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Two forms of simian-virus-40-specific T-antigen in abortive and lytic infection

(polyacrylamide–sodium dodecyl sulfate gels/tryptic peptide fingerprinting/protein modification)

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ABSTRACT **Simian-virus-40-specific T-antigen was isolated by immunoprecipitation. From other studies we have proof that the T-antigen described in this work is coded by the viral DNA. The molecular weight estimated from electrophoretic mobility in sodium dodecyl sulfate–polyacrylamide gels of T-antigen isolated from nonpermissive mouse cells in abortive infection is 86,000 and from permissive monkey cells in lytic infection is 82,000. The 86 kilodalton T-antigen is readily converted *in vitro* into an 82 kilodalton form by incubation with extracts from permissive monkey cells but not with extracts from nonpermissive mouse or hamster cells. This and the results of fingerprinting analysis of tryptic peptides suggest that T-antigen may be processed in permissive cells.**

Simian virus 40 (SV40) contains a double-stranded circular DNA of about 3 megadaltons. The viral DNA codes an early (19 S) and two late (16 S and 19 S) mRNAs. The late messengers direct synthesis of the viral capsid proteins (1–4). Early 19S mRNA is synthesized throughout lytic and abortive infection and also in virus-transformed cells; it is the transcript (1, 4,‡) of a part of SV40 DNA ("early region") that comprises about 45% of total length (5, 6). Evidence discussed elsewhere suggested that early 19S mRNA directs synthesis of a single, rather large protein, the SV40-specific T-antigen, which is required to induce lytic and abortive infection, to initiate and maintain cell transformation, and probably also to induce primary tumors in animals (details in refs. 4 and 7).

In the present work we isolated T-antigen by immunoprecipitation. The T-antigen isolated in this way is shown to be coded by the viral DNA. We compared the molecular properties of T-antigen in abortive and lytic infection. Our results confirm that SV40 T-antigen is indeed a large protein. They further show that T-antigen in lytic infections of permissive monkey cells is smaller than in abortive infections of nonpermissive mouse cells.

MATERIALS AND METHODS

Cell cultures were plated in 85 mm diameter plastic petri dishes and grown to confluency in 10 ml of reinforced Eagle's medium containing 10% bovine serum. Confluent *primary* mouse kidney (MK) cultures were prepared according to Winocour (8). Confluent cultures contained $12 \pm 2 \times 10^6$ cells per dish. They were infected 2–6 days after confluency. Primary monkey (vervet) kidney cell cultures were obtained from Mérieux (Lyon). Several continuous monkey kidney cell lines were used: CV-1, a gift from Prof. B. Hirt (Lausanne); BSC from Dr. N. Salzmann (National Institutes of Health); and Vero from Dr. M. F. Paccaud (Geneva). To exclude a possible contamination with mycoplasma or other bacterial contaminants, we performed parallel experiments with the same cultures that had been kept for 1–4 weeks before use (and during infection) in the presence of 1% gentamicin (Schering) and/or 1% anti-mycoplasma agent (Tylocine, GIBCO) and also with CV-1 cultures freshly obtained from Flow Laboratories and presumed to be free of mycoplasma. Under standard conditions monkey kidney cultures were infected 3–5 days after confluency.

Hamster cell lines, one (BHK) obtained from Dr. I. Maxwell, another derived in our laboratory from a primary, polyoma-induced subcutaneous tumor, were grown to confluency under the same conditions.

Infection with SV40 was performed with twice plaque-purified, nondefective, wild-type SV40. All viral preparations contained about 10^9 plaque-forming units (PFU)/ml. The results were the same whether crude viral lysates or highly purified viral preparations were used for infection. Virus (0.3 ml per dish) was adsorbed at 37° for 90 min; the cultures were then covered with 10 ml of medium containing 10% bovine serum; in most experiments primary mouse kidney cultures were covered with serum-free medium (4). Parallel cultures were mock-infected with 0.3 ml of medium and then treated as SV40-infected cultures.

