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Adenovirus 3 Fiber Polypeptide Gene: Implications for the Structure of the Fiber Protein

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The nucleotide sequence of a 1,330-base-pair-long DNA segment located between map coordinates 88.5 and 92.3 in the adenovirus type 3 (Ad3) genome was determined. Transcripts from the r-strand of the region were mapped by S1 nuclease analysis and by in vitro translation of RNA, selected by filter hybridization. The results revealed that the sequenced region encodes the Ad3 fiber polypeptide with a molecular weight of 34,800. A comparison between the predicted amino acid sequences of the Ad3 and the Ad2 fiber polypeptides revealed that they have almost identical secondary structures, consisting of a tail, a shaft, and a knob. A striking difference between Ad2 and Ad3 fibers was that the shaft of the Ad3 fiber was significantly shorter, containing only 6 repeat units compared with 22 in the Ad2 fiber. The secondary structure suggests that the fiber is a dimeric structure, as proposed earlier (N. M. Green, N. G. Wrigley, W. C. Russell, S. R. Martin, and A. D. McLachlan, EMBO J. 2:1357–1365, 1983), with the size of the polypeptide determining the length of the fiber protein.

The adenoviruses comprise a family of viruses with an icosahedral capsid consisting of 252 capsomers. Of these capsomers, 240 are hexons which form the surface of the virion, whereas the remaining 12 are designated pentons and occupy the vertices of the iscosahedron (for a review see reference 21). The pentons consist of two distinct structural entities: the penton base, which anchors the pentons to the capsid, and the fiber, which forms an elongated structure protruding from the vertices. The fiber, which is responsible for the attachment of adenoviruses to the cell surface, contains, together with the hexon, the major antigenic determinants of the virion and determines the serotype specificity of the virus (19). The length of the fiber varies considerably between members from different adenovirus subgroups, ranging from ca. 10 nm for subgroup B viruses up to ca. 30 nm for subgroup C viruses (20). The fibers from adenovirus type 2 (Ad2) and Ad5, belonging to subgroup C, are the only fibers which so far have been purified and studied thoroughly. By sequence analysis of the gene, it has been shown that the Ad2 fiber polypeptide consists of 581 amino acids and has a molecular weight of 61,925 (10, 11). The predicted value is in excellent agreement with that obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, giving values between 60,000 and 64,000 (5, 15, 26). The native fiber protein has an estimated molecular weight of 150,000 to 205,000, based on sedimentation equilibrium centrifugation and neutron scattering analysis (4, 5, 26). These results thus suggest that the fiber protein exists as either a dimeric or a trimeric structure. Recently, evidence in favor of a dimeric structure of the Ad2 fiber has been presented (4, 8). In the study by Green et al. (8) it was proposed that the shaft portion of the fiber is composed of two parallel β-sheet structures.

The model contradicts previous observations which indicate that the short fibers of subgroup B members consist of polypeptide subunits of approximately the same size as those of the long fibers of subgroup C members (see reference 31). To resolve the apparent contradiction, we sequenced the fiber gene from Ad3 (subgroup B) and compared the predicted amino acid sequence with that of the Ad2 fiber gene (subgroup C).

MATERIALS AND METHODS

Cells, viruses, and DNA. Ad3 was grown in HeLa cell suspension cultures as previously described (6), except that the cells were harvested at 70 h postinfection. Virus was purified and dialyzed against 20 mM Tris-hydrochloride (pH 7.8)–1 mM $MgCl_2$–10% glycerol and stored frozen at −70°C. Viral DNA was prepared from purified virus by proteinase K digestion and phenol extraction as previously described (22).

Construction of plasmid pAd3fib. Ad3 DNA was cleaved with restriction endonucleases *Hin*dIII and *Xma*I. The resulting fragment mixture was cloned into plasmid pKGO007 (27), cleaved with the same enzymes (16). Colonies containing the Ad3 *Hin*dIII-*Xma*I fragment located between coordinates 83.5 and 97.4 (29, 30) were isolated by colony hybridization by using the nick-translated Ad3 *Eco*RI-B fragment as a probe (9). Large-scale purification of plasmid DNA was as described by Tanaka and Weisblum (28).

