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Genomic Arrangement of an Adenovirus-Simian Virus 40 Hybrid Virus, Ad2$^+$ND$_{4del}$

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Ad2$^+$ND$_{4del}$ is an adenovirus type 2-simian virus 40 hybrid virus nondefective for growth in human cells. The virus was first observed when stocks of Ad2$^+$ND$_4$, a hybrid isolated from primary monkey kidney cells, were propagated in human cells. This paper describes the DNA sequence at two sites of DNA recombination, the site of the left adenovirus type 2-simian virus 40 junction and the site of a deletion of internal simian virus 40 sequences. Since the deletion was observed when the virus was switched from monkey to human cells, an analysis of gene expression in the region of DNA rearrangement may prove useful for the elucidation of molecular events that accompany virus growth in different hosts.

Ad2$^+$ND$_4$ is a recombinant between human adenovirus type 2 (Ad2) and simian virus 40 (SV40) which originated and was plaque purified in cultures of primary monkey kidney cells (16, 17). The virus is nondefective (ND) for adenovirus function; i.e., it grows well in cell cultures of human origin, despite the fact that it lacks Ad2 DNA sequences between map positions 81 and 86. At the place of this deletion, the virus carries a portion of early SV40 DNA inserted in rightward polarity of transcription (10, 12, 20, 22). The expression of at least that part of the integrated SV40 DNA sequences corresponding to the C-terminal end of T antigen is thought to enable the virus to grow to high titers in simian cells (6, 8, 17). A portion of the integrated SV40 sequences is found deleted from some of the progeny of Ad2$^+$ND$_4$ grown in human cells (11, 21, 31). The resulting virus, Ad2$^+$ND$_{4del}$, has been plaque isolated from human cells (H. W. Gerry, A. M. Lewis, Jr., M. Johnson, and H. Westphal, unpublished data).

Expression of the integrated SV40 sequences is under Ad2 control. Human cells productively infected with Ad2$^+$ND$_4$ and Ad2$^+$ND$_{4del}$ contain a number of T-antigen-related polypeptides which are translation products of chimeric mRNA's. At least a majority of these RNAs initiate at the major *r*-strand promoter of Ad2 and terminate at the polyadenylate addition site within the integrated SV40 sequence, their diversity being generated by splice variations both in the Ad2 and in the SV40 portions of their sequences (11, 14, 18, 31). Most interestingly, the normal SV40 splice sites at positions 0.53 and 0.60 are contained in the sequence deleted from Ad2$^+$ND$_{4del}$, and cells infected with this virus present in the inoculum produce chimeric RNAs with a novel SV40 splice site (11, 31).

The Ad2$^+$ND virus system offers important insights into the molecular mechanisms that accompany virus growth in different hosts. It is in this context that I report here on the DNA structure of Ad2$^+$ND$_{4del}$.

MATERIALS AND METHODS

Ad2$^+$ND$_{4del}$ DNA was a gift of H. W. Gerry. A *Hin*dIII subclone in pBR322 was used for sequence analysis by the method of Maxam and Gilbert (19). Ad2 and SV40 DNA probes for Southern blotting (29) were prepared by nick translation (26). Hybridization of DNA in preparation for electron microscopy was described earlier (31).

RESULTS

The DNA structures of Ad2$^+$ND$_{4del}$ analyzed in this study were (i) the Ad2-SV40 junction near Ad2 map position 81 and (ii) the deletion of internal SV40 sequences. The Ad2 DNA sequences upstream from the SV40 insertion, the SV40 DNA sequence, and the size and position of the SV40 deletion are known (7, 9, 11, 21, 25, 31). This facilitated the selection of a restriction fragment that carried both structural features and was suitable for DNA sequence analysis. Figure 1 shows the *Hin*dIII digests of Ad2$^+$ND$_{4del}$ and of Ad2 DNA. The fragment labeled LJ, or left junction, contains sequences upstream from the SV40 insertion (i.e., left of SV40 in the conventional map) and the SV40 sequences flanking the deletion. AD2 *Hin*dIII fragment E, the fragment corresponding to the region of the SV40 DNA insertion in the Ad2$^+$ND viruses, was naturally missing from the

