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Simian virus 40 and polyoma virus stimulate overall cellular RNA and protein synthesis

(viral tumor antigens/mitogens/growth-promoting polypeptide and steroid hormones)

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ABSTRACT **In lytic infection with simian virus 40 and polyoma virus of monkey and mouse cells in tissue culture, synthesis of the viral tumor (T) antigens (T antigens) is rapidly followed by a mitogenic response of the host cell. The latter begins with virus-induced stimulation of overall cellular RNA and protein synthesis, leading to a substantial increase in cytoplasmic and nuclear RNA and protein. Stimulation begins within 1 hr after onset of T-antigen synthesis and also occurs if virus-induced DNA synthesis is blocked by metabolic inhibitors. The broad spectrum of biological and molecular effects induced by simian virus 40 and polyoma virus is, at least phenotypically, reminiscent of the pleiotropic impact exerted on target cells by nonviral mitogens and by certain growth-promoting steroid and polypeptide hormones.**

Simian virus 40 (SV40) and polyoma virus induce a lytic infection in permissive cells and an abortive ("transforming") infection in nonpermissive cells. These infections exhibit considerable similarity (for details and references, see ref. 1). Expression of the early viral gene—i.e., synthesis of virus-specific early 19S mRNAs and of the tumor antigens (T antigens), is rapidly followed by a mitogenic reaction of the host cell. This reaction includes virus-induced stimulation of overall cellular RNA synthesis and an increase in total, mainly ribosomal RNA, activation of the cellular DNA-synthesizing apparatus and duplication of the host cell chromatin (S phase). In nonpermissive cells, virus-induced S phase is followed by prophase and mitosis but no viral DNA is replicated. In permissive cells, S phase is paralleled by replication of viral DNA as a nucleohistone and by production of progeny virus and is followed by cell death (lysis). The early events of infection, including the activation of the cellular DNA-synthesizing apparatus, also occur if virus-induced DNA synthesis is blocked by metabolic inhibitors such as 1-β-D-arabinofuranosylcytosine (araC) or FdUrd.

In this paper we report that, in lytic infection, SV40- and polyoma-induced stimulation of cellular RNA synthesis is paralleled by stimulation of cellular protein synthesis which also occurs in the presence of araC or FdUrd.

MATERIALS AND METHODS

Primary mouse kidney (2), secondary monkey kidney, and CV-1 (a monkey kidney cell line) cultures were grown in 10-cm-diameter plastic dishes in reinforced Eagle's medium ("culture medium") containing 10% fetal bovine serum (GIBCO) (3, 4). For infection, we used twice-plaque-purified wild-type SV40 or polyoma virus at an input multiplicity of 25–50 plaque-forming units per cell. In all experiments, parallel cultures were mock-infected with culture medium and then treated in the same way as the virus-infected cultures. araC (20 μg/ml; Sigma) or FdUrd (15 μg/ml; Hoffmann–La Roche) was present in the culture medium added after the adsorption of the virus (90 min). Polyoma-infected cultures were incubated at 37°C in serum-free culture medium (4); SV40-infected cultures were incubated in medium containing 5% serum.

Intranuclear SV40- and polyoma T antigen was visualized by the immunofluorescence reaction. The relative number of DNA-synthesizing cells was determined by autoradiography of cultures pulse-labeled for 1 hr with [^{3}H]dThd (3, 4). Radioimmunoassays for SV40, and polyoma T antigens and viral capsid proteins were performed according to Schwyzer (5).

Cultures were pulsed-labeled (with and without araC or FdUrd) for 1 hr with 60 μCi of [^{35}S]methionine (500–1000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels; Radiochemical Centre, Amersham) or with 100 μCi of [^{3}H]leucine (137 Ci/mmol) in 2 ml of methionine- or leucine-free culture medium (with and without serum).

To separate cells into cytoplasmic and nuclear fractions, cultures were incubated for 10 min at 4°C in lysis buffer (300 mM sucrose/10 mM Tris·HCl, pH 7.4/5 mM NaCl/3 mM $MgCl_2$/0.5% Nonidet P-40), 1 ml per dish. The cells were scraped from the plates and passed through a syringe (20-gauge needle) seven times, and the lysate was centrifuged at 3000 × *g* for 15 min at 4°C.