DNA sequence analysis. Base-specific chemical degradation was carried out as described by Maxam and Gilbert (17). 5′-End labeling was performed with polynucleotide kinase and [γ-^{32}P]ATP (New England Nuclear Corp.), and 3′-end labeling was performed with the Klenow fragment of DNA polymerase I (New England Biolabs) and [α-^{32}P]dCTP or [α-^{32}P]TTP (16).

RNA preparation and S1 analysis. Late viral RNA was prepared from Ad3-infected cells at 20 h postinfection as described by Pettersson et al. (23). The 5′- or 3′-end labeled DNA fragments were hybridized to total cytoplasmic RNA and subjected to S1 nuclease cleavage as previously described (1, 32). The S1 nuclease-resistant material was analyzed by electrophoretic separation through thin 4 or 8% polyacrylamide gels containing 8.3 M urea (24).

RNA selection and in vitro translation. Total cytoplasmic RNA isolated at 20 h postinfection was purified by hybridization selection (18) to nitrocellulose filters containing the Ad3 *Hpa*I-D fragment (coordinates 89.8 to 90.7). The selected RNA was translated in a mRNA-dependent rabbit reticulocyte cell-free system (New England Nuclear Corp.).

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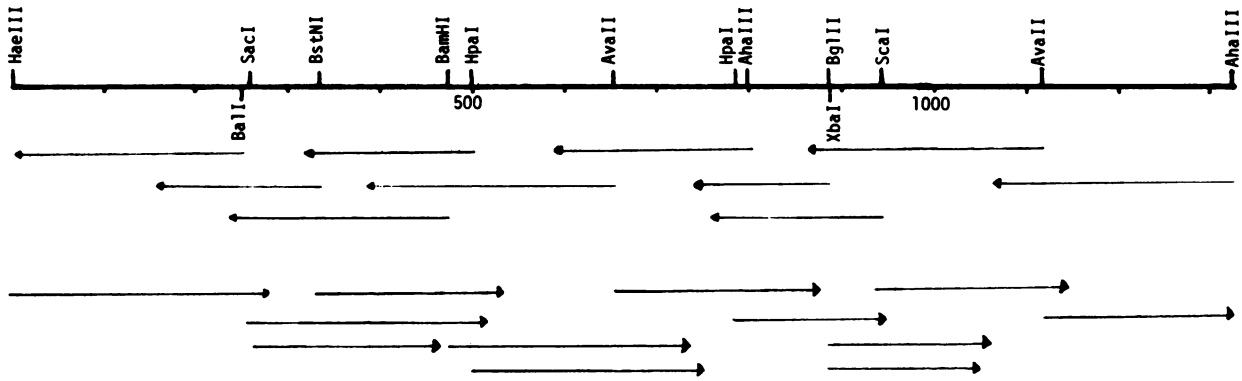

FIG. 1. Sequence strategy. The arrows show the direction and the amount of sequence information that was collected from individual restriction enzyme cleavage sites.

The translation products were resolved on a 13% sodium dodecyl sulfate-polyacrylamide gel, followed by fluorography.

RESULTS

Nucleotide sequence analysis of the Ad3 fiber gene. For most of the sequence determinations a clone containing the *Hin*dIII-*Sma*I fragment (pAd3Fib), located between coordinates 83.5 and 97.4 (29, 30), was used. In some sequence studies the end-labeled fragments were isolated directly from Ad3 virion DNA. All restriction enzyme cleavage sites used for end labeling were sequenced across in overlapping fragments to exclude the possibility that very short fragments were overlooked (Fig. 1). The sequence was determined independently for both complementary DNA strands, and the two sequences were in perfect agreement. The established 1,330-base-pair (bp)-long nucleotide sequence located between map coordinates 88.5 (*Hae*III site) and 92.3 (*Aha*III site) is shown in Fig. 2.

Comparison of DNA and protein sequences of the Ad2 and Ad3 fibers. The r-strand sequence contains only two open translational reading frames (ORFs) which have the capacity to encode proteins exceeding 7,000 in molecular weight (Fig. 3). The shorter of the two ORFs, which specifies a 12,000-molecular-weight (12K) polypeptide, lacks a suitable initiation codon and is most likely not functional in vivo. The larger ORF specifies a polypeptide with a hypothetical molecular weight of 34,800. This ORF most likely encodes the Ad3 fiber polypeptide since it shows extensive homology with the Ad2 fiber gene (Fig. 2 and Table 1). The l-strand sequence contains one ORF with the capacity to code for a 14K polypeptide. The right-hand part of the established sequence includes an ORF which is equivalent to ORF7 in region E4 of Ad2 (7, 11). The leftmost part encodes the equivalent of the E3-14K polypeptide of Ad2, as revealed by a comparison with the corresponding Ad2 sequence (10).

