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Block to Multiplication of Adenovirus Serotype 2 in Monkey Cells

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The block to adenovirus 2 (Ad2) multiplication in monkey cells can be overcome by coinfection with simian virus 40 (SV40). To identify this block we have compared the synthesis of Ad2 proteins in monkey cells infected with Ad2 alone (unenhanced) or with Ad2 plus SV40 (enhanced). Synthesis of viral proteins in enhanced cells was virtually identical to that found for permissive infection of human cells by Ad2 alone. In contrast, the unenhanced cells were strikingly deficient in the production of the IV (fiber) and 11.5K proteins whereas the synthesis of 100K and IVa_2 was normal. Synthesis of a number of other proteins such as II, V, and P-VII was partially reduced. A similar specific reduction in synthesis of these proteins was found when their messages were assayed by cell-free translation. This result suggests that the block to Ad2 protein synthesis is at the RNA level rather than with the translational machinery of monkey cells. Analysis of the complexity and the concentration of Ad2-specific RNAs, using hybridization of restriction endonuclease fragments of the Ad2 genome to increasing concentrations of RNA, shows that although all species of late Ad2 mRNA are present, the concentration of several species is reduced sevenfold or more in unenhanced monkey cells as compared with enhanced cells. These species come from regions of the genome known to encode the deficient proteins. A model for the failure of adenovirus to multiply in monkey cells, based on abnormal processing of specific adenovirus messages, is presented.

Adenovirus infection of African green monkey kidney (AGMK) cells results in a very low level of virus production in comparison to a similar infection of human cells. However, Rabson et al. (30) reported in 1964 that coinfection with simian virus 40 (SV40) enhanced the replication of adenovirus in monkey cells to a level comparable to that found in human cells. This release of the block to productive replication of adenovirus in monkey cells by SV40 is termed enhancement.

The nature of the block to adenovirus multiplication in AGMK cells is not yet clearly understood. The adsorption and entry of adenovirus into monkey cells appears normal (11). Both the onset and the rate of viral DNA synthesis are comparable in enhanced and unenhanced infections (14, 21, 31). Early proteins, including T antigen, are synthesized in unenhanced cells, as determined by immunofluorescence, complement fixation, and DNA binding techniques (11, 14, 36). However, the synthesis of some late adenovirus proteins, in particular virion proteins, is reduced in the unenhanced cells (4, 10, 14, 18, 21, 24). Adenovirus mRNA metabolism has been reported to be normal with respect to rate and time of synthesis after infection, polyadenylation, and complexity of cytoplasmic RNA in unenhanced cells compared to enhanced cells (12, 13, 21, 24). In contrast, there are fewer species of adenovirus mRNA in polysomes from unenhanced cells than in those from enhanced cells (13, 21). Recently, Nakajima et al. (26) showed that 80S initiation complexes with adenovirus RNA failed to form in ribosomal preparations from unenhanced cells. Formation of this complex was restored by addition of a high salt wash of ribosomes from enhanced cells to a ribosomal preparation from unenhanced cells.

Published data suggest that the block to productive adenovirus replication in AGMK cells is at the level of initiation of late adenovirus message translation. Improper initiation may be due to a defect in the viral RNA or in the host translation system. Defects in adenovirus mRNA which affect translatability may be due to partial destruction of or lack of appropriate modification of the mRNA. Alternatively, the failure of monkey cells to translate certain adenovirus serotype 2 (Ad2) mRNA species may be

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due to the presence of a factor which prevent translation, or to the absence of a factor required for adenovirus mRNA translation. If the defect is at the RNA level, then for the simplest case one would predict that RNA extracted from unenhanced monkey cells and translated in a cell-free system would give the same results found in vivo, namely, reduced synthesis of several late adenovirus proteins. On the other hand, if the defect is in the translation system of the unenhanced cells, then in the simplest model adenovirus RNA extracted from these cells should be translatable in a fractionated mammalian cell-free system, since this system translates all known Ad2 mRNA species efficiently without the use of any specific factors for Ad2 mRNA (2).

In this paper we report reduced synthesis in vitro as well as in vivo of several late Ad2 proteins from RNA extracted from unenhanced cells. These results conflict with those of Eron et al. (10), which showed that RNA extracted from BSC-1 cells infected with Ad2 or Ad2 plus SV40 was equally active in programming Ad2 protein synthesis in a murine Krebs II ascites S30 cell-free translation system. Results from experiments designed to explain this discrepancy are discussed. We also report the results of studies on the complexity and level of Ad2-specific RNAs in enhanced versus unenhanced cells, using hybridization of restriction endonuclease fragments of the Ad2 genome to increasing concentrations of RNA. These studies show that, although the complexity of Ad2-specific RNA is similar, the concentrations of several species of Ad2 RNA are reduced several-fold in unenhanced versus enhanced cells. Our results suggest that the block to adenovirus replication in monkey cells may be due to abnormal metabolism of adenovirus RNA, rather than to a defect in the translation system.

MATERIALS AND METHODS

Cells and viruses. CV_1 cells, an established line of AGMK cells, were obtained from J. Mertz. Three sublines of BSC-1 cells, also an established line of AGMK cells, were obtained from G. Khoury and from R. Pollack. HeLa cells were obtained from J. F. Williams. The monkey cells were cultivated in Dulbecco's modification of Eagle minimal essential medium (Microbiological Assn. catalogue no. 11-305) supplemented with 10% fetal bovine serum (Reheis Chemical Co.), 100 μg of streptomycin (Sigma) per ml, and 100 μg of penicillin (Sigma) per ml. HeLa cells were cultivated in a similar medium, with 10% calf serum (GIBCO) replacing fetal bovine serum.

Ad2 was obtained from U. Pettersson. Immediately before infection, a sample of the stock (3×10^{10} PFU/ml) was incubated with 100 μg of trypsin (GIBCO) per ml for 30 min at 37 C. SV40, strain 776, was obtained from J. Sambrook. Plaque titrations were performed as described by Grodzicker et al. (18).

Confluent plates (90 mm, Nunc) of cells ($\sim 6 \times 10^6$ cells/plate) were split approximately 1 to 2.5, 14 to 18 h before infection, and had reached a semiconfluent state by the time of infection. After removal of the medium, cells were infected with Ad2 (5 PFU/cell) or Ad2 (5 PFU/cell) plus SV40 (3 to 6 PFU/cell) in 0.5 ml of phosphate-buffered saline. Mock-infected cells received 0.5 ml of phosphate-buffered saline in place of virus. After adsorption of the virus at 37 C for 1 h, fresh medium plus 20 μg of mycostatin (GIBCO) per ml was added. Cells were labeled with [^{35}S]methionine or harvested for RNA extraction 30 h post-infection (pi), unless otherwise indicated.

Preparation of RNA. RNA was prepared according to a modified procedure of Kumar and Lindberg (22). Cells were harvested by scraping and centrifugation, washed with phosphate-buffered saline plus 1 mM EDTA, and suspended in approximately 3 volumes of TSM (10 mM Tris-hydrochloride, pH 7.6, 150 mM NaCl, 1.5 mM $MgCl_2$) plus 0.5% (vol/vol) Triton X-100. After 3 to 5 min on ice, the nuclei were removed by centrifugation at 1,500 $\times$ g for 3 min. The cytosol was centrifuged again to remove any residual nuclei. An equal volume of TSE (10 mM Tris-hydrochloride, pH 7.6, 150 mM NaCl, 5 mM EDTA) plus 1% sodium dodecyl sulfate (SDS) was added to the cytosol, which was then extracted four times, alternating with a mixture of phenol, chloroform, and isoamyl alcohol (24:24:1) saturated with a pH 6.0 buffer containing 10 mM sodium acetate, 100 mM NaCl, 1 mM EDTA, or with a mixture of chloroform and isoamyl alcohol (24:1). The aqueous phase was ethanol precipitated three times and then used for in vitro protein synthesis or further treated before use in RNA-DNA hybridization. The pelleted nuclei were washed once in TSM plus 0.5% Triton X-100 and once in TSM. After resuspension in 1 volume of TSM, the nuclei were lysed with 25 volumes of nuclei lysis buffer (2% SDS, 7 M urea, 350 mM NaCl, 1 mM EDTA, 10 mM Tris-hydrochloride, pH 8.0). After sonic treatment to decrease the viscosity, the solution was extracted with phenol, chloroform, and isoamyl alcohol and was ethanol precipitated in a manner similar to that described for cytoplasmic RNA.

The RNA for hybridization was treated as described by Sharp et al. (34) with minor modifications. It was dissolved in 50 mM Tris-hydrochloride, pH 7.6, 10 mM NaCl, and 10 mM $MgCl_2$ and treated with 25 μg of DNase 1 (electrophoretically pure, Worthington) per ml, which had been passed through a 1-ml column of agarose-5'-(4-aminophenyl-phosphoryl-uridine-2'-[3']-phosphate) (Miles Laboratories, Inc.) to remove residual RNase. The digestion was for 1 to 2 h at 37 C. NaCl and SDS were added to give a final concentration of 300 mM and 0.1%, respectively. The RNA was extracted with the phenol-chloroform-isoamyl alcohol mixture described above and then ethanol precipitated. The nuclear RNA solution was redigested, extracted, and precipitated a second time.

The treated RNA was dissolved in 10 mM Tris-hydrochloride, pH 7.6, 10 mM NaCl, and 0.01% SDS and passed through a column (45 by 1.3 cm) of Sephadex G-100 in the buffer described above. RNA eluting in the void volume of the column was concentrated by ethanol precipitation. RNA treated in this fashion was free of contaminating viral DNA, by the criterion that all detectable hybridization to separated viral DNA strands was destroyed when samples were treated with 0.3 N NaOH for 12 h at 37 C.

