JOURNAL OF VIROLOGY, Feb. 1981, p. 759-769
0022-538X/81/020759-11$02.00/0

Vol. 37, No. 2

Adenovirus Type 2 Expresses Fiber in Monkey-Human Hybrids and Reconstructed Cells

GLENN A. ZORN AND CARL W. ANDERSON*

Biology Department, Brookhaven National Laboratory, Associated Universities, Inc., Upton, New York 11973

Adenovirus type 2 protein expression was measured by indirect immunofluorescence in monkey-human hybrids and in cells reconstructed from monkey and human cell karyoplasts and cytoplasts. Monkey-human hybrid clones infected with adenovirus type 2 expressed fiber protein, whereas infected monkey cells alone did not. Hybrids constructed after the parental monkey cells were infected with adenovirus type 2 demonstrated that fiber synthesis in these cells could be rescued by fusion to uninfected human cells. Thus, human cells contain a dominant factor that acts in *trans* and overcomes the inability of monkey cells to synthesize fiber. Cells reconstructed from infected human karyoplasts and monkey cytoplasts expressed fiber, whereas cells reconstructed from infected monkey karyoplasts and human cytoplasts did not. These results are consistent with the hypothesis that the block to adenovirus replication in monkey cells involves a nuclear event that prevents the formation of functional mRNA for some late viral proteins including fiber polypeptide. Furthermore, they suggest that the translational apparatus of monkey cells is competent to translate functional fiber mRNA synthesized in human cells.

Human adenovirus type 2 (Ad2) has a double stranded, linear DNA genome (32) that encodes about 30 proteins. Ad2 replicates efficiently in the nuclei (6) of primary human fibroblasts and established human cell lines (e.g., HeLa). Approximately 10^4 to 10^6 infectious particles per cell are produced. Human adenovirus-infected monkey kidney cells yield about 1,000-fold less virus than do infected human cells; however, adenovirus yields in monkey cells can be enhanced by coinfection with simian virus 40 (SV40) (34), by covalent insertion of SV40 sequences into the adenovirus genome so as to form an adenovirus-SV40 hybrid that expresses the carboxy-terminal portion of SV40 (large) T-antigen (11, 18, 26, 27), by infection with mutants of adenovirus containing mutations which map in the structural gene for the virus-encoded 72,000-M_r DNA binding protein (21, 24, 25) and, to a lesser degree, by treatment of monkey cells with several small molecules, including iododeoxyuridine (20, 39) and dimethyl sulfoxide in the presence of serum (Zorn and Anderson, manuscript in preparation). The reason for reduced adenovirus yields in monkey cells is not precisely known. Virus adsorption and entry into cells is normal (10), as are the onset and rate of viral DNA synthesis (14, 19, 36). Baum et al. (3) provided the initial evidence that a majority of Ad2-specific RNA found in infected HeLa cells is also present in infected monkey cells. Later studies demonstrated that the RNAs were made in proper sequence (13, 40) and at rates similar to the rates found in productive infections (28). Lucas and Ginsberg (28) showed by RNA competition experiments and Klessig and Anderson (22) showed by RNA hybridization to restriction enzyme-produced genome fragments that all early and late Ad2 RNA sequences are present in infected monkey cells. However, several late RNA sequences were found to be present in reduced amounts (up to 20-fold) in both the nuclei and cytoplasm of infected cells, whereas other late RNA sequences were present at concentrations equivalent to the concentrations found in infected HeLa cells (9, 22). This result suggests that in monkey cells some late RNAs are synthesized at reduced rates or are degraded prematurely. Reduced RNA concentrations alone have not been considered sufficient to account for the 1,000-fold-lower virus yields in comparison with enhanced infections.

An examination of the proteins synthesized in adenovirus-infected monkey cells by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a general reduction in the synthesis of many late viral proteins (2) and a drastic reduction in the synthesis of several late proteins, including fiber, IIIa, and 11.5K (17, 22). Cytoplasmic RNA extracted from infected human cells supports fiber polypeptide synthesis in vitro (1, 8), and Eron et al. (8) reported that cytoplas-

mic RNA extracted from infected monkey cells also supports fiber polypeptide synthesis in vitro. In contrast, Klessig and Anderson (22) found that cytoplasmic RNA extracted from Ad2-infected monkey cells did not support fiber polypeptide synthesis in vitro. The results of Klessig and Anderson suggest that infected monkey cells fail to synthesize functional fiber mRNA, but the possibility that fiber mRNA is inactivated upon entering monkey cell cytoplasm could not be excluded. Late viral mRNA's were reported to be absent from polyribosomes of infected monkey cells (19, 30), and a factor from the high-salt wash of ribosomes from SV40-infected monkey cells was capable of significantly stimulating 80S ribosome formation with Ad2 late mRNA's (31). These results were interpreted to mean that monkey cell ribosomes were normally not competent to translate adenovirus late mRNA but that they could be modified by an SV40-specific product in such a way that they became competent.

Fiber production correlates well with the inability of adenovirus to grow efficiently in monkey cells, and although other blocks that limit adenovirus production may exist, fiber production may be used as an indicator of at least one aspect of abortive infection. Fiber might not be synthesized efficiently in monkey cells (i) because fiber mRNA is not synthesized properly, (ii) because fiber mRNA is not recognized by monkey translational components, or (iii) because fiber mRNA is specifically inactivated upon entering the cytoplasm of a monkey cell. In addition to the evidence cited above that less fiber mRNA is produced in monkey cells, Klessig and Chow (23) have shown recently that the splicing of fiber mRNA in monkey cells is incomplete.

The mechanisms cited above are not mutually exclusive; despite difficulties in the interpretation of in vitro translation experiments, there is a possibility that an additional defect(s) in the fiber biosynthetic pathway exists in monkey cell cytoplasm, as suggested by Nakajima and colleagues (31). Therefore, we approached the question of whether the block to adenovirus replication in monkey cells involves both nuclear and cytoplasmic defects by using the techniques of cell hybridization and cell reconstruction.

MATERIALS AND METHODS

Cells, virus, and infection. HeLa cells for suspensions were obtained from J. Flint, Princeton University. They were grown in Joklik modified minimal essential medium containing penicillin, streptomycin, and 5% calf serum. HeLa cells for monolayers were obtained from J. Williams, Carnegie-Mellon University. They are grown in the Dulbecco modification of Eagle medium containing a high glucose concentration (4.5 g/liter); this medium was supplemented with 10% calf serum, penicillin, and streptomycin. CV-C cells, which were derived from TC7 cells (37), were obtained from P. Tegmeyer, State University of New York at Stony Brook. They were grown in the Dulbecco modification of Eagle medium, supplemented with 10% fetal calf serum, penicillin, and streptomycin. Monolayer cultures were maintained at 37°C in a 5% CO_2 atmosphere and were subcultured twice weekly.