As shown in Fig. 2, extensive homologies exist at both the DNA and the protein sequence levels. The homologies are, however, interrupted by large deletions in the Ad3 sequence (or large duplications in the Ad2 sequence). The nucleotide sequence homology of the whole region is 57%, whereas the amino acid sequence homology is only 41%. There are many deletions in the Ad3 sequence as compared with Ad2 (Fig. 2). All large deletions are located within the region specifying the shaft of the fiber or in the 5′ or 3′ noncoding sequences, which flank the fiber gene (Fig. 2).

S1 nuclease analysis. To locate the splice site and the polyadenylate [poly(A)] addition site of the Ad3 fiber mRNA, we analyzed the structure of early and late Ad3 RNAs by S1 nuclease mapping. To map the 5′ splice site where the tripartite leader is attached to the body of the Ad3 fiber mRNA (13), early and late Ad3 RNAs were hybridized to a *Bam*HI-*Hin*dIII fragment 5′ labeled at the *Bam*HI cleavage site (Fig. 3). The hybrids were treated with S1 nuclease. Two RNA species were detected in late RNA preparations, both of which were absent from early RNA (Fig. 4). The 5′ end of the most prominant species maps some 240 bp upstream of the *Bam*HI cleavage site. To determine the precise structure of this RNA, S1 nuclease analysis was performed by using a 5′-end-labeled *Bst*NI-*Nco*I fragment (Fig. 3) as a DNA probe. The nuclease-resistant material was separated in parallel with the corresponding DNA sequence ladder (32) on a denaturing polyacrylamide gel (data not shown). By this strategy we could show that the 5′ end of the major RNA maps to the AG dinucleotide located immediately upstream of the first ATG in the ORF encoding the 34.8K polypeptide.

The longer RNA species maps ca. 650 bp further upstream, and its 5′ border is located in the E3 region; it probably coincides with the splice acceptor site which is used to generate the E3-14K mRNA (preliminary data not shown). It is thus likely to be a transcript from the major late promoter which uses an E3 splice acceptor site. A corresponding RNA was not seen in early RNA preparations.

Five hypothetical poly(A) addition signals (AATAAA) exist within the established sequence, three in the r-strand sequence and two in the l-strand sequence (Fig. 3). To map the poly(A) addition site for the fiber mRNA, early and late RNA samples were hybridized to a 3′-end-labeled *Bam*HI-*Sma*I fragment located between coordinates 89.7 and 97.4 (29, 30) (Fig. 3). Again, no bands were observed with early RNA, and only one band was detected with late RNA (Fig. 4). The position of the 3′ end was mapped at the nucleotide level by S1 nuclease analysis by using a 3′-end-labeled *Ava*II-*Hin*fI fragment (Fig. 3) as a DNA probe. The S1-resistant material was separated in parallel with a corresponding DNA sequence ladder on a denaturing polyacrylamide gel (data not shown). By this strategy we could locate the poly(A) addition site of the fiber mRNA to nucleotides 1240 through 1243. The hexanucleotide sequence AATAAA is located 23 to 26 nucleotides upstream. The poly(A) addition site for the fiber mRNA is thus located ca. 770 bp downstream from the *Bam*HI cleavage site. The additional AATAAA sequence which is located within the coding region of the fiber polypeptide at nucleotide 1069 (Fig. 3) does not seem to be used, since no 3′ end was detected in the vicinity of this position (Fig. 4).