Cell-free protein synthesis. The synthesis of Ad2 proteins in the fractionated mammalian cell-free system developed by Schreier and Staehelin (33) has been previously described (2; J. F. Atkins, J. B. Lewis, C. W. Anderson, and R. F. Gesteland, J. Biol. Chem., in press). The preparation of murine Krebs II ascites S30 for cell-free protein synthesis was according to the methods previously described for the preparation of ribosomal subunits (2), with the following modifications. Twenty minutes after the start of polysome run-off, the S30 was chilled on ice and centrifuged for 10 min at 12,000 × *g*. The supernatant was passed through a column of Sephadex G-25 in 0.5 mM dithiothretitol, 3 mM magnesium acetate, 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, pH 7.2, 100 mM KCl. The S30 eluting in the void volume was stored at −70 C in 0.2-ml aliquots, after quick freezing in liquid N_2. The reaction mixture for the Krebs II ascites cell S30 protein synthesizing system contained the same concentration of components as described for the fractionated mammalian cell-free translation system (2), with the following exceptions. The ribosomes, initiation factors, and the pH 5 enzyme fraction of the fractionated system were replaced by 30 μl/50μl of reaction mixture of Krebs II ascites S30; the final concentrations of Mg^{2+} and K^+ were 3 and 100 mM, respectively. Oligo(dT)-selected RNA (3) was used to program this system for protein synthesis in an amount equivalent to 8 μg of unselected cytoplasmic RNA per 50 μl of reaction mixture.

Analysis of Ad2 proteins. The synthesis of Ad2 proteins in vivo was followed by labeling of cells with [^{35}S]methionine for 1 to 2 h as previously described (1). The products were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography (1). Proteins synthesized in the cell-free translation systems were treated as follows. After synthesis, EDTA and RNase A were added to the reaction mixture to a final concentration of 10 mM and 20 μg/ml, respectively, and it was incubated for 10 to 20 min at 37 C. The proteins were precipitated with 10 volumes of 80% acetone, dissolved in 50 μl of sample buffer (100 mM dithiothreitol, 80 mM Tris-hydrochloride, pH 6.8, 10% glycerol, 2% SDS, 0.004% bromophenyl blue), and boiled for 3 to 5 min. Three to 5 μl of each sample was analyzed by electrophoresis and autoradiography as above.

Preparation of single strands of ^{32}P-labeled restriction endonuclease fragments of Ad2 DNA. ^{32}P-labeled Ad2 DNA with a specific activity of approximately 2 × 10^6 counts/min per μg was prepared from infected human KB cells (28). The DNA was cleaved (27) with EcoRI or BamHI restriction endonucleases (a gift of P. Myers and R. J. Roberts). The resulting fragments were resolved by electrophoresis on 1.4% (EcoRI fragments) or 1% (BamHI fragments) agarose gels as previously described (23). The purified fragments were recovered from the gel by electrophoresis into dialysis tubing and residual agarose was removed by phenol extraction (27). After concentration of the DNA by ethanol precipitation, the strands were separated by denaturing in 0.2 N NaOH followed by electrophoresis on 0.7% (EcoRI fragments) (34, 35) or 1% (BamHI fragments) agarose gels. After staining in aqueous ethidium bromide (0.5 μg/ml) for 10 min, the gel was examined with UV illumination (35). The two single strands were well resolved for all fragments except EcoRI-A, BamHI-A, and BamHI-D. The lack of distinct bands of single-stranded DNA for the largest fragments, EcoRI-A and BamHI-A, was probably the result of breakdown of the DNA caused by radiolysis. These two fragments were not used as probes in RNA-DNA hybridization, since small restriction endonuclease fragments covering the same region were available (Fig. 1). BamHI-D single strands formed a single broad band on agarose gels after denaturation. This band was divided into equal halves and treated in the same manner as single-stranded bands from other fragments. The strand with the greater electrophoretic mobility on an agarose gel was labeled the "fast" strand, and the other was designated the "slow" strand. Sharp et al. (34) have shown that the fast strands of EcoRI-B-F and

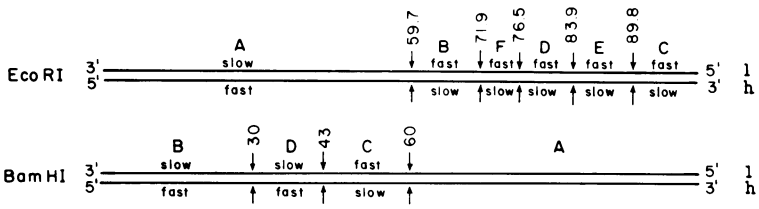

FIG. 1. *Restriction endonuclease map of the Ad2 genome. The top half of the figure shows the DNA fragments (designated by capital letters) generated by restriction endonuclease EcoRI. The cleavage sites are denoted by arrows and numeral coordinates (25). The lower half of the figure shows the DNA fragments generated by restriction endonuclease BamHI. The arrows and numbers give the approximate cleavage positions (C. Mulder, personal communication). Heavy (h) and light (l) refer to the density of the separated strands on a CsCl gradient in the presence of poly(U,G) (34). Fast and slow denote the relative electrophoretic mobilities of the two strands of each fragment on agarose gels (34; unpublished data).*

the slow strand of EcoRI-A correspond to the light strand with respect to density on a CsCl gradient in the presence of poly(U,G), whereas the complementary strand is referred to as the heavy (h) strand (Fig. 1). The slow strands of BamHI-B and BamHI-D and the fast strand of BamHI-C also correspond to the light strand, as determined by hybridization of separated strands of ^{32}P-labeled BamHI fragments B, C, and D to separated strands of EcoRI-A (Mathews and Klessig, unpublished data).

The bands of agarose containing the single strands were dissolved in two volumes of 5 M sodium perchlorate (Fischer, filtered before use) at 60 C (16, modified). NaCl and sodium phosphate buffer (PB), pH 6.8, were added to give a final concentration of 1 M and 50 mM, respectively. This mixture was incubated at 50 C for at least 20 times its C_0t 1/2, to allow annealing of any contaminating complementary strands. The solution was diluted fourfold with 5 mM PB, pH 6.8, and passed through a 1-ml column of hydroxylapatite at 60 C. The column was washed with 15 ml of 50 mM PB, pH 6.8. Single strands of DNA were eluted from the column with five 0.5-ml washes of 140 mM PB, pH 6.8, and 0.4% SDS. The fraction containing the largest amount of DNA was then tested for contaminating double-stranded DNA by annealing a small aliquot of the DNA under the conditions described below for RNA-DNA hybridization. If greater than 95% of the ^{32}P-labeled DNA failed to self-anneal, the remaining DNA in that fraction was used for RNA-DNA hybridization. In each case it was shown that greater than 90% of the ^{32}P-labeled single-stranded DNA from each fragment hybridized to a 1,000-fold excess of unlabeled Ad2 DNA under the conditions described below.

RNA-DNA hybridization. The hybridization of RNA to separated strands of ^{32}P-labeled restriction endonuclease fragments of the Ad2 genome was carried out at 68 C for 24 h in a 0.15-ml reaction mixture containing 1 M NaCl, 0.4% SDS, and 100 mM PB, pH 6.8. Increasing amounts of RNA from 3 to 750 μg/ml were added to 250 to 400 counts/min (depending on the strand employed) of ^{32}P-labeled DNA strands, where specific activity ranged from 0.6×10^6 to 1.5×10^6 counts/min per μg in the three different DNA preparations. After hybridization, the samples were diluted to 1 ml with 140 mM PB, pH 6.8, plus 0.4% SDS and analyzed by chromatography on hydroxlapatite as described by Sambrook et al. (32).

RESULTS

In vivo protein synthesis and virus production. To try to define the block to adenovirus multiplication in monkey cells, we first examined synthesis of proteins in vivo. The nonpermissive CV_1 cells infected with Ad2 or Ad2 plus SV40 were labeled with [^{35}S]methionine and compared with similarly infected HeLa cells which are permissive (see above). The labeled proteins were analyzed by electrophoresis on SDS-polyacrylamide gels followed by autoradiography (1; Fig. 2, Table 1). Under the conditions of these experiments the synthesis of host proteins is only partially reduced. Above this background of host proteins several Ad2-coded proteins can be seen. Ad2 protein synthesis in enhanced monkey cells was similar to that found in Ad2-infected HeLa cells. However, the synthesis of several of these Ad2 proteins were greatly reduced in unenhanced monkey cells compared to enhanced monkey cells, as had been previously reported (4, 10, 14, 18, 21, 24). However, not all late Ad2 proteins were synthesized in reduced amounts (Table 1). For example, proteins designated 100K and IVa_2 were made in approximately equal amounts in both enhanced and unenhanced cells. In contrast, synthesis of IV (fiber) and 11.5K was undetectable in unenhanced cells. The remaining known proteins fell into a continuum between these two extremes.

The observed results may, however, depend on the time after infection at which the cells were labeled with [^{35}S]methionine. To test the possibility that Ad2-specific RNA and/or protein metabolism in unenhanced monkey cells lags behind similar metabolism in enhanced cells, both types of cells were labeled in vivo with [^{35}S]methionine for 2 h at 6-h intervals from 22 to 52 h pi. Their proteins were analyzed as before on SDS-polyacrylamide gels (Fig. 3). Clearly, the differences seen at 28 h pi between enhanced and unenhanced cells in pattern of protein synthesis increased rather than decreased with time after infection.

Production of Ad2 in these experiments was similar in human and enhanced monkey cells; approximately 300 PFU of Ad2 per cell were produced. On the other hand, adenovirus production in unenhanced monkey cells was 600- to 1,500-fold lower (Table 2).

