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Altered mRNA splicing in monkey cells abortively infected with human adenovirus may be responsible for inefficient synthesis of the virion fiber polypeptide

(primer extension/synthetic oligodeoxynucleotide/post-transcriptional gene regulation)

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ABSTRACT **Messenger RNA encoding the fiber protein of the human adenovirus serotype 2 (Ad2) capsid is inefficiently translated in abortively infected African green monkey kidney cells. The amount of fiber mRNA present in the cytoplasm of abortively infected monkey cells is less than that in productively infected cells by a factor of 5–10 but synthesis of the fiber polypeptide is reduced by a factor of more than 100. Evidence from a variety of experiments indicates that the defect does not lie in the translational apparatus of the monkey cell but may best be explained by differences in the fiber messages made in abortively versus productively infected cells. Here we report that fiber mRNA isolated from abortively infected monkey cells is processed differently than that made in productively infected cells. Primer extension analysis of the 5′ ends of fiber messages from several different productive and abortive infections shows a direct correlation between synthesis of the fiber polypeptide *in vivo* and the presence of the "x" and/or "y" ancillary leaders on messages encoding the fiber polypeptide. Of all the mRNAs encoded by the major late transcriptional unit of Ad2 only the fiber message can contain the x and y leaders, and the fiber protein is the only late Ad2 protein reported to be glycosylated. We speculate that these leader sequences play a role in the synthesis of this glycoprotein, as well as that of the Ad2 19-kilodalton glycoprotein encoded by early region 3, whose mRNA also contains the x and y leaders.**

Human adenovirus serotype 2 (Ad2) is a nuclear replicating DNA virus with a linear double-stranded genome of approximately 35 kilobase pairs. Ad2 replicates efficiently in cells derived from its normal human host but grows poorly in monkey cells, resulting in reduction by a factor of at least 500 in the yield of infectious virus. The block to replication of Ad2 in monkey cells can be overcome by coinfection with simian virus 40 (SV40; ref. 1) or by infection with Ad2–SV40 hybrid viruses (e.g., Ad2$^+$ND1) that contain a segment of the SV40 genome encoding only the carboxyl terminus of the SV40 large tumor antigen (2–5). In addition, mutants of human adenovirus have been isolated (Ad2hr400-403, Ad5hr404, Ad2$^+$ND3hr600-603) that grow efficiently in monkey cells as well as human cells (6–8). All nine of these independent isolates have achieved their altered host range phenotype as a result of identical point mutations in the amino terminus of the adenovirus DNA binding protein (refs. 9 and 10; unpublished work).

Early RNA synthesis (11) and viral DNA replication (12–14) proceed normally in abortively infected monkey cells, but the steady-state levels of cytoplasmic messenger RNAs encoding late viral proteins are reduced by a factor of 2–10, resulting in a comparable reduction in the synthesis of most late viral proteins (15). However, the synthesis of at least one late protein (the fiber polypeptide of the viral capsid) is reduced by a factor of >100 in abortively infected CV-1 cells even though the steady-state level of mRNA encoding this protein is reduced by a factor of only 5–10 (16, 17).

The discrepancy between fiber mRNA level and fiber protein synthesis in abortively infected CV-1 cells is probably not due to inability of the ribosomes of the monkey cell to utilize functional fiber mRNA as a template (18, 19) but rather is more likely the result of differences between the fiber mRNAs of abortively and productively infected cells that could affect the ability of fiber message to serve as an efficient template for fiber synthesis. However, alterations that might account for the differences in translatability have been difficult to detect. Fiber mRNAs isolated from both abortively and productively infected cells are polyadenylylated, contain the same 5′ capped undecanucleotide, and migrate identically on denaturing methylmercury hydroxide/agarose gels (16, 17). In addition, fiber message isolated from abortively infected CV-1 cells serves just as efficiently as a template for fiber synthesis *in vitro* as message from productively infected cells and even cosediments with polyribosomes isolated from abortively infected cells (17). However, Klessig and Chow (16) showed, using electron microscopy, that ≈15% of fiber message in abortively infected CV-1 cells contains long segments of RNA between the tripartite leader and the fiber-encoding portion of the message, which are normally removed by splicing in a productive infection. The presence of these RNA species suggests that splicing of the fiber message in abortively infected CV-1 cells may be somewhat anomalous.