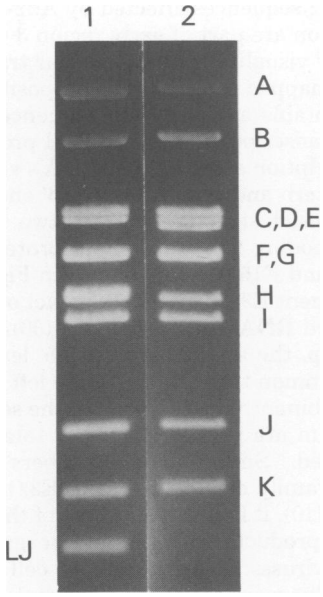

FIG. 1. *HindIII digest of Ad2+ND4del DNA (lane 1) and Ad2 DNA (lane 2). The fragments were separated by electrophoresis through a 1% agarose gel. The positions of Ad2 fragments A through K and of LJ, unique for Ad2+ND4del, are shown in the margins. Two smaller fragments, Ad2 L and a 526-base-pair fragment cut from the SV40 insert, are not included in this display.*

Ad2+ND4del digest. The band on top of fragment H in lane 1 corresponded in size to the right Ad2-SV40 junction, located downstream from LJ.

LJ was a chimeric fragment, consisting of Ad2 and SV40 sequences (Fig. 2). *Hin*dIII fragments of Ad2, SV40, and Ad2+ND4del migrating faster than fragment I were blotted onto two replica nitrocellulose filters and hybridized with either SV40 or Ad2 ^{32}P-labeled probe DNA. LJ hybridized with both probes. Besides LJ, the Ad2+ND4del *Hin*dIII digest contained one other SV40-specific fragment which comigrated with the 526-base-pair *Hin*dIII fragment D of SV40 and was cut from within the SV40 sequences present in Ad2+ND4del.

For further analysis, LJ was inserted into the plasmic vector pBR322 and amplified in *Escherichia coli*. The cloned LJ fragment hybridized with SV40 *Hin*dIII fragment B (Fig. 3). In the tracing, the dashed line indicates the Ad2 portion of the LJ sequence. The deletion of SV40 sequences bridged by LJ is seen as a loop.

In preparation for sequencing studies, two unique restriction sites within the LJ fragment were determined. LJ was cleaved once by *Hae*III and by *Hin*fI (data not shown). Fragments were 5′ end labeled at either the *Hae*III or the *Hin*fI site and were sequenced by the Maxam and Gilbert method. Figure 4 shows the portion of the sequencing gel containing the site of the SV40 deletion (panel 1) and the site of the Ad2-SV40 sequence junction (panel 2). These sequences were aligned with those of the parental viruses (Fig. 5). A deletion of 528 base pairs of SV40 DNA in Ad2+ND4del resulted in the linkage of nucleotides 4365 and 4893 or 4366 and 4894. The Ad2-SV40 junction took place between nucleotide 1863 of Ad2 *Eco*RI fragment D and nucleotide 4974 of SV40. No obvious homology exists in these regions of recombination. The sequence determined in this study does not deviate from corresponding Ad2 and SV40 sequences communicated by others (7, 9, 25). Based on published sequence information and the experiment of Fig. 4, I drew the structure of the LJ fragment (Fig. 6). The positions of the Ad2-SV40 junction (Ad2 81.3-SV40 0.626) and the deletion (SV40 0.611/0.509) were closely predicted by a previous examination of Ad2+ND4

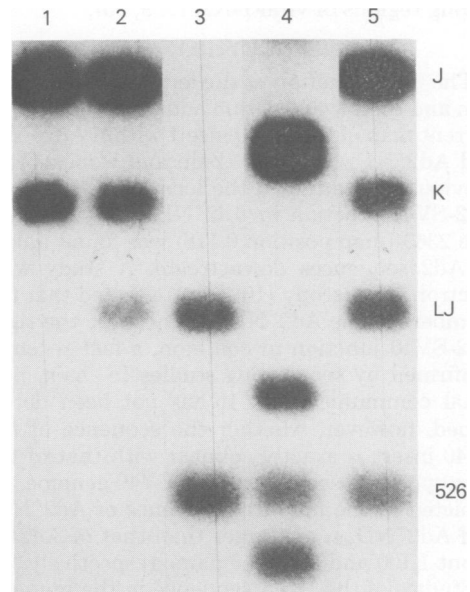

FIG. 2. *SV40 and Ad2 sequences of HindIII fragments identified by probe hybridization. An agarose gel (similar to that of Fig. 1) contained HindIII digests of Ad2 and Ad2+ND4del and a partial HindIII digest of SV40. Fragments migrating faster than Ad2 HindIII fragment I were blotted onto two replica nitrocellulose filters, hybridized to either an SV40 or an Ad2 ^{32}P-labeled DNA probe, and autoradiographed. Lane 1, Ad2 digest, Ad2 probe; lane 2, Ad2+ND4del digest, Ad2 probe; lane 3, Ad2+ND4del digest, SV40 probe; lane 4, SV40 digest, SV40 probe; lane 5, lanes 2 and 3 superimposed. The positions of Ad2 fragments J and K, of LJ, and of a 526-base-pair fragment of SV40 are indicated in the margin.*