In vitro protein synthesis. Does the failure to synthesize some Ad2 proteins reside at the mRNA level, or is it due to a defect in the translation machinery of the monkey cell? This question was answered by testing for active mRNA species by cell-free translation of cytoplasmic RNA that was prepared in parallel with the in vivo labeled protein preparations described above. Translation of these RNAs in the fractionated mammalian cell-free system (33), followed by analysis of the synthesized proteins on SDS-polyacrylamide gels, showed that the synthesis of several late Ad2 proteins was also specifically blocked (Fig. 2, Table 1). In fact, the pattern of reduction of protein synthesis in vitro with RNA from unenhanced versus enhanced monkey cells was strikingly similar to that found in vivo. For instance, again the synthesis of IV and 11.5K was undetectable, whereas the synthesis of 100K and IVa_2 was similar from RNA prepared from unen-

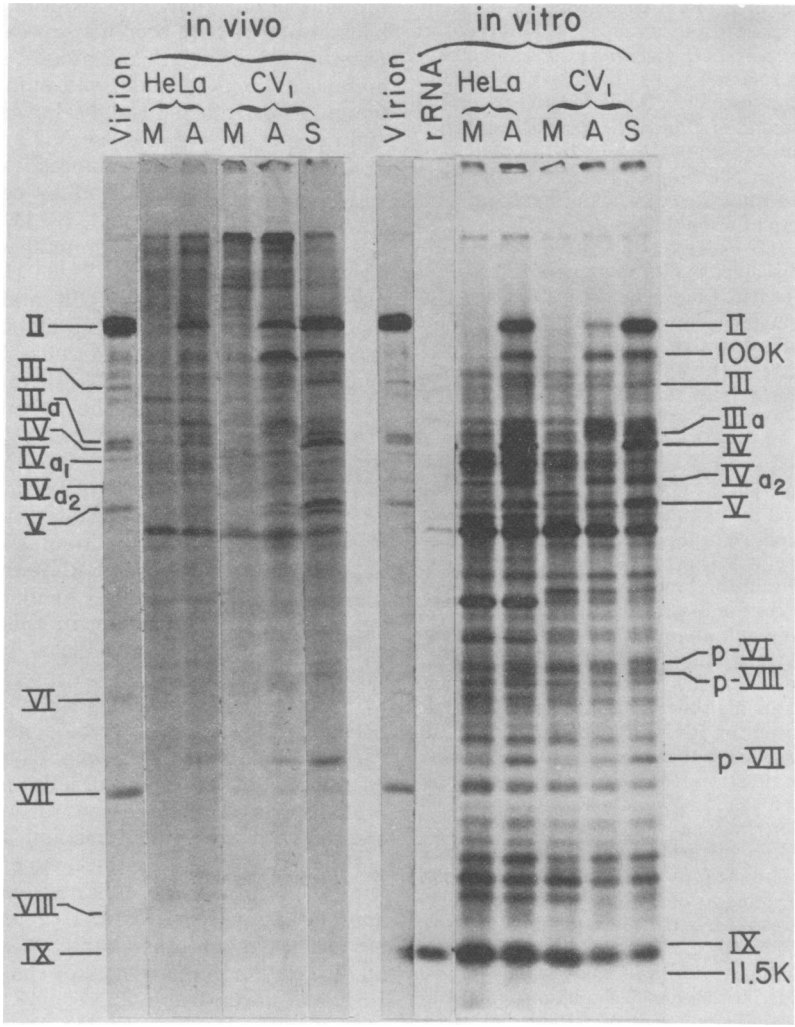

FIG. 2. *Autoradiogram of in vivo and in vitro synthesized proteins from infected human and monkey cells electrophoresed on a 17.5% SDS-polyacrylamide gel. Cells infected with Ad2 (A) or Ad2 plus SV40 (S) or mock-infected (M) were labeled with [^{35}S]methionine or harvested for extraction of cytoplasmic RNA 30 h pi. The extracted RNA was translated in the fractionated mammalian cell-free system (see text). Equal amounts of cell extract (equivalent to 1/100 of a 90-mm plate) or in vitro extract (equivalent to 3 μl of a 50-μl reaction mixture) were prepared as described in the text and applied to the sample wells. The in vivo tracks shown come from a 2- or 4-day exposure, whereas the in vitro tracks all come from an 8-h exposure.*

hanced versus enhanced cells. Cell-free translation of RNA, prepared in parallel with labeled proteins in the time course experiment discussed above, showed that the amount of active Ad2 mRNA in unenhanced cells in comparison with enhanced cells decreased with time after infection (Fig. 4), just as was seen for the synthesis of Ad2 proteins in vivo (Fig. 3). In vitro synthesis of Ad2 proteins was similar for Ad2-infected HeLa cells and for CV_1 cells infected with Ad2 plus SV40 (Fig. 2). Thus, the synthesis of Ad2 proteins in vitro from RNA prepared from CV_1 cells infected with Ad2 or Ad2 plus SV40 mimicks that seen in vivo.

Complexity and concentration of Ad2 RNA in the cytoplasm of unenhanced versus enhanced monkey cells. The results described above suggested that the defective synthesis of several late Ad2 proteins is reflected in the amount of translatable mRNA species. Perhaps the mRNA species encoding those proteins, synthesized in very reduced amounts in unenhanced cells, are absent, or present in much lower concentrations, in these cells than in en-

TABLE 1. *Relative level of synthesis of Ad2 proteins in unenhanced versus enhanced cells*[a]

In vivo[b]	In vitro[c]
Group A (little or no reduction)	
100K	100K
IVa_2	IVa_2
Group B (moderate reduction [~2–7-fold])	
II	II
V	V
P-VII	P-VII
III, IX	Background interferes with analysis
Group C (not detected [>5.5-fold])	
IV	IV
11.5K	11.5K

[a] Enhanced and unenhanced CV_1 cells were labeled with [^{35}S]methionine or harvested for RNA extraction at 30 h pi. The RNA was used to program a fractionated mammalian cell-free translation system. Labeled proteins synthesized in vivo and in vitro were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography (see text). The autoradiograms were scanned with a Joyce Loebl densitometer and the areas under peaks corresponding to Ad2 proteins were integrated. The numerical values refer to the ratio of synthesis in the enhanced versus unenhanced infections. These values were determined from the ratios of the areas of corresponding peaks from appropriate gel tracks after subtracting the areas from corresponding regions from mock-infected cells. Differences in loading of the gel tracks were corrected for by assuming that the rate of 100K and IVa_2 synthesis in the enhanced and unenhanced infections was the same and then normalizing the data so that a value of 1.0 was obtained for the average of the ratios of these two components. Owing to difficulties in analyzing the complex protein patterns obtained and in evaluating the degree of host protein shut-off, the values obtained are only rough quantitations of the differences observed and probably underestimate such differences.

[b] Four of the six independent experiments were quantitated by densitometry.

[c] Two of the eight independent experiments using different preparations of RNA were quantitated by densitometry.

hanced cells. This possibility was tested by hybridization experiments. Several groups (5, 12, 13, 21, 24) have already reported that adenovirus RNA complexity in unenhanced cells versus enhanced cells was similar. However, the most sensitive method employed in those studies was competition hybridization, using the entire adenovirus genome. With this method, it would be difficult to detect either the absence of a few adenovirus RNA species or a moderate reduction in the amount of some adenovirus-specific RNA in unenhanced versus enhanced cells. Consequently a study was undertaken of the complexity and concentration of Ad2 RNA from enhanced versus unenhanced cells, using fragments from specific parts of the genome. By hybridization of increasing amounts of RNA to separated strands of ^{32}P-labeled Ad2 DNA restriction fragments, the complexity and concentration of RNA complementary to each strand of each fragment was determined (34; see above). The complexity of the RNA or the portion of the DNA probe being transcribed is given by the percentage of ^{32}P-labeled DNA in hybrids at the saturation or plateau level.

The results of the hybridization of cytoplasmic RNA to the DNA probes (Fig. 5 and 6, Table 3) suggest that all species present in the enhanced cells were also present in the unenhanced cells. The plateau values given in Table 3 are taken from one experiment. The hybridization analysis was done with two to four different pairs of RNA preparations from enhanced and unenhanced cells and three separate preparations of ^{32}P-labeled DNA. For all preparations, those RNAs found in enhanced cells were also present in unenhanced cells, with the following exception: the plateau level for EcoRI-Bl was 20% lower in one out of four preparations of RNA from unenhanced cells, compared to enhanced cells. At present we have no explanation for this descrepancy. The plateau level of some strands of restriction fragments (EcoRI-Bl and EcoRI-El) varied significantly from one experiment to another; however, within a given experiment the plateau level with RNA from enhanced and unenhanced cells was the same. Thus, the significance of the differences in Ad2 transcription pattern between monkey and human cells shown in Table 3 is questionable, since the range of plateau values found in different experiments for CV_1 cells encompassed the values found by Sharp et al. (34) and Philipson et al. (29) for human cells.

An estimate of the relative concentrations of the adenovirus RNA can be determined from the amount of RNA required to reach 50% of the plateau level. The results from such an analysis of the data shown in Fig. 5 and 6 are summarized in Table 4. Clearly, there was a considerable range of differences between enhanced and unenhanced cells in the concentration of cytoplasmic RNA complementary to a given DNA probe. For some probes, such as EcoRI-Bh, EcoRI-Ch, BamHI-Bh, and BamHI-Bl, little or no difference in the concentration of comple-

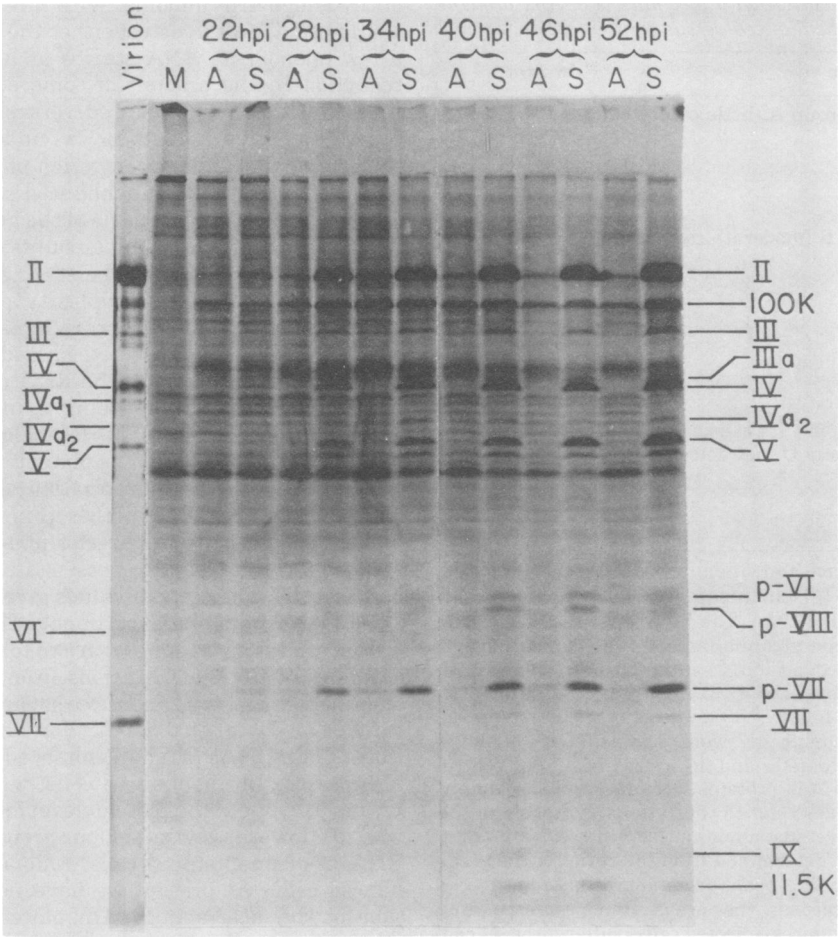

FIG. 3. *Autoradiogram of a 17.5% SDS-polyacrylamide gel displaying proteins synthesized in Ad2 (A) or Ad2 plus SV40 (S) infected CV_1 cells at 6-h intervals from 22 to 52 h pi. Cells were labeled with [^{35}S]methionine for 2 h. Proteins from mock-infected CV_1 cells (M) labeled at 30 h pi are shown for comparison.*

TABLE 2. *Ad2 production in human cells and monkey cells*[a]

Cell	Virus	Production of PFU of Ad2/cell
HeLa[b]		$<2 \times 10^{-6}$
HeLa[b]	Ad2	3×10^{2}
CV_1[b]		$<2 \times 10^{-6}$
CV_1[b]	Ad2	2×10^{-1}
CV_1[b]	Ad2 + SV40	3×10^{2}
CV_1[c]	Ad2	8×10^{-1}
CV_1[c]	Ad2 + SV40	5×10^{2}

[a] Cells were harvested at the times stated below and opened by freeze-thawing and sonic treatment. Adenovirus was titered on HeLa cells as previously described (18). SV40 does not plaque on HeLa cells.

