MOLECULAR AND CELLULAR BIOLOGY, Oct. 1985, p. 2870–2873
0270-7306/85/102870-04$02.00/0

Vol. 5, No. 10

Microinjection of mRNA Enhances Translational Efficiency of Human Adenovirus Fiber Message in Monkey Cells

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Received 25 February 1985/Accepted 25 June 1985

In monkey cells abortively infected with human adenovirus serotype 2, the synthesis of the fiber polypeptide of the virion capsid is reduced by at least a factor of 100 when compared with that in monkey cells productively infected with a host range mutant of adenovirus serotype 2 (Ad2hr400). However, the steady-state level of fiber-encoding mRNA present in abortively infected monkey cells is only reduced by a factor of 5 to 10. When mRNA isolated from abortively and productively infected monkey cells was microinjected into the cytoplasms of uninfected or abortively infected monkey cells, no differences in the efficiency of translation of the fiber messages from these two sources were observed. These results suggest that the block to synthesis of the fiber polypeptide in abortively infected monkey cells does not reside in the translational machinery of the abortively infected cells themselves but may involve compartmentalization of the fiber message within the cells or an altered processing of the fiber message which prevents correct presentation to the ribosomes.

Human adenovirus serotype 2 (Ad2) is a nuclear replicating DNA virus which grows efficiently in cells derived from its normal human host but poorly in many lines of cultured African green monkey kidney cells. The reduction in the yield of infectious virus from these cells (at least 500-fold) can be overcome by coinfecting the cells with simian virus 40 (13) or by using host range mutants of Ad2 (e.g., Ad2hr400 or Ad5hr404) (1, 7, 10). In monkey cells abortively infected with Ad2, the synthesis of the fiber polypeptide of the virion is reduced by at least a factor of 100 over that in productively infected monkey cells. However, the steady-state level of fiber-encoding mRNA in abortively infected monkey cells is only reduced by a factor of 5 to 10 (2, 9). Pulse-chase experiments show that this discrepancy is not due to an instability of the fiber polypeptide in abortively infected monkey cells (K. P. Anderson and D. F. Klessig, unpublished data). The fiber message present in abortively infected monkey cells translates in vitro with an efficiency comparable to that of the fiber message isolated from productively infected monkey cells when assayed either as an exogenous message in a standard nuclease-treated rabbit reticulocyte lysate or as an endogenous message in S10 cytoplasmic extracts prepared from infected monkey cells (2). The block to translation of the fiber message in abortively infected monkey cells is therefore manifested in vivo but not in vitro.

To compare the ability of fiber messages isolated from productively and abortively infected cells to be translated in a system more closely approximating that found in vivo, we used microinjection technology to introduce mRNA into the cytoplasms of intact cells. Richardson and Anderson (14) used this method to show that the fiber message isolated from productively infected HeLa cells can be translated when introduced into uninfected or abortively infected monkey cells. However, their experiments did not address the question of the functionality of the fiber message synthesized in abortively infected monkey cells.

For the microinjection experiments reported here, cytoplasmic poly(A)$^+$ RNA was isolated from abortively (Ad2) infected and productively (Ad2hr400) infected CV1 cells, an established line of African green monkey kidney cells, at 37 h postinfection (2, 8). For the determination of the relative amounts of fiber mRNA present in each preparation, samples of each were fractionated on denaturing formaldehyde-agarose gels, blotted onto nitrocellulose paper, and hybridized with a nick-translated probe specific for the main body of the fiber message (a *Bam*HI fragment of the Ad2-simian virus 40 hybrid virus Ad2$^+$ND1dp2 containing Ad2 sequences from 0.86 to 0.87 map units) (3). Direct autoradiography of the blot followed by densitometric analysis showed that the amount of fiber message present in Ad2-infected CV1 cells was reduced by a factor of 5 over that in Ad2hr400-infected CV1 cells (Fig. 1A).

To monitor in vivo fiber polypeptide synthesis in this experiment, we infected CV1 cells in parallel with those used for the RNA preparations and labeled them for 1.5 h in the presence of 50 μCi of L-[^{35}S]methionine per ml beginning at the time of RNA extraction. Labeled fiber polypeptide was immunoprecipitated from cell extracts by use of the immunoglobulin G fraction of rabbit antiserum raised against purified native fiber protein (2) and was detected by autoradiography of sodium dodecyl sulfate (SDS)-polyacrylamide gels. A comparison of the amount of label incorporated into Ad2- and Ad2hr400-infected cells showed that fiber synthesis was reduced by at least a factor of 125 in Ad2-infected cells (Fig. 1B).

