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Translation of Adenovirus 2 Late mRNAs Microinjected into Cultured African Green Monkey Kidney Cells

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Adenovirus 2-infected monkey cells fail to synthesize fiber, a 62,000 M_r virion polypeptide expressed at late times in productively infected cells. Yet these cells contain fiber mRNA that, after isolation, can be translated in vitro. The reason for the failure of monkey cells to translate fiber mRNA has been approached by microinjecting adenovirus mRNA into the cytoplasm of cultured monkey cells. Late adenovirus 2 mRNA, isolated from infected HeLa cells, was efficiently expressed when microinjected into the African green monkey kidney cell line CV-C. Expressed viral proteins identified by immunoprecipitation included the adenovirus fiber polypeptide. This result demonstrates that the monkey cell translational apparatus is capable of recognizing and expressing functional adenovirus fiber mRNA. Microinjection of late virus mRNA into cells previously infected with wild-type adenovirus 2 failed to increase significantly the yield of infectious virus.

Cell lines derived from African green or rhesus monkey kidneys produce 100- to 1,000-fold fewer virions when infected by group C human adenoviruses than comparably infected human HeLa cells (for review, see reference 24). Although early virus functions and virus DNA replication proceed normally, a three- to fivefold reduction in the expression of many late proteins has been observed (8, 14). Most striking, however, is the near-total absence of the adenovirus fiber polypeptide (10, 14). Monkey cells infected by both adenovirus and simian virus 40 (SV40) (21), by adenovirus-SV40 hybrids that express the carboxy-terminal portion of the SV40 large tumor (T) antigen (10, 11, 17, 18), or by adenovirus host range mutants altered in their DNA binding protein (1, 2, 16) grow adenovirus as efficiently as human HeLa cells and express normal amounts of the late viral proteins.

Two explanations have been offered for the reduced expression of late virus proteins and the specific absence of fiber protein in infected monkey cells. Klessig and Anderson (14) found reduced amounts of some late mRNAs, but the reduction in fiber mRNA has been found to be at least 10-fold less than the reduction in fiber polypeptide (4, 14). However, Klessig and Chow (15) have presented evidence for the incomplete or aberrant splicing of fiber mRNA in infected monkey cells, and Zorn and Anderson (26) found that the block to fiber expression in monkey cells was associated with the cell nucleus. Thus, one possible explanation for the reduction in late protein synthesis and the absence of fiber polypeptide in adenovirus 2 (Ad2)-infected monkey cells is that incomplete or abortive mRNA processing leads to a reduction in the cytoplasmic concentration of functional late mRNAs. Fiber mRNA may be particularly sensitive to improper processing.

A second explanation for the reduced levels of late polypeptides may be that the monkey cell translational machinery does not efficiently recognize late adenovirus mRNAs. Hashimoto et al. (12) found that Ad2-infected monkey cell polysomes were deficient in late adenovirus mRNA as compared with Ad2-infected monkey cells expressing SV40 T-antigen. Eron et al. (7) reported that RNA extracted from infected monkey cells could be translated in vitro to produce the fiber polypeptide. Recently, Anderson and Klessig (4) have reported that fiber mRNA isolated from infected monkey cells could be translated as efficiently in vitro as fiber mRNA isolated from infected human cells. These results suggest that otherwise functional fiber mRNA may not be properly recognized by the monkey cell translational apparatus. To test this hypothesis, we microinjected late adenovirus mRNAs into the cytoplasm of cultured monkey cells and examined the polypeptides subsequently synthesized.

The propagation of human HeLa and KB cells and the monkey cell lines CV-1 and CV-C, the growth of wild-type Ad2, and the preparation of cytoplasmic RNA from Ad2-infected cells have been described previously (1, 2, 7, 23, 26). Human A143 cells (a thymidine kinase-negative cell line) were obtained from Carlo Croce, Wistar Institute, Philadelphia, Pa. The microinjection procedure and the analysis of products synthesized after microinjection have been described previously (22, 23). For each time point, approximately 200 cells were injected with a preparation of polyadenylated RNA isolated from Ad2-infected HeLa cells harvested 16 h after infection. At the various times after microinjection, the culture medium was removed and replaced with [^{35}S]methionine-containing medium; labeling was for 2 h at 37°C. After labeling, cultures were washed and lysed in a Nonidet P-40-containing buffer, and a portion of each culture was immunoprecipitated with a mixture of sera specific for several Ad2 late polypeptides (see legends to Fig. 1 and 2).