[b] Titer of infected cell extracts harvested at 60 h pi from the experiments shown in Fig. 2.

[c] Average titer of infected cell extracts harvested at 74 and 96 h pi from the experiment shown in Fig. 3 and 4.

mentary RNA was observed. In contrast, the concentration of cytoplasmic RNAs complementary to probes EcoRI-Cl, EcoRI-El, and BamHI-Cl was 7- to 8.5-fold lower in unenhanced cells than in enhanced cells. This is easily visualized in Fig. 7, which is an enlargement of the graph for EcoRI-El shown in Fig. 5.

The results from cell-free translation of RNA extracted at 6-h intervals from 22 to 52 h pi suggested that the levels and/or translatability of certain Ad2 mRNA's in unenhanced cells by comparison with enhanced cells persisted late in infection. The persistence of lower levels of specific mRNA's in unenhanced cells was confirmed by hybridization of RNA, from the same preparation used for cell-free translation, to EcoRI-Bh, EcoRI-El, and BamHI-Cl (Fig. 8, 9, 10). Essentially no difference between enhanced and unenhanced cells in the amount of cytoplasmic RNA complementary to EcoRI-Bh

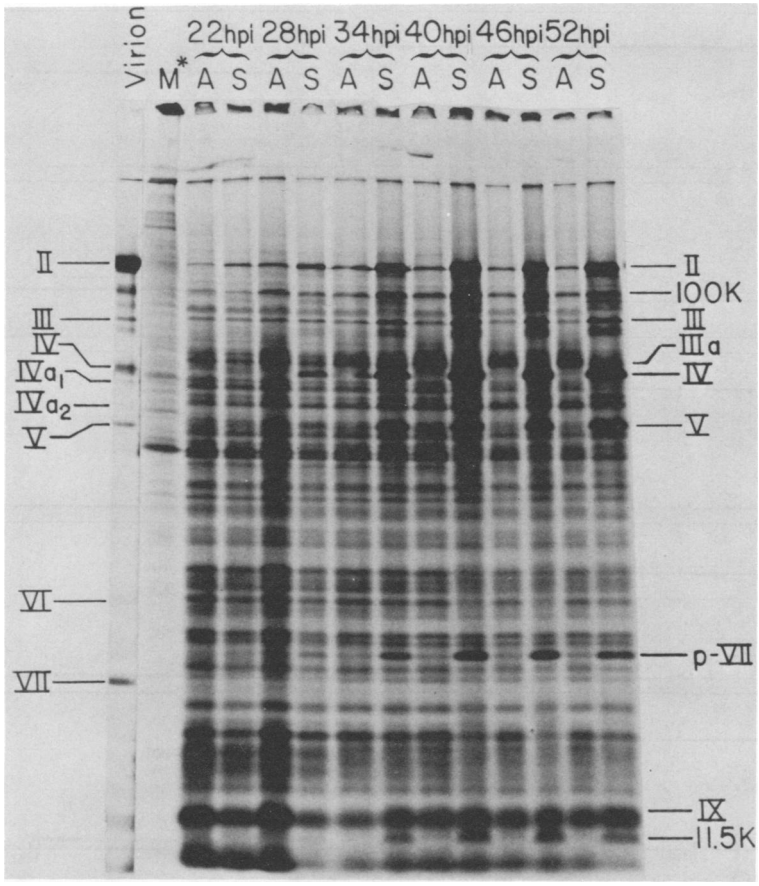

FIG. 4. *Autoradiogram of a 17.5% SDS-polyacrylamide gel displaying proteins synthesized in the fractionated mammalian cell-free system programmed by RNA extracted from Ad2 (A) or Ad2 plus SV40 (S) infected CV_1 cells at 6-h intervals from 22 to 52 h pi. Proteins from mock-infected CV_1 cells (M) labeled at 30 h pi are shown for comparison.*

was seen, either at 30 h pi in previous experiments or at 22 to 52 h pi in this experiment. In earlier experiments, at 30 h pi a difference of 6- to 16-fold was found between enhanced and unenhanced cells for RNA complementary to EcoRI-El. In this experiment, a 9- to 23-fold difference was observed for RNAs extracted at various times after infection. Similarly, the difference in amount of complementary RNA to probe BamHI-Cl at 30 h pi in previous experiments was six- to eightfold, whereas the difference observed for the various time points in this experiment ranged from 6- to 10-fold. Furthermore, a comparison of the concentration of RNA extracted from enhanced cells at a relatively early stage in infection (28 h pi) with RNA extracted from unenhanced cells late in infection (52 h pi) also showed a large excess (sixfold) of RNA complementary to EcoRI-El and BamHI-Cl in enhanced cells. Thus, all three parameters measured (in vivo protein synthesis, activity of mRNAs in a cell-free translation system, and concentration of complementary RNA) suggest that RNA and protein metabolism in Ad2-infected monkey cells does not simply lag behind similar metabolism in cells infected with Ad2 plus SV40.

Complexity and concentration of Ad2 RNA in the nuclei of unenhanced versus enhanced monkey cells. To test whether the differences in relative complexity and concentration of Ad2 cytoplasmic RNA were reflected in the nucleus, a similar hybridization analysis was performed with nuclear RNA. The results show that although all RNA species found in the nuclei of enhanced cells were also present in the nuclei of unenhanced cells, several species were missing from the nuclei of enhanced cells (Fig. 11 and 12, Table 3). This is evident from the plateau levels for EcoRI-Bh and EcoRI-Ch (Fig. 11, Table 3). The nuclear RNA complementary to these two probes from enhanced cells

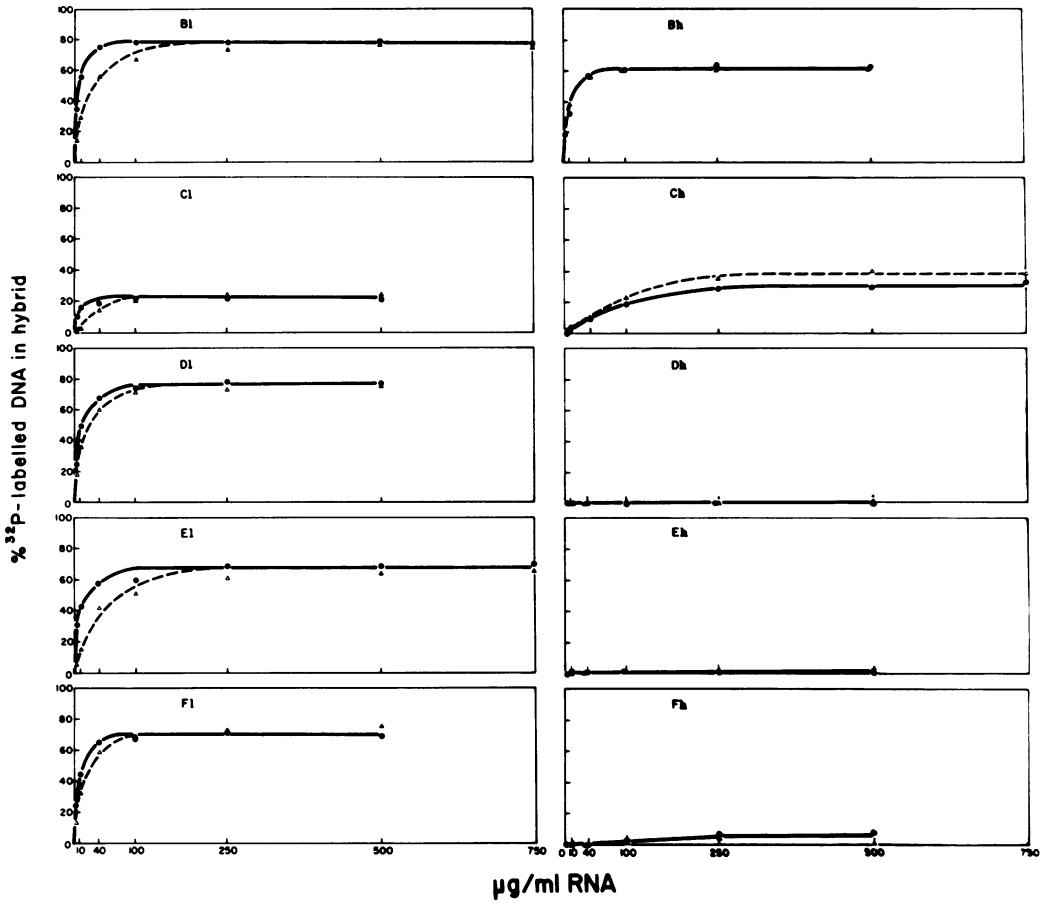

FIG. 5. *Hybridization of cytoplasmic RNA extracted from enhanced and unenhanced cells to separated strands of EcoRI fragments of the Ad2 genome. CV_1 cells infected with either Ad2 or Ad2 plus SV40 were harvested at 30 h pi. The cytoplasmic RNA was purified and hybridized to separated strands of ^{32}P-labeled Ad2 DNA fragments generated by EcoRI (see text). The fraction of the DNA entering hybrids was determined by hydroxylapatite chromatography (31). Graphs of the percentage of DNA in hybrids versus concentration of RNA from unenhanced cells (△) or from enhanced cells (●) are shown for separated strands of five of the six EcoRI fragments. The percentage of DNA in hybrids for each concentration of RNA added was determined by dividing the counts per 20 min in the 0.4 M PB elutant by the counts per 20 min in the 0.14 M PB plus 0.4 M PB elutants, and by subtracting the percentage of DNA in 0.4 M PB elutant with no added RNA. No correction for hybridizability of the probes was made since greater than 90% of the probes hybridized to a 1,000-fold excess of Ad2 DNA.*

was approximately 900 nucleotides lower in complexity than that from unenhanced cells.