In vitro translation. To demonstrate that the 34.8K ORF, identified in the r-strand sequence, indeed encodes a protein

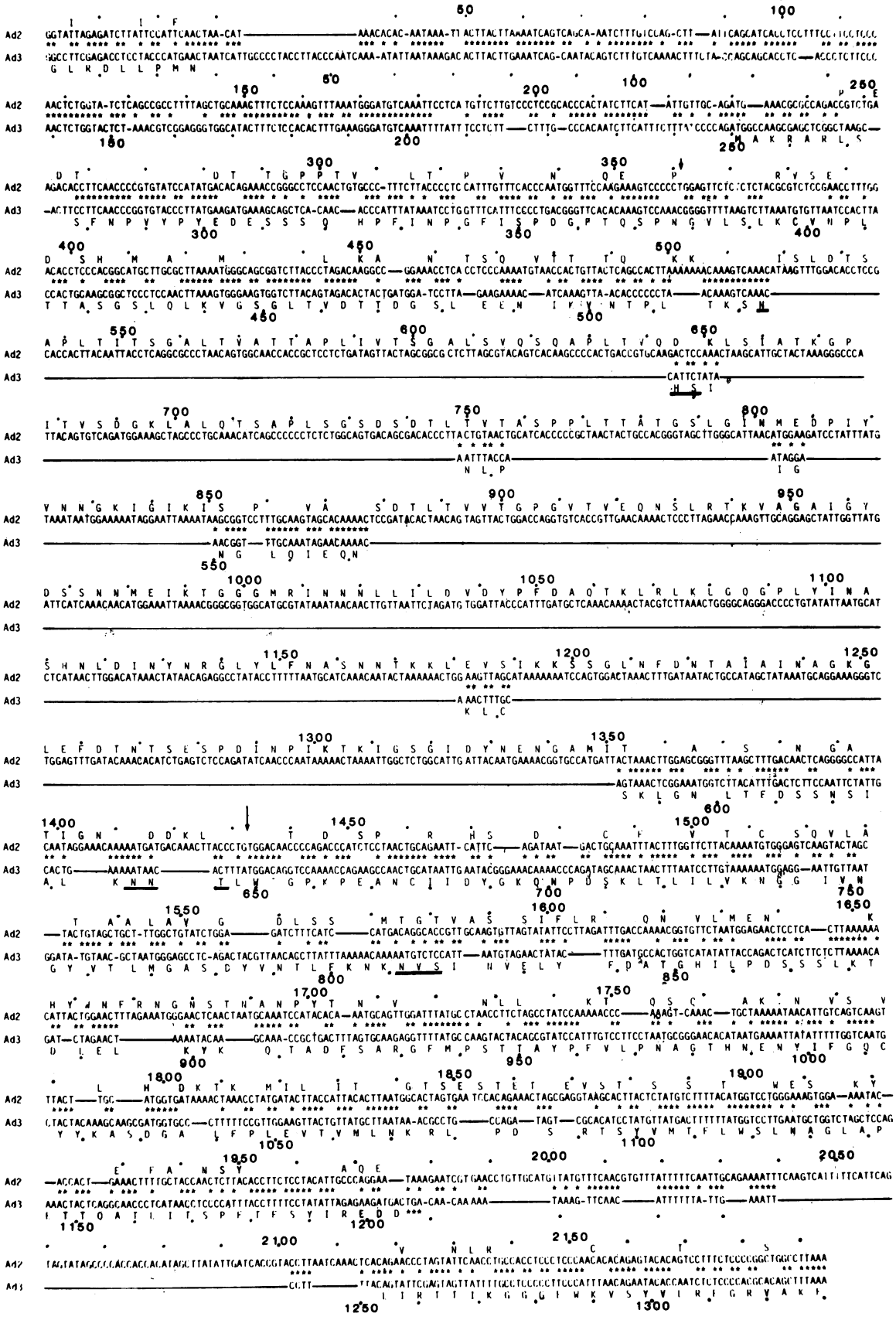

FIG. 2. DNA sequence comparison between the genes for the Ad2 and the Ad3 fiber polypeptides. The Ad3 sequence starts at the *Hae*III restriction enzyme cleavage site, as indicated in Fig. 1. Nucleotides which are identical in the Ad2 and Ad3 sequences are indicated with asterisks, and deletions are indicated with dashes. The amino acid sequence deduced for the Ad3 fiber polypeptide is indicated with a one-letter code. Amino acids which are different from those in Ad3 are indicated in the Ad2 sequence. Potential glycosylation sites are underlined in the sequence.