Ad2 was provided originally by Ulf Pettersson, Uppsala University, Uppsala, Sweden. Stocks were grown on HeLa cell suspensions as described by Pettersson et al. (33) and were stored at −70°C. Ad2 stocks were titrated on HeLa cell monolayers as described by Williams (42), except that 5% fetal calf serum was used in the overlay medium. The Enders strain of Sendai virus was obtained from J. Lucas, State University of New York at Stony Brook. Sendai virus was prepared by infecting 10-day-old fertilized hen eggs, using the procedures of Lucas and Kates (29). Virus stocks were assayed on sheep erythrocytes and had titers of 2,000 to 5,000 hemagglutinating units per ml. The virus was inactivated by exposure to UV light from a General Electric G8T5 bulb for 5 min at a distance of 15 cm just before use.

CV-C cells or HeLa cells were infected in monolayer cultures by removal of the medium and addition of an appropriate dilution of virus in phosphate-buffered saline. Ad2 (always more than 250 PFU/cell) volumes were 0.1 ml/6-cm dish or 0.5 ml/9-cm dish containing enucleation sheets. Adsorption was for 1 h at 37°C in a 5% CO_2 atmosphere before fresh medium was added.

Cell enucleations. Cells in monolayers were enucleated into karyoplasts (nucleus-containing bodies) and cytoplasts as previously described (29, 43). Briefly, cells were grown on plastic sheets (120 by 67 mm) cut from the bottoms of Falcon tissue culture flasks. These sheets were sterilized for 15 min under UV light and placed in 9-cm culture dishes for cell growth. Enucleation was by a three-step procedure. First, the CV-C cells grown on plastic sheets were centrifuged in medium at 10,400 × *g* (HB-4 swinging bucket rotor; Dupont-Sorvall) at 37°C for 15 min to remove loosely attached cells. Second, the sheets were incubated immediately in medium containing 10 μg of cytochalasin B (Aldrich Chemical Co.) per ml at 37°C for 30 min. Finally, the cells were enucleated by centrifugation in medium containing 10 μg of cytochalasin B per ml, as described above (10,400 × *g*), except that centrifugation was for 45 min.

For enucleation of HeLa cells (infected and uninfected) and infected CV-C cells, cultures were grown on plastic sheets coated with a 4% solution of collagen (Ethicon) in 0.5% acetic acid. Collagen-coated sheets were not required for the enucleation of cells in uninfected CV-C cultures.

Karyoplasts migrated to the bottoms of the tubes during centrifugation. They were resuspended gently in fresh medium and repelleted by centrifugation at 1,000 × *g* for 5 min to remove excess cytochalasin B. After suspension in fresh medium, karyoplasts were plated into tissue culture dishes and incubated for 1 to

2 h at 37°C before cell reconstruction. During this incubation period, contaminating whole cells attached to the tissue culture dishes. Cytoplasts, which remained attached to the plastic enucleation sheets, were washed in fresh medium and incubated for 1 to 2 h at 37°C. Cytoplasts regained the morphology of whole cells during this interval. After the recovery period, a portion of the cytoplast preparation was Giemsa stained to determine the percentage of enucleation; karyoplasts were stained for viability with trypan blue (43). Enucleation values were more than 95% for CV-C cells and more than 85% for HeLa cells. For all preparations more than 95% of the CV-C karyoplasts and more than 50% of the HeLa karyoplasts excluded trypan blue.

Cell hybridization and nuclear transplantation. Whole-cell hybridizations utilized the procedures of Rao and Johnson (35) and Lucas and Kates (29), with some modifications. At 2 days before hybridization, whole cells were placed in 10 ml of medium containing approximately 8×10^9 fluorescent latex beads (fluorescein or rhodamine conjugated; Polysciences). These latex beads were readily engulfed into the cytoplasm of the cells and served as convenient markers for particular cell types. Monkey cultures were washed extensively in phosphate-buffered saline, trypsinized, suspended in Earle balanced salt solution at pH 8.5, and chilled at 4°C for 10 min. UV-inactivated Sendai virus was added to a final concentration of 400 hemagglutinating units per 10^6 cells in a volume of 0.5 ml. After incubation at 4°C for 15 min, cultures were warmed at 37°C for 45 min and then gently centrifuged to remove excess virus. Cells were plated sparsely onto glass slides in 9-cm dishes. The cells were allowed to form clones and then were infected with Ad2 8 days after fusion. Hybrid clones were identified by the presence of both fluorescein- and rhodamine-conjugated latex beads within the clone.

Hybrids between uninfected and infected cells were produced 18 h after infection by using similar procedures. They were fixed for immunofluorescence 8 h after plating onto glass slides.

We performed cell reconstructions between uninfected and infected cells in monolayers by using the procedures of Lucas and Kates (29) 17 to 18 h after Ad2 infection.

Antibody preparation and indirect immunofluorescence. Ad2 fiber and hexon were prepared from the "top component" of a virus preparation, as described by Boulanger and Puvion (5). Female New Zealand white rabbits (4 to 5 lb [1.7 to 2.3 kg]) were injected intradermally with 300 to 800 μg of purified protein emulsified in complete Freund adjuvant at four sites (0.3 to 0.4 ml/site), and each rabbit received an intramuscular booster injection of 200 μg of purified protein emulsified in incomplete Freund adjuvant approximately 4 weeks later. Rabbits were bled about 9 and 15 days after a booster injection, and booster injections were repeated at 4- to 6-week intervals. Blood was allowed to clot for 2 h at 37°C, and serum was prepared by centrifugation to remove cells and the clot. Serum was stored at −70°C. Each bleeding was tested for specificity by Ouchterlony analysis; no significant precipitin lines were observed between heterologous sera and purified proteins, whereas each serum gave a strong precipitin line with its homologous purified protein. Each serum was also tested by indirect immunofluorescence for reactivity with uninfected cells; no significant staining of uninfected cells was observed with dilutions that gave bright nuclear fluorescence with infected cells fixed 24 to 28 h after infection.

For immunofluorescence, cells were grown on glass cover slips or glass slides. At an appropriate time the cells were washed for 10 min in phosphate-buffered saline at room temperature, fixed for 10 min in 100% methanol at −20°C, air dried, and processed immediately or stored at −70°C for up to 7 days. Air-dried cells were washed at room temperature in phosphate-buffered saline and covered with an appropriate dilution of antiserum. After incubation at 37°C for 30 min, the cells were washed three times for 7 min with phosphate-buffered saline. This procedure was repeated for staining by fluorescein-conjugated antirabbit antibody (Cappell). Stained cells were washed in distilled water and mounted in 25% glycerol. Slides were examined with a Universal Zeiss microscope equipped with a 50-w mercury lamp and filters for fluorescein and rhodamine microscopy. Photomicrographs were taken with high-speed Polaroid film (ASA 3000) or 35-mm Kodak Ektachrome (ASA 800).