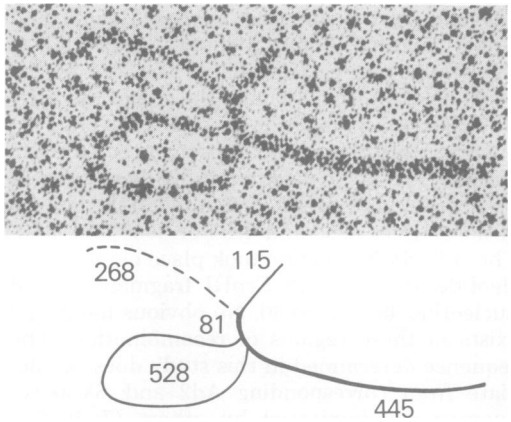

FIG. 3. *Electron micrograph of LJ hybridized to SV40 HindIII fragment B. In the tracing, distances are given in base pairs. The dashed line indicates Ad2; the solid lines indicate SV40 sequences. The hybrid portion is marked by a heavy line.*

and Ad2$^+$ND$_{4del}$ genomes in an electron microscope (20, 31). Both sites of recombination affect coding regions of viral DNA (7, 9, 25).

DISCUSSION

The exact location of the left Ad2-SV40 junction and the SV40 deletion adds precision to the current map of the SV40 insert within Ad2$^+$ND$_4$ and Ad2$^+$ND$_{4del}$ (Fig. 7). Zain and Roberts (33) previously determined the location of the right Ad2-SV40 junction in Ad2$^+$ND$_1$. SV40 nucleotide 2383 (map position 0.118) was found linked to Ad2 sequences downstream. A study with electron microscopy (10) has suggested that the members of the Ad2$^+$ND family share the right Ad2-SV40 junction in common, a fact recently confirmed by sequencing studies (S. Zain, personal communication). It has not been determined, however, whether the sequence of the SV40 insert is exactly colinear with that of the corresponding segment of the SV40 genome. As depicted in the map, the genomes of Ad2$^+$ND$_4$ and Ad2$^+$ND$_{4del}$ are larger than that of Ad2 by about 1,100 and 600 base pairs, respectively.

Studies of the DNA sequence in the immediate vicinity of the two recombination sites examined in this study reveal only chance homologies (Fig. 5). A computer analysis of these sequences failed to detect distinctive features, such as direct or inverted repeats or palindromes. The situation is similar to that described for the right Ad2-SV40 junction (33) and implies that signals other than sequence homology or prominent features of secondary structure have directed recombination at these sites. Attention is naturally focused on the genetic information encoded in the SV40 insert and the surrounding Ad2 sequences.

The Ad2 sequences affected by Ad2-SV40 recombination are part of early region 3 (E3). We previously visualized the E3 nuclear transcripts (13) and mapped their 5′ end near position 76.6, which contains a recognition sequence for the start of transcription (9). The final products of E3 transcription are a set of mRNA's varying in splice pattern and position of the 3′ end (4, 13). The proposed structures (9) of two of these mRNA's coding for two distinct protein moieties, E14 and E16 (23), are shown in Fig. 7. The more frequent E3/16 mRNA is in fact one of the first spliced RNAs ever visualized (30). As seen in the map, the sequences of the 5′ leader and intron, common to both RNAs, are left intact in the recombinant viruses, whereas the sequences downstream are partly (E3/16) or totally (E3/14) deleted. Since other members of the Ad2$^+$ND family delete the entire E3/16 coding sequence (10), it follows that none of the known E3 gene products is required for growth of Ad2$^+$ND viruses, at least in those cell cultures used for their propagation. The fact that E3 sequences are dispensable makes them naturally susceptible for deletions and substitutions of the type seen in the Ad2$^+$ND viruses.