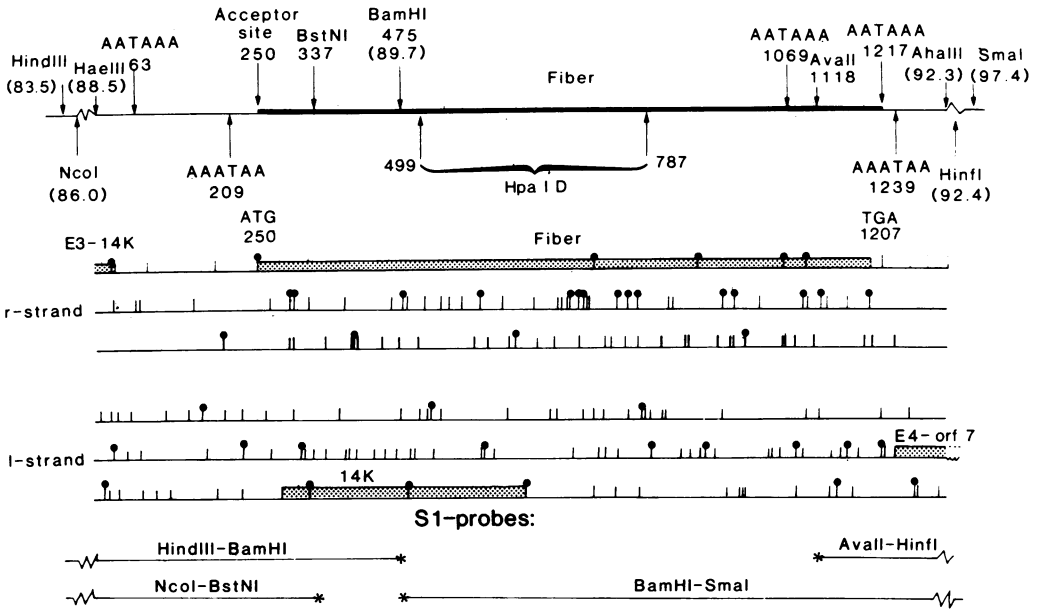

FIG. 3. Organization of the Ad3 fiber region. The upper part indicates the gene for the Ad3 fiber polypeptide (bold line). Selected restriction enzyme cleavage sites are also indicated, as well as the acceptor splice site used for generation of the fiber mRNA. The positions of the hexanucleotide sequence, AATAAA, are also shown. The middle part of the figure outlines the location of the ORFs in the r-strand and l-strand sequences. The ORF encoding the fiber polypeptide, as well as a hypothetical 14K polypeptide, is indicated. Vertical bars indicate termination codons. ATGs are also indicated (↑). Fragments used as probes for the S1 analysis are shown in the lower part of the figure.

product, we analyzed the mRNAs from this region of the Ad3 genome by in vitro translation. Late cytoplasmic RNA was selected by hybridization to a 288-bp-long Ad3 *Hpa*I fragment previously overlooked when restriction enzyme cleavage maps were constructed (29, 30) (Fig. 3) and subjected to in vitro translation. A ca. 35K polypeptide was detected when *Hpa*I-D-selected RNA was translated (Fig. 5). It corresponds in all likelihood to the Ad3 fiber polypeptide, since the DNA sequence predicts a polypeptide of this size, showing clear-cut homology with the Ad2 fiber polypeptide. A close examination of polypeptides present in purified virions revealed the presence of a 35K polypeptide which most likely is identical with the fiber polypeptide characterized in this study (data not shown).

TABLE 1. Amino acid composition of Ad2 and Ad3 fibers

Amino acid	Mol%[a]	
	Ad2	Ad3
Thr	11.7	9.1
Ser	10.7	10.0
Leu	9.6	11.0
Asn	8.1	8.5
Gly	7.9	7.2
Ala	6.4	5.0
Lys	5.8	6.3
Pro	5.8	6.3
Val	5.7	5.0
Ile	5.5	5.0
Asp	5.0	4.7
Gln	3.1	2.8
Glu	3.1	4.1
Phe	2.9	4.7
Tyr	2.9	4.1
Met	2.1	1.6
Arg	1.7	1.9
His	0.9	1.3
Trp	0.7	0.6
Cys	0.5	1.3

[a] The molecular weight of Ad2 is 61,900, and that of Ad3 is 34,800.