The difference in concentration of Ad2 nuclear RNA between enhanced and unenhanced cells was in several cases less pronounced and in other cases about the same as that found for cytoplasmic RNA (Fig. 11 and 12, Table 4). There appears to be a lower concentration of RNA complementary to probes EcoRI-Bh and EcoRI-Ch in the nucleus of enhanced cells, compared to unenhanced cells. However, since there were fewer RNA species complementary to these two probes in the nuclei of enhanced versus unenhanced cells, it is difficult to determine whether there is a difference in the concentration of those RNA species common to both.

DISCUSSION

Protein synthesis. The results presented above confirm previous reports (4, 10, 14, 18, 21, 24) concerning the reduction of synthesis of several late proteins in unenhanced cells compared to enhanced cells. In addition, we have shown that the synthesis of several late Ad2 proteins in a cell-free translation system programmed

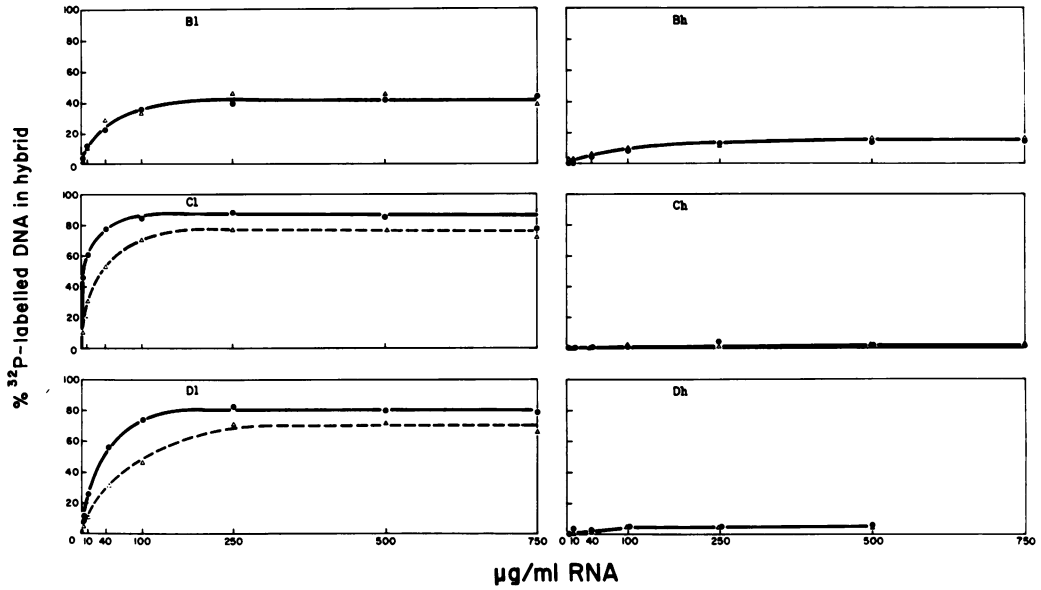

FIG. 6. *Hybridization of cytoplasmic RNA extracted from enhanced and unenhanced cells to separated strands of BamHI fragments of the Ad2 genome. The RNA and DNA preparations used in this experiment were the same as those described in Fig. 5. The fraction of ^{32}P-labeled DNA entering hybrids after annealing to RNA from unenhanced cells (△) or from enhanced cells (●) is shown for separated strands of three of the four Ad2 fragments generated by BamHI.*

TABLE 3. *Percentage of restriction endonuclease fragments of Ad2 genome complementary to cytoplasmic RNA and nuclear RNA*[a]

^{32}P-labeled DNA probe	^{32}P-labeled DNA (%) in hybrid (plateau level) at saturating concn of cytoplasmic RNA from:				^{32}P-labeled DNA (%) in hybrid (plateau level) at saturating concn of nuclear RNA from:			
	Unenhanced CV_1	Enhanced CV_1	KB[b]	HeLa[c]	Unenhanced CV_1	Enhanced CV_1	KB[b]	HeLa[c]
EcoRI-								
Bh	60	60	35	40	50	25	40	15
Bl	80	80	65	52	90	90	95	88
Ch	40	35	50	50	50	25	30	19
Cl	25	25	25	31	85	85	90	87
Dh	<10	<10	0	5	<10	<10	10	3
Dl	80	80	100	82	80	80	100	91
Eh	<10	<10	10	12	<10	<10	10	3
El	70	70	85	82	70	70	80	88
Fh	<10	<10	0	11	<10	<10	15	7
Fl	70	70	95	84	70	70	100	87
BamHI-								
Bh	20	20			20	15		
Bl	45	45			65	65		
Ch	<10	<10			<10	<10		
Cl	80	90			90	90		
Dh	<10	<10			<10	<10		
Dl	70	80			90	90		

[a] RNA extracted from unenhanced and enhanced CV_1 cells 30 h pi was hybridized to separated strands of ^{32}P-labeled restriction endonuclease fragments of Ad2 genome. The fractions of ^{32}P-labeled DNA entering hybrids were determined by chromatography on hydroxylapatite (see text). The plateau levels or values were taken from data shown in Fig. 5, 6, 11, and 12.

[b] The plateau values shown are taken from Sharp et al. (34).

[c] The plateau values shown are taken from Philipson et al. (29).

TABLE 4. *Relative concentration of complementary RNA to Ad2 DNA fragments from unenhanced versus enhanced cells*[a]

^{32}P-labeled DNA probe	Concn (ratio) of cytoplasmic RNA from unenhanced cells to enhanced cells required to reach 50% of the plateau level	Concn (ratio) of nuclear RNA from unenhanced cells to enhanced cells required to reach 50% of the plateau level
EcoRI-		
Bh	1.0	0.3
Bl	4.3	3.2
Ch	0.8	0.3
Cl	7.0	6.9
Dh	S	S
Dl	2.0	3.0
Eh	S	S
El	8.5	3.4
Fh	S	S
Fl	2.0	1.1
BamHI-		
Bh	1.0	0.3
Bl	1.0	1.2
Ch	S	S
Cl	7.9	2.9
Dh	S	S
Dl	3.0	2.8

[a] Preparation of the DNA and RNA and their subsequent hybridization are described in the text and the footnote to Table 3. The ratios were determined from the data shown in Fig. 5, 6, 11, and 12. S denotes our inability to measure the ratios due to the very small amount of RNA complementary to these probes.

with RNA extracted from unenhanced cells is also reduced compared to similar synthesis using RNA from enhanced cells. The different late Ad2 proteins varied in the amount of reduction of their synthesis in unenhanced cells. For example, there was little or no reduction in the synthesis of 100K and IVa_2 for the unenhanced infection, whereas synthesis of IV (fiber) and 11.5K was undectable in unenhanced cells. The synthesis of other late Ad2 proteins fell into a continuum between these extremes; reduction in vitro was similar to that found in vivo.

We have no explanation for the variation in the extent to which different late genes are expressed in the unenhanced infection of monkey cells by Ad2. Baum et al. (4) had shown that major virion components were only weakly expressed (if at all) in unenhanced cells, whereas Grodzicker et al. (18) found that most late Ad2 proteins, with the exceptions of IV, P-VI, P-VII, and P-VIII, were synthesized in nearly normal amounts in unenhanced cells. Our results appear to be intermediate in comparison with these two reports. Such differences may be a reflection of the different cell lines, virus stocks, etc., used by the various investigators. For instance, we have observed little or no synthesis of most of the late Ad2 proteins in one subline of BSC-1 cells singly infected with Ad2, whereas protein synthesis in the other two sublines tested was very similar to that found for CV_1 cells (see below).

In contrast to our findings, Eron et al. (10) recently reported that synthesis of the late Ad2 proteins in a Krebs II ascites S30 cell-free system using RNA extracted from unenhanced and enhanced BSC-1 cells was similar even though in vivo synthesis of these proteins in unenhanced BSC-1 cells was reduced. The discrepancy between our results and those of Eron et al. (10) is not due to differences in the method of RNA preparation, type of cell-free translation system, or level of serum after infection (unpublished data). H. Westphal and G. Khoury kindly provided us with the RNA used in their experiments, and we obtained a result similar to theirs when translating the RNA in our fractionated mammalian cell-free system or Krebs II ascites S30; i.e., synthesis of most late Ad2 proteins was similar, whereas fiber production was slightly reduced with RNA from unenhanced cells compared to that from enhanced cells. However, when we prepared cytoplasmic RNAs from their subline of BSC-1, 31

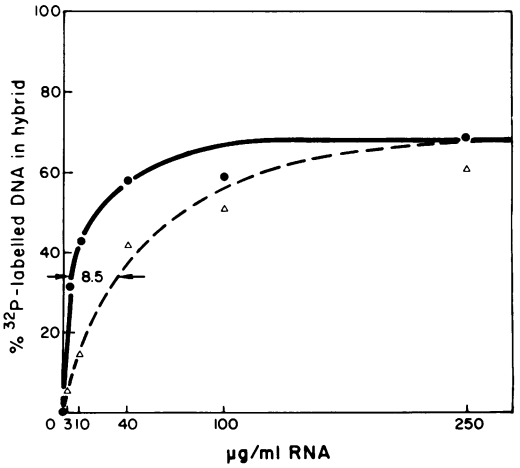

FIG. 7. *Hybridization of cytoplasmic RNA from enhanced and unenhanced cells to EcoRI-El. This figure is an enlargement of the graph taken from Fig. 5 of RNA hybridizing to the l-strand of EcoRI-E. The arrows denote the positions on the curves used to determine the ratio of the RNA concentration from unenhanced cells (△) compared to that from enhanced cells (●) required to reach 50% of the final plateau level. The number between these arrows indicates the value of this ratio.*