Figure 1 shows the results of microinjecting polyadenylated late Ad2 RNA into cultured CV-C monkey cells as a function of time after microinjection. Adenovirus hexon, penton base, IIIa, and fiber polypeptides were clearly synthesized in the period from 1 to 3 h after microinjection, and these polypeptides continued to be synthesized beyond 7 h after microinjection. By 11 h after microinjection, the amount of each of the viral polypeptides synthesized was considerably reduced, and by 18 h after microinjection the synthesis of viral polypeptides was no longer detected. Examination of the autoradiogram shown in Fig. 1 reveals that the apparent maximum synthetic rate of hexon and IIIa occurs between 7 and 9 h after microinjection. Fiber poly-

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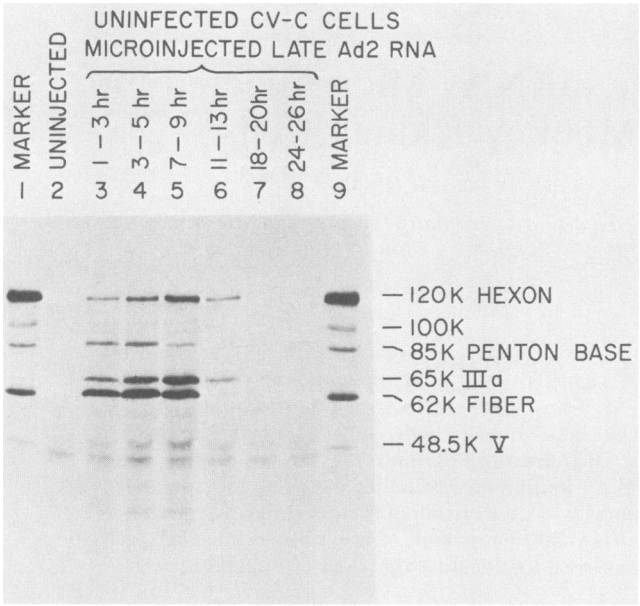

FIG. 1. Production of late Ad2 proteins in CV-C cells microinjected with late Ad2 mRNA. Islands of about 200 CV-C cells were microinjected as previously described (23) with RNA selected with oligodeoxythymidylate-cellulose from cytoplasmic RNA isolated from Ad2-infected HeLa cells harvested 16 h after infection. About 10 fl of RNA at 1.5 mg/ml were injected into each cell. At the times indicated, the medium was removed, cell islands were washed with methionine-free medium, and islands were then covered with 10 μl of [^{35}S]methionine-containing medium; incubation at 37°C was then continued for 2 h. Cell islands were then washed three times with ice-cold phosphate-buffered saline, drained, and lysed in 15 μl of 0.5% Nonidet P-40–0.1 M NaCl–5 mM EDTA–0.1 M Tris-hydrochloride (pH 7.2)–50 μg of tosylamide-2-phenylethyl chloromethyl ketone per ml. Approximately 100,000 cpm of each sample was diluted into lysis buffer for immunoprecipitation, as previously described, with a mixture of antisera directed against late Ad2 proteins. The mixture consisted of rabbit antisera directed against Ad2 hexon (26), fiber (26), 100K protein (generously provided by E. Oosterom-Dragon), and component IIIa (generously provided by P. Boulanger). Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Shown is a photograph of an autoradiogram. The marker was [^{35}S]methionine-labeled cell extract harvested late after Ad2 infection.

peptide had a more uniform synthetic rate between 1 and 9 h after microinjection. The synthesis of penton base appeared to reach maximum between 3 and 5 h after microinjection. Whether these results actually reflect protein synthesis initiation rates and not immunoprecipitation efficiency has not been determined. In particular, note that antiserum to penton base was not included in the antiserum mixture. We presume that penton base is being detected because of its association with hexon. The apparent rate of penton base accumulation may, therefore, reflect an assembly process rather than a true rate of synthesis.

The important conclusion from the above results is that fiber polypeptide is efficiently synthesized in CV-C monkey cells; in fact, fiber appears to accumulate at a greater rate than other late viral polypeptides. Thus, the monkey cell translational apparatus must be able to recognize the adenovirus fiber mRNA at least as well as other late adenovirus mRNAs. A similar conclusion has been reached by Quinlin and Klessig (20) who examined fiber synthesis in cell-free extracts prepared from uninfected, abortively infected, and productively infected monkey cells. Our approach of injecting intact cells offers the reassurance that, since no cell disruption and fractionation are involved, any message-discriminatory factors cannot have been lost. The amount of RNA injected in our experiments is estimated to be about 100 to 1,000 copies of the major late mRNA species per cell (22) or an amount significantly less than that found in infected HeLa cells at late times (9). Thus, fiber protein synthesis is unlikely to have resulted from overloading a negative discriminatory factor in the monkey cell cytoplasm. We might also note that the cell line chosen for microinjection is considerably less permissive with respect to adenovirus production (1) and fiber polypeptide synthesis (26) than most cultured monkey kidney lines.