Cultures were labeled with [^{35}S]methionine (200–350 Ci/mmol; The Radiochemical Centre, Amersham, England) by addition of 3 ml per dish of prewarmed (37°) medium (± serum) which contained only 2 μg/ml of unlabeled methionine (instead of 30 μg) and 50 μCi/ml of [^{35}S]methionine. At the end of the labeling period the cultures were washed twice with 5 ml of cold (2°) isotonic phosphate buffer; then the cells were gently scraped off in 1 ml of the same phosphate buffer with a silicone policeman and centrifuged for 5 min at $500 \times g$.

Preparation of "Soluble Extracts." The pellets were suspended at concentrations varying between 10^7 and 10^8 cells in 5 ml of extraction buffer (2.5 mM Tris·HCl, pH 7.4, 0.5 M LiCl, 1 mM EDTA), sonicated (MSE 100 W Ultrasonic Disintegrator) four times for 15 sec in an ice-water bath, and then immediately centrifuged for 30 min at $30,000 \times g$ at 4°. The supernatant was removed and is referred to as "soluble extract." In early experiments phenylmethylsulfonyl fluoride (0.1–0.3 mg/ml) and dithiothreitol (1 mM) were present in the extraction buffer.

Immunoprecipitation. Most experiments were performed with sera (anti-T) obtained from Syrian hamsters bearing tumors induced by inoculation of an SV40-transformed hamster cell line (9). One preparation was a gift of Drs. E. and P. May, others were made in our laboratory. Hamster anti-T sera (batches 3×1888 and 4×21) were received from the National Cancer Institute (Bethesda, Md.). The anti-T sera used had specific complement fixing titers of 160–640. Control serum was a pool of 15 normal adult Syrian hamster

Abbreviations: SV40, simian virus 40; PFU, plaque-forming units; kDal, kilodaltons; MK, mouse kidney.

‡ E. May, P. May, and R. Weil, manuscript in preparation.

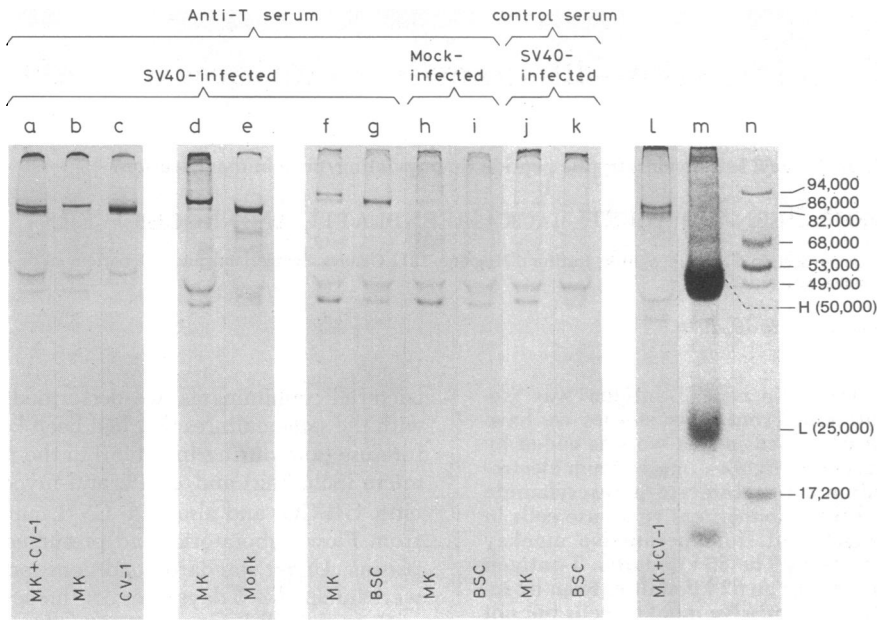

FIG. 1. Identification of SV40 T-antigen synthesized during lytic and abortive infection. Primary mouse kidney (MK) cultures (nonpermissive), or CV-1, BSC, and primary monkey kidney cultures (permissive) were infected with SV40 or mock-infected and labeled with [^{35}S]methionine (50 μCi/ml) during the following times after infection: 11–24 hr (b, c); 14–17.5 hr (d); 41–44 hr (e); 15–19 hr (f, h, j); 21–24 hr (g, i, k). Soluble extracts were reacted with anti-T or control serum (*Materials and Methods*), and precipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The cellular origins of the various soluble extracts are indicated in the lower part of the gel patterns: Monk stands for primary monkey kidney cultures. Samples a to l were autoradiographed. Sample m is a duplicate of l, but stained with Coomassie brilliant blue. Sample n, which shows molecular weight standards described in *Materials and Methods*, is also stained with Coomassie blue. H and L are the heavy and light chains of immunoglobulin. Note that controls are shown only for BSC and MK. Similar controls made with CV-1 and primary monkey kidney cultures gave indistinguishable patterns. Finally, samples a to c, d and e, f to k, and l to n were on four different gels.