To extract proteins, unfractionated cultures were suspended, at 1.0 ml per dish, in 1% $NaDodSO_4$/1 mM NaH_2PO_4, pH 8.5; nuclear pellets were suspended in 0.25 ml per dish. To cytoplasmic fractions (1 ml per dish) 50 μl of 20% (wt/vol) $NaDodSO_4$ was added. The lysates were then passed 10 times through a syringe (tuberculin needle) and protein was quantitated colorimetrically according to Lowry *et al.* (6). Bovine serum albumin (Calbiochem) was used as standard. Cellular RNA and DNA were extracted by a modified Schneider procedure (4, 7) and quantitated colorimetrically with orcinol (RNA) or diphenylamine (DNA) (7, 8). For every experimental point, two or three virus- or mock-infected cultures were used.

Aliquots of $NaDodSO_4$-extracted protein to be analyzed by one-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis were mixed with sample buffer and denatured by boiling for 2 min (9). Staining of the gels with Coomassie brilliant blue and autoradiography were performed as described (10). The films were exposed to the dried gels for different periods to ascertain that the exposure response was linear to the amount of radioactivity (optical density at 630 nm = 1.2). Stained gels (before drying) and autoradiographs were routinely scanned with a Joyce–Loebl MK IIIc microdensitometer. For quantitative measurements, autoradiographs were scanned and simultaneously analyzed in a Hewlett–Packard (model 3385) integrator.

^{35}S-Labeled nuclear and cytoplasmic fractions to be analyzed in two-dimensional polyacrylamide gels were prepared as de-

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Abbreviations: SV40, simian virus 40; T antigens, tumor antigens; araC, 1-β-D-arabinofuranosylcytosine.

scribed above and then immediately freeze-dried. Nuclear and cytoplasmic extracts were resuspended in "urea lysis buffer" (11) at 0.25 or 1.0 ml per dish, respectively. Protein content was determined on parallel samples that had been extracted with $NaDodSO_4$. Electrophoresis on two-dimensional gels was performed according to O'Farrell (11), with the exceptions that the cathodal electrolyte contained 200 mM NaOH and the anodal one contained 100 mM H_3PO_4 and that the $NaDodSO_4$/polyacrylamide gel of the second dimension contained a uniform concentration of 12.5% acrylamide and 0.33% bisacrylamide. ^{35}S-Labeled proteins were detected by autoradiography on X-Omat R film (XR-5, Kodak) after exposure for 2–3 weeks.

RESULTS

Time course of virus-induced cellular RNA and protein synthesis

Lytic Infection with SV40. We infected and mock-infected superconfluent CV-1 cultures (16–18 × 10^6 cells per dish) in the presence of araC. The time course of synthesis of early viral mRNA(s) and of T antigen was similar to that observed in secondary monkey kidney cultures (1, 3): by 6–8 hr after infection, synthesis of early 19S mRNA(s) could be detected by molecular hybridization to SV40 DNA, and synthesis of large and small SV40 T antigens could be detected by radioimmunoassay. The immunofluorescence reaction revealed intranuclear T antigen in 1–2% of the cells by 9–10 hr and in 95–100% by 24 hr. DNA, RNA, and protein were extracted from total cultures at different times between 12 and 70 hr after infection and quantitated colorimetrically.

All results reported in this paper are representative of at least three independent experiments. As expected, cell number and DNA content remained unchanged throughout the experiments. However, by 17–22 hr, SV40-infected cultures contained 5% more RNA and protein than did mock-infected controls. At this time, 50–80% of the nuclei exhibited an immunofluorescence reaction for SV40 T antigen. Thereafter, RNA and protein slowly increased, reaching a maximum plateau (60–70%) around 60 hr (Figs. 1 and 2). In mock-infected cultures (with or without araC), DNA, RNA, and protein content remained virtually unchanged.