The SV40 insert in Ad2$^+$ND$_4$ contains almost the entire early region, including the polyade-

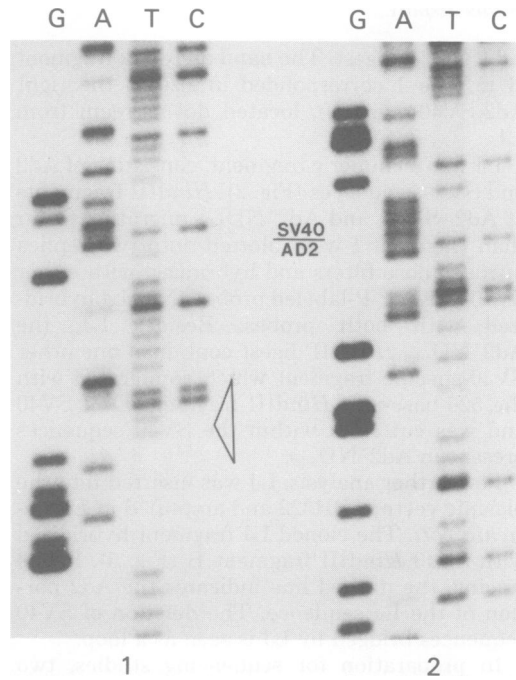

FIG. 4. *Ad2$^+$ND$_{4del}$ sequences at the site of the SV40 deletion (panel 1) and at the left Ad2-SV40 junction (panel 2). See text for details. Nucleotides were read from individual lanes as indicated on top.*

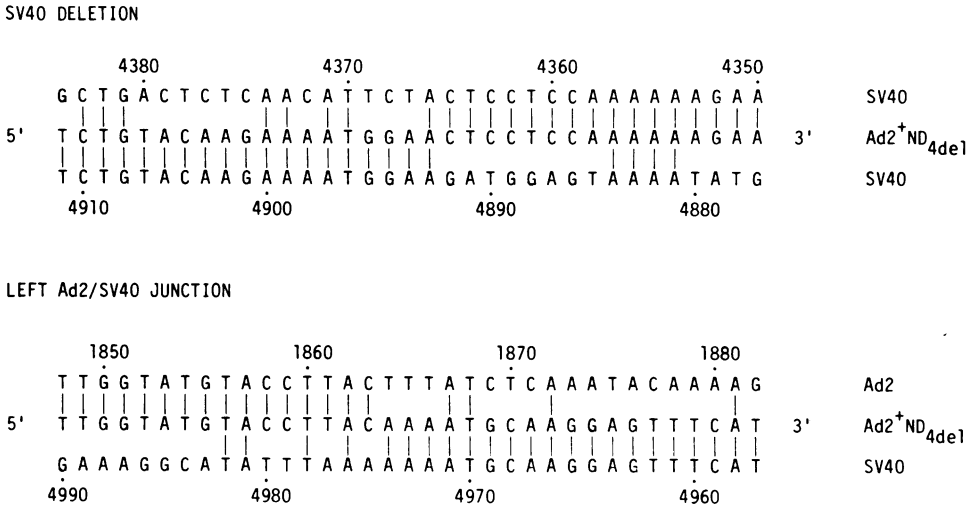

FIG. 5. *DNA sequence comparison of Ad2, SV40, and Ad2+ND4del in the immediate vicinity of the SV40 deletion and the left Ad2-SV40 junction. The sense strand is shown. Nucleotides are numbered as previously published (9, 25). Homologies are indicated by vertical lines.*

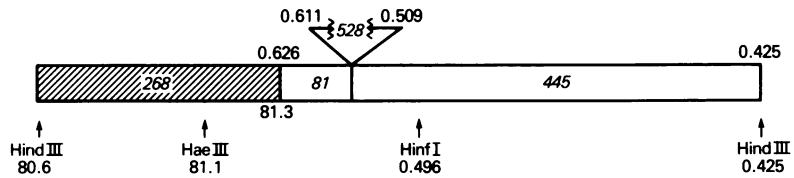

FIG. 6. *Structure of Ad2+ND4del HindIII fragment LJ. Ad2 sequences are shaded; SV40 sequences are white. Distances (italics) are in base pairs. Conventional notations are used for Ad2 (e.g., 80.6) and SV40 (e.g., 0.626) map positions.*

nylate addition site of the early RNAs. Only the leading end of the early DNA carrying the signals for DNA replication and for initiation of early RNA synthesis (7, 24, 25) is not present in the hybrid virus, and, as a result, the expression of the integrated SV40 sequences is firmly under Ad2 control. The structure of the two early SV40 mRNA's and the location of the t- and T-antigen codons (7, 24, 25) are shown in Fig. 7. In Ad2+ND4, we see Ad2 sequences encoding the N-terminal end of the E3/16 polypeptide linked to the truncated SV40 t- and T-antigen codons. The deletion-generating Ad2+ND4del encompasses the introns of both SV40 mRNA's plus some sequences on either side, leaving only 81 base pairs of the common leader plus the T-antigen codons downstream from position 0.51.