sera. Anti-hamster immunoglobulin serum was prepared in rabbits by repeated injection of purified hamster IgG. [^{35}S]Methionine-labeled soluble extracts (1 ml) were allowed to react either with anti-T or control sera (10 μl) at 4° for 1.5 hr. Excess rabbit antiserum to hamster immunoglobulin was added and the samples were incubated at 4° for 3 hr. Then the samples were centrifuged at 1000 × *g* for 15 min at 4° and the precipitates were washed three times with 9 ml of extraction buffer and once with 0.01 M sodium phosphate buffer, pH 7.1.

Gel Electrophoresis and Molecular Weight Estimation. The immunoprecipitates were dissolved in 100 μl of electrophoresis sample buffer [0.01 M sodium phosphate buffer, pH 7.1, 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 15% (vol/vol) glycerol 0.002% bromophenol blue] and heated for 5 min at 95°. Samples of 10–15 μl (4000–20,000 cpm) were applied to the gel [slab-gel, composed of 10% acrylamide, 0.27% *N,N'*-methylene-bis(acrylamide) in 0.1 M sodium phosphate buffer, pH 7.1, 0.1% sodium dodecyl sulfate]. Electrophoresis was carried out at room temperature at 40–60 mA for 12–8 hr. The gel was fixed (20% trichloroacetic acid, 4 hr), stained with Coomassie blue (0.1% in 20% trichloroacetic acid, 1 hr), destained (7.5% acetic acid, 10% methanol), vacuum dried, and autoradiographed on Kodirex x-ray films for about 24 hr. Molecular weights were estimated (10) by using phosphorylase a (94,000), BSA (68,000), glutamate dehydrogenase (53,000), fumarase (49,000), and myoglobin (17,200) as markers.

Fingerprinting Analysis of Tryptic Peptides. [^{35}S]Methionine-labeled bands were cut from dry gels and eluted electrophoretically into dialysis bags. Bovine serum albumin (100–200 μg) was added as carrier and the eluates were precipitated with 20% trichloroacetic acid, and washed in 5% trichloroacetic acid and finally in an ether–ethanol (volume ratio 4:1) solution. The precipitates were oxidized with performic acid and digested with trypsin, and the resulting peptides were separated by two-dimensional chromatography (fingerprinting analysis) as described previously (11). The labeled peptides were revealed by autoradiography.

Immunofluorescence Assays were performed on glass coverslips (20 × 20 mm) present in the same cultures used to isolate T-antigen. T-antigen and capsid antigen were determined by the immunofluorescence technique used earlier (12). Hamster anti-T sera were the same as used for immunoprecipitation. The rabbit serum directed against purified SV40 capsid protein was a gift of Drs. E. and P. May.

RESULTS

Time course of appearance of SV40-specific T-antigen as detected by the immunofluorescence technique

Abortive Infection. The molecular and cytological events of the abortive infection in mouse kidney (MK) cells have been described (4, 12, 13). Under the conditions used in this study (10^9 PFU/ml; 37°) a weak immunofluorescence reaction for T-antigen could be detected in 1–2% of the nuclei 7–8 hr after infection; later the relative number of positive nuclei (and the intensity of the immunofluorescence reaction) rapidly increased, reaching by 24 hr a plateau of 35% ± 10%. In mock-infected parallel cultures no T-antigen or capsid antigen was detected.