DISCUSSION

The nucleotide sequence of the region which encodes the Ad3 fiber polypeptide has been determined and compared with the corresponding sequence in Ad2. The comparison revealed that the sequences flanking the fiber gene are better conserved than the protein-coding sequences. This finding was unexpected but can be explained in several ways. One explanation is that the flanking sequences contain important RNA processing signals. The 5′ flanking sequence contains the poly(A) addition sites for the E3 mRNAs (data not shown) and the splice signals for the fiber mRNA. The 3′ flanking sequences include the polyadenylation signals for both the fiber and the E4 mRNAs (Fig. 3 and 4). These structures have probably imposed evolutionary constraints on the noncoding sequences bordering the fiber gene. There may also be a selection pressure in favor of changes in the fiber polypeptide, since the fiber contains one of the major antigenic determinants exposed on the adenovirus particle (19). The coding region of the fiber gene shows a higher degree of nucleotide sequence homology than of amino acid sequence homology, suggesting that there has been little selection pressure to retain the amino acid sequence of the fiber polypeptide.

Three AATAAA sequences are encoded by the r-strand sequence presented in Fig. 2. In analogy with the Ad2 E3 sequence (10, 25), the first AATAAA, which is located at position 63, is probably used for polyadenylation of the E3 mRNAs. The third hexanucleotide sequence, located at position 1217, is used to generate the fiber mRNA, as revealed by S1 nuclease analysis (Fig. 4). The hexanucleotide which most likely is used for polyadenylation of the E4 mRNAs is located on the opposite strand 9 bp downstream

of the AATAAA sequence, used for the generation of the fiber mRNA.

Thus, it appears likely that in Ad3 the fiber mRNA and the E4 mRNAs overlap each other, unlike the situation at the corresonding position in the Ad2 genome (14). We have not been able to detect mRNAs which use the second AATAAA sequence (position 1069) in either early or late mRNA preparations (Fig. 4). Therefore, as has been previously noted, the sequence AATAAA alone is apparently not sufficient to signal polyadenylation.

The l-strand sequence specifies one AATAAA sequence which is preceded by an ORF, theoretically encoding a 14K polypeptide (Fig. 3). We have not determined whether l-strand-specific transcripts corresponding to this reading frame exist. An ORF encoding a hypothetical 13K polypeptide is found at approximately the same position in the Ad2 sequence (10). This polypeptide has not been shown to be functional in Ad2 and does not share significant sequence homology with the predicted Ad3 14K polypeptide.

In vitro translation of RNA selected by hybridization to the fiber region revealed a weak band corresponding to a

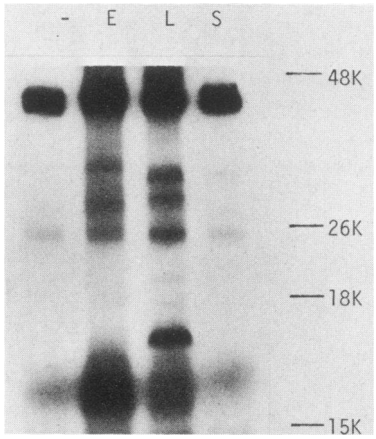

FIG. 5. In vitro translation of Ad3 mRNA. Early (E), late (L), and no (—) RNAs were added. S, Late RNA hybridization selected on the 288-bp fragment (a fragment located within the coding sequence for the fiber polypeptide [Fig. 3]). A molecular weight marker is also indicated.