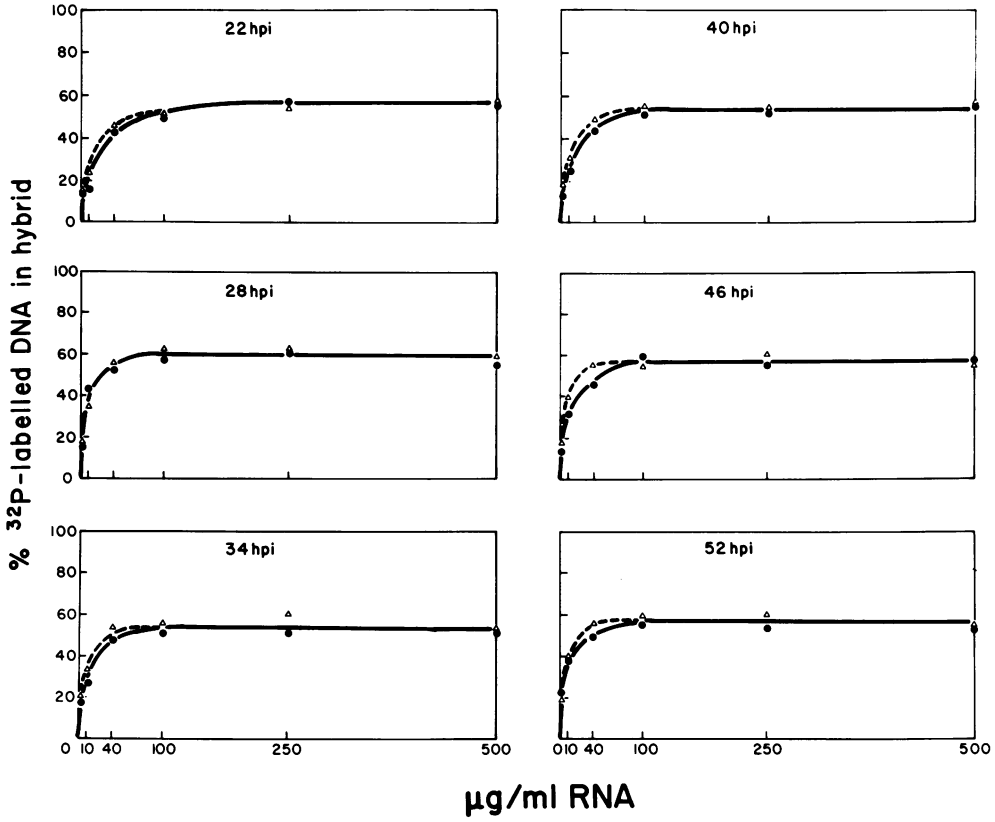

FIG. 8. *Hybridization of EcoRI-Bh of cytoplasmic RNA extracted from enhanced and unenhanced cells at various times after infection. Cytoplasmic RNA was extracted from Ad2-infected (△) or Ad2 plus SV40-infected (●) CV_1 cells at 6-h intervals from 22 to 52 h pi. The RNA was purified and hybridized to the h-strand of EcoRI-B (see text).*

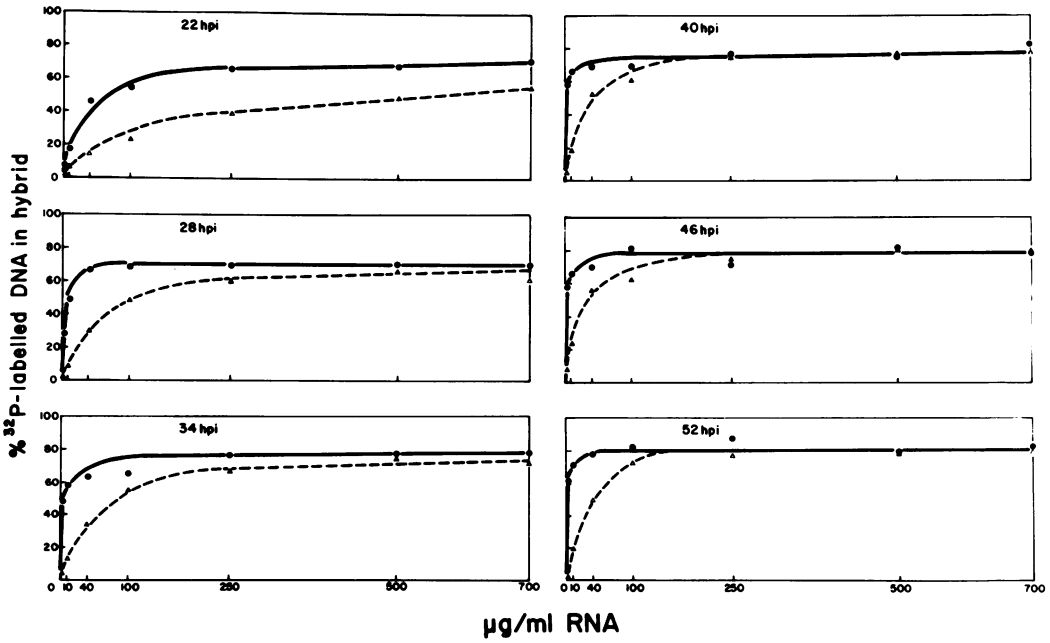

FIG. 9. *Hybridization to EcoRI-El of cytoplasmic RNA extracted from enhanced and unenhanced cells at various times after infection. Cytoplasmic RNA was extracted from Ad2-infected (△) or Ad2 plus SV40-infected (●) CV_1 cells at 6-h intervals from 22 to 52 h pi. The RNA was purified and hybridized to the l-strand of EcoRI-E (see text).*

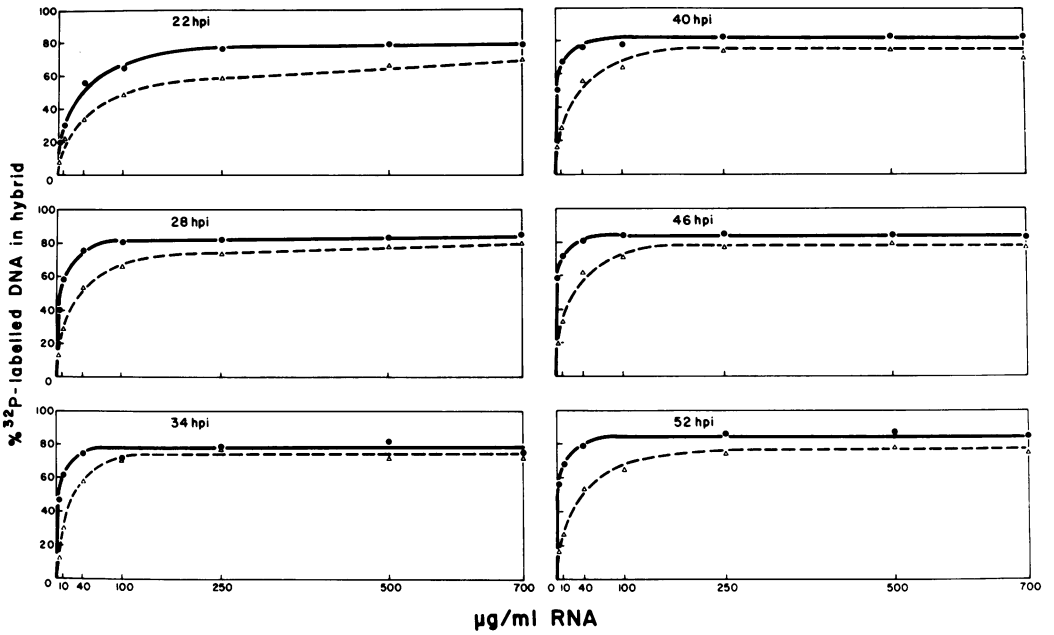

FIG. 10. *Hybridization to BamHI-Cl of cytoplasmic RNA extracted from enhanced and unenhanced cells at various times after infection. Cytoplasmic RNA was extracted from Ad2-infected (△) or Ad2 plus SV40-infected (●) CV_1 cells at 6-h intervals from 22 to 52 h pi. The RNA was purified and hybridized to the l-strand of BamHI-C (see text).*

to 33 h pi, as well as from two other sublines of BSC-1 obtained from R. Pollack, and used these RNAs to program the fractionated mammalian cell-free system, results similar to those reported in this paper for CV_1 cells were obtained, i.e., little or no synthesis of fiber and reduced synthesis of several other late Ad2 proteins. At present there is no obvious explanation for the discrepancy between their results and our results.

Our results suggest that the enhancement of Ad2 protein synthesis in monkey cells is not due to a factor operating at the level of translation. If SV40 were supplying or inducing a positive factor (either a protein or RNA) which allowed the translation of Ad2 RNA in monkey cells, then we would have expected normal translation in vitro since our cell-free system translates all known Ad2 mRNA's without the requirement for a specific factor. This has been shown by the efficient translation of oligo(dT)- or hybridization-selected Ad2 mRNA (16). A negative control factor which prevents the synthesis of Ad2 mRNA in monkey cells has been ruled out by the following mixing experiments. RNA from unenhanced infections was mixed with RNA from enhanced infections in ratios from 1 to 10 and 10 to 1, and no reduction in the synthesis of specific Ad2 proteins was found in comparison to a control experiment using KB cell cytoplasmic RNA.

RNA complexity and concentration. Our in vitro translation results suggest that the defect in protein synthesis may be attributable to reduced levels of certain Ad2 mRNA's in unenhanced cells, which may result from improper synthesis of, processing of, or modification of specific mRNA's. This possibility is supported by our analysis of the presence of Ad2 RNA sequences and their levels in enhanced and unenhanced cells.