RESULTS

Hexon and fiber are Ad2 capsid proteins synthesized in large amounts during the late phase of virus growth. Previous studies have shown that hexon is synthesized in Ad2-infected monkey cultures in somewhat reduced amounts compared with hexon synthesis in infected human cultures or in SV40-coinfected (enhanced) monkey cultures (17, 22). However, fiber synthesis is greatly reduced in infected monkey cultures and often has not been detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Fiber synthesis serves as a simple indicator of virus growth in monkey cells, as fiber protein can be detected readily in individual cells by indirect immunofluorescence.

A preliminary experiment with CV-1 cells (a monkey cell line derived from African green monkey kidney cells) indicated that by 30 h after infection, 10% of the cells fluoresced when stained for fiber protein. Thus, a significant fraction of the CV-1 cells synthesized sufficient fiber protein to be detected by indirect immunofluorescence (more than 5,000 molecules per cell) (15, 16) despite the very low level of fiber produced in the cell population as a whole. Because this high background of fluorescent cells severely limited the sensitivity with which hybrids or reconstructed cells could be analyzed for fiber production, a survey of established monkey cell lines was initiated. In contrast to secondary African green monkey kidney cell cultures, most

established cell lines, like CV-1, were found to be semipermissive for adenovirus growth. CV-C cells, which were derived from CV-1 cells, were substantially less permissive for growth than CV-1 cells (less than 0.01 PFU/cell for CV-C cells and 10 to 100 PFU/cell for CV-1 cells). CV-C cultures coinfected with SV40 (or infected with Ad2$^+$ND1) produced yields of Ad2 (10^3 to 10^5 PFU/cell) that were comparable to the yields of virus produced by infected HeLa cells. Less than 0.5% of the Ad2-infected CV-C cells were stained by fiber antiserum (Fig. 1). Similar results were obtained with primary African green monkey kidney cells (data not shown). CV-C cells were chosen for subsequent experiments because the cells were more easily obtained and better characterized than primary or secondary cultures of African green monkey kidney cells.

For some experiments, enucleations and hybridizations had to be performed before late antigens were expressed in the infected cells. A time course study of fiber and hexon expression indicated that neither HeLa nor CV-C cells produced detectable fiber or hexon before 12 h after infection, as determined by immunofluorescence. Fluorescence from the staining of both proteins was observed in a small number of HeLa cells by 18 h after infection. The largest percentage of hexon-expressing cells was observed 42 h after infection. At later times, late-antigen-expressing cells were lost preferentially from the cover slips, and a modest decrease in the percentage of fiber- and hexon-expressing cells was observed.

Fiber expression paralleled hexon expression in HeLa cells, but no more than 0.5% of the CV-C cells were positive by 30 h after infection. Even at later times, when more than 50% of the CV-C cells were hexon positive, few expressed fiber (Fig. 1). We observed no significant difference in the time course of hexon expression in CV-C and HeLa cells, and comparable experiments with SV40-coinfected cells and Ad2$^+$ND1-infected cells (data not shown) indicated that productively infected CV-C cells expressed fiber in parallel with hexon.

Hexon and fiber are expressed in monkey-human hybrid clones. Ad2-infected monkey cells may fail to express fiber protein either because the cells possess a factor which prevents fiber synthesis or because a factor required for efficient fiber expression is absent in monkey cells. If the latter is true, hybridization of monkey and human cells would probably result in cells capable of producing fiber because the human cells would supply the missing factor. However, if monkey cells possess a factor that inhibits fiber synthesis, we would expect fiber syn-

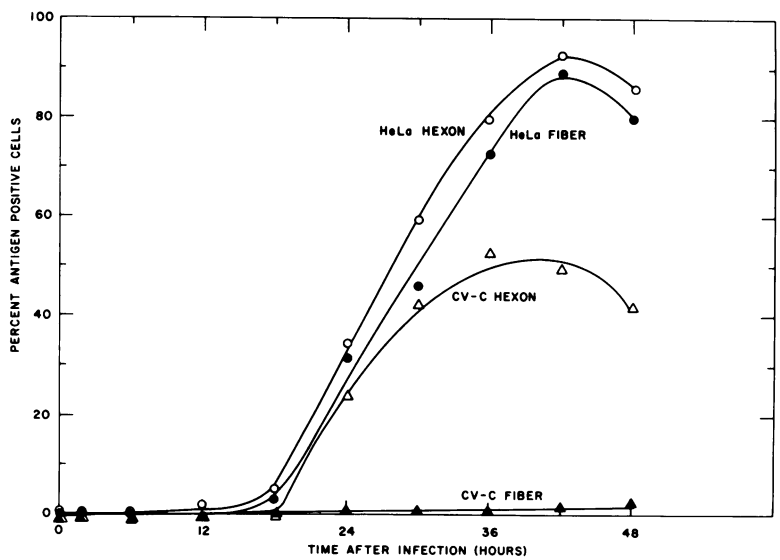

FIG. 1. *Hexon expression and fiber expression as assayed by indirect immunofluorescence at varying times after infection. Cells washed with phosphate-buffered saline were infected with Ad2 at a PFU/cell ratio of 250. Virus was adsorbed for 1 h before fresh medium was added. Cells were fixed and processed for immunofluorescence at the indicated times, as described in the text. For each time point a minimum of 500 cells were counted. Symbols: ○, hexon expression in HeLa cells; ●, fiber expression in HeLa cells; △, hexon expression in CV-C cells; ▲, fiber expression in CV-C cells. At later times (beyond 30 h after infection) a significant number of cells of both cell types were lost from cover slips during fixation and staining.*

thesis to be inhibited in monkey-human hybrids.

Monkey-human hybrids were obtained by mixing equal numbers of CV-C and HeLa cells with UV-inactivated Sendai virus. CV-C cells were grown in the presence of rhodamine-conjugated beads for 48 h before fusion. HeLa cells were grown in the presence of fluorescein-conjugated beads. After fusion, the cells were cloned on glass slides. When the clones reached a size of 50 to 100 cells, they were infected with Ad2 (250 PFU/cell). As controls, parental cultures were mixed (without UV-inactivated Sendai virus), cloned, and infected simultaneously. Approximately 28 h after infection, the clones were fixed and stained for immunofluorescence. All of the cells of a particular clone were not labeled with latex beads. Often the latex beads were clustered in only a few cells. Hybrid clones were identified by the presence of both rhodamine-conjugated beads and fluorescein-conjugated beads within the cytoplasm of the cells in a clone. In every hybrid clone examined, at least one cell within the clone contained both types of colored beads.

