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Post-Transcriptional Restriction of Human Adenovirus Expression in Monkey Cells

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Infection of the continuous simian cell lines CV-1 and BSC-1 with human adenovirus type 2 (Ad2) is abortive. However, the restriction of Ad2 reproduction in these cells can be overcome by increasing the Ad2 infectious dose or by coinfection with simian virus 40. Vero, another established simian cell line free of detectable endogenous simian virus 40 DNA, is not restricted in its ability to promote Ad2 growth even at low input multiplicities of Ad2 and in the absence of SV40 helper. The amount of structural Ad2 proteins in total cell extracts of enhanced BSC-1 cells is at least two orders of magnitude higher than that of unenhanced cells. In contrast, comparable quantities of Ad2 mRNA specifying these proteins are found in both the enhanced and the unenhanced cell. Both sets of mRNA can be translated in a cell-free system with equal efficiency.

The growth of human adenovirus in simian cells is restricted. This restriction can be overcome by coinfection with simian virus 40 (SV40). In the restricted system, adenovirus 2 (Ad2) T antigen is produced (8, 25), viral DNA is replicated (3, 28), and viral RNA synthesis appears to be unaffected (9, 10). The defect is thought to reside in the production of structural components of the adenovirion, which are found to be greatly reduced in the restricted system (2, 11, 15).

We have analyzed the biological activity of Ad2 mRNA in a cell-free, protein-synthesizing system. The results indicate that equal amounts of RNA specifying late Ad2 products are present both in the restricted and in the SV40-enhanced cells.

MATERIALS AND METHODS

Viruses and cells. Ad2, free of adeno-associated virus, was obtained from J. Rose (National Institutes of Health) and propagated in KB cell spinner culture (6). Infectivity was determined by measuring the cytopathic effect on monolayers of human embryonic kidney cells (16). Plaque-purified small-plaque SV40 strain 776, obtained from K. Takemoto (National Institutes of Health), was propagated on Vero cell monolayers. Titers were determined by plaque assay on primary African green monkey kidney cells. Monolayers of the established monkey kidney cell lines (BSC-1) (17), Vero (5), and CV-1 (18) were grown in Eagle minimal essential medium supplemented with 0.03% (wt/vol) glutamine and 10% (vol/vol) fetal calf serum. Cells were infected with virus multiplicities of infection (MOI) given in the test. After 1 h of adsorption at room temperature, the monolayers were drained, rinsed with fresh medium, and incubated at 37 C in medium containing 2% (vol/vol) fetal calf serum.

A search for endogenous SV40 DNA in Vero cells was conducted as follows. Unlabeled DNA was extracted from confluent cell cultures and sheared to an average size of 3×10^5 daltons (12). The DNA was denatured and hybridized in excess (2 mg/ml) with either of the separated strands of ^{32}P-labeled SV40 DNA (4 ng/ml) in 1.0 M NaCl/0.02 M sodium phosphate (pH 6.8) at 68 C for 24 h. The formation of SV40 DNA hybrids was monitored on hydroxyapatite columns. This procedure is sensitive enough to detect the presence of less than one copy of SV40 DNA per diploid cell genome (19). No SV40 DNA was detected in the Vero cell population.

Extraction of polysomes. At 32 h postinfection, the cells were labeled for 2 h at 37 C with 75 to 100 μCi of [^{3}H]uridine (New England Nuclear, 40 Ci/mmol) per ml of culture medium. Cycloheximide (10 μg/ml) was added 2 h later, and the incubation was terminated 140 min post-pulse. After scraping from the glass surface, the cells were washed twice with cold phosphate-buffered saline containing 10 μg of cycloheximide per ml and lysed (27) in 2 volumes of 0.01 M Tris-hydrochloride (pH 7.5), 0.01 M KCl, 1.5 mM $MgCl_2$, 0.05 mg of dextran sulfate 500 (Pharmacia) per ml, 0.5% (vol/vol) Triton X-100, and 1 mM dithiothreitol with 10 strokes of a tight-fitting glass homogenizer. Nuclei were removed by low-speed centrifugation, and the polysomes were sedimented through a cushion of 1 M sucrose in 0.01 M Tris-hydrochloride (pH 7.5), 0.15 M KCl, 5 mM magnesium acetate, 3 mM $CaCl_2$, and 0.1 mM EDTA for 90 min at 3 C in the Spinco Ti-60 rotor at 60,000 rpm.