Microinjection of the isolated RNA was performed by a modification of the procedure of Richardson and Westphal (15). Oligo(dT)-selected RNA dissolved in distilled water was filtered and injected into the cytoplasms of 350 cells growing as a colony on a glass cover slip. A glass microcapillary tube and a constant-flow microinjection apparatus (described in reference 4) were used to deliver an approximate volume of 50×10^{-15} liters to each cell (based on the estimates of Kopchick et al. [11]). RNA from Ad2-infected cells was injected at a concentration of 5 mg/ml, and that from Ad2hr400-infected cells was injected at a concentration of 1 mg/ml, so that the number of fiber mRNA molecules

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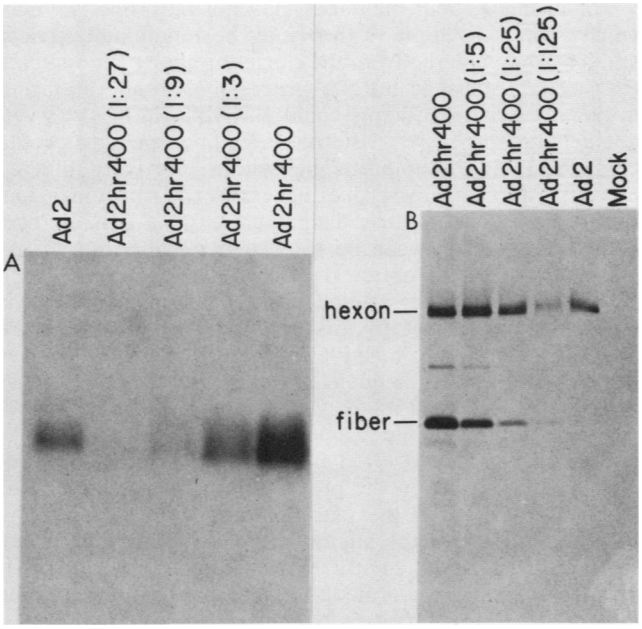

FIG. 1. Comparison of the steady-state levels of fiber mRNA and in vivo fiber synthesis in abortively and productively infected CV1 cells. (A) Cytoplasmic poly(A)$^+$ RNA isolated from Ad2- or Ad2hr400-infected CV1 cells was fractionated by electrophoresis on denaturing agarose gels, blotted onto nitrocellulose paper, and probed for fiber sequences. Equal amounts of RNA from Ad2- or Ad2hr400-infected cells (0.2 μg) or threefold serial dilutions of Ad2hr400 RNA were loaded in each well of the gel. The blot was exposed directly to film without intensifying screens and the resulting autoradiograph is shown in panel A. (B) CV1 cells propagated and infected in parallel with those used for RNA isolation were labeled at the time of RNA extraction with L-[^{35}S]methionine. Fiber polypeptide was quantitatively immunoprecipitated from cellular extracts and fractionated by electrophoresis on SDS-polyacrylamide gels. Panel B shows a direct autoradiograph of the dried gels. Equivalent numbers of cells were used for the immunoprecipitation of mock-, Ad2-, and Ad2hr400-infected cell extracts. Fivefold serial dilutions of the Ad2hr400-infected cell extract were prepared before immunoprecipitation. Nonquantitative precipitation of the hexon polypeptide of Ad2 in this and other figures was due to the presence of antihexon antibodies that were present in the serum used for immunoprecipitation and that probably resulted from small amounts of contaminating hexon polypeptide in the fiber preparation used for the immunization of rabbits.

injected into each cell was approximately the same. The total number of fiber mRNA molecules injected into each cell was approximately 10% of the number of fiber mRNA molecules estimated to be present in productively infected HeLa cells (~20,000 copies per cell) (6). After injection, the cells were incubated for 4 h at 37°C and then labeled for 2 h in the presence of 3.5 mCi of L-[^{35}S]methionine per ml. Cells were harvested from cover slips by two 20-μl washes of 0.15 M NaCl–50 mM Tris hydrochloride (pH 7.4)–5 mM EDTA–0.6% Nonidet P-40, and the fiber polypeptide was immunoprecipitated from the resulting cell lysates. Synthesis was analyzed by autoradiography of SDS-polyacrylamide gels.