As expected, most of the clones expressed hexon (Table 1). When these clones were examined for bead content, we observed hexon-producing clones that contained only fluorescein-conjugated beads, hexon-producing clones that contained only rhodamine-conjugated beads, and hexon-producing clones that contained both types of fluorescent beads. As expected, we found that clones containing fluorescein beads resembled HeLa cells, that clones containing rhodamine beads resembled CV-C cells, and that clones containing both bead colors (hybrids) had a distinct morphology, which was clearly intermediate between the two parental types (Fig. 2). Few clones containing fluorescein and rhodamine beads were observed in simple mixing experiments without Sendai virus.

When examined by immunofluorescence for fiber synthesis, most clones containing only fluorescein beads (presumptive HeLa cells) expressed fiber, and almost all of the clones containing only rhodamine beads (presumptive CV-C cells) did not. Most clones containing beads that fluoresced with both rhodamine and fluorescein (presumptive hybrid cells) also stained positive for fiber synthesis (Table 1). Again, there was a strong correlation between fluorescent beads and cell morphology (Fig. 2). We

TABLE 1. *Hexon expression and fiber expression in cloned cells and hybrids*[a]

Presumed cell type[b]	Fused with Sendai virus before cloning	Latex bead type in clone[c]	No. of clones examined with each antiserum	% of hexon-positive clones	% of fiber-positive clones
Experimental[d]					
CV-C	Yes	Rhodamine	500	100.0	3.0[e]
HeLa	Yes	Fluorescein	500	100.0	100.0
CV-C–HeLa hybrid	Yes	Rhodamine and fluorescein	50	98.0	82.0
Control[f]					
CV-C	No	Rhodamine	500	100.0	<0.2
HeLa	No	Fluorescein	500	100.0	100.0
CV-C and HeLa mixed clones[g]	No	Rhodamine and fluorescein	1 (Hexon) 2 (Fiber)	100.0	50.0

[a] CV-C cells were labeled with rhodamine-conjugated latex beads, and HeLa cells were labeled with fluorescein-conjugated latex beads 48 h before cloning, as described in the text. Clones were infected with Ad2 when they contained 50 to 100 cells. Clones were considered positive if more than 10% of the cells stained by immunofluorescence. In most clones more than 50% of the cells stained; antigen-positive hybrid clones contained at least one fluorescent cell with beads of both colors. The data shown are from one of three comparable experiments.

[b] Cell type was determined from bead color.

[c] Clones had to contain a minimum of 10 beads to be counted and at least 3 beads of each type to be counted as hybrid or mixed clones. Beads also tended to segregate together. Therefore, all cells in a clone were not labeled with beads.

[d] Equal numbers of CV-C and HeLa cells were mixed, treated with UV-inactivated Sendai virus, and cloned on slides. About 30% of the cells fused.

[e] The high background of fiber-positive cells may have been due to fusion of CV-C cells with HeLa cells that did not ingest beads.

[f] Equal numbers of CV-C and HeLa cells were mixed and cloned on slides. Sendai virus fusion was omitted.

[g] Clones with both bead colors were presumed to be formed from either two or more cells (mixed CV-C and HeLa) or from a hybrid cell formed by spontaneous fusion; 3 such clones (1 from hexon-stained slides, 2 from fiber-stained slides) were found in the 1,003 clones examined.