Preparation and cell-free translation of viral

mRNA. RNA extracted from polysomes was adsorbed to oligo(dT)-cellulose to select poly(A)-containing sequences (6). Cell-free translation in a system utilizing the S30 fraction of murine Krebs II ascites cells was performed as described earlier (6), with the following modifications. Incubation was for 2 h at 37 C. The reaction mixture contained 2.9 mM magnesium acetate. The KCl concentration was raised from 67 mM to 110 mM 5 min after the onset of incubation (29); [^{35}S]methionine-labeled products of the in vitro reaction were analyzed by electrophoresis through 13% sodium dodecyl sulfate (SDS)-polyacrylamide slab gels, separating polypeptides in the size range of 120,000 to 18,000 daltons.

RESULTS

Optimal conditions for SV40-induced enhancement of Ad2 expression in monkey cells. Two continuous lines of African green monkey kidney cells, CV-1 (3) and BSC-1 (21), have previously been shown to promote Ad2 growth when coinfected with SV40. We therefore employed these two cell lines plus an additional established simian cell line, Vero, for our studies of the SV40-induced enhancement of Ad2 expression in monkey cells. When infected simultaneously with Ad2 and SV40, all three cell lines, BSC-1, CV-1, and Vero, produced structural Ad2 proteins which were easily recognized in SDS-polyacrylamide gel electropherograms of total cell extracts (Fig. 1). An Ad2 MOI of 1 was sufficient to visualize this effect; however, the polypeptide bands appeared to be much stronger when the infectious dose was increased fivefold. As shown previously (2), in unenhanced infections, i.e., in the absence of SV40, little if any Ad2 structural proteins were detected in CV-1 and in BSC-1 cells. In contrast, Vero cells appeared to be independent of the SV40 helper function in their ability to produce Ad2 proteins. A search (see above) for endogenous SV40 DNA, which might substitute for exogenously added helper, remained negative under conditions sensitive enough to detect <1 SV40 genome per cell.

Friedman et al. (11) reported that the block of Ad2 growth in monkey cells can be overcome, in the absence of SV40, by high doses of virus. We confirm that, when the input multiplicity of Ad2 was raised from 5 to 50, BSC-1 cells contained comparable amounts of Ad2 structural components whether or not they were coinfected with SV40 (Table 1). At low MOI, hexon synthesis in CV-1 and BSC-1 cells was strictly dependent on SV40 helper function,