Lytic Infection. Most experiments reported here were performed with crowded CV-1 cultures (18 ± 3 × 10^6 cells per dish), since under these conditions the variation in cell number was negligible and appearance of T-antigen was very reproducible. The temporal and quantitative relation between synthesis of SV40-specific early 19S mRNA and of

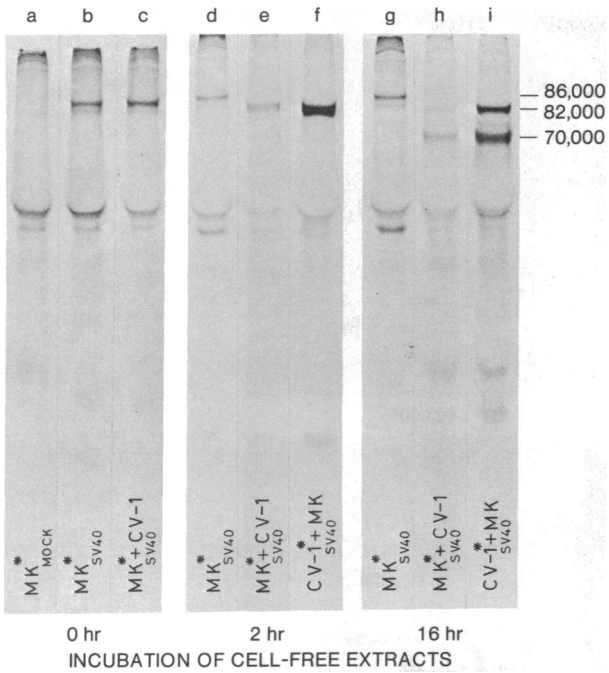

FIG. 2. Conversion *in vitro* of 86 kDal T-antigen to 82 kDal T-antigen by incubation in cell-free extracts of permissive cells. MK and CV-1 cells were labeled with [^{35}S]methionine (50 μCi/ml) from 14 to 17 hr and 20 to 24 hr after infection, respectively. Infected and ^{35}S-labeled MK cells (5×10^7) were mixed with unlabeled, uninfected CV-1 cells (7×10^7); infected, ^{35}S-labeled CV-1 cells were mixed with unlabeled uninfected MK cells. The resulting cell pellets were disrupted by ultrasonication (see *Materials and Methods*) and the sonicates were incubated at 4°. Aliquots were removed from the sonicates either immediately (0 hr) or after 2 and 16 hr and centrifuged (120,000 × *g*), and the resulting soluble extracts reacted with anti-T serum as described in *Materials and Methods*. Precipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Description of the samples in the lower part of the figure: (a) uninfected MK*; (b) infected MK*; (c) infected MK* + uninfected CV-1; (d) same as b; (e) same as c; (f) infected CV-1* + uninfected MK; (g) same as b; (h) same as e; (i) same as f.

* Indicates that the cells were labeled with [^{35}S]methionine.

T-antigen was closely similar to that observed during the abortive infection of MK cultures (unpublished results). Under the conditions used (10^9 PFU/ml; 37°) 1–5% of the nuclei contained T-antigen by 8–9 hr after infection; later the relative number of positive nuclei (and the intensity of the immunofluorescence reaction) rapidly increased, reaching 25–50% by 15 hr and 90–100% by 20–24 hr after infection. Time course of infection was similar in confluent BSC and Vero monkey kidney cell lines and also in confluent primary monkey kidney cell cultures. In mock-infected parallel cultures no T- or capsid antigen was detected.

In some experiments, lytic infection took place in the presence of cytosine arabinonucleoside (20 μg/ml added 1.5 hr after infection), which prevents viral and cellular DNA replication and synthesis of capsid proteins (14). Kinetics of appearance, determined by immunofluorescence, and the molecular properties of T-antigen were the same as observed without the inhibitor (unpublished observations).