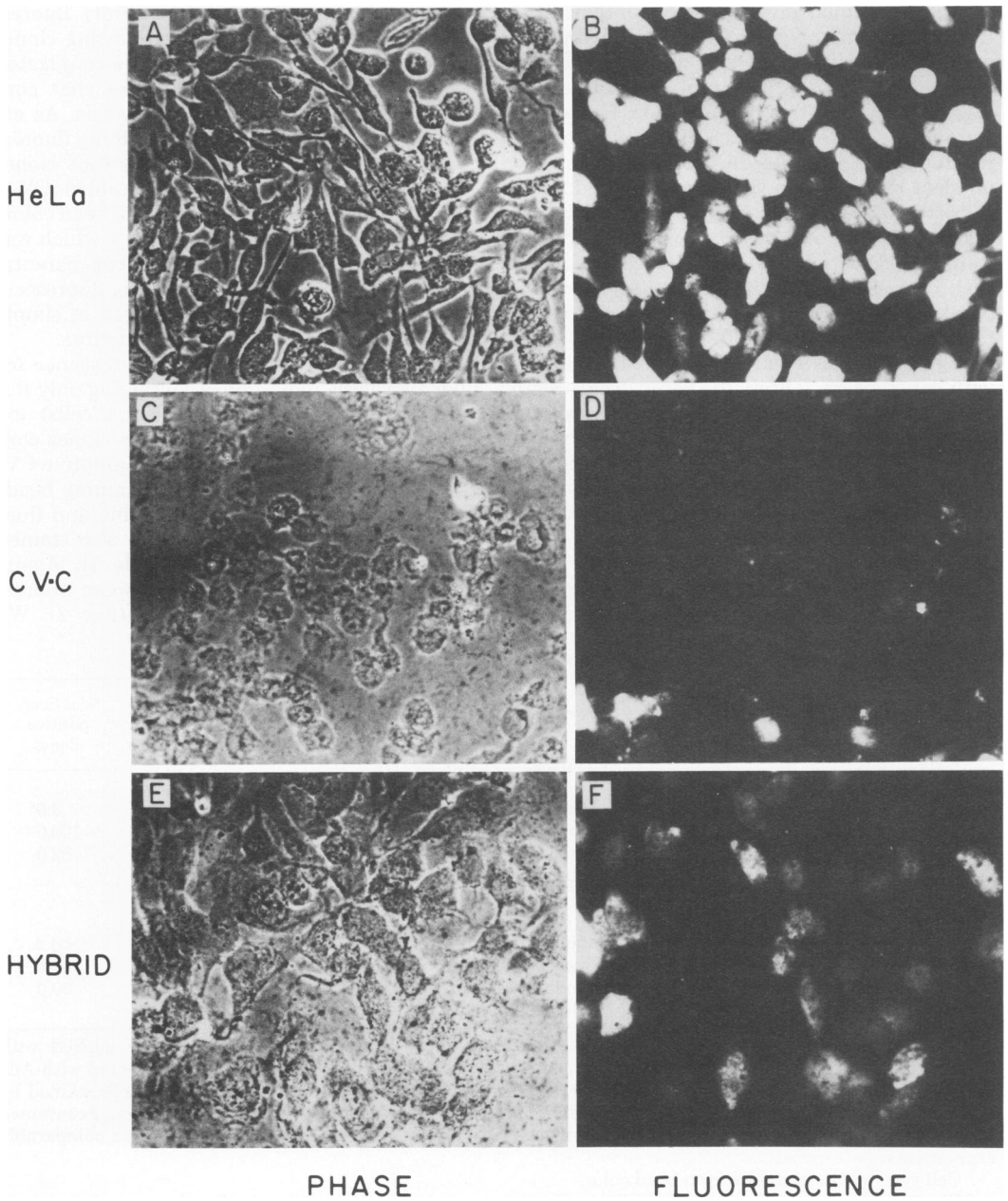

FIG. 2. *Ad2 fiber expression in parental and CV-C–HeLa hybrid clones. Cells were labeled and treated as described in Table 1, footnotes a and d. (A, C, and E) Phase-contrast micrographs of three morphologically distinct Ad2-infected clones observed 28 h after infection. (B, D, and F) Micrographs of the same fields observed with fluorescent microscopy after indirect staining for the fiber protein. The cells in (B) and (F) stained positive for the fiber protein, whereas a majority of the cells in (D) did not. In addition, the cells in (B) contained only fluorescein latex beads (presumptive HeLa cells); the cells in (D) contained only rhodamine latex beads (presumptive CV-C-cells), and the cells in (F) contained both fluorescein and rhodamine latex beads (presumptive CV-C-HeLa hybrid cells).*

conclude that hybrid cells derived from monkey and human parents efficiently express adenovirus fiber protein.

Expression of fiber in monkey-human hybrids formed from uninfected and infected cells. We produced hybrids between uninfected and Ad2-infected cells to characterize further the nature of the complementation observed in clones of monkey-human cell hybrids. Adenovirus-infected cells were fused 18 h after infection, at which time little or no fiber or hexon was detected by indirect immunofluorescence, and the cultures were fixed for immunofluorescence 8 h after fusion (26 h after infection) (Table 2). An examination of a Giemsa-stained sample indicated that approximately 30% of the cell products had two or more nuclei (cells containing more than two nuclei were never observed).

In preliminary experiments, we attempted to identify individual monkey-human hybrid cells by tagging the parental cells with fluorescent latex beads as described above, but we could not find conditions for bead uptake that allowed each hybrid cell to be tagged and yet did not interfere with the assessment of antibody fluorescence. We were also not confident in our ability to identify cell hybrids formed by the fusion of cells tagged with nonfluorescent beads of different sizes. Therefore, in the experiments reported below, we made no attempt to distinguish between the different products obtained after cell fusion, and the results are reported as changes in percentages of the total cell population.

TABLE 2. *Ad2 antigen expression in whole cells and whole-cell hybrids*

Cell or hybrid	% Hexon positive[a]	% Fiber positive[a]
Uninfected HeLa cells	<0.2	<0.2
Ad2-infected HeLa cells	45.0	45.0
Ad2-infected HeLa cells × Ad2-infected HeLa cells	47.0	49.6
Ad2-infected HeLa cells × uninfected HeLa cells	27.0	22.0
Uninfected CV-C cells	<0.2	<0.2
Ad2-infected CV-C cells	31.0	<0.2
Ad2-infected CV-C cells × Ad2-infected CV-C cells	29.0	<0.2
Ad2-infected CV-C cells × uninfected CV-C cells	17.0	<0.2
Ad2-infected CV-C cells × uninfected HeLA cells	17.8	2.0 (all binucleate)[b]
Ad2-infected HeLa cells × uninfected CV-C cells	24.0	20.0

[a] Cells were fixed for immunofluorescence 8 h after hybridization and 26 h after Ad2 infection.

[b] Because only 30% of the cell products after fusion represented hybrid cells and only 50% of the infected HeLa control cells expressed fiber at the time of fixation, a maximum of only approximately 7% of the cell products were expected to express fiber (i.e., 50% of one-half the fusion index). In two separate experiments the percentages of fiber-positive cell products found after fusion of Ad2-infected CV-C cells with uninfected HeLa cells were 1.8 and 2.4% (percentages of hexon-positive products were 22.0 and 17.0, respectively).

As Table 2 shows, fusion had a minimal effect on antigen expression when infected cell populations were self-fused. However, when equal numbers of infected and uninfected cells were mixed and fused, the fraction of antigen-expressing cells was expected to decrease due to the presence of self-fused and unfused uninfected cells. The expected decrease in antigen-expressing cells could not be determined precisely because we did not know that all fusion events were equally possible. However, fusion of an infected cell with an uninfected cell of the same type did not change the fraction of cells expressing fiber or hexon beyond the change expected from dilution alone. When Ad2-infected CV-C cells were fused with uninfected HeLa cells, the fraction of cells that expressed fiber protein increased at least 10-fold. Fluorescence was observed in both nuclei of cell hybrids; therefore, fiber protein accumulated in both the infected and uninfected nuclei of hybrid cells. The fraction of fiber-expressing cells (2% in the experiment shown) was about one-third the number expected if all monkey-human cell hybrids expressed fiber as efficiently as the infected HeLa cells in a parallel control experiment (Table 2). We conclude that a substantial fraction of infected monkey cells became capable of fiber expression within 8 h after exposure to a human cell component. Unfortunately, because hybrid cells could not be specifically recognized independent of fiber expression, we cannot conclude from this experiment that uninfected monkey cells do not inhibit fiber expression in monkey-human hybrids when fiber expression is directed by the nucleus of an infected HeLa cell.

Fiber expression in cells reconstructed from monkey and human karyoplasts and cytoplasts. Infected cells were reconstructed from cytoplasts and karyoplasts of different parental types in order to determine whether the block to fiber expression in monkey cells is due to an event (or lack of an event) that is primarily nuclear, primarily cytoplasmic, or both. This approach also allowed us to pursue the question of whether monkey cells contain an inhibitor of fiber protein synthesis.

Because we were concerned that the exposure

of cells to one or more of the substances (cytochalasin B, dimethyl sulfoxide, UV-inactivated Sendai virus) used to produce reconstructed cells might alter fiber expression, these substances were tested individually for the ability to alter fiber expression in adenovirus-infected cells. None altered fiber expression under the conditions used for cell reconstruction (data not shown). However, dimethyl sulfoxide at 10-fold-higher concentrations caused a substantial increase in the number of CV-C cells that expressed fiber, as detected by immunofluorescence. The effect of dimethyl sulfoxide on the expression of fiber in monkey cells has been investigated further and will be reported elsewhere (Zorn and Anderson, manuscript in preparation).