Studies to date suggest that adenovirus fiber expression in infected monkey cells is probably controlled at two separate steps. Most abortively infected monkey cells clearly have lower amounts of fiber mRNA than productively infected monkey or human cells. For primary monkey kidney cells and for the stringently restricted cell line CV-C, the lack of functional fiber mRNA may be the critical factor that limits virus production (but see below). The failure of fiber mRNA export in abortively infected monkey cells is similar and possibly related to the inhibition of host cell mRNA export to the cytoplasm in productively infected human cells (5, 6). In less stringently restricted cell lines such as CV-1 and BSC-1, fiber synthesis may be limited by a post-transcriptional control mechanism as is suggested by the evidence from Anderson and Klessig (4). Our data and those of Quinlin and Klessig (20) argue that in these cases the inability to translate fiber mRNA is not a property of the translational apparatus itself. Instead, we suggest that the critical event may be the way fiber mRNA is presented to the translational apparatus. Fiber mRNA presented by an intact nucleus appears not to be translated, whereas that supplied exogenously to the cytoplasm or to cytoplasmic extracts can be translated. A possible mechanism of fiber mRNA discrimination could be an interaction in the nucleus of fiber mRNA with a cellular component that prevents polypeptide elongation. This interaction might be analogous to the block to protein translation that occurs for some secreted proteins in the absence of the 7S translocation factor (25). Alternatively, interaction of the nascent fiber polypeptide chain with a cellular component might block further elongation, but only for mRNA transported from a nucleus. These hypotheses predict that fiber mRNA microinjected into the monkey cell nucleus and subsequently transported to the cytoplasm will not be translated.

Whereas the absence of the fiber polypeptide is the most obvious defect that prevents adenovirus reproduction in monkey cells, and whereas this deficiency is sufficient to account for the decreased production of infectious virions, the lack of fiber (or other late virus protein) production may not be the only block to efficient virus reproduction. Other less obvious deficiencies could influence the level of virus produced. In an attempt to address this question, we microinjected early or late adenovirus mRNA into cells that previously had been infected with wild-type Ad2.

Early Ad2 mRNA was microinjected 2 h before infection with 10 PFU of Ad2 per cell; late Ad2 mRNA was microinjected at 20 h after infection with Ad2. Both groups of cells were harvested 48 h after infection. Human A143 cells, infected with Ad2 and harvested 48 h after infection, served as a positive control. Each lysate was passaged once on human KB cells to amplify any yield of virus; the KB cell lysates were assayed for plaque-forming ability on HeLa and CV-1 monolayers.

TABLE 1. Yield of Ad2 from cells microinjected with RNA

Condition	Virus yield[a]	
	Expt 1	Expt 2
Uninfected A143 cells	ND	0
Ad2-infected A143 cells	2.0×10^7	3.4×10^8
Ad2-infected CV-C cells	2.5×10^3	2.2×10^4
Ad2-infected CV-C cells microinjected with late mRNA	1.2×10^4	5.6×10^4
Ad2-infected CV-C cells microinjected with early RNA	7.6×10^3	5.2×10^3

[a] Cells were infected and microinjected as described in the legend to Fig. 2. They were harvested 48 h after infection by freeze-thawing three times in 20 μl of serum-free culture medium. The original cell lysates were used to infect KB cells grown in 60-mm culture dishes, and these were harvested 48 h after infection. After disruption by freeze-thawing, the KB cell lysates were titrated on HeLa and CV-1 cell monolayers as described previously (1). HeLa cell titers are reported; titers on CV-1 cells were about 1,000-fold less for each culture.

In two independent experiments, only a marginal, if any, increase in Ad2 titer was observed in CV-C cells microinjected with Ad2 RNA in comparison with Ad2-infected CV-C cells that had not been microinjected with RNA (Table 1). Infected A143 cells produced a substantial yield of virus as expected.

Analysis of duplicate cell groups, labeled with [^{35}S]-methionine and processed by immunoprecipitation as previously described (Fig. 1), demonstrated that Ad2 fiber polypeptide was synthesized in infected cells microinjected with late Ad2 mRNA (Fig. 2). Fiber polypeptide was not synthesized in Ad2-infected CV-C cells injected with early Ad2 mRNA. Hexon, 100K, and a polypeptide migrating with the mobility of component IIIa were observed in infected CV-C cells whether or not they were microinjected with RNA. The strong band at the IIIa position and the band just below 100K in Fig. 2 are probably hexon degradation products as they were not observed when hexon antiserum was omitted from the mixture used for immunoprecipitation. Other experiments have suggested that IIIa might not be efficiently synthesized in Ad2-infected monkey cells (26). These aberrant products have only been observed in infected monkey cells; the reason for their production is unknown.