Identification of T-antigen by polyacrylamide gel analysis

CV-1, BSC, and Vero cell lines, primary monkey kidney cell cultures (all permissive), and primary mouse kidney cell cultures (nonpermissive) were infected with SV40 and labeled with [^{35}S]methionine for 2–16 hr at various times following infection (from 1.5 to 48 hr). Mock-infected control cultures were labeled in the same way. Soluble extracts were prepared (*Materials and Methods*) and allowed to react with either hamster anti-T or control sera, and the immunoprecipitates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Fig. 1 shows that a major polypeptide was selectively precipitated by anti-T serum from extracts of SV40-infected permissive or nonpermissive cells that contained T-antigen detectable by immunofluorescence. This polypeptide was absent before appearance of T-antigen and in extracts from mock-infected cultures. As seen in Fig. 1, the apparent molecular weight of this major polypeptide, presumed to be T-antigen, depends on its source. Isolated from the three monkey kidney cell lines (and Vero, not shown) and primary monkey kidney cultures, T-antigen migrates as a polypeptide of 82 kilodaltons (kDal) between phosphorylase a (94 kDal) and bovine serum albumin (68 kDal). In contrast, T-antigen from nonpermissive MK cells behaves on electrophoresis as a larger polypeptide of 86 kDal. The same results were observed with the different batches of anti-T sera tested (*Materials and Methods*).

Pulse experiments with [^{35}S]methionine (1–3 hr at different times from 1.5 to 48 hr after infection) showed that, as little as 2 hr after its appearance, T-antigen in monkey cells is an 82 kDal polypeptide and in mouse cells is a polypeptide of 86 kDal. Use of protease inhibitors and/or reducing agents, or of monkey kidney cell lines from different laboratories (*Materials and Methods*), did not affect this pattern. *A priori*, this result would imply that either (*i*) the 86 kDal T-antigen is the primary gene product and it is rapidly cleaved in monkey cells or, (*ii*) the 82 kDal T-antigen is the primary gene product and it is modified (by some unknown addition) in nonpermissive MK cells into an 86 kDal polypeptide. To test these hypotheses, we mixed and incubated extracts containing ^{35}S-labeled T-antigen made in one type of cells with unlabeled extract from a different type of cells, precipitated with anti-T, and analyzed the resulting immunoprecipitates by polyacrylamide gel electrophoresis. As can be seen in Fig. 2, the labeled 86 kDal T-antigen of MK is converted into an 82 kDal form by a 2 hr incubation with an unlabeled extract of uninfected CV-1 cells. Parallel experiment using extracts of infected CV-1 cells and other experiments using BSC cells gave the same result (not shown). We, therefore, tentatively conclude that the 86 kDal T-antigen is the primary translation product and that it is cleaved in monkey cells to yield the 82 kDal species. Under the same conditions, a 16 hr incubation of labeled 86 kDal T-antigen with CV-1 extract generates, in addition to the 82 kDal form, a polypeptide of about 70 kDal (Fig. 2h). This 70 kDal polypeptide also appears when labeled extracts of infected CV-1 cells are incubated alone or mixed with extracts of unlabeled MK cells. By contrast, the 86 kDal T-antigen in labeled extracts of infected MK, alone (Fig. 2d and g) or in the presence of extracts of nonpermissive hamster cells (not shown), is unaltered by this incubation procedure.

Several other minor polypeptides are present in the gels. In particular, the samples of infected MK cells (Figs. 1–3) exhibit a polypeptide migrating almost as the 82 kDal T-antigen. However, an 83 kDal polypeptide is also present both in control samples of mock-infected mouse and monkey kidney cells. The assumption that it is a contaminant of cellular origin is confirmed by fingerprinting analysis which was also used to identify the other minor polypeptides seen in Figs. 1 and 2 (see below).