Cytoplasts were isolated from infected HeLa cells enucleated 18 h after infection and incubated for 8 to 12 h to allow attachment to glass slides and expression of mRNA's before fixation for immunofluorescence. They were only slightly more fluorescent than uninfected parental cells. Cells reconstructed from infected HeLa cytoplasts and karyoplasts from uninfected cells also were only slightly fluorescent when fixed and stained for fiber or hexon 8 to 12 h after reconstruction. These results indicate that little fiber or hexon mRNA had been transferred to the cytoplasm by the time of enucleation. Thus, reconstructed cells that stained intensely for fiber or hexon could be assumed to have acquired functional mRNA from the infected karyoplast, which, was subsequently transported to and expressed in the cytoplasm. Similar results were observed for cytoplasts from infected CV-C cells, except that none of the cytoplasts observed expressed detectable fiber. Some whole-cell contamination was observed (Tables 3 and 4), but the amount of whole-cell contamination observed was not sufficient to obscure the significant findings. The efficiency of cell reconstruction in these experiments was estimated to be 30%, but because the remaining cytoplasts without nuclei could not be differentiated readily from nonfluorescent reconstructed cells (by fluorescence or phase-contrast microscopy), results are reported as percentages of the entire population.

When karyoplasts from infected HeLa cells were fused with cytoplasts from uninfected CV-C cells, about 30% of the population expressed fiber and hexon. This was about the number expected from the efficiency of reconstruction and from the fact that more than 90% of the infected HeLa control culture expressed fiber and hexon. This result indicates that a CV-C cytoplasm can support fiber protein synthesis if it is provided with functional fiber mRNA from a HeLa cell nucleus. We found no evidence for the inhibition of fiber expression in these reconstructed cells that indicated the presence of a monkey cell cytoplasmic inhibitor capable of specifically inactivating fiber mRNA. If such a factor were present, we would have expected the fraction of fiber-expressing cells to be substantially less and also to be less than the fraction of hexon-expressing cells.

When karyoplasts from infected CV-C cells were fused with cytoplasts from uninfected HeLa cells, we found no cells that expressed fiber by 28 h after infection and 10 h after

TABLE 3. *Nuclear transplantation: Ad2-infected HeLa × uninfected CV-C*[a]

Reconstructed cell or cell part	% Hexon positive	% Fiber positive
HeLa cells	94.0	97.0
CV-C cells	<0.2	<0.2
HeLa cytoplasts	10.0[b]	7.2[b]
CV-C cytoplasts	<0.2	<0.2
Ad2-infected HeLa cytoplasts × CV-C karyoplasts	8.0[b]	8.0[b]
CV-C cytoplasts × Ad2-infected HeLa karyoplasts	31.8	27.0
CV-C cytoplasts and Ad2-infected HeLa karyoplasts	<0.2[c]	<0.2[c]

[a] Cells were enucleated 18 h after infection for cell reconstruction by fusion with UV-inactivated Sendai virus. Fixation for immunofluorescence was at 28 h after infection.

[b] Approximately 84% of the infected HeLa cells were enucleated; therefore, about 16% of the reconstructed cell population was composed of parental HeLa cells. See also Table 4 and text.

[c] Cells were not fused with Sendai virus (indicates lack of whole cells in karyoplasts).

TABLE 4. *Nuclear transplantation: Ad2-infected CV-C × uninfected HeLa*[a]

Reconstructed cell or cell part	% Hexon positive	% Fiber positive
HeLa cells	<0.2	<0.2
CV-C cells	48.0	<0.2
HeLa cytoplasts	<0.2	<0.2
CV-C cytoplasts	3.0[b]	<0.2
HeLa cytoplasts × Ad2-infected CV-C karyoplasts	10.0	<0.2
Ad2-infected CV-C cytoplasts × HeLa karyoplasts	6.0[b]	<0.2

[a] Cells were enucleated 18 h after infection for cell reconstruction by fusion with UV-inactivated Sendai virus. Fixation for immunofluorescence was at 28 h after infection.

[b] Approximately 96% of the infected CV-C cells were enucleated; therefore, about 4% of the reconstructed cell population represented parental CV-C cells.

reconstruction; 10% of the reconstructed cell population expressed hexon. We conclude from these results that the primary block to fiber expression in monkey cells is associated with a nuclear event. The simplest hypothesis consistent with these experiments and previous reports is that monkey cells export nonfunctional fiber RNA sequences to the cytoplasm.

DISCUSSION

Comparisons between abortively infected and productively infected monkey cells with respect to events in adenovirus replication have shown that many of the steps in virus replication are indistinguishable in these two circumstances and are also indistinguishable from events in the productive infection of human cells. However, the expression of at least three virus-encoded late polypeptides (fiber, IIIa, and 11.5K) is drastically reduced in abortively infected monkey cells, as is virus production. An explanation for the lack of virus growth in monkey cells could reside in a failure to express one or all of these virus polypeptides. Because reduced fiber protein expression in monkey cells correlates well with reduced infectious virion production and because fiber expression can be measured easily both in mass cultures and in single cells, we and others (23, 41) have used fiber expression as an indicator of the ability of monkey cells to support virus growth. We recognize that fiber expression has not been shown to be the rate-limiting step in virion production. Thus, there may be some circumstances in which fiber expression is indicative of only one aspect of the block to adenovirus growth in monkey cells.

Several hypotheses to account for the failure of adenovirus growth in monkey cells have been proposed. These models assume that the block to adenovirus growth is due to the lack of expression of one or more late virus functions. The proposed mechanisms include (i) a failure to produce some functional late mRNA's, (ii) a failure of the monkey cell translational apparatus to recognize and translate late viral mRNA's, and (iii) the presence of a monkey cell factor that inhibits the translation of or inactivates some late viral mRNA's. These proposed hypotheses are not necessarily mutually exclusive because the block to adenovirus growth may be due to more than a single event. If adenovirus replication and assembly were a simple process that could be reproduced in vitro, we would be able to distinguish between the fundamentally different ways that virus production could be suppressed in monkey cells by employing traditional biochemical procedures. Obviously, at this time many of the steps in adenovirus reproduction cannot be duplicated in vitro. Therefore, we looked for a way to localize the critical event that prevents adenovirus replication in monkey cells and to determine the nature of that event without resorting to in vitro systems. The principle techniques available for cell fractionation and mixing in vivo are whole-cell fusion and cell reconstruction.