Figure 2 shows an autoradiograph of one microinjection experiment which includes duplicate microinjection samples for both RNA types. The comparable intensities of fiber bands resulting from the injection of RNA isolated from either Ad2-infected or Ad2hr400-infected CV1 cells shows that these messages were translated with comparable efficiencies even when introduced into intact growing cells. Similar results were obtained when CVC cells (another line of African green monkey kidney cells even more restrictive to the growth of human adenoviruses) were used for microinjection. The injection of RNA from Ad2- or Ad2hr400-infected cells at concentrations fivefold lower than those indicated above resulted in proportional reductions in the amount of fiber synthesis, indicating that the translational machinery of the cell was not saturated by the injected mRNA.

As the environment of an infected monkey cell may differ considerably from that of an uninfected monkey cell, experiments were devised to examine the translation of fiber mRNA in an infected-cell environment. For these experiments, CV1 cells were infected with Ad5 48 h before injection with mRNA (48 h after infection is the optimum time for analysis of late protein synthesis in this system.) Ad5 causes an abortive infection in monkey cells very similar to that caused by Ad2, and as the fiber polypeptide of Ad5 migrates with a slightly faster mobility on SDS-polyacrylamide gels than the fiber polypeptide of Ad2, it is

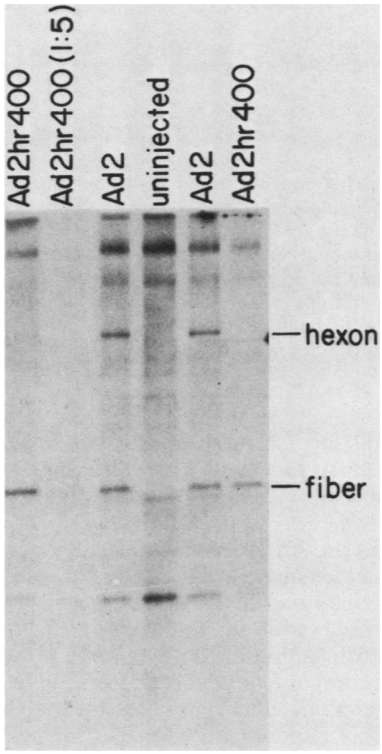

FIG. 2. Fiber synthesis in uninfected CV1 cells microinjected with infected-cell RNA. Cytoplasmic poly(A)$^+$ RNA from infected CV1 cells (Fig. 1) was microinjected into the cytoplasms of 350 uninfected CV1 cells; fiber synthesis was monitored by immunoprecipitation of L-[^{35}S]methionine-labeled extracts. Injections were performed in duplicate with RNA from Ad2-infected CV1 cells at a concentration of 5 mg/ml and RNA from Ad2hr400-infected cells at a concentration of 1 mg/ml so that the amount of fiber message injected would be approximately equal. A direct autoradiograph of a dried SDS-polyacrylamide gel of the immunoprecipitated products is shown. A control sample of uninjected cells was prepared in parallel with experimental samples and is shown for comparison. In addition, an immunoprecipitation experiment with one-fifth of the amount of extract from cells microinjected with RNA from Ad2hr400-infected CV1 cells is shown.

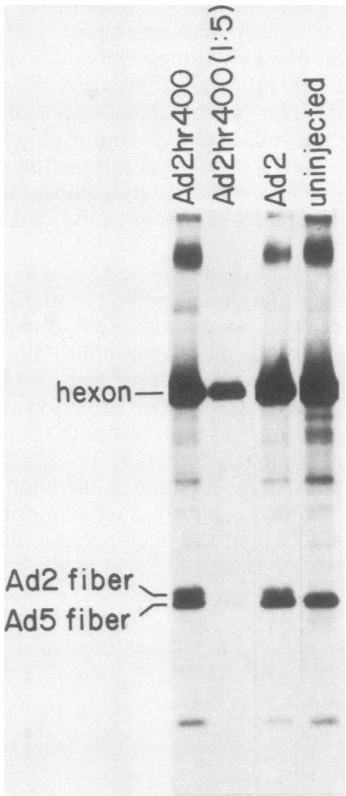

FIG. 3. Translation of microinjected fiber mRNA in abortively infected CV1 cells. CV1 cells were infected with Ad5 and microinjected 48 h later with cytoplasmic poly(A)$^+$ RNA from Ad2- or Ad2hr400-infected CV1 cells. RNA concentrations and fiber immunoprecipitation were as described in the legend to Fig. 2. A direct autoradiograph of the dried polyacrylamide gel is shown. Synthesis from endogenous Ad5 message is represented by the band labeled Ad5 fiber, and synthesis from the microinjected Ad2 message is represented by the band labeled Ad2 fiber.

possible to distinguish between the synthesis of Ad5 fiber polypeptide from an endogenous fiber message of the infected cell and the synthesis of Ad2 fiber polypeptide from injected mRNA.