In this paper, the 5′ ends of fiber messages isolated from productively and abortively infected cells have been examined in detail with the hope of detecting minor processing differences which could affect the translatability of the fiber message *in vivo*. The status of the "x", "y", and "z" ancillary leaders on fiber messages of abortively infected CV-1 cells was of particular interest since these leaders are encoded by sequences within early region 3 (see Fig. 1) and do not occur in any other late messages except the fiber mRNA.

MATERIALS AND METHODS

Virus Infections. Confluent monolayers of CVC cells, CV-1 cells, and HeLa cells were infected as described (17) using 20 plaque-forming units per cell for adenovirus or 40 plaque-forming units per cell for SV40.

RNA Preparation. Cytoplasmic RNA was prepared from infected monolayers of cells as described by Klessig and Anderson (15).

Primer Extension Analysis of Fiber mRNA. A 20-nucleotide primer with the sequence 5′ T-C-T-T-C-A-G-A-C-G-G-T-C-T-G-G-C-G-C-G 3′ was synthesized by New England Bio-

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Abbreviations: Ad2, human adenovirus serotype 2; SV40, simian virus 40.

labs. This primer is complementary to sequences beginning 7 nucleotides downstream from the splice junction at the 5′ end of the main body of the fiber message. Since the initiator AUG for the fiber message occurs exactly at the splice junction, these sequences are within those coding for the fiber polypeptide.

The procedure used for analysis was essentially that of Ghosh *et al.* (20). The single-stranded primer was dephosphorylated with bacterial alkaline phosphatase and then labeled at the 5′ end using T4 polynucleotide kinase and [γ-^{32}P]ATP. Labeled primer (0.2 μg) was combined with 50 μg of oligo(dT)-selected cytoplasmic RNA from infected cells in 0.1 ml of 65% formamide/0.4 M NaCl/0.1 M Hepes, pH 8.0/5 mM EDTA, heated to 80°C for 5 min, and incubated at 37°C for 6–10 hr. The hybridization mixture was then diluted with 1 ml of 0.5 M NaCl/10 mM Tris·HCl, 7.6/1 mM EDTA, and mRNA-primer hybrids were separated from unhybridized primer by passage over oligo(dT)-cellulose. The poly-(A)$^+$ fraction was ethanol precipitated and then dissolved in 0.2 ml of 0.14 M KCl/0.1 M Tris·HCl, pH 8.3/10 mM $MgCl_2$/4 mM dithiothreitol/1 mM dATP/dCTP/dGTP/dTTP. After incubation for 2 hr at 42°C in the presence of 40 units of avian myeloblastosis virus (AMV) reverse transcriptase (Life Sciences, St. Petersburg, FL), RNA was hydrolyzed and the reaction was stopped by addition of 0.05 ml of 1.0 M NaOH/100 mM EDTA followed by heating to 52°C for 30 min. The mixture was then neutralized with 0.125 ml of 1.0 M Hepes, and the cDNA was phenol and chloroform extracted and ethanol precipitated. The labeled single-stranded cDNA products of the reaction were electrophoresed in denaturing 8% polyacrylamide/7 M urea gels and detected by autoradiography. Kinase-digested *Hpa* II and *Hae* III fragments of pBR322 were used as size standards.

***In Vivo* Protein Synthesis and Immunoprecipitation.** Labeling and analysis of proteins synthesized *in vivo* by NaDodSO$_4$/polyacrylamide gel electrophoresis were carried out as described by Klessig and Anderson (15). Fiber polypeptide was immunoprecipitated as described by Anderson and Klessig (17).

RESULTS

Fiber Messages Isolated from Abortively Infected CV-1 Cells Lack the Ancillary x and y Leaders. The 5′ ends of fiber messages from productively and abortively infected human and monkey cells were analyzed by a primer extension procedure. Single-stranded radiolabeled synthetic oligodeoxynucleotide primer complementary to the 5′ end of the main body of the fiber message was hybridized to RNA from productively or abortively infected cells and elongated with AMV reverse transcriptase to make complete cDNA copies of the 5′ ends of fiber messages (Fig. 1). The labeled cDNA copies were then fractionated according to size by electrophoresis on denaturing polyacrylamide gels and detected by autoradiography. Since the sizes of all of the leader segments are known from sequence analysis (21–25), the presence of each of these leaders in a population of RNA molecules can be detected by the appearance of a labeled cDNA band of appropriate electrophoretic mobility on the gel.