In monkey and mouse cell cultures infected with SV40 or polyoma virus in the presence of inhibitors of DNA synthesis, the relative amounts of virus-coded RNAs always remains <1%

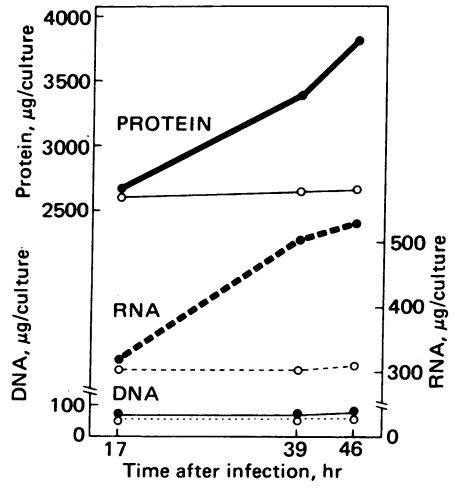

FIG. 1. CV-1 cultures were infected with SV40 (●) or mock-infected (plus araC) (○). Total DNA, RNA, and protein were quantitated colorimetrically at the times indicated in the figure.

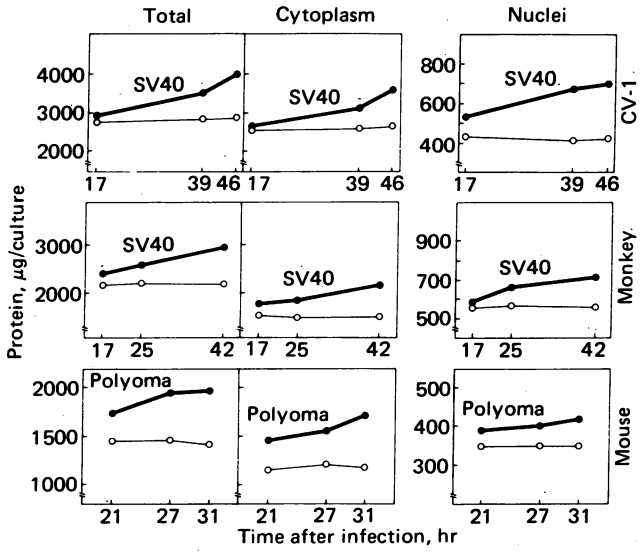

FIG. 2. Protein content of total cultures, isolated nuclei, and cytoplasm was determined colorimetrically at different times after infection of CV-1 and secondary monkey kidney cultures with SV40 (plus araC) and of primary mouse kidney cultures with polyoma virus (plus FdUrd) (●). Mock-infected parallel cultures (plus araC or FdUrd) (○) were analyzed in the same way.

of total RNA (1, 3, 4) and virus-encoded proteins (T antigens) account for no more than 0.1% of total protein (see below). In most experiments, SV40-infected and mock-infected cultures (with and without araC) were labeled with [^3H]uridine or [^{35}S]methionine (or [^3H]leucine) for 1 hr before extraction. Rate of incorporation of both precursors into RNA and protein, respectively, increased simultaneously around 8–9 hr*—i.e., within 1 hr after onset of T-antigen synthesis, detectable by radioimmunoassay. Rate of incorporation of the precursors reached a maximum plateau around 50 hr when it was 2–3 times higher in SV40-infected cultures. Stimulated incorporation of radioactive amino acids into SV40-infected BSC cultures has been observed by Kiehn (12).

In several experiments (with araC) we separated nuclei and cytoplasm at different times between 15 and 60 hr after infection. Nuclear preparations from mock-infected cultures contained virtually total cellular DNA and about 25% of RNA (not shown) and 20% of protein (Fig. 2) present in unfractionated cultures. In SV40-infected cultures the time course of the increase in cytoplasmic protein (Fig. 2) and RNA (not shown) was similar to that in total cultures whereas nuclear protein content increased earlier; as a result, nuclei from SV40-infected CV-1 cultures (with araC) at ≈20 hr contained about 25% more protein than did nuclei from mock-infected controls.