Using cell reconstruction, we were able to combine the cytoplasm of an uninfected monkey cell with the nucleus of an adenovirus-infected human cell. This reconstructed cell was as competent to express fiber (as determined by immunofluorescence) as a parental infected human cell. If the monkey cell translational apparatus were incapable of recognizing functional adenovirus mRNA's or if monkey cell cytoplasm contained a potent inhibitor or inactivator of adenovirus mRNA, we would have expected the fraction of reconstructed cells that expressed fiber to be substantially less. This experiment does not support, the hypotheses that the monkey cell translational apparatus is incompetent to recognize late adenovirus mRNA's (8, 30, 31) and that monkey cells contain a factor which inhibits the translation of viral mRNA's or inactivates viral mRNA's (22). We cannot rigorously exclude the possibility that HeLa cell nuclei supply a missing factor required for the translation of viral mRNA's in monkey cells, but by the time of cell reconstruction (18 h after infection) the transport of host cell mRNA's out of the nucleus is inhibited severely, if not completely (4). Thus, human cell nuclei probably do not supply an mRNA for the synthesis of such a factor. Karyoplasts do retain small percentages of cytoplasmic components (43; J. White, J. Bruno, and J. J. Lucas, submitted for publication), and although these are not sufficient to support HeLa karyoplast regeneration, we cannot be certain that the retained human cytoplasmic components are not sufficient to allow viral mRNA expression in the presence of monkey cytoplasm. These criticisms are valid for all cell reconstruction experiments which have been accomplished with the present technology. However, our contention that monkey cell cytoplasm is capable of supporting fiber protein synthesis is supported by recent studies of C. W. Anderson and W. D. Richardson (unpublished data). These workers have found that fiber protein is synthesized after microinjection of polyadenylic acid-containing RNA from adenovirus-infected HeLa cells into the cytoplasm of CV-C monkey cells.

When cells were reconstructed from infected monkey karyoplasts and uninfected human cytoplasts, fiber expression was not observed. This result suggests that monkey cell nuclei are not normally capable of exporting functional fiber

mRNA and is consistent with the theory that monkey cells fail to process some late adenovirus mRNA's properly (23). Incomplete processing could account for the reduced number of fiber sequences found in the nuclei and cytoplasm of infected monkey cells (22) and could also account for the discrepancy between the amount of fiber protein observed (1,000-fold reduction) and the modest reduction (5- to 20-fold) in the number of cytoplasmic fiber sequences (23). If correct, this hypothesis predicts that either the carboxy-terminal portion of SV40 T-antigen (U-antigen) interacts directly with the monkey cell mRNA-processing apparatus or U-antigen induces a monkey cell component that provides the proper processing specificity for the production of functional fiber mRNA. SV40 U-antigen has been reported to have a perinuclear location (27), and mRNA processing may well be associated with mRNA transport to the cytoplasm. As will be reported elsewhere (Zorn and Anderson, manuscript in preparation), we have found significant changes in the ability of monkey cell lines to express fiber protein when they are exposed to agents that are known to affect the cytoplasmic accumulation of functional, stable mRNA species in other systems (38). These observations are all consistent with the hypothesis that monkey cells fail to express constitutively a nuclear factor required for the correct processing of some late adenovirus mRNA's; this mechanism alone would be sufficient to account for the 1,000-fold reduction in virus yield in monkey cells compared with human cells.

If correct, the hypothesis described above also predicts that human cells constitutively express a factor which allows proper processing of adenovirus fiber mRNA and which might complement the missing function in monkey cells. We found that when uninfected human cells were fused with infected monkey cells, fiber expression was increased at least 10-fold within 8 h after fusion. This observation is consistent with the existence of a human factor that can complement the monkey cell deficiency. Because the block to fiber expression appears to represent a nuclear event, the complementing human function presumably acts on or within the monkey cell nucleus (i.e., in *trans*) to allow the export of functional fiber mRNA. We also found that established monkey-human hybrid clones of 50 to 100 cells were competent to express adenovirus fiber protein. This result suggests that the complementing human factor is expressed dominantly in monkey-human hybrids, although it is also possible that the complementing factor derived from the human parent persists for five to seven cell generations. Studies with appropriately marked cell lines (to permit the isolation of stable monkey-human hybrids) should permit a definitive determination of dominance, as well as allow a correlation between fiber expression in hybrid cells and the production of infectious virions. If the complementing factor proves to be expressed dominantly, microenucleation of human diploid cells (7, 12) and the subsequent fusing of human microcells (which contain limited numbers of chromosomes) to monkey cells would permit us to map genetically the human function which allows synthesis of the fiber protein. Fiber production may also provide an assay for the eventual isolation and characterization of the human cell factor that allows the production of functional fiber mRNA.

ACKNOWLEDGMENTS

We are grateful to J. J. Lucas for information concerning the enucleation of HeLa and CV-C cells and to Anita Lewis for technical assistance. This research was carried out at Brookhaven National Laboratory under the auspices of the U.S. Department of Energy.

This work was supported by Public Health Service Training Grant CA09176 from the National Institutes of Health (to G.A.Z.) and by the United States Department of Energy.

LITERATURE CITED

1. **Anderson, C. W., J. B. Lewis, J. R. Atkins, and R. F. Gesteland.** 1974. Cell-free synthesis of adenovirus 2 proteins programmed by fractionated messenger RNA: a comparison of polypeptide products and messenger RNA lengths. Proc. Natl. Acad. Sci. U.S.A. **71**:2756–2760.
2. **Baum, S. G., M. Horwitz, and J. Maizel, Jr.** 1972. Studies on the mechanism of enhancement of human adenovirus infection in monkey cells by simian virus 40. J. Virol. **10**:211–219.
3. **Baum, S. G., W. H. Wiese, and P. R. Reich.** 1968. Studies on the mechanism of enhancement of adenovirus 7 infection in African green monkey cells by simian virus 40: formation of adenovirus-specific RNA. Virology **34**:373–376.
4. **Beltz, G., and J. Flint.** 1979. Inhibition of HeLa cell protein synthesis during adenovirus infection. Restriction of cellular messenger RNA sequences to the nucleus. J. Mol. Biol. **131**:353–373.
5. **Boulanger, P. A., and F. Puvion.** 1973. Large-scale purification of soluble adenovirus hexon, penton, and fiber antigens. Eur. J. Biochem. **39**:37–42.
6. **Brandon, F. B., and I. W. McLean.** 1962. Adenovirus. Adv. Virus Res. **9**:157–193.
7. **Ege, T., H. Hamberg, V. Krondahl, J. Ericsson, and N. R. Ringertz.** 1974. Characterization of minicells (nuclei) obtained by cytochalasin enucleation. Exp. Cell Res. **87**:365–377.
8. **Eron, L., H. Westphal, and G. Khoury.** 1975. Post-transcriptional restriction of human adenovirus expression in monkey cells. J. Virol. **15**:1256–1261.
9. **Farber, M. S., and S. G. Baum.** 1978. Transcription of adenovirus RNA in permissive and non-permissive infections. J. Virol. **27**:136–148.
10. **Feldman, L. A., J. S. Butel, and F. Rapp.** 1966. Interaction of a simian papovavirus and adenovirus. I. Induction of adenovirus tumor antigen during abortive infection of simian cells. J. Bacteriol. **91**:813–818.
11. **Fey, B., J. B. Lewis, T. Grodzicker, and A. Bothwell.**