When RNA from HeLa cells productively infected with Ad2 or Ad2hr400 was used in this reaction, several prominent bands were observed whose sizes corresponded with the predicted sizes of the 5′ ends of fiber messages containing different leader segments (Fig. 2). The most-prominent band exhibited an electrophoretic mobility of approximately 230 nucleotides, corresponding to fiber message containing only the 203-nucleotide tripartite leader (since the primer is 20 nucleotides long and is complementary to a sequence beginning with the seventh nucleotide from the 5′ splice junction of the main body of the fiber message, the cDNA copy should migrate with a size 26 nucleotides longer than the leader). Other prominent bands exhibited mobilities of approximately 360, 375, and 410 nucleotides, corresponding to fiber messages containing the tripartite leader plus the ancillary leaders x (133 nucleotides), z (145 nucleotides), and y (184 nucleotides), respectively. Other larger bands probably correspond to fiber messages containing the tripartite leader in combination with more than one of the ancillary leaders or with the "i" leader (26, 27). However, size determinations in this area of the gel were not precise enough to allow unambiguous assignment of the origins of these bands. All of the bands observed are presumed to represent copies of the 5′ ends of fiber messages because no background bands were seen when RNA from uninfected cells was used in the primer extension reaction. The pattern of bands observed was the same as that reported by Uhlen *et al.* (25), who also confirmed the assignment of bands corresponding to the tripartite leader alone, the tripartite leader plus y, and the tripartite leader plus z by hybridization and sequence analysis. The nucleotide size of the x leader has been predicted from sequencing data of the area of the Ad2 genome shown to encode the x leader by electron microscopy (23, 28).

When RNA isolated from CV-1 cells productively infected with the host range mutant Ad2hr400 was used in the primer extension reaction, a similar pattern of bands was observed.

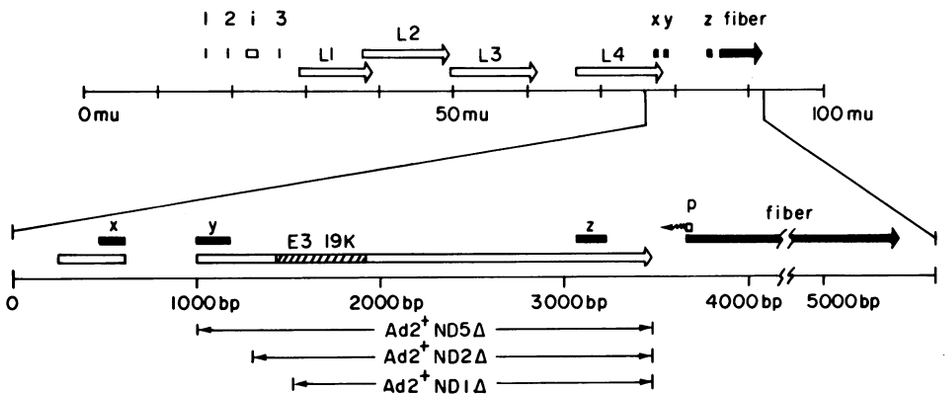

FIG. 1. Sequences encoding the fiber message of Ad2. Fiber messages are represented as solid arrows. Other messages of interest are shown as open arrows. All fiber messages contain the tripartite leader (1 + 2 + 3) while some also contain the x, y, or z ancillary leaders. The synthetic oligodeoxynucleotide used for primer extension is represented by the open box labeled p. The coding sequences for the early region 3 19-kilodalton glycoprotein are represented as the shaded area on that message. The thin arrows at the bottom of the figure show the region of the Ad2 chromosome deleted in the Ad2–SV40 hybrid viruses indicated. The top scale is in map units while the bottom scale shows the number of base pairs (bp) from the *Eco*RI site at 75.9 map units (m.u.).