We also infected and mock-infected CV-1 cultures in the absence of araC. As determined by autoradiography, (unpublished data), mock-infected cultures contained a background of 2–3% DNA-synthesizing cells. In SV40-infected cultures the number of DNA-synthesizing cells increased between 15 and 20 hr and by 40 hr, close to 100% of the cells were engaged in DNA synthesis. Determined colorimetrically, the increases in total cellular RNA and protein were similar to those observed in cultures infected in the presence of araC. However, later than 30–35 hr, the amounts of nuclear DNA and protein were higher than in cultures infected in the presence of araC. By 50 hr, nuclear preparations from cultures infected without araC contained at least 50% more DNA and protein; this was mainly due to replication of host and viral chromatin and the pro-

* Matter, J.-M. (1978) Dissertation (University of Geneva).

duction of viral capsid proteins (unpublished data). CV-1 cultures infected without araC began to lyse after 50–60 hr, precluding further comparative chemical analyses.

Extending earlier studies (1, 3) on SV40-induced stimulation of cellular RNA synthesis in confluent, secondary monkey kidney cultures (10–12 × 10^6 cells per dish), we determined total DNA, RNA, and protein content at different times between 10 and 50 hr after infection in the presence of araC. Cell number and DNA content remained unchanged throughout the experiments. By 15–20 hr, SV40-infected cultures (50–80% T-antigen-positive nuclei) contained 5% more RNA and protein than did mock-infected controls. RNA and protein content reached a maximum plateau (30%) around 35–40 hr after infection. The time course of the increase was similar when protein and RNA were quantitated in nuclear and cytoplasmic preparations (Fig. 2 and unpublished data). Labeling of the cultures for 1 hr with [^{3}H]uridine or [^{35}S]methionine (or [^{3}H]-leucine) at different times between 5 and 50 hr after infection (with or without araC) revealed a simultaneous increase in the rate of incorporation of the precursors into RNA and protein by 8–9 hr—i.e., within 1 hr after onset of T-antigen synthesis. Rate of incorporation reached a maximum plateau around 25–30 hr when it was 2–3 times higher in SV40-infected cultures.

In several parallel experiments we infected and mock-infected cultures in the absence of araC. Determined by autoradiography, SV40-induced DNA synthesis began around 15–20 hr and reached a maximum plateau by 30–35 hr when >90% of the cells synthesized DNA. The time course of the increase in total RNA and protein was similar to that observed in cultures infected in the presence of the inhibitor. However, by 30–40 hr, nuclear preparations from normally infected cultures contained 30–60% more DNA and protein than did parallel cultures infected in the presence of araC (data not shown).

Lytic Infection with Polyoma Virus. Confluent primary mouse kidney cultures (10–11 × 10^6 cells per dish) were infected with polyoma virus in the presence of FdUrd (15 μg/ml) (1, 4): synthesis of early 19S polyoma mRNA(s) and of polyoma T antigens (radioimmunoassay) became detectable by 6–8 hr after infection. By 10 and 24 hr, about 0.5% and 90–100%, respectively, of the nuclei exhibited an immunofluorescence reaction for polyoma T antigen. Throughout the experiments (with FdUrd), cell number and DNA content of the cultures remained unchanged (1, 4). However, by 15 hr, infected cultures contained 5% more RNA (1, 4) and protein, and a maximum plateau (30%) of RNA (1, 4) and protein (Fig. 2) was reached by 25–30 hr. A similar increase in RNA (not shown) and protein (Fig. 2) was observed in isolated nuclei and cytoplasm.

In parallel cultures infected without FdUrd present the time course of the increase in total RNA and protein was similar; however, by 25–30 hr nuclear preparations contained 50–60% more DNA and protein than did parallel cultures infected in the presence of FdUrd (1). As determined by autoradiography, polyoma-induced DNA synthesis started in about 1% of the cells