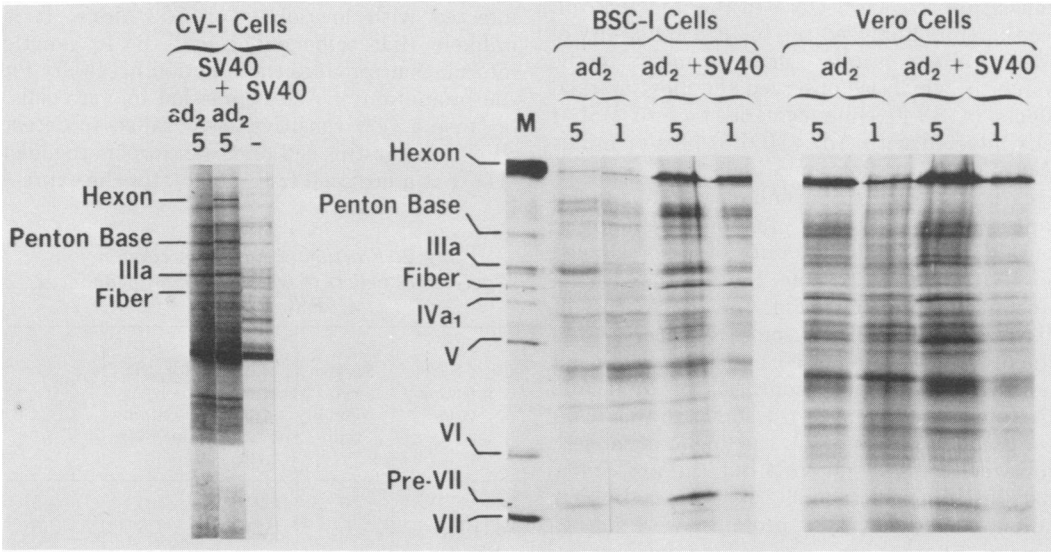

FIG. 1. *SDS-polyacrylamide gel electrophoresis of proteins extracted from infected CV-1, BSC-1, and Vero cells. The various monkey cell lines (3 × 10^6 cells) were infected with Ad2 (MOI as indicated), SV40 (MOI 10), or both Ad2 and SV40 and pulse-labeled at 30 h postinfection, the time of maximal viral protein synthesis. After incubation with 250 μCi of [^{35}S]methionine (Amersham, 250 Ci/mmol) in 1 ml of methionine-free medium for 1 h at 37 C, the cells were washed and incubated for another 1 h in medium containing unlabeled methionine. The cells were scraped from the glass surface, washed twice with cold phosphate-buffered saline, boiled 5 min in SDS sample buffer (1) at a concentration of 3 × 10^5 cells per 10 μl, applied to a slot of a 13% SDS-polyacrylamide slab gel, and subjected to electrophoresis as described elsewhere (6). Ad2 virion polypeptides (6) with known molecular weight (1) were included as size markers (M). Pre-VII refers to the precursor of core protein VII (1). The gel was autoradiographed for 1 day.*

TABLE 1. *Ad2 hexon synthesis in enhanced and in unenhanced monkey cell lines*[a]

MOI		Counts/min		
Ad2	SV40	BSC-1	CV-1	Vero
5		180	100	12,580
75		6,700	ND	11,690
5	10	7,500	985	9,600
	10	150	75	125

[a] [^{35}S]methionine-labeled proteins from infected cells were fractionated by SDS-polyacrylamide gel electrophoresis as indicated in the legend to Fig. 1. The radioactivity contained in polypeptide bands comigrating with hexon was determined as described elsewhere (6). ND, Not determined.

whereas at high MOI it was not. Vero cells, in contrast, produced near equal amounts of hexon in the presence or in the absence of coinfecting SV40, even at low Ad2 MOI. The yield of infectious Ad2 paralleled that of hexon in these experiments (data not shown).

Effect of SV40 coinfection on the production of biologically active Ad2 mRNA in BSC-1 cells. Previous reports (9, 10, 24) suggested that the restriction of Ad2 growth in monkey cells acts after, rather than prior to, transcription of viral DNA in the infected cell. We therefore analyzed the biological activity of Ad2 mRNA, confining our experiments to the system in which we observed the most marked effects of SV40 enhancement, i.e., to BSC-1 cells infected with Ad2 (MOI 5) plus or minus SV40 (MOI 10). Polysomal pellets prepared as described above from enhanced and unenhanced cells at 34 h postinfection were found to contain near equal amounts of pulse-labeled poly(A)-Ad2 RNA (Table 2). In either case a substantial proportion of the RNA cosedimented with >80*S* polysomal structures from which it could be released by EDTA (data not shown). Thus neither synthesis of total Ad2 RNA nor attachment of ribosomes to this RNA seemed to be restricted in the unenhanced cell. However, this did not rule out the possibility that, in the restricted cell, mRNA's coding for individual adenovirion proteins were either missing or not properly assembled in polysomes.

The former alternative was tested in two experiments. First, we determined the genetic complexity of pulse-labeled poly(A)-Ad2 RNA extracted from polysomal pellets which were derived from the unenhanced or the enhanced system (Fig. 2). As was suggested previously (24) by a different method, the amount of viral genetic information present in either RNA was similar to that of a corresponding RNA preparation from productively infected KB cells (7).

More specifically, a second experiment compared the messenger activities of the RNA preparations derived from polysomal pellets of the unenhanced and the enhanced system. Polypeptides comigrating with all major adenovirion polypeptides in an SDS-polyacrylamide gel, including those identified (7) as viral gene products, were found to be synthesized in cell-free assays programmed with either RNA. Moreover, no difference could be observed in the relative quantities of these polypeptides which were directly related to the RNA input (Fig. 3). Therefore, the production of mRNA's coding for these adenovirion proteins appears to be both quantitatively and qualitatively normal, whereas their translation in vivo is restricted in the unenhanced cell.