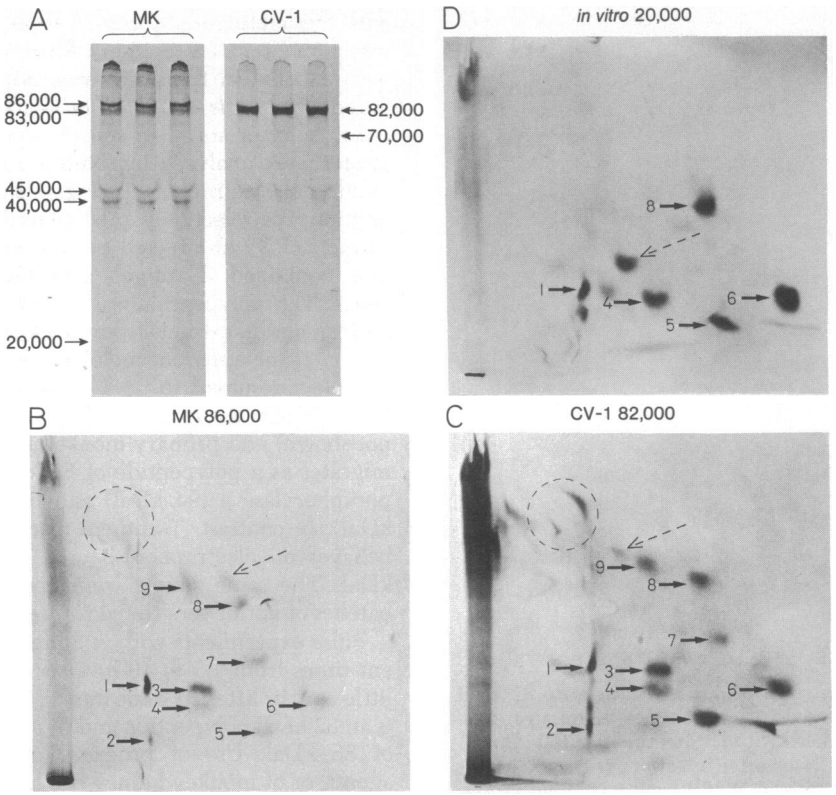

FIG. 3. Characterization of polypeptides by fingerprinting analysis. Polypeptides shown in A were eluted from the gel electrophoretically, except 70,000, which was eluted from the gel in Fig. 2i. Tryptic peptides were prepared and resolved as indicated in *Materials and Methods*. Nine peptides shared by MK 86,000 (B) and CV-1 82,000 (C) are indicated by arrows. Dashed arrows point to a peptide migrating differently in the two samples. The dashed circles roughly delimit an area where poorly resolved peptides (at least three) are consistently seen. Preparation of *in vitro* T-antigen in a DNA-dependent protein synthesizing system will be presented elsewhere (Greenblatt and Allet, manuscript in preparation). The actual *in vitro* sample was a polypeptide migrating on gels as a 20 kDal species. It was eluted from the gel and prepared as the *in vivo* samples.

Identification of the polypeptides by fingerprinting analysis

The different polypeptides separated by gel electrophoresis (Fig. 3A) were analyzed by two-dimensional chromatography of their tryptic peptides (see *Materials and Methods*). As can be seen in Fig. 3B and C, the patterns obtained for the 86 kDal T-antigen of MK cells and the 82 kDal T-antigen of CV-1 cells are very similar, as nine common peptides are unambiguously detected. One peptide present in the pattern of MK (dashed arrow) is missing in CV-1. Conversely, another major peptide is seen only in the pattern of CV-1. Whether this unique peptide difference results from differences in the primary structure of 86 kDal and 82 kDal T-antigen needs further investigation.

As seen in Figs. 1, 2, and 3, all the gel patterns include a polypeptide (83 kDal) migrating almost with the 82 kDal T-antigen. That this is a contaminant cellular polypeptide was confirmed by two-dimensional fingerprinting (not shown). Its occurrence in all extracts from mouse and monkey cells possibly explains the appearance of minor spots in the fingerprinting of CV-1 82 kDal T-antigen, because the latter was probably cut from the gel together with the contaminant.

Two-dimensional fingerprinting analysis of the minor polypeptides revealed that the 40 kDal polypeptide is also unrelated to T-antigen. By contrast, all others (70 kDal, 45 kDal, and 20 kDal) exhibited various sets of typical T-antigen tryptic peptides. In addition, the polypeptide of about 45 kDal from CV-1 cultures (infected for 20 hr or longer), but not that from MK cultures, generated the same tryptic peptides as the viral capsid protein.

Fig. 3D, taken from data to be presented elsewhere (Greenblatt and Allet, manuscript in preparation) shows the tryptic fingerprint of a 20 kDal polypeptide made *in vitro* with SV40 DNA as template in an *Escherichia coli* DNA-dependent protein-synthesizing system (15). Clearly the *in vitro* sample has tryptic peptides in common with the *in vivo* T-antigen. More complete characterization of the various products revealed that T-antigen made *in vitro* contains eight of the nine T-antigen tryptic peptides indicated in Fig. 3C (Greenblatt and Allet, manuscript in preparation). Therefore, the material we call T-antigen in this work must be coded by the viral DNA.