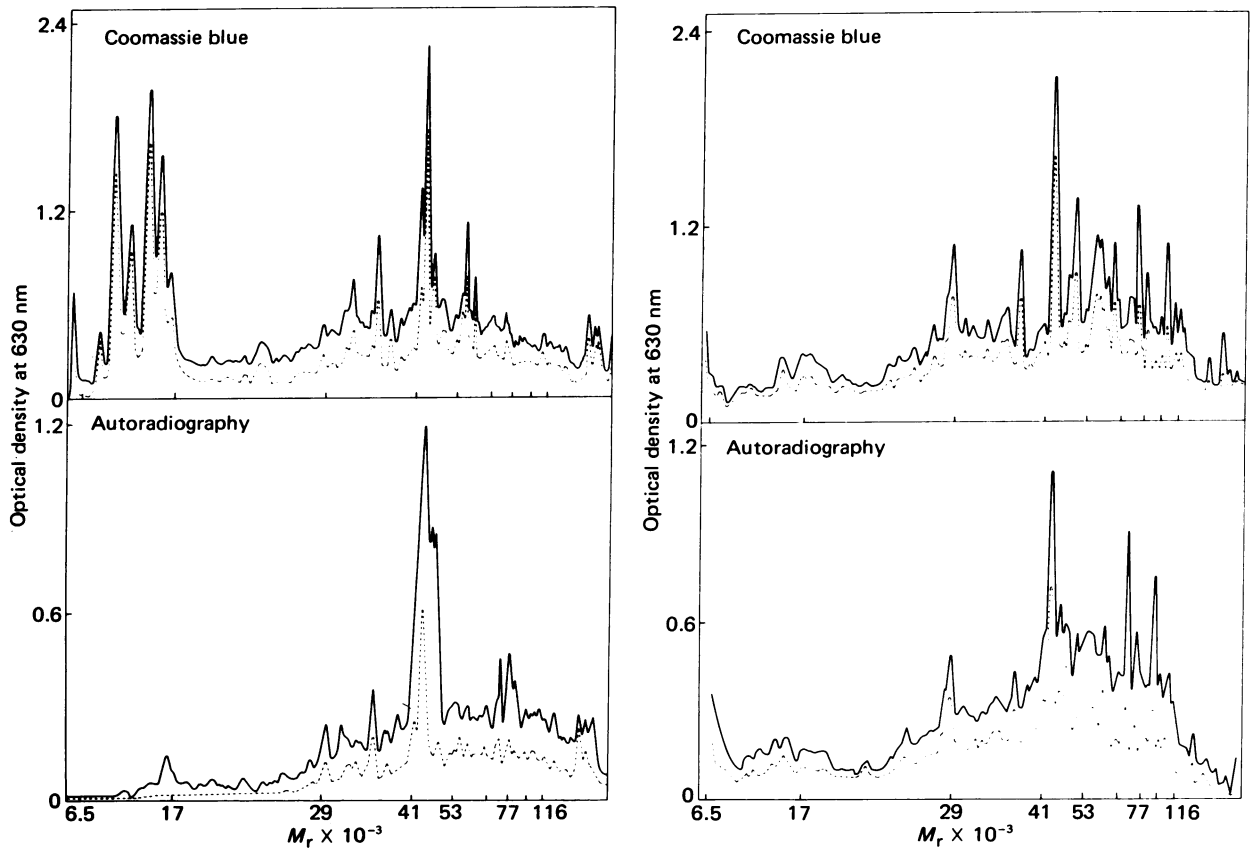

FIG. 3. Analysis, on a one-dimensional NaDodSO$_4$/polyacrylamide gel (12.5% acrylamide), of cytoplasmic (*Right*) and nuclear (*Left*) proteins from CV-1 cultures infected with SV40 (—) or mock-infected (- - -) for 46 hr (with araC; see also Fig. 2). Cultures were labeled with [^{35}S]methionine from 45–46 hr after infection. Aliquots (25 μl) from nuclear or cytoplasmic extracts were applied to the slots. Aliquots from mock-infected and SV40-infected cytoplasm contained 3 × 10^4 and 6.8 × 10^4 cpm (52 and 72 μg of protein), respectively; aliquots from mock-infected and SV40-infected nuclei contained 1.5 × 10^4 and 4.9 × 10^4 cpm (36 and 56 μg of protein), respectively. The figures show the densitometer tracings of gels stained with Coomassie blue and of their corresponding autoradiographs (3-day exposure). Molecular weight markers: β-galactosidase, 116,000; phosphorylase a, 96,500; transferrin, 77,000; bovine serum albumin, 69,000; glutamate dehydrogenase, 53,000; alcohol dehydrogenase, 41,000; carbonic anhydrase, 29,000; myoglobin, 17,000; trasylol, 6500.