DISCUSSION

The restriction of Ad2 expression in BSC-1 and CV-1 cells can be overcome by coinfection with SV40 (3, 11). There is, however, no absolute requirement for SV40 helper function to render monkey cells permissive for Ad2 growth, for high input multiplicities of Ad2 alone will also release the restriction. Moreover, the Vero line of monkey kidney cells is permissive even if infected with low doses of Ad2 alone. It is unlikely that some endogenous SV40 genetic information provides the function necessary for the promotion of Ad2 expression in Vero cells, because a very sensitive assay failed to detect SV40 DNA in this cell line. We support the idea (11) that a host cell factor regulates the expres-

TABLE 2. *Fraction of Ad2 RNA contained in polysomal pellets of enhanced and unenhanced BSC-1 cells*[a]

Infecting virus	Packed cell volume (ml)	A_{260} of RNA extracted from polysomal pellets	A_{260} of poly(A)-containing RNA	Ad2-specific RNA[b]
Ad2	6	240	4.0	7.5
Ad2 plus SV40	6	240	3.5	7.0
SV40	6	190	3.0	<0.1

[a] RNA was extracted from polysomal pellets as described in Materials and Methods, adsorbed to oligo(dT)-cellulose to select for poly(A)-containing sequences (6), and hybridized with excess amounts of denatured Ad2 DNA immobilized on filters (13). A_{260}, Absorbancy at 260 nm.

[b] Percent of radioactivity of poly(A)-containing RNA.

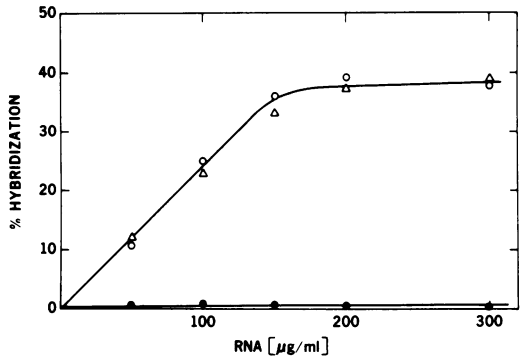

FIG. 2. *Kinetics of the reassociation of Ad2 DNA and polysomal RNA derived from enhanced and unenhanced BSC-1 cells. Hybridization assays contained 12 ng of sonically treated, heat-denatured ^{3}H-labeled Ad2 RNA and increasing amounts of unlabeled, poly(A)-containing RNA. The RNA had been harvested from BSC-1 cells 34 h after infection with (○) Ad2 (MOI 1) or (△) Ad2 (MOI 1) plus SV40 (MOI 10), or (●) SV40 (MOI 10). Nucleic acids were incubated in 100 μl of 0.14 M sodium phosphate (pH 6.8) and treated with 0.1 N NaOH for 18 h at 37 C to hydrolyze more than 99.9% of the RNA. The amount of ^{3}H-labeled Ad2 DNA in each fraction was assayed by trichloroacetic acid precipitation onto membrane filters (Millipore), followed by counting in a toluene-based scintillation fluid.*

sion of Ad2. If it acts as a positive control element, this factor would be present in sufficient amounts in Vero cells but require induction in BSC-1 and CV-1 cells. Alternatively, as an inhibitor of Ad2 expression, the factor would be inactive in Vero cells while responding to repression by SV40 or by high doses of Ad2 in BSC-1 and CV-1 cells. Although the nature of this putative cellular factor is not known, it must respond to an early SV40 gene product, for Ad2-SV40 hybrid viruses which express only early SV40 functions (23) and contain mostly early SV40 DNA sequences (22) grow in monkey