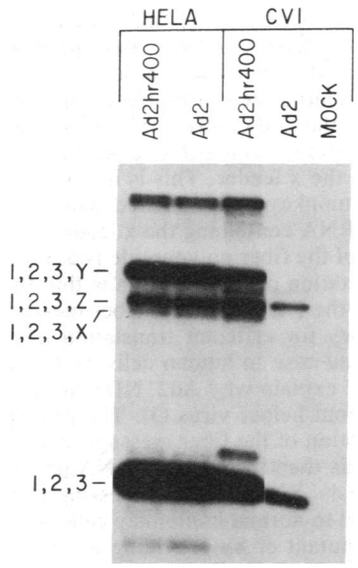

FIG. 2. Primer extension analysis of the 5′ ends of fiber messages isolated from productively and abortively infected cells. Equal portions of each reaction mixture were loaded in separate wells in the gel and, after electrophoresis, the gel was subjected to direct autoradiography. RNA for each reaction mixture was isolated from cells infected with Ad2hr400 or Ad2 or mock infected. Bands corresponding to fiber messages containing different 5′ leader segments are labeled.

However, in this case additional bands specific for the CV-1 cell RNA were also detected. The two most prominent of these bands migrated with sizes of 240 and 390 nucleotides. Their origin is at present unknown, although it is presumed that they represent the 5′ ends of fiber messages that are produced in CV-1 cells but not HeLa cells.

RNA from CV-1 cells abortively infected with Ad2 results in a strikingly different pattern of cDNA bands. Bands corresponding to the tripartite leader alone and the tripartite leader plus z showed a diminished intensity on autoradiograms, undoubtedly reflecting the factor of 5–10 reduction in levels of fiber mRNA present in abortively infected CV-1 cells. More surprising, however, was the almost complete absence of bands representing the tripartite leader plus the x or y ancillary leaders. This is in striking contrast to the situation found for productive RNA, where the band corresponding to the tripartite leader plus y is the second-most-prominent band on the gel. The 240- and 390-nucleotide bands observed when RNA from productively infected CV-1 cells was used, but not when that from productively infected HeLa cells was used, were also observed when RNA from abortively infected CV-1 cells was used as template. Thus the presence of these two bands depended on the type of cell infected and did not depend on whether the infection was productive or abortive.

Role of the y Leader in Translation of the Fiber Message. The striking reduction in the presence of the x and y ancillary leaders on fiber messages of abortively infected CV-1 cells correlates with the inability of abortively infected CV-1 cells to synthesize the fiber polypeptide and suggests that the x and/or y leaders may play an important role in translation of the fiber mRNA *in vivo*. To test the effect of the y leader on translation of the fiber message in monkey cells, we took advantage of the observation that the mutations in the DNA-binding protein of adenovirus host range mutants (e.g., Ad2hr400 or Ad5hr404) will act in *trans* to allow expression in monkey cells of the fiber gene carried by a coinfecting wild-type adenovirus (7). This observation was facilitated by the fact that the fiber polypeptides of the closely related Ad2 and adenovirus serotype 5 (Ad5) migrate with slightly different electrophoretic mobilities in $NaDodSO_4$/polyacrylamide gels. In our experiment, CVC cells (a subclone of CV-1 cells more restrictive for growth of Ad2) were coinfected with Ad5hr404 and either Ad2 or a variant of Ad2, $Ad2^+ND5$. $Ad2^+ND5$ is an Ad2–SV40 hybrid virus that grows poorly in monkey cells. In this virus, some nonessential early region 3 sequences of the Ad2 genome are replaced with SV40 sequences (Fig. 1; ref. 4). This results in loss of those sequences encoding the y and z leaders. If the y leader is really essential for translation of the fiber message in monkey cells then only Ad5 fiber should be synthesized in a coinfection with $Ad2^+ND5$ and Ad5hr404. In the control experiment, coinfection with Ad2 and Ad5hr404 at equal multiplicities resulted in the synthesis of both Ad2 and Ad5 fiber as expected (Fig. 3). In contrast, a parallel coinfection using $Ad2^+ND5$ and Ad5hr404 at equal multiplicities resulted in a considerably lower level of synthesis of Ad2 fiber from the $Ad2^+ND5$ genome. However, the synthesis of Ad2 fiber in the $Ad2^+ND5$/Ad5hr404 coinfection was significantly greater than that observed when monkey cells were infected with $Ad2^+ND5$ alone or with $Ad2^+ND5$ and wild-type Ad5 together.