DISCUSSION

Estimated from electrophoretic mobility, the molecular weight of T-antigen synthesized in permissive monkey kidney cells is smaller (82,000) than in nonpermissive mouse kidney cells (86,000). Conceivably, a differential modification of the same polypeptide might affect its migration rate in the gels. That the mobility differences seen in this work were not due to such an effect is suggested by the observation that the 86 kDal T-antigen can be readily converted into an 82 kDal form by incubation with cell-free extracts of permissive monkey but not of MK and other nonpermissive cells. Moreover, large differences in the modification pattern should have been detected by the fingerprinting analyses.

The 86 kDal T-antigen is stable in extracts of nonpermissive mouse and hamster cells, since it is not detectably altered by a 16 hr incubation (Fig. 2). By contrast, incubation of the 82 kDal T-antigen (whether made in monkey cells or resulting from *in vitro* conversion of 86 kDal T-antigen) in extracts of monkey cells yields smaller products, in particular a polypeptide of about 70 kDal (Fig. 2h and i). Whether this reflects different protease activities in the extracts, or a difference in the stability of 86 kDal and 82 kDal T-antigens, or both, is unknown, as is the biological significance of the smaller polypeptides. In this context it should be recalled that peptides containing as few as three amino acids may exert important biological functions (16).

Several lines of experimental observations suggested that SV40 T-antigen is coded by the viral genome (12, 13, 17, 18). Recently more direct evidence in support of this hypothesis was reported by Roberts *et al.* (19). They showed that SV40-DNA-directed polypeptides synthesized in a cell-free extract of wheat germ were selectively precipitated by anti-T serum. Direct and complete evidence that the T-antigen we have isolated is indeed coded by the viral DNA came from the fingerprinting analysis of polypeptides made *in vitro* from SV40 DNA I in an *E. coli* cell free system (Greenblatt and Allet, manuscript in preparation), as shown in Fig. 3.

The lower molecular weight estimate (70,000) for natural T-antigen reported by others (20) can possibly be accounted for by our observation that the 82,000 T-antigen readily yields a 70,000 form. Higher values (up to 100,000) have also been reported (21); they may be overestimates resulting from nonlinearity between mobility and logarithm of the molecular weight in the upper part of the gels. The results in Fig. 1 clearly show that both 86 kDal and 82 kDal T-antigens migrate faster than phosphorylase a (94 kDal) and slower than bovine serum albumin (68 kDal).

It must be noted that an 86 kDal polypeptide corresponds to a coding capacity in the range of the estimated size of virus-specific early 19S mRNA (4). These results, and the existence of a single "early" complementation group (22), support the notion that early 19S mRNA codes a single protein. However, we cannot exclude the possibility that early 19S mRNA may, in addition, code one or more very small polypeptides which would not be detected by the techniques used in this work.

In this paper we have shown that T-antigen is processed from 86 kDal to 82 kDal in several kinds of permissive monkey cells. For technical reasons the only nonpermissive cells in which the stability of T-antigen was directly analyzed were the MK cells. However, in mixed incubation experiments, the molecular weight of T-antigen remains 86,000 not only in MK cell extracts, but also in the presence of extracts of other kinds of nonpermissive cells derived from the hamster. Earlier observations suggested that SV40- (and polyoma-) induced host chromatin replication may be necessary but is not sufficient for viral DNA replication; hence it was postulated that permissive cells may contain a specific factor(s) absent in nonpermissive cells (4, 23). Without proving it, our results are compatible with the hypothesis that this factor is a protease(s): 86 kDal T-antigen synthesized in nonpermissive cells would be able to induce a mitogenic response (leading to chromatin replication and mitosis), but it would allow autonomous replication of the viral DNA only if cleaved to the 82 kDal form. This hypothesis might also explain "rescue" of infective SV40 from virus-free, nonpermissive, SV40-transformed cells after fusion with uninfected permissive cells (24). It remains to be determined whether other types of host-induced posttranslational modifications (such as glucosylation and phosphorylation, etc.) occur.

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