The control experiments described above demonstrated that most of the late adenovirus polypeptides, including fiber polypeptide, were synthesized in Ad2-infected CV-C cells microinjected with late Ad2 RNA. The amount of radioactively labeled fiber precipitated from microinjected CV-C cells was comparable to that immunoprecipitated from a similar number of productively infected human cells labeled in the same way. Our failure to detect an increased level of infectious virus production thus suggests that the block to fiber protein synthesis is not the only reason for the failure of monkey cells to produce large amounts of infectious virions. Unfortunately, without nonsense or deletion mutants deficient in fiber synthesis, an adequate control for this experiment is difficult to design. We have not explored the influence of several parameters (e.g., time of microinjection) that might influence virus yield. The microinjection of RNA that specifies the carboxy-terminal portion of SV40 T-antigen has been shown to allow fiber production (assayed by immunofluorescence) in Ad2-infected monkey cells (19); however, in this instance, SV40 T-antigen may function in a catalytic manner.

That microinjection of adenovirus early mRNA did not result in the subsequent appearance of fiber polypeptide suggests that the absence of fiber synthesis in infected monkey cells is not due to a failure to synthesize an early viral product. This result is therefore in agreement with observations of Anderson and Klessig (3) who found no significant differences by Northern blot analysis between human and monkey cells with respect to the expression of early mRNAs from regions E1, E2, E3, and E4.

That at least two separate events appear to regulate Ad2 fiber protein synthesis in infected monkey cells suggests that subtle, multiple controls may operate in mammalian cells to regulate gene expression. An understanding of the molecular mechanisms regulating adenovirus gene expression in monkey cells may provide new insights into this area of fundamental importance.

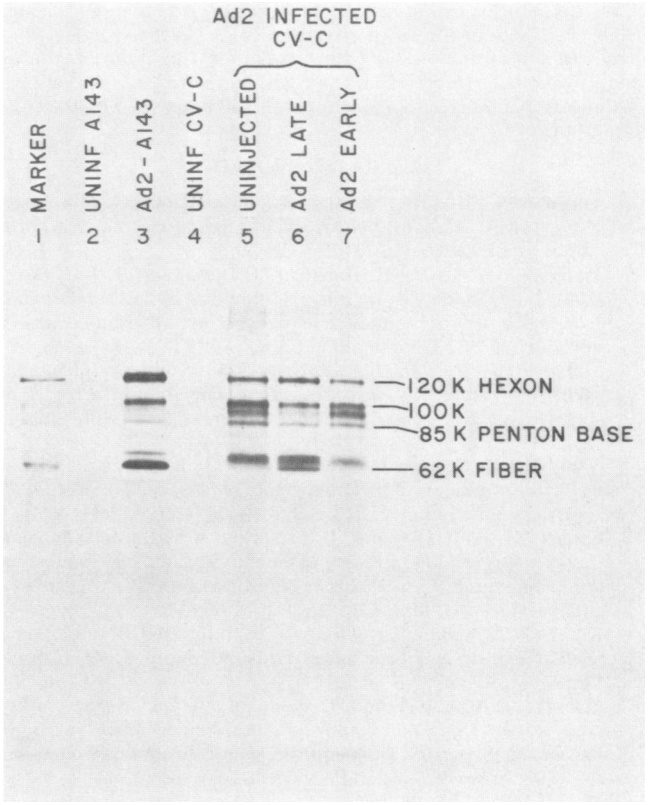

FIG. 2. Polypeptides synthesized in cells infected with Ad2 and microinjected with Ad2 mRNA. Cultured A143 human cells or CV-C cells were infected with Ad2 at 10 PFU/cell. Two hours before infection, two islands of CV-C cells were microinjected with early adenovirus mRNA. Early mRNA was RNA selected with oligodeoxythymidylate-cellulose from cytoplasmic RNA obtained from Ad2-infected HeLa cells incubated in the presence of 100 μg of cycloheximide per ml and harvested 9 h after infection. Another two islands of Ad2-infected CV-C cells were microinjected with late Ad2 mRNA at 20 h after infection. Late mRNA was the RNA selected with oligodeoxythymidylate-cellulose from cytoplasmic RNA obtained from Ad2-infected HeLa cells harvested 16 h after infection. At 24 h after infection, one set of cells was labeled with [^{35}S]-methionine and processed by immunoprecipitation as described in the legend to Fig. 1. A second set was harvested 48 h after infection and assayed for virus yield as described in Table 1. Shown is an autoradiogram of a sodium dodecyl sulfate-polyacrylamide gel upon which the immunoprecipitates were analyzed.

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