The results of this experiment imply that, although the presence of the y leader may play some role in enhancing translation of the fiber message in monkey cells, it is not absolutely essential for fiber synthesis. This point is further substantiated by the fact that fiber synthesis (Fig. 3) as well as virus production (3) are enhanced in CVC cells coinfected with $Ad2^+ND5$ and SV40. Although it is possible that complementation by SV40 occurs via a different mechanism than that used by Ad5hr404, analysis of the RNA species synthesized in $Ad2^+ND5$-infected cells suggests that the presence of the x leader may also enhance translation of the fiber message in monkey cells (see below).

Presence of the x and/or y Ancillary Leaders Correlates with Productive Infection and Synthesis of the Fiber Polypeptide *in Vivo*. To determine whether the x and y leaders are always present on a significant portion of fiber encoding messages when abundant levels of fiber polypeptide are synthesized *in vivo*, RNAs from a variety of different productive and abortive infections in both CV-1 and CVC cells were

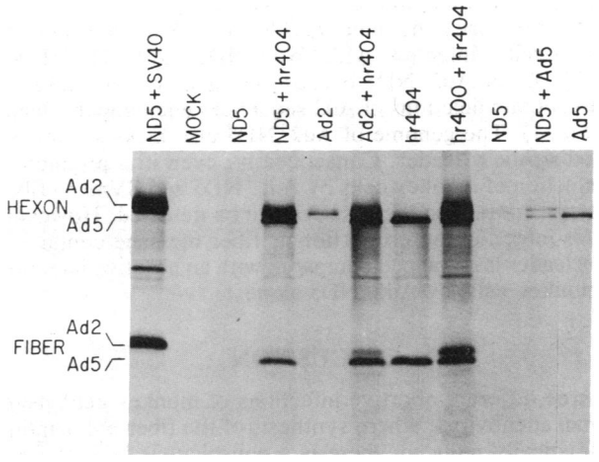

FIG. 3. Fiber synthesis in CVC cells coinfected with $Ad2^+ND5$ and Ad5hr404. CVC cells were infected with various combinations of viruses so that total input adenovirus was 40 plaque-forming units per cell. Infected cells were labeled 30 hr later with L-[^{35}S]methionine at 75 μCi/ml (1 Ci = 37 GBq) for 1.5 hr and the fiber polypeptide was immunoprecipitated and electrophoresed on a $NaDodSO_4$/polyacrylamide gel that was directly autoradiographed. The positions of Ad2 and Ad5 fiber polypeptides as well as of contaminating Ad2 and Ad5 hexon polypeptides are indicated.

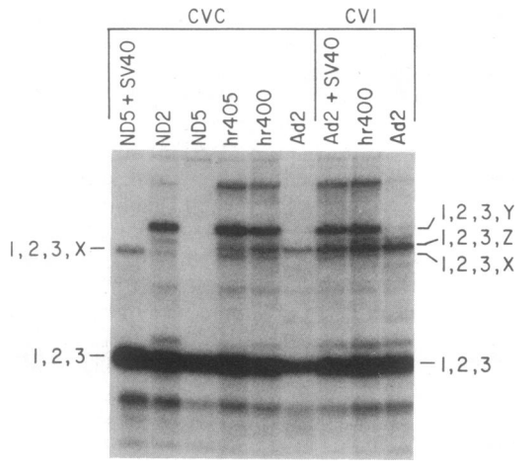

FIG. 4. Correlation of the presence of the x and y ancillary leaders on fiber message with synthesis of the fiber polypeptide. Details are as in Fig. 2, except that material containing equal numbers of cpm were loaded in individual wells of the gel to more clearly demonstrate the proportion of fiber message containing different leader sequences in each infection.

analyzed by the primer extension method. In the experiments shown in Fig. 4, amounts of reaction mixtures containing equal numbers of cpm were loaded in separate wells of the gel to more clearly demonstrate the proportion of fiber messages exhibiting the different leaders in each infection.