When RNAs from Ad2-infected and Ad2hr400-infected cells were injected into the cytoplasms of Ad5-infected CV1 cells and assayed for fiber synthesis, no significant differences were observed in the intensities of fiber bands resulting from microinjection of the two RNA preparations (Fig. 3). Microinjection of less concentrated samples showed that the translational machinery of the infected cell was not saturated. This experiment was also repeated with Ad5-infected CVC cells, yielding similar results.

The results presented here extend the data of previous reports (2, 12, 14, 16) and conclusively demonstrate that the fiber message made in abortively infected cells is functional and that the protein-synthesizing machinery of monkey cells as it exists in intact cells is capable of translating the fiber message of either abortively or productively infected cells with equal efficiency. This fact implies that the block to synthesis of the fiber polypeptide in abortively infected monkey cells is not at the level of translation of the message itself but rather may be at a step which can affect the manner of presentation of the message to the ribosomes or the amount of the message available to the ribosomes for translation.

For instance, fiber messages may not be properly "activated" for translation or they may be functionally inactivated as they emerge from the nuclei of abortively infected CV1 cells. Aberrant mRNA processing or alterations of mRNA-associated proteins could play roles in these as-yet-unelucidated processes. Alternatively, fiber messages could be sequestered in compartments within abortively infected cells that do not allow proper access to the ribosomes as a result of these alterations. This could explain why endogenous fiber messages can be translated in vitro in extracts prepared by the disruption of abortively infected cells (2). Fiber messages microinjected into the cytoplasms of cells might not be subject to this compartmentalization or to possible activation or inactivation steps that might occur as RNA emerges from the nucleus. It is interesting to note that in abortively infected monkey cells, the proportion of fiber messages containing the X and Y ancillary leader sequences is drastically reduced (3). Perhaps these 5′ noncoding sequences play a role in the proper compartmentalization or activation of the fiber message for translation.

It is possible that the microinjection procedure itself can perturb the cell sufficiently to relieve the block to fiber synthesis. Studies performed to determine the optimum time for the labeling of cells after microinjection revealed a general reduction of protein synthesis in cells immediately after injection which gradually returned to normal over the next several hours. This could be the result of mechanical disruption of the cells, perturbation of the chemical and ionic balances in the cells, or dilution of the factors necessary for normal translation. If translation of the fiber message in vivo is dependent upon these variables, then translation of the fiber message after microinjection could be affected. Injections into CV1 cells abortively infected with Ad5, however, did not alter the fiber synthesis from an endogenous message (Fig. 3), indicating that these factors probably did not significantly affect the results of these experiments.

It is obvious from the results presented here that caution must be applied in interpreting the data obtained from RNA microinjection experiments. Even though the cytoplasms of intact growing cells were the environment used for this translation assay, we obtained results which did not reflect the true in vivo situation. It is difficult to conceive of an in vitro system which more closely approximates the conditions found in vivo than this microinjection system, but perhaps the use of cells permeabilized with detergents or compounds such as lysolecithin (5) will provide an in vitro system that can mimic the block to fiber synthesis seen in vivo in abortively infected monkey cells and allow experimental manipulation to uncover its molecular basis.

This work was supported by Public Health Service grant AI19755 from the National Institutes of Health and by a G. D. Searle & Co. scholarship to D.F.K. from the Chicago Community Trust. K.P.A. was supported by postdoctoral fellowship AI06870 from the National Institutes of Health. E.A.W. was supported in part by American Chemical Society grant PF1995 and by Public Health Service grant GM 09678 from the National Institutes of Health. D.F.K. was supported by faculty research award 270 from the American Cancer Society.

We thank Mario Capecchi for expert advice and for the use of his laboratory facilities for microinjection.

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