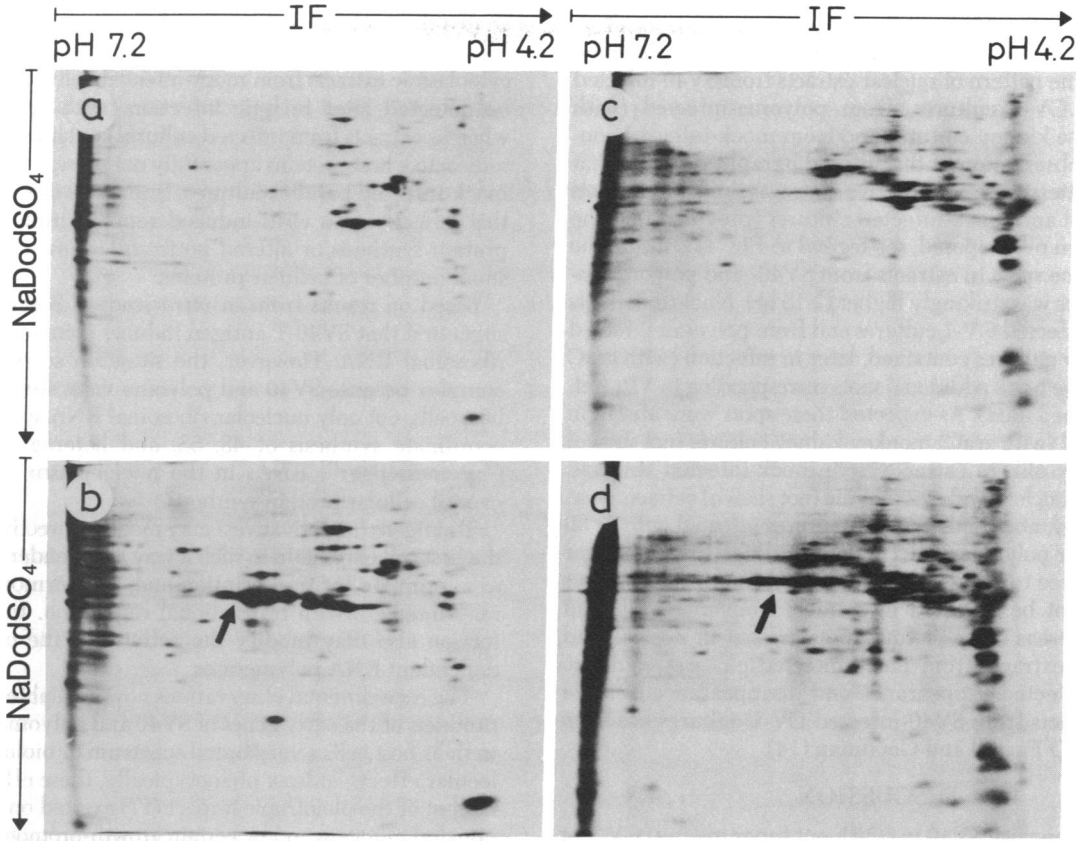

FIG. 4. Analysis on two-dimensional polyacrylamide gels of ^{35}S-labeled nuclear proteins. (*a*) Mock-infected (with araC) CV-1. (*b*) SV40-infected (with araC) CV-1. (*c*) Mock-infected (with FdUrd) primary mouse kidney cultures. (*d*) Polyoma-infected (with FdUrd) primary mouse kidney cultures. SV40-infected cultures (*a* and *b*) were labeled with [^{35}S]methionine from 45 to 46 hr; polyoma-infected cultures (*c* and *d*) were labeled from 30 to 31 hr. The films were exposed for 3 weeks; this led to overexposure which was required, however, to reveal numerous proteins synthesized at lower rates in extracts from mock-infected cultures. The arrows indicate the spots corresponding to VP_1.

by 12–13 hr; by 25–30 hr, about 80% of the cells were synthesizing DNA (1). In numerous experiments (with and without FdUrd) we labeled the cultures with [^3H]uridine or [^{35}S]methionine (or [^3H]leucine) for 1 hr before extraction.* Increased incorporation of the precursors into RNA and protein in infected cultures began by 8–9 hr—i.e., within 1 hr after detectable onset of T-antigen synthesis. A maximum plateau was reached around 25 hr when polyoma-infected cultures (with or without FdUrd) incorporated the precursors at a rate 2–3 times higher.