Productive infections of CV-1 or CVC cells by Ad2hr400, Ad2hr405 (a host range mutant exhibiting a larger plaque size and virus burst size than Ad2hr400 on monkey cells; see ref. 29), or Ad2 plus SV40 yielded significant populations of fiber mRNA molecules containing the x and y leaders. Productive infections of monkey cells using the Ad2–SV40 hybrid viruses Ad2$^+$ND2 or Ad2$^+$ND1 (data not shown) yielded similar results. Both of these viruses grow well in monkey cells (2, 3). In contrast, abortive infections of monkey cells by Ad2, Ad2$^+$ND5, or Ad2$^+$ND1H140 (an Ad2$^+$ND1 derivative containing an amber mutation in the SV40 sequences responsible for growth on adenovirus in monkey cells; ref. 30; data not shown) showed only barely detectable levels of fiber messages containing the x and y leaders.

Fiber message containing the z leader could not be detected in cells infected with Ad2$^+$ND1, Ad2$^+$ND1H140, Ad2$^+$ND2, or Ad2$^+$ND5 because the genomes of these viruses contain deletions of Ad2 sequences encoding this leader (Fig. 1). The genome of Ad2$^+$ND5 also lacks sequences encoding the y leader. Consequently, even in a productive coinfection of monkey cells by Ad2$^+$ND5 and SV40 no fiber message containing the y leader can be detected. However, in this infection the proportion of fiber message containing the x leader is enhanced compared with an abortive infection of monkey cells by Ad2$^+$ND5 alone.

DISCUSSION

In three different abortive infections of monkey cells using human adenovirus, where synthesis of the fiber polypeptide is drastically reduced, there is a conspicuous lack of fiber messages containing the x or y ancillary leaders. In contrast, all productive adenovirus infections, of both human and monkey cells, exhibit both efficient synthesis of the fiber polypeptide and a significant portion of fiber messages containing either the x or the y leader. The reduction in synthesis of the Ad2 fiber polypeptide when Ad2$^+$ND5 rather than wild-type Ad2 is used in a coinfection of monkey cells with Ad5hr404 suggests that the y leader (whose encoding sequences are deleted in the Ad2$^+$ND5 genome) enhances the efficiency of translation of the fiber mRNA. However, the presence of the y leader on fiber message is not absolutely essential for translation because at least some Ad2$^+$ND5 fiber protein is synthesized in monkey cells coinfected with this virus and either Ad5hr404 or SV40. At least in the latter case, where analysis was feasible, a portion of the fiber messages contains the x leader. This is in contrast to the situation found in monkey cells infected with Ad2$^+$ND5 alone where fiber mRNA containing the x leader is not detectable and synthesis of the fiber polypeptide is drastically reduced.

Our interpretation of these results is that the presence of either the x or the y leader on the fiber message of adenovirus is necessary for efficient translation of that message. This may be the case in human cells as well as in monkey cells and could explain why Ad2$^+$ND5 can grow well in human cells without helper virus (3). The primary defect preventing translation of the fiber message in abortively infected monkey cells then would be in RNA processing and not protein synthesis. When RNA processing of the fiber message is restored to normal in monkey cells, by infection with a host range mutant or by providing SV40 helper function, efficient synthesis of the fiber polypeptide is also restored.

This hypothesis, while attractive, has not yet been rigorously proven. In fact, some experimental data appear to be in conflict with it. For instance, in both productive and abortive infections, a majority of the fiber message contains only the tripartite leader (Figs. 2 and 4). The presence of the tripartite leader by itself on other late Ad2 messages appears to be sufficient for efficient synthesis of the Ad2 late proteins encoded by these messages. Futhermore, Dunn *et al.* (31) have shown that messages encoding an Ad2 fiber–SV40 large tumor antigen hybrid protein are translated efficiently *in vitro* whether the message contains the tripartite leader alone or in combination with the y leader.

