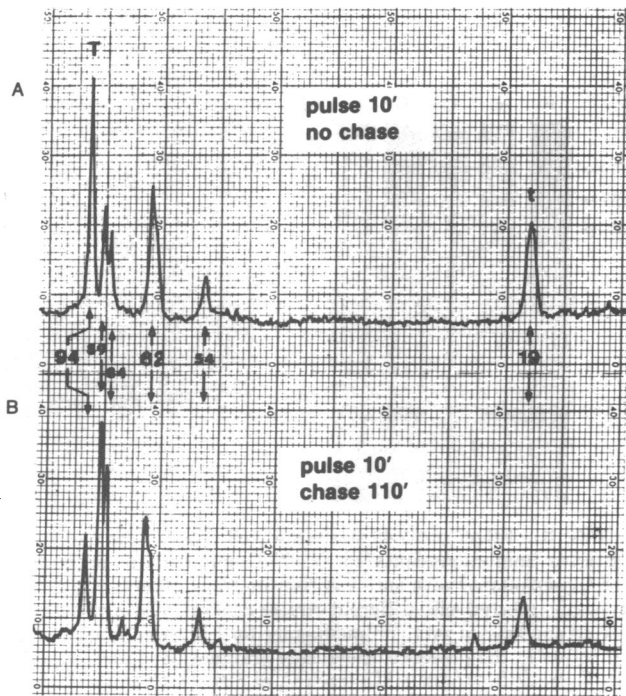


FIG. 4. Optical density scan showing the effect of pulse and pulse-chase on the stability of SV40 tumor antigens. CV-1 cells infected with tsA30 at 41°C were labeled with [³⁵S]methionine at 34 hr after infection as follows: (A) 10-min pulse, no chase; (B) 10-min pulse, 110-min chase. Equal volumes of each extract were immunoprecipitated with anti-T antiserum. The gel was scanned with a Gilford 250 spectrophotometer equipped with a linear transport accessory and recorder.

dioactive 94-kDal peptide was converted to 84- and 89-kDal species, but not to the 62-kDal species (results not shown). These results suggest that the 62-kDal polypeptide species are synthesized and degraded independently of the 94-kDal species.

Partial Peptide Maps of 62-kDal Polypeptide and 94-kDal SV40 T Antigen. To test the relatedness of the 62- and 94-kDal tumor antigens, the polyacrylamide gel profiles of peptides generated by three concentrations of staphylococcal V8 protease (35) from the 62-kDal polypeptide, which could occasionally be resolved into a doublet designated a and b polypeptides, were compared with those of the 94-kDal T antigen. The profiles were very similar (Fig. 5). A mixture of the a and b polypeptides contained all of the partial peptides of less than 62 kDal observed for the a polypeptide alone and the SV40 T antigen alone. Differences in the intensities of some of the partial peptide bands at various V8 protease concentrations probably represent different stages of digestion because it was impossible to precisely control the ratio of substrate to protease. These results suggest that the 62-kDal and 94-kDal polypeptides precipitated by SV40 anti-T antiserum are closely related, at least in structural features, that confer sensitivity to partial proteolytic digestion by *S. aureus* V8 protease.

DISCUSSION

We have shown that the synthesis of early SV40-specific polypeptides at early times after infection of CV-1 and BSC-1 cells is inhibited by treatment with interferon prior to infection. This has also recently been shown for T antigen under similar conditions (8) and for T and t antigens under quite different conditions of interferon treatment of infected cells late in the infection cycle (6). We have extended these observations by