Nature of the virus-induced proteins

CV-1 cultures and secondary monkey and primary mouse kidney cultures were infected with SV40 or polyoma virus and mock-infected in the presence of araC or FdUrd. The cultures were pulse-labeled for 1 hr with [^{35}S]methionine (or [^3H]leucine) at different times after infection. Extracts were prepared from unfractionated cultures and from isolated nuclei and cytoplasm. Protein content of all preparations was determined colorimetrically and aliquots were analyzed on one-dimensional $NaDodSO_4$/polyacrylamide gels (9, 10). Visual observation and scanning of stained gels (e.g., the results in Fig. 3) revealed an increase in cytoplasmic and nuclear proteins in extracts from infected cultures. This increase became noticeable in polyoma-infected cultures around 15 hr and in SV40-infected cultures around 20 hr, except that in cytoplasmic proteins from SV40-infected CV-1 cultures a distinct increase could be observed no sooner than by 25 hr. As expected (1), histones did not show a detectable increase in Coomassie blue staining and, as judged by autoradiography, incorporated little if any [^{35}S]-methionine (or [^3H]leucine). Computer analyses of the scanned autoradiographs supported and extended the visual observation that essentially the same proteins were synthesized in infected cultures as in mock-infected controls; however, the rate of synthesis was increased.

The increase in labeling detectable by autoradiography coincided in time with the increase in the rate of incorporation of radioactive amino acids. Later than about 15 hr after infection with SV40 or polyoma virus, several (groups of) proteins ($M_r \approx$ 120,000, 95,000, 76,000–72,000, 55,000–52,000, 37,000, and 23,000) were 1.5 to 3 times more labeled than the other proteins. Autoradiographs of nuclear extracts from CV1 cultures infected with SV40 (with araC) and from primary mouse kidney cultures infected with polyoma virus (with FdUrd) exhibited a band (or a doublet) corresponding to VP_1, the major capsid protein ($M_r \approx$ 45,000; Fig. 3 and unpublished results obtained by immunoprecipitation) which could not be detected on stained gels. Synthesis of very small amounts of late viral mRNAs coding for SV40 or polyoma virus capsid proteins, in the presence of araC or FdUrd, has been reported (1, 13). In contrast, no evidence for synthesis of viral capsid proteins could be detected in secondary monkey kidney cultures infected with SV40 in the presence of araC.

We were unable to visualize, in nuclear and cytoplasmic extracts, SV40 or polyoma T antigens either by Coomassie blue staining or by autoradiography, unless they had first been immunoprecipitated with anti-T antisera. Because, later in lytic infection ($\geq$30 hr; with or without araC) SV40 T antigen accounts for about 0.1% of total protein (M. Schwyzer, personal communication), we concluded that the resolution of the proteins was rather limited. We therefore used two-dimensional gels (11) to analyze ^{35}S-labeled nuclear and cytoplasmic extracts

from SV40- and polyoma-infected cultures (with araC or FdUrd). The proteins were revealed by autoradiography only. Fig. 4 shows the pattern of nuclear extracts from SV40-infected (with araC) CV-1 cultures, from polyoma-infected (with FdUrd) mouse kidney cultures and from mock-infected controls. Visual observation of the autoradiographs revealed that the overall pattern of the radioactive spots was similar in extracts from infected and mock-infected cultures (provided that the films had been overexposed; see legend to Fig. 4) but that the intensity of the spots in extracts from SV40- and polyoma-infected cultures was strikingly higher (≥15 hr). Nuclear extracts from SV40-infected CV-1 cultures and from polyoma-infected mouse kidney cultures contained, later in infection (with araC or FdUrd), five or six additional spots corresponding to VP_1 (ref. 14; unpublished data). As expected these spots were absent in SV40-infected (with araC) monkey kidney cultures (not shown) and obviously also in extracts from mock-infected controls. However, all nuclear and cytoplasmic (not shown) extracts from CV-1, monkey, and mouse kidney cultures infected with SV40 (with araC) or polyoma virus (with FdUrd) for 15 hr or longer contained three to five distinct additional (host protein) spots that could not be detected in extracts from mock-infected cultures, whereas three or four spots present in nuclear and cytoplasmic extracts from mock-infected cultures could no longer be detected. Appearance and disappearance of some spots in extracts from SV40-infected CV-1 cultures has been reported by O'Farrell and Goodman (14).