For our analysis we employed a technique, developed by Sharp et al. (34), which uses separated strands of restriction endonuclease fragments of the Ad2 genome as probes in RNA-DNA hybridization. With the 16 probes used on our experiment, this method was at least several-fold more sensitive with respect to determining the complexity of Ad2 RNA in unenhanced versus enhanced cells than the previously employed method of competition hybridization. In most cases this technique would have detected the absence of mRNA for individual Ad2 proteins. In addition, it provides data regarding Ad2 RNA concentration not obtainable by other techniques. The sensitivity of the method we employed to detect missing species depends on the reproducibility with which the

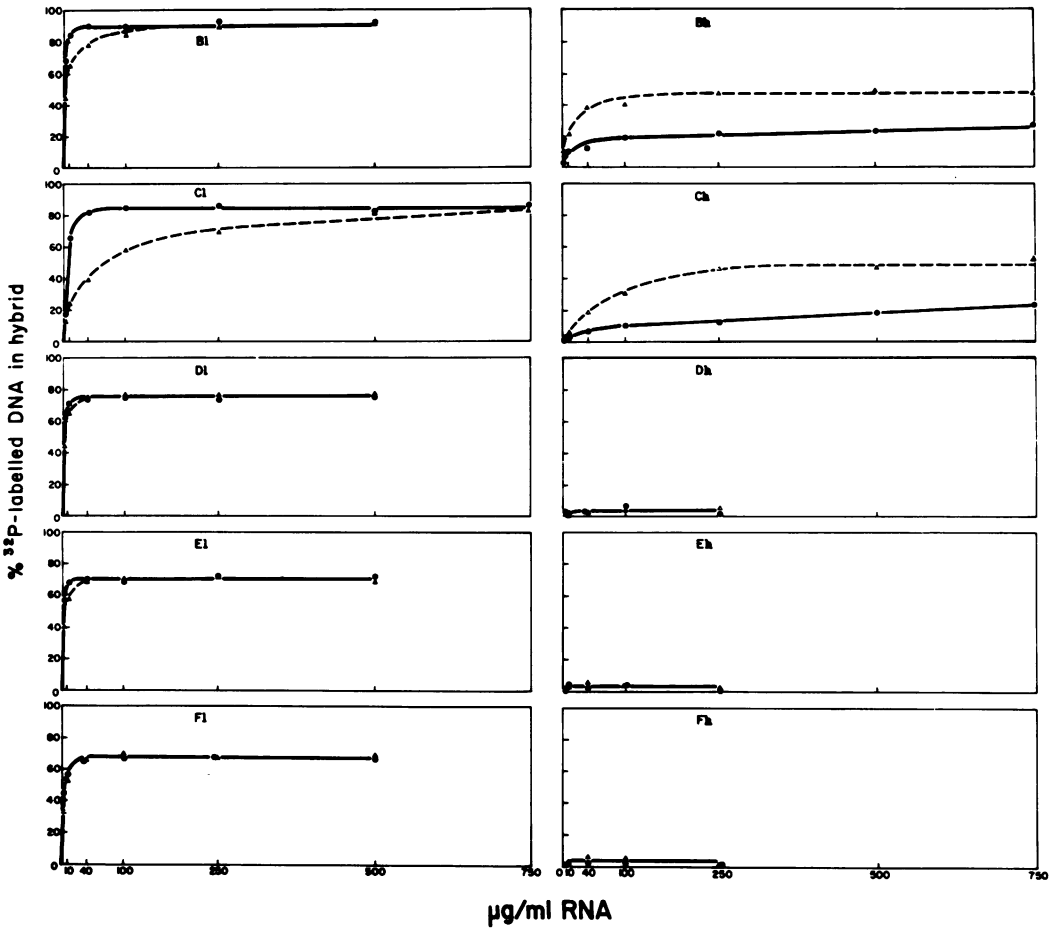

FIG. 11. *Hybridization of nuclear RNA from enhanced and unenhanced cells to separated strands of EcoRI fragments of the Ad2 genome. The nuclear RNA was from the same cell preparations as described in Fig. 5 (see text). Hybridization was to separated strands of ^{32}P-labeled Ad2 DNA fragments described in Fig. 5. The fraction of DNA entering hybrids after annealing to RNA from unenhanced cells (△) or from enhanced cells (●) is shown for separated strands of five of the six EcoRI fragments.*

plateau levels can be determined. We estimate that the plateau level is reproducible to within ±10% for a given preparation of DNA and RNA. This means that in the worst case (i.e., BamHI-B, which represents approximately 30% of the Ad2 genome) a difference in complexity of complementary Ad2 RNA sufficient to encode a 30,000- to 35,000-dalton protein could go undetected. Obviously, the analysis is better with smaller probes, since the probability that an alteration in the transcriptional pattern would occur entirely within the region covered by the probe increases with decreasing probe size.

Within the limits of this technique, our results show that there was little or no difference in the total complexity of cytoplasmic adenovirus RNA between enhanced and unenhanced cells, with the following possible exceptions. The RNAs complementary to BamHI-Cl and BamHI-Dl showed differences in complexity between enhanced and unenhanced cells sufficient to code for a 10,000- to 15,000-dalton protein (see Fig. 6); but these differences were near the limits of resolution of this technique and were not observed with a second preparation of RNA. When nuclear RNA was examined, similar results were obtained with most probes. However, the complexities of nuclear RNAs complementary to probes EcoRI-Bh and EcoRI-Ch were approximately twofold less in enhanced than in unenhanced cells. The reason for this difference is not entirely clear. From work by Sharp et al. (34) it is known that early RNAs are transcribed from EcoRI-Bh and EcoRI-Ch. Thus, it is possible that the differ-

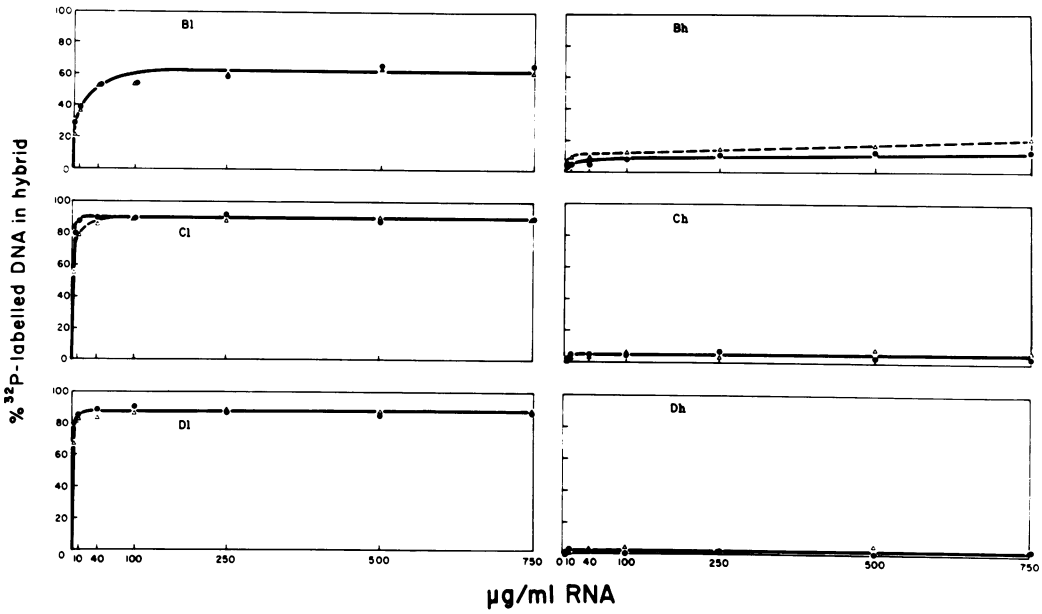

FIG. 12. *Hybridization of nuclear RNA from enhanced and unenhanced cells to separated strands of BamHI fragments of the Ad2 genome. The RNA and DNA preparation used in this experiment were the same as those described in Fig. 11. The fraction of DNA entering hybrids after annealing to RNA from unenhanced cells (△) or from enhanced cells (●) is shown for separated strands of three of the four BamHI fragments.*

ence in complexity of RNA in the nucleus may be due to turn-off of transcription of some early regions in the enhanced but not in the unenhanced cells. In fact, it appears from our data shown in Fig. 3 and 4 that there was more synthesis of an early protein (72K) encoded by fragment EcoRI-B (J. B. Lewis, J. F. Atkins, P. R. Baum, R. F. Gesteland, and C. W. Anderson, submitted for publication) in unenhanced cells than in enhanced cells at late times. In Ad2-infected human cells, Craig et al. (8) have observed that these early RNAs encoded by EcoRI-B and EcoRI-C are turned off late, and Sharp et al. (34) have found that early RNAs disappear from the cytoplasm late (30 h) after infection. The absence of a difference in the plateau levels for cytoplasmic RNA may be due to the stability of early transcripts in the cytoplasm but not the nucleus. On the other hand, Philipson et al. (29) and Sharp et al. (34) reported that the Ad2 genome may be symmetrically transcribed in some regions. If a difference in the levels of symmetrical RNA occurred between enhanced and unenhanced cells, this RNA could selectively remove some early nuclear transcripts by RNA:RNA hybridization, giving the impression of a difference in complexity. Such differences might not be found for cytoplasmic RNA because of the lability of the symmetric transcript or its failure to be transported to the cytoplasm.

Considerable variation in plateau levels between experiments using different preparations of DNA and/or RNA was observed for several probes (EcoRI-B1 and EcoRI-E1). There may be several reasons for such variation. Some variability may be due to differences in the average size of the DNA, since single-stranded DNA tails were not removed from hybrids before chromatography on hydroxylapatite and since size affects the rate of hybridization. An analysis of the plateau levels for probes which represent a small percentage of the genome and which have low plateau levels (e.g., EcoRI-C1) indicates that the average size of the DNA fragments is less than 1% of the genome (600 to 700 nucleotides). Biological variables such as multiplicity of infection and the state of the cells may also contribute to the different plateau levels observed in different experiments. The same virus stock was used throughout these experiments to minimize the variation in PFU-to-particle ratio, and an effort was made to keep the PFU-to-cell ratios constant. Other factors such as serum, cell confluency, and temperature may also influence the multiplication of adenovirus in monkey cells.

The similarities in complexity of cytoplasmic Ad2-specific RNA between enhanced and unenhanced cells suggest that the differences

in protein synthesis both in vivo and in vitro cannot be explained by the total absence of specific messages. Comparison of the RNA concentration from the cytoplasm of unenhanced cells to that from enhanced cells required to reach 50% of the plateau level indicates a considerable range of differences in the amount of particular Ad2 RNA species between enhanced and unenhanced cells. Differences in concentration of some RNA species as large as 23-fold have been found between enhanced and unenhanced cells, whereas little or no difference in concentration of other species was detected. These differences are minimum estimates of the ratio of the concentration of specific RNA species between enhanced and unenhanced cells, since the DNA probe in many cases encodes more than one RNA species.