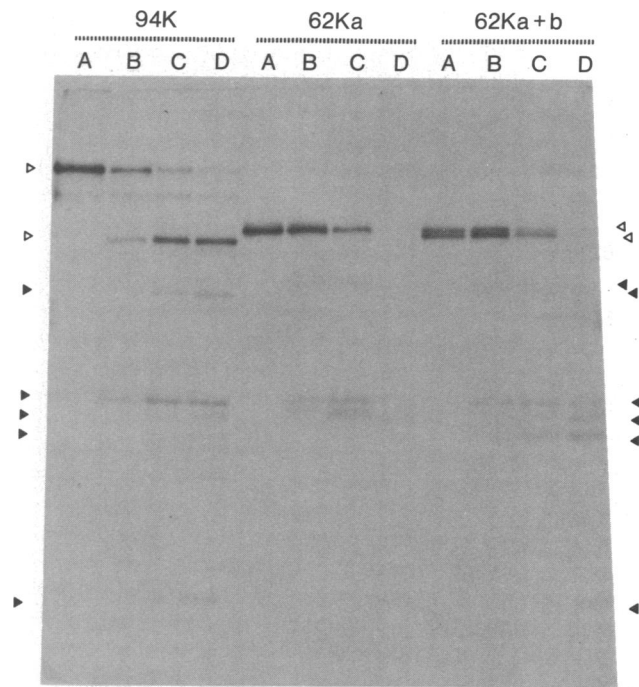


FIG. 5. Comparison of the partial peptide maps of the 62-kDal polypeptide and the 94-kDal T antigen. The 94-kDal T antigen (94K), the 62-kDal a polypeptide (62 Ka), and a mixture of the 62-kDal a and b polypeptides (62Ka+b) were digested with 0 (tracks A), 2 (tracks B), 6 (tracks C), and 20 (tracks D) μ g of *S. aureus* V8 protease per ml and analyzed.

showing that t antigen synthesis is sensitive to interferon-mediated inhibition in pretreated cells and by showing that an additional polypeptide, possibly a virus-coded middle T antigen of 62 kDal, is also interferon sensitive. Furthermore, all three early polypeptides (T, t, and the 62-kDal polypeptide) displayed comparable dose-dependent sensitivities to interferon. The comparable interferon sensitivities of the three SV40 early polypeptides differs, however, from the situation with some other viruses; for example, different reovirus polypeptides such as λ_1 and μ_{NS} that are encoded by separate primary transcripts possess quite different interferon sensitivities (34). The similar sensitivities of T and t antigens might be explained by the fact that although they are encoded by separate early RNA species, these differ only in splicing patterns (14, 15) and that the parameters important in initiation of translation, a step that appears to be inhibited by interferon (40), may be similar because the polypeptides possess identical amino-terminal amino acid sequences (41).

The nature of the primary transcript for the 62-kDal polypeptide is unknown. It could be encoded by initiation near 0.65, similar to T and t, of an additional RNA species with novel splice junctions. Alternatively, a 62-kDal protein could be generated by initiation of translation beyond map position 0.54 because the region between 0.54 and 0.17 contains sufficient coding capacity for a protein in excess of 70 kDal (42). Such initiation apparently occurs in *in vitro* systems programmed with SV40 cRNA that is unspliced, and the major product is a 60-kDal polypeptide (43).

The viral origin of middle T antigens has not been proven, but our studies of the interferon sensitivity of the 62-kDal polypeptide lend support to this idea. First, interferon has little effect on total cell proteins analyzed before immunoprecipitation (unpublished results) or those proteins apparently fortuitously precipitated (Fig. 1), whereas the 62-kDal protein was clearly inhibited by interferon. Second, the dose-dependent

characteristics of the interferon-mediated inhibition of the 62-kDal species are the same as for well-established virus-coded polypeptides such as T and t antigens (Fig. 2). Finally, in transformed cells where T (refs. 8 and 9; Fig. 3) and t (Fig. 3) antigens are unaffected by interferon, the 62-kDal polypeptide is also resistant to inhibition. Additional arguments suggesting the viral origin of the 62-kDal species are as follows: (i) They are detected only in virus-infected cells and are selectively precipitated with SV40 anti-T antiserum. (ii) The presence of the 62-kDal species is not dependent upon the nature of the host cell; it was observed in immunoprecipitates from permissive CV-1 and BSC-1 cells and from transformed 3T3 cells. (iii) The level of production of the 62-kDal polypeptide relative to T antigen is dependent on the virus strain used, tsA30 infection yielding higher levels of the 62-kDal species as compared to, for example, wt 708 infection. (iv) The partial peptide maps generated by V8 protease digestion of the 94- and 62-kDal polypeptides are very similar.

It is unlikely that the 62-kDal polypeptide species is generated by post-translational cleavage of the T polypeptide because in pulse-chase experiments no precursor-product relationship was detected between the 94- and 62-kDal polypeptides. It is also unlikely that the 62-kDal polypeptides are detected because of coprecipitation with T or t antigens, because immunoprecipitates of a mixture of unlabeled tsA-infected CV-1 extract and ³⁵S-labeled uninfected CV-1 extract did not yield the 62-kDal polypeptide; likewise, a mixture of SV3T3 extract (containing little 62-kDal polypeptide but large amounts of T and t antigens) and labeled uninfected CV-1 extract did not yield a greater amount of 62-kDal polypeptide than observed with the SV3T3 extract alone (unpublished results).

Unequivocal proof for the viral origin of a particular polypeptide, however, depends upon the production of an altered polypeptide with mutant virus and the ability to synthesize the polypeptide *in vitro* with template RNA that specifically hybridizes to the viral genome.

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