DISCUSSION

Lytic infection with SV40 in confluent (nongrowing) CV-1 or secondary monkey kidney cultures and with polyoma virus in confluent primary mouse kidney cultures stimulates cellular protein synthesis; this stimulation coincides in time with the virus-induced stimulation of overall cellular RNA synthesis. The resulting increase in nuclear and cytoplasmic cellular RNA and protein exhibits essentially the same kinetics when virus-induced DNA synthesis is blocked with araC or FdUrd.

Stimulation of cellular RNA and protein synthesis becomes noticeable by 8–9 hr after infection—i.e., within 1 hr after onset of T-antigen synthesis, detectable by radioimmunoassay. Recently, Wintersberger and Pöckl observed that DNA-dependent RNA polymerase activity in isolated nuclei from primary mouse kidney cultures increased by 5–8 hr after infection with SV40 or polyoma virus (E. Wintersberger, personal communication). As determined colorimetrically, there is 5% more RNA and protein in polyoma-infected cultures by 15 hr and in SV40-infected cultures by 15–20 hr; at these times, 50–80% of the nuclei exhibit an immunofluorescence reaction for T antigen. A maximum plateau (30%) of RNA and protein is reached in polyoma- and SV40-infected mouse and monkey kidney cultures around 30 and 40 hr, respectively. In SV40-infected CV-1 cultures the maximum plateau of RNA and protein is higher (60–70%) and is reached later (around 60 hr). In polyoma-infected cultures the maximum number of DNA-synthesizing cells, determined by autoradiography, and the maximum plateau of RNA and protein coincide in time whereas in SV40-infected secondary monkey kidney and CV-1 cultures, RNA and protein contents increase for another 10 and 20 hr, respectively, after virus-induced DNA synthesis has reached its maximum.

Polyacrylamide gel electrophoresis in one or two dimensions showed that most proteins synthesized in mock-infected controls are also synthesized in infected cultures, although at an increased rate. In cultures infected with SV40 or polyoma virus for 15 hr or longer, synthesis of several (groups of) host proteins is more stimulated than that of the remainder. Autoradiography of two-dimensional gels revealed, furthermore, that a small fraction of the radioactive host proteins present in nuclear and cytoplasmic extracts from mock-infected cultures can no longer be detected later in lytic infection (with araC or FdUrd) whereas extracts from infected cultures contain some additional radioactive host proteins apparently not present in extracts from mock-infected parallel cultures. It remains unknown whether this reflects some virus-induced reprogramming of cellular protein synthesis or altered posttranslational processing of a small number of cellular proteins.

Based on results from *in vitro* studies, Baserga *et al.* (15) suggested that SV40 T antigen induces primarily synthesis of ribosomal RNA. However, the situation seems to be more complex because SV40 and polyoma virus stimulate, in their host cells, not only nucleolar ribosomal RNA synthesis but also coordinate synthesis of 4S, 5S, and heterogeneous nuclear ("premessenger") RNAs in the nucleoplasm (1, 16, *) and overall cellular protein synthesis.

T antigens (or derivatives) may modify, directly or indirectly, the host cell chromatin in such a way as to render it more active as a template for transcription and, furthermore, T antigens may interfere with translational regulation. In addition, infection also may modify the activity of the cellular DNA-dependent RNA polymerases.

The experimental observations now available show that the products of the early genes of SV40 and polyoma virus induce in their host cells a very broad spectrum of biological and molecular effects; at least phenotypically, these effects are reminiscent of the pleiotropic impact (17) exerted on target cells by nonviral mitogens and by certain growth-promoting steroid and polypeptide hormones (1, 17–20).

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