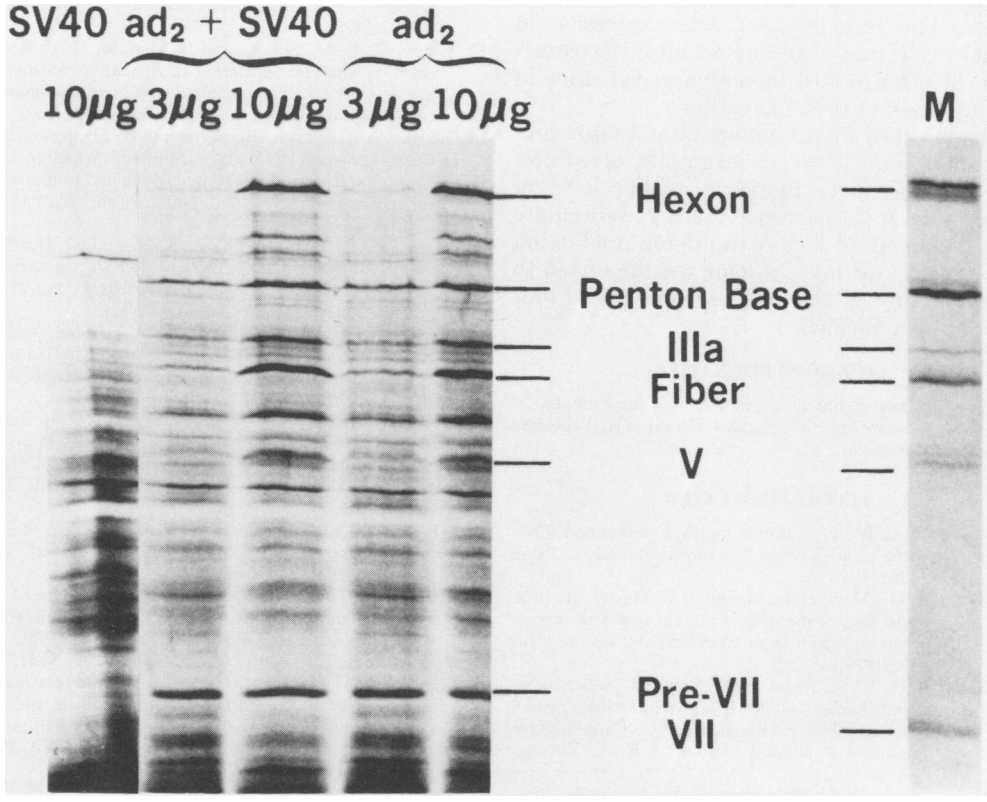

FIG. 3. *SDS-polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled polypeptides synthesized in a cell-free system derived from murine Krebs II ascites cells. One milliliter of assay mixture (see Materials and Methods) was programmed by either 3 or 10 μg of poly(A)-containing polysomal RNA from BSC-1 cells harvested 34 h after infection with Ad2 (MOI 1), Ad2 (MOI 1) plus SV40 (MOI 10), or SV40 (MOI 10). The equivalent of 15 μl of assay mixture was applied to one gel slot. After electrophoresis, the gel was autoradiographed for 3 days. Marker (M) proteins (see legend to Fig. 1) were derived from Ad2 virions.*

cells which are not permissive for wild-type Ad2.

We have reinvestigated the molecular level at which Ad2 expression is controlled in monkey cells. BSC-1 and CV-1 cells infected with low doses of Ad2 do not produce Ad2 structural proteins despite the fact that they contain Ad2 mRNA capable of specifying these proteins in a cell-free, protein-synthesizing system. Simultaneous infection with SV40 induces the synthesis of Ad2 structural proteins in these cells, yet no increase in the net amount of the corresponding mRNA is observed. It appears, therefore, that the SV40-induced production of Ad2 in BSC-1 cells is controlled at a post-transcriptional level.

In both the presence and the absence of SV40 coinfection, Ad2 RNA in BSC-1 cells is found attached to ribosomes from which it can be released with EDTA. However, recent observations by Hashimoto et al. (14), Fox and Baum (10), and Nakajima et al. (26) suggest that in the absence of SV40 this RNA fails to be incorporated into functionally active polysomes. The restriction of Ad2 expression in monkey cells may therefore act after the association of mRNA with monosomes, yet prior to the initiation of protein synthesis.

The effect of interferon on animal virus production is one of a few examples of control mechanisms likely to occur at the level of translation. It is therefore tempting to speculate that the apparent lack of interferon production in Vero cells (4) may in some way be linked to the capability of this monkey cell line to produce human adenovirus.

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