1979. Characterization of a fused protein specific by the adenovirus type 2-simian virus 40 hybrid Ad2+ND1 dp2. J. Virol. 30:201-217.
12. Fournier, R. E. K., and F. H. Ruddle. 1977. Microcell-mediated transfer of murine chromosomes into mouse, Chinese hamster, and human somatic cells. Proc. Natl. Acad. Sci. U.S.A. 74:319-323.
13. Fox, R., and S. Baum. 1972. Synthesis of viral ribonucleic acid during restricted adenovirus infection. J. Virol. 10: 220-227.
14. Friedman, M. P., M. J. Lyons, and H. S. Ginsberg. 1970. Biochemical consequences of type 2 adenovirus and simian virus 40 double infection of African green monkey kidney cells. J. Virol. 5:586-597.
15. Graessmann, M., and A. Graessmann. 1976. "Early" simian-virus-40-specific RNA contains information for tumor antigen formation and chromatin replication. Proc. Natl. Acad. Sci. U.S.A. 73:366-370.
16. Graessmann, M., A. Graessmann, and C. Mueller. 1977. Regulatory function of simian virus 40. DNA replication for late viral gene expression. Proc. Natl. Acad. Sci. U.S.A. 74:4831-4834.
17. Grodzicker, T., C. W. Anderson, P. A. Sharp, and J. Sambrook. 1974. Conditional lethal mutants of adenovirus 2-simian virus 40 hybrids. I. Host range mutants of Ad2+ND1. J. Virol. 13:1237-1244.
18. Grodzicker, T., J. B. Lewis, and C. W. Anderson. 1976. Conditional lethal mutants of adenovirus type 2-simian virus 40 hybrids. II. Ad2+ND1 host-range mutants that synthesize fragments of the Ad2+ND1 30K protein. J. Virol. 19:559-571.
19. Hashimoto, K., K. Nakajima, K. Oda, and H. Shimojo. 1973. Complementation of translation defect for growth of human adenovirus type 2 in simian cells by a simian virus 40-induced factor. J. Mol. Biol. 81:207-223.
20. Jerkofsky, M., and F. Rapp. 1975. Stimulation of adenovirus replication in simian cells in the absence of a helper virus by pretreatment of the cells with iododeoxyuridine. J. Virol. 15:253-258.
21. Klessig, D. F. 1977. Isolation of a variant of human adenovirus serotype 2 that multiplies efficiently in monkey cells. J. Virol. 21:1243-1246.
22. Klessig, D. F., and C. W. Anderson. 1975. Block to multiplication of adenovirus serotype 2 in monkey cells. J. Virol. 16:1650-1658.
23. Klessig, D. F., and L. T. Chow. 1980. Incomplete splicing and deficient accumulation of the fiber mRNAs in monkey cells infected by human adenovirus type 2. J. Mol. Biol. 139:221-242.
24. Klessig, D. F., and T. Grodzicker. 1979. Mutations that allow human Ad2 and Ad5 to express late genes in monkey cells map in the viral gene encoding the 72K DNA binding protein. Cell 17:957-966.
25. Klessig, D. F., and J. A. Hassell. 1978. Characterization of a variant of human adenovirus type 2 which multiplies efficiently in simian cells. J. Virol. 28:945-946.
26. Lewis, A. M., Jr., M. J. Levin, W. H. Wiese, C. S. Crumpacker, and P. H. Henry. 1969. A non-defective (competent) adenovirus-SV40 hybrid isolated from the Ad2-SV40 hybrid population. Proc. Natl. Acad. Sci. U.S.A. 63:1128-1135.
27. Lewis, A. M., Jr., and W. P. Rowe. 1971. Studies on nondefective adenovirus-simian virus 40 hybrid viruses. I. A newly characterized simian virus 40 antigen induced by the Ad2+ND1 virus. J. Virol. 7:189-197.
28. Lucas, J. J., and H. Ginsberg. 1972. Transcription and transport of virus-specific ribonucleic acid in African green monkey kidney cells abortively infected with type 2 adenovirus. J. Virol. 10:1109-1117.
29. Lucas, J. J., and J. R. Kates. 1976. The construction of viable nuclear cytoplasmic hybrid cells by nuclear transplantation. Cell 7:397-405.
30. Nakajima, K., H. Ishitsuka, and K. Oda. 1974. An SV40-induced initiation factor for protein synthesis concerned with regulation of permissiveness. Nature (London) 252:649-653.
31. Nakajima, K., and K. Oda. 1975. The alteration of ribosomes for mRNA selection concerned with adenovirus growth in SV40-infected simian cells. Virology 67: 85-93.
32. Pereira, H. G., R. J. Huebner, H. S. Ginsberg, and J. van der Veen. 1963. A short description of the adenovirus group. Virology 20:613-620.
33. Pettersson, U., L. Philipson, and S. Hoglund. 1967. Structural proteins of adenovirus. I. Purification and characterization of the adenovirus type 2 hexon antigen. Virology 33:575-590.
34. Rabson, A. S., G. T. O'Conor, I. K. Berezesky, and F. J. Paul. 1964. Enhancement of adenovirus growth in African green monkey kidney cell cultures by SV40. Proc. Soc. Exp. Biol. Med. 116:187-190.
35. Rao, P. N., and R. T. Johnson. 1972. Cell fusion and its application to studies on the regulation of the cell cycle. Methods Cell Physiol. 5:75-126.
36. Reich, P. R., S. G. Baum, J. A. Rose, W. P. Rowe, and S. M. Weissman. 1966. Nucleic acid homology studies of adenovirus type 7-SV40 interactions. Proc. Natl. Acad. Sci. U.S.A. 55:336-341.
37. Robb, J., and R. G. Martin. 1972. Genetic analysis of simian virus 40. III. Characterization of a temperature-sensitive mutant blocked at an early stage of productive infection in monkey cells. J. Virol. 9:956-968.
38. Ross, J., Y. Ikawa, and P. Leder. 1972. Globin messenger-RNA induction during erythroid differentiation of cultured leukemia cells. Proc. Natl. Acad. Sci. U.S.A. 69:3620-3623.
39. Staal, S. P., and W. P. Rowe. 1975. Enhancement of adenovirus infection in WI-38 and AGMK cells by pretreatment of cells with 5-iododeoxyuridine. Virology 64: 513-519.
40. Thomas, D. C., and M. Green. 1969. Biochemical studies on adenovirus multiplication. XV. Transcription of the adenovirus type 2 genome during productive infection. Virology 39:205-210.
41. Tjian, R., G. Fey, and A. Graessmann. 1978. Biological activity of purified simian virus 40 antigen proteins. Proc. Natl. Acad. Sci. U.S.A. 75:1279-1283.
42. Williams, F. J. 1970. Enhancement of adenovirus plaque formation on HeLa cells by magnesium chloride. J. Gen. Virol. 9:251-255.
43. Zorn, G. A., J. J. Lucas, and J. R. Kates. 1979. Purification and characterization of regenerating mouse L929 karyoplasts. Cell 18:659-672.