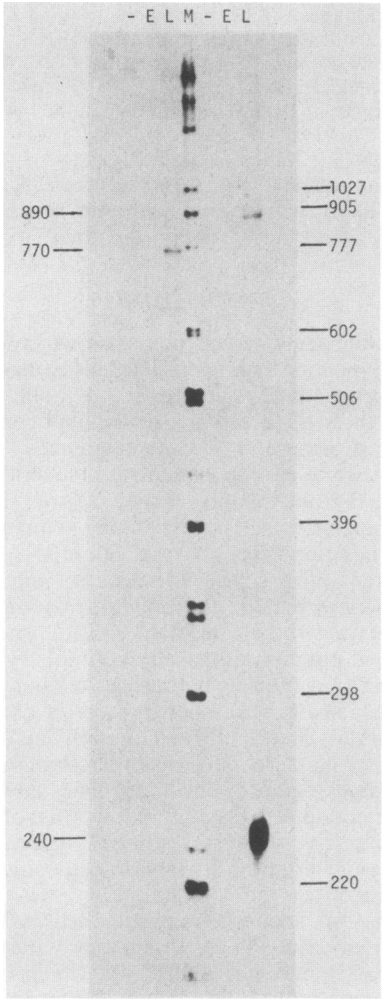

FIG. 4. S1 nuclease analysis of the Ad3 fiber mRNA. The right part shows the position of the splice acceptor site, and the left part shows the poly(A) addition site for the fiber mRNA. Early (E), late (L), and no (—) Ad3 RNAs were used for the analysis. pBR322 marker fragments (M) are also shown.

14K polypeptide (data not shown). The origin of this polypeptide is unknown. It may correspond to the l-strand-specific 14K polypeptide. A second, more likely, possibility is that the 14K polypeptide is translated from a late E3 mRNA that continues beyond its usual poly(A) site and coterminates with the fiber mRNA (Fig. 4).

A surprising finding was that the Ad3 fiber polypeptide had a molecular weight of only 34,800. Previously, a 70K polypeptide observed in purified Ad3 virions was assumed to be the fiber polypeptide, due to its relative abundance and similar mobility to the Ad2 and Ad5 fiber polypeptides (31). The fiber is a glycoprotein containing two residues of *N*-acetylglycosamine per polypeptide chain (2, 3, 12). The functional significance of the carbohydrate moiety is at present

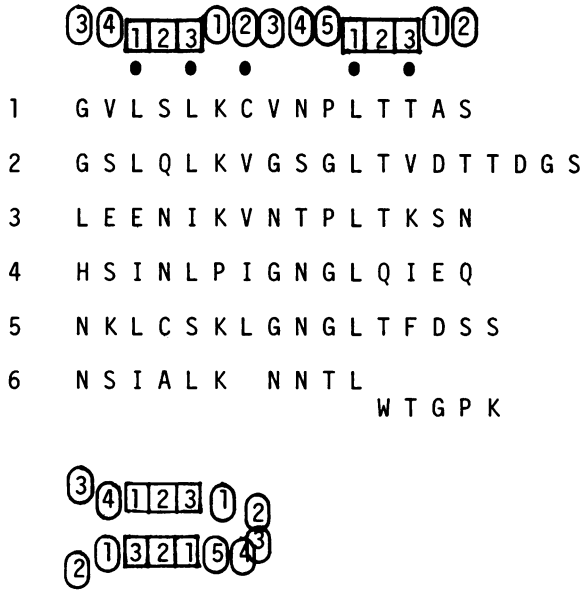

FIG. 6. Repeating segments from the shaft region of the Ad3 fiber protein. The structural patterns of two strands and two bends in each segment are shown. Closed circles designate the positions of hydrophobic amino acids. The segment number is shown on the left.

unknown, since a viable mutant which fails to glycosylate fibers at the nonpermissive temperature has been isolated (3). Three possible sites for glycosylation (Asn-X-Ser/Thr)