The lower concentration of Ad2 RNA in the cytoplasm of unenhanced cells may be due to leakage of Ad2 RNA from the nuclei of enhanced but not unenheanced cells, if the nuclear membrane becomes quite fragile in productive but not in abortive infection. For nuclear leakage to give the observed results, there must be a large excess of a particular RNA in the nucleus by comparison with the cytoplasm. The relative amount of Ad2-specific RNA can be estimated by comparing the concentration of cytoplasmic RNA to nuclear RNA required to reach the plateau level for any given DNA probe, and from the relative amount of total RNA in the nucleus and the cytoplasm. The concentration of Ad2 RNA is on the average fivefold higher in nuclear RNA than in cytoplasmic RNA, but the cytoplasm contains about 2.5-fold more total RNA than the nucleus. Thus, on the average, the nucleus contains about twice as much Ad2 RNA as the cytoplasm. This twofold difference is also true for those RNAs complementary to DNA probes, such as EcoRI-El, which show large differences in concentration of Ad2-specific RNA between the cytoplasms of enhanced and unenhanced cells. Thus, even complete nuclear breakdown in enhanced cells could not account for differences of sevenfold or more.

A similar analysis of the amount of complementary RNA sequences in the nuclei of unenhanced versus enhanced cells shows that the differences in concentration of these RNAs between these two cell types were often less pronounced in the nucleus than in the cytoplasm. This suggests that some control beyond the level of transcription must be occurring in the unenhanced cells. The less pronounced difference for nuclear RNA may be due to a faster rate of degradation of these RNAs in the cytoplasm of unenhanced cells and/or a slower rate of transport of these RNAs across the nuclear membrane of the unenhanced cells.

Protein synthesis versus RNA concentration. If the reduction of synthesis of several of the late Ad2 proteins in unenhanced cells is due to the lower concentration of the coding mRNA in unenhanced cells, then one would expect a correlation between the relative amount of synthesis of particular proteins and the relative concentrations of the coding mRNA in enhanced versus unenhanced cells. Since many of the late Ad2 proteins have been mapped on the Ad2 genome by RNA-DNA hybridization followed by cell-free translation of the hybridized RNA (23; C. W. Anderson, J. F. Atkins, P. R. Baum, R. F. Gesteland, and J. B. Lewis, personal communication), we can make this comparison (Fig. 13), but two main problems arise. First, the coding strands for the proteins have not been determined. However, in many cases we can deduce the sense strand, since RNA hybridized to only one of the strands for five of the eight restriction endonuclease fragments used as probes. For the remaining three restriction fragments (EcoRI-B, EcoRI-C, and BamHI-B) the assignment is more difficult.

Proteins designated 100K and II are probably coded by the light (l) strand of EcoRI-B, for the following reasons. Protein 100K also maps on EcoRI fragments D and F, of which only stable RNAs are transcribed from the l strand. By a similar argument, II must map on the l strand of EcoRI-B, since it also maps on BamHI-C and only the l strand of BamHI-C is transcribed. Again by this type of reasoning, IV (fiber) maps on EcoRI-Cl, for it also maps on EcoRI-E, of which only the l strand is transcribed into stable RNA. We are so far unable to predict which proteins are coded by each strand of BamHI-B.

The second problem is that many of the restriction endonuclease fragments encode more than one protein. This means that in these cases a meaningful analysis can be done only if all the proteins encoded by the particular DNA fragment are similar with respect to the amount of reduction of their synthesis in enhanced versus unenhanced cells. Moreover, since the amount of RNA complementary to several of the DNA fragments is greater than the size of the RNAs known to encode particular proteins that map on these DNA fragments (2), caution must be exercised even when only one known protein is encoded by a DNA fragment.

Given these reservations, there does appear to be a correlation between a reduction in the synthesis of specific proteins and a reduction in the concentrations of the encoding mRNA's in unenhanced versus enhanced cells. The strong-

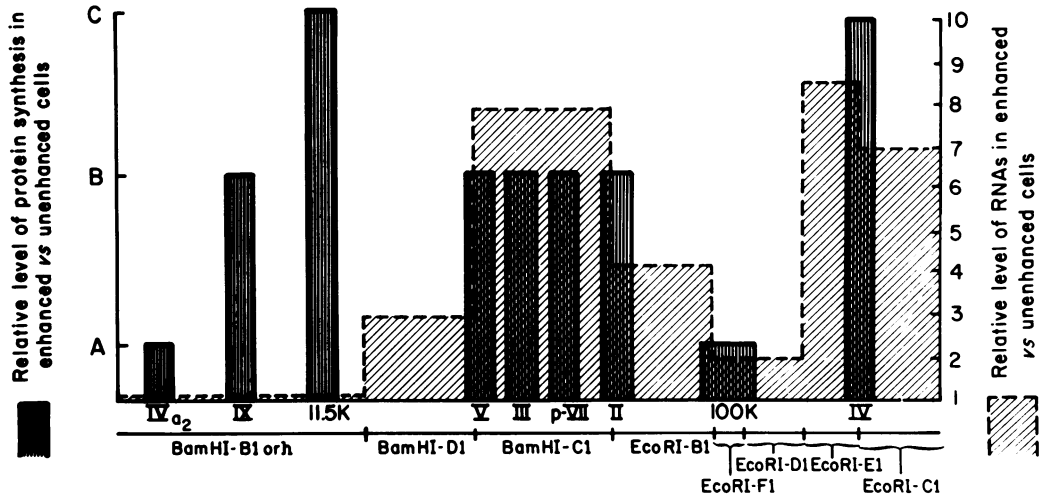

FIG. 13. *Diagrammatic representation of the correlation between levels of synthesis of Ad2 proteins and the concentration of RNA complementary to their encoding restriction endonuclease fragments in unenhanced versus enhanced cells. The extent of expression of each Ad2 protein has been assigned one of three arbitrary values based on information given in Table 1 and is indicated by a solid bar. The ratios of concentrations of RNA complementary to the restriction endonuclease fragments for enhanced versus unenhanced cells represented by dashed boxes is taken from Table 4. The location on the Ad2 genome of the mRNA's encoding the proteins have been determined (23; Anderson et al., personal communication). The order of the proteins whose message lies entirely within a restriction fragment has not been determined, e.g., IVa$_2$, IX, and 11.5 K or III and P-VII. The determination of the sense strand of each fragment is described in the text.*

est correlation is for IV (fiber). Its synthesis was undetectable both in vivo and in vitro. Its mRNA maps on EcoRI-Cl and EcoRI-El, which both show the largest difference (6- to 23-fold) in concentration of complementary RNA between enhanced and unenhanced cells.

A meaningful correlation can be made between protein synthesis and RNA concentration for 100K, since only the mRNA of 100K can be encoded by EcoRI-Fl because it is also partially coded by the two adjacent restriction fragments. Synthesis of 100K in vivo and in vitro is approximately the same in enhanced and unenhanced cells, and there is about a twofold lower concentration of RNA complementary to EcoRI-Fl in unenhanced cells.

The mRNA's for II, III, V, and P-VII all hybridize to BamHI-Cl and account for more than the coding capacity of this fragment. The synthesis of all four of these proteins is moderately reduced, and the concentration of complementary RNA is 6- to 10-fold lower in unenhanced cells than in enhanced cells.

The proteins (IVa$_2$, IX, 11.5K) encoded by BamHI-B show considerable variation in their relative levels of synthesis in enhanced versus unenhanced cells, whereas the concentration of RNA complementary to each strand is approximately the same in these two types of infected cells. This apparent lack of correlation is probably due to our inability to more finely analyze this complex region, which encodes several early proteins (Lewis et al., submitted for publication) as well as these three late proteins.

Thus, in general, there seems to be a reasonable correlation between the relative levels of protein synthesis in vivo and in vitro and the relative concentrations of the RNAs complementary to the restriction endonuclease fragments that encode the proteins. Although it is reasonable to suppose that a reduced level of mRNA's would result in reduced synthesis of the corresponding proteins, no direct evidence for this exists. In fact, it is equally plausible that the lower concentration of complementary RNA is the indirect result rather than the cause of reduced protein synthesis. Furthermore, SV40 may have several effects on Ad2 multiplication in monkey cells; for example, it may affect independently both RNA metabolism and the protein-synthesizing machinery.

Ribosome-associated nuclease hypothesis. The following results should be taken into consideration in the construction of a hypothesis to explain the block to multiplication of adenovirus in monkey cells. (i) All adenovirus RNA species found in enhanced cells are also present in unenhanced cells (5, 12, 13, 21, 24). (ii) Several species of adenovirus RNA present in polysomes from enhanced cells are not found in polysomes from unenhanced cells (13, 21). (iii) A high salt wash of polysomes from en-

hanced, but not from unenhanced, cells enables ribosomal subunits to form 80S initiation complexes for protein synthesis with late adenovirus RNA from unenhanced cells, whereas a high salt wash of polysomes from either enhanced or unenhanced cells allows formation of the 80S complex with poly(U) or SV40 RNA (26). (iv) Synthesis of several late adenovirus proteins in vivo (4, 10, 14, 18, 21, 24) and in vitro is very much reduced or absent for unenhanced versus enhanced cells. (v) This reduction of protein synthesis in unenhanced cells, in general, correlates with a lower concentration of RNA complementary to the encoding DNA probe. A nuclease specific for certain late adenovirus RNAs and associated with the ribosomes from unenhanced cells could account for the above results.

Nakajima et al. (26) reported degradation of late adenovirus RNA when it was incubated with a high salt wash of polysomes from unenhanced but not from enhanced cells. This degradation may be due to the nuclease activity suggested in our hypothesis. It is noteworthy that Bothwell and Altman (6, 7) have recently reported the discovery of a ribosome-associated nuclease which exhibits a moderate degree of specificity for unstable RNAs.

Eron et al. (10) and G. Khoury (personal communication) have already noted the possible relation between interferon and inhibition of late adenovirus protein synthesis in monkey cells, based on the observation that Vero cells, the only monkey cell line known to be permissive for adenovirus multiplication, cannot produce interferon (9). In addition, recent reports show that the impairment of protein synthesis in S30 prepared from interferon-treated cells is caused by one or more inhibitors which are bound to the ribosomes (15, 19); and Gupta et al. (19) have evidence that this impairment may be due to the defectiveness of certain tRNA's. Perhaps inhibition of viral protein synthesis in interferon-treated cells is due to partial degradation of certain tRNA's required for viral protein synthesis, as previously suggested (19), whereas impairment of synthesis of particular proteins in adenovirus-infected monkey cells is due to a similar degradation of the encoding RNAs. SV40 may enhance adenovirus multiplication in nonpermissive monkey cells by inhibiting the synthesis or activity of interferon or a hypothetical interferon-induced nuclease.

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