However, there are compelling arguments supporting the idea that these leaders may play an important role in synthesis of the fiber polypeptide *in vivo*. For example, the inefficiency of translation of fiber message in abortively infected monkey cells is seen only *in vivo*. Fiber message isolated from abortively infected monkey cells (most of which contains only the tripartite leader) serves as efficient template for synthesis of fiber *in vitro* (17). Therefore, the results of Dunn *et al.* (31) are not inconsistent with our hypothesis. The observation that the presence of the x or y leaders may be important for efficient translation of fiber mRNA *in vivo*, but not *in vitro*, is not unexpected since the specificity of the host translational machinery appears to be altered during the late phase of adenovirus infection (32–34).

In addition, the fiber message differs from other late messages in two important aspects. First, the initiation codon for the fiber polypeptide occurs at the extreme 5′ end of the main body of the fiber message so that the first three nucleotides downstream of the splice junction form the initiating AUG (25). This would place leader sequences immediately upstream of this AUG, where they could have a profound effect on the efficiency of translation of the message. In contrast, cDNA sequencing of the 5′ end of the hexon message of Ad2 has shown that this message contains 36 nucleotides between the 3′ end of the tripartite leader and the initiating AUG in the main body of the message (21). Genomic sequence analysis has predicted similar spacer regions between the tripartite leader and the initiating codons of several other messages of the late transcriptional unit. These data suggest that perhaps other sequences in addition to those present in the tripartite leader may be necessary for efficient translation of late Ad2 messages. In the case of the fiber message, these additional sequences may be provided by the x and/or y leaders.

Second, fiber is the only late Ad2 protein reported to be glycosylated (35). This observation is particularly intriguing

because the other major reported glycoprotein of Ad2, the 19-kilodalton glycoprotein of early region 3, is translated from a message that also contains sequences of both the x and y ancillary leaders at a position upstream from the coding portion of the message (23, 36). Perhaps the x and/or y leaders contain information that can direct the fiber or early region 3 19-kilodalton messages (or both) to the proper compartment in the cell for translation and glycosylation of their encoded polypeptides. The failure to translate fiber mRNA even though it is associated with polyribosomes in abortively infected monkey cells (17) is reminiscent of the inhibition, by the signal recognition particle, of translational elongation of proteins destined for the endoplasmic reticulum (37, 38). The synthesis and glycosylation of fiber, however, differs from that of secreted or membrane-bound proteins in two important aspects. First, the fiber protein is neither secreted nor membrane bound but migrates to the nucleus of the infected cell, where it forms the spikes at the vertices of the nonenveloped capsids (39). Second, the postulated signal carried in the x and/or y leaders is expressed only at the RNA level, rather than in any nascent polypeptide chain. Neither the tripartite leader nor the y leader contains AUG codons, and the only AUG codon present in the x leader is not in frame with the coding sequences of the fiber polypeptide.

Regulation of translation of the Ad2 fiber message as described here may not be peculiar to infected monkey cells but may operate as a normal regulatory mechanism in productively infected human cells as well. Pettersson *et al.* (40) estimated that the amount of fiber polypeptide present in Ad2-infected human (KB) cells is less than the amount of hexon present in the same cells by a factor of at least 10. In contrast, messages encoding the hexon and fiber polypeptides are present in comparable amounts in productively infected cells (41, 42). Since only a fraction of fiber messages in productively infected cells contain the x or y leaders (Fig. 2 and 4; ref. 25), a requirement for these sequences in translation of the fiber message could account for the discrepancy between the amounts of hexon and fiber present in Ad2-infected human cells.

Regardless of the possible role of the ancillary leaders on translation of the fiber polypeptide, we have demonstrated specific and reproducible differences in splicing of the Ad2 fiber messages in productively versus abortively infected cells. Since the pattern of fiber mRNA splicing differs when the same wild-type adenovirus infects cells derived from human cervical tissue (HeLa cells) or cells originating from monkey kidneys (CV-1 and CVC cells), the mRNA processing machinery from different tissues and/or species must vary somewhat. The presence of two spliced species of fiber mRNA that occur in monkey cells, regardless of whether the infection is productive or abortive, but not human cells also supports this contention. In addition, our observations indicate that viral proteins may modulate the activity or alter the specificity of the host cell RNA processing machinery because mutations in the adenovirus DNA-binding protein or the presence of the SV40 large tumor antigen can alter the pattern of fiber mRNA splicing in infected monkey cells.

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