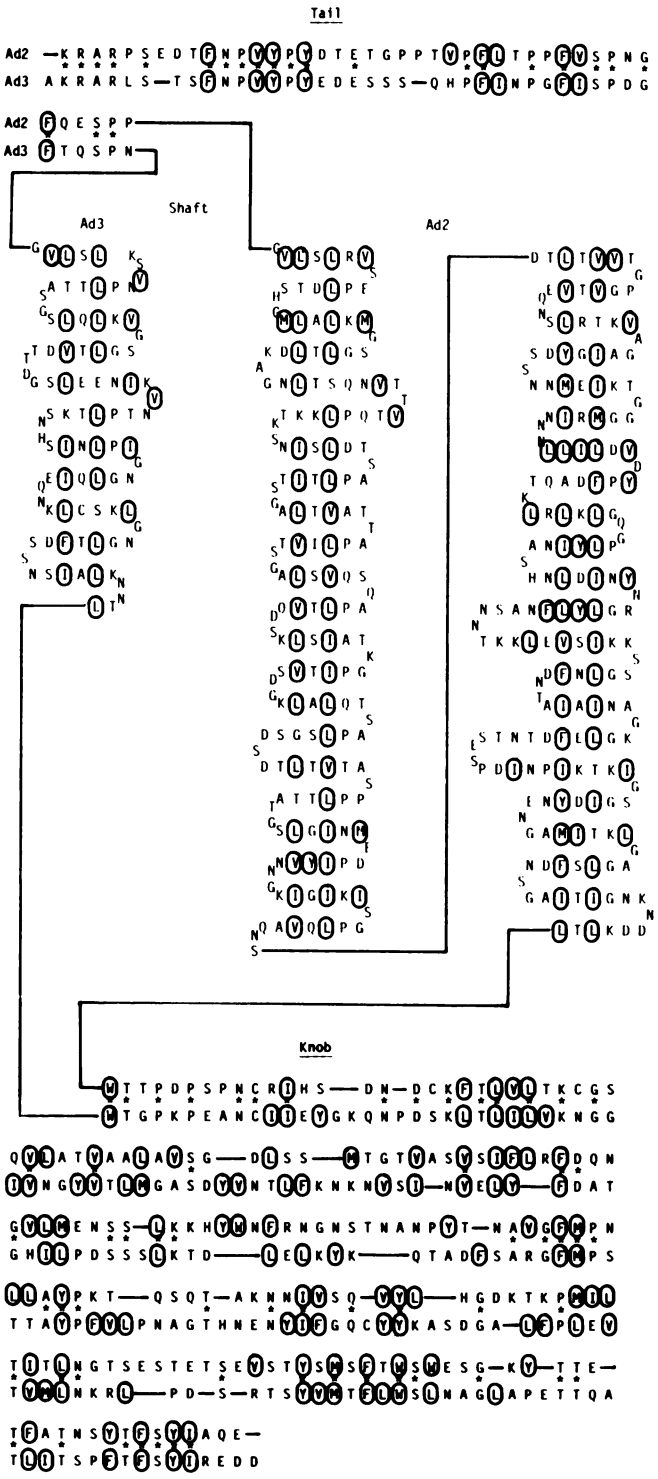

FIG. 7. Structural organization of the subunits of the Ad2 and Ad3 fiber proteins. Each subunit comprises a short N-terminal tail separated from a distal knob by a repetitive sequence which forms the shaft. All hydrophobic residues are indicated with circles. The part of the figure which illustrates the organization of the Ad2 fiber is taken from Green et al. (8).

were found in the sequence of the Ad3 fiber polypeptide (Fig. 2). None of these is, however, present in the Ad2 fiber polypeptide.

Recently Green et al. (8) demonstrated that the amino acid sequence of the Ad2 fiber contains a 15-residue motif, which is repeated 22 times. The repeated units form two alternating β-strands and β-bends and generate together an amphipathic β-sheet structure. Based on these results, a model was proposed in which the shaft of the native fiber is composed of parallel β-sheets which are stabilized by dimer formation.

An important finding from our sequence studies was that the 15-residue repeat unit present in the Ad2 fiber also is found in the primary sequence of the Ad3 fiber polypeptide (Fig. 6). However, in the Ad3 fiber only six repeat units appear to be present, thus making the shaft of the Ad3 fiber shorter than that of the Ad2 fiber. This finding was expected since the Ad3 fibers are much shorter than the Ad2 fibers, as revealed by electron microscopy (20). It is noteworthy that the deletions in most cases occur in multiples of repeat units, thus leaving the 15-residue repeat unit intact. The only exception is segment two (Fig. 7), in which a lysine residue in Ad2 has been changed to a hydrophobic amino acid (isoleucine) to maintain the general feature of the repeat unit.

As is evident from Fig. 7, the primary sequence of the Ad3 fiber is compatible with the fiber structure proposed by Green et al. (8). The tail and the knob are almost identical in size between the two serotypes, but the shaft has been shortened considerably in the Ad3 fiber. The model predicts that the total length of the Ad3 fiber is 10 to 11 nm, a value which is in good agreement with the previous electron microscopic measurements (20). The result furthermore lends additional support to a dimeric structure of the native fiber protein and suggests that the length of the native fiber is related to the length of the polypeptide and not to the number of polypeptide subunits.

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