The expression of the SV40 insert has been most extensively studied in human cells at late stages of productive infection with a mixture of Ad2+ND4 and Ad2+ND4del. A number of mRNA's have been characterized by electron microscopy (31) or gel analysis (11). The proposed fine structure of some of these mRNA's is shown in the top of Fig. 7. Each of the chimeric RNAs starts with the familiar tripartite leader (map positions 16.6, 19.6, and 26.6) characteristic of the late Ad2 mRNA's (1, 5), followed by various combinations of short exons preceding the SV40 sequences (31). Of these short exons, the first is from position 75.0 to 75.6, outside the region shown in Fig. 7. The next two coincide with the X and Y leaders found in part of the fiber mRNA's (3) (see bottom of Fig. 7). Their exact map location is as proposed by Hérissé et al. (9), i.e., on the basis of sequence studies (9, 32–34) and of rules established for consensus sequences of splice donors and acceptors (2, 15, 27, 28). Note that the Y sequence also coincides with the second exon of the Ad2 E3/14 mRNA. From one or another of these exons, the RNAs are spliced to SV40 sequences. RNA A is the most frequently observed class of chimeric mRNA's. It is likely to be transcribed from Ad2+ND4del, and its splice point at SV40 map unit 0.46 was recently sequenced (R. Dhar, personal communication). The proposed structure of RNA classes B through E require confirmation by direct sequencing. I drew the splice point joining the leader segments with the body of the

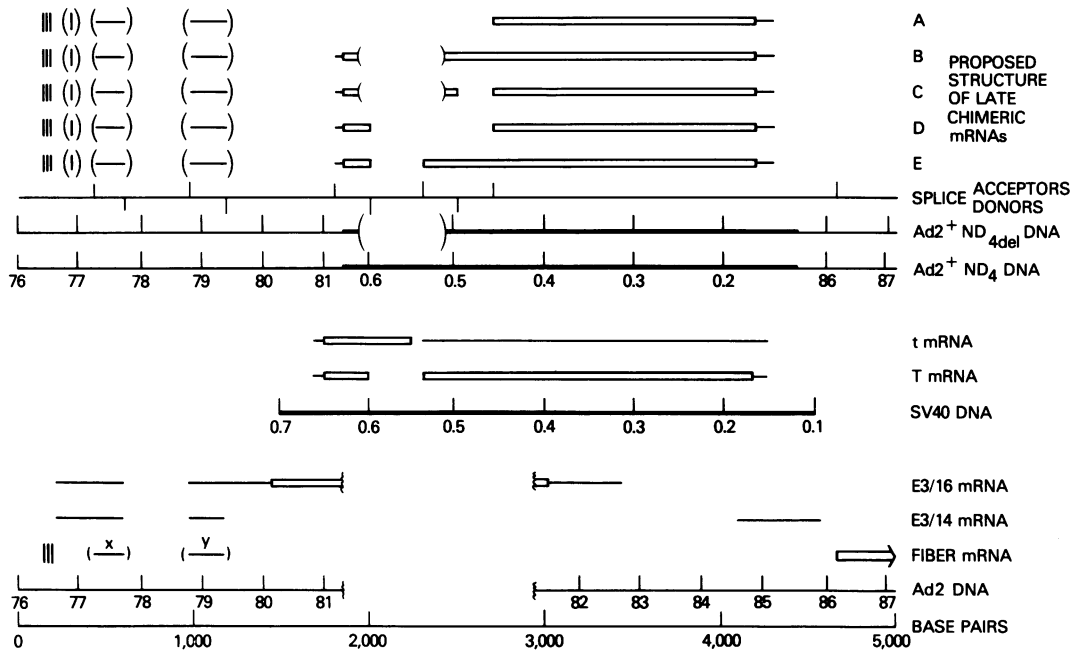

FIG. 7. *Map of DNA and mRNA's in the region of SV40 inserts in Ad2$^+$ND$_4$ and Ad2$^+$ND$_{4del}$. The DNA maps show the SV40 sequences as heavy lines. The Ad2 DNA map is interrupted at the site of the left Ad2-SV40 junction so that Ad2 sequences on either side of the SV40 insert line up. The size of the gap in the Ad2 DNA map indicates the net gain of DNA sequences present in Ad2$^+$ND$_4$. Transcription is in the rightward direction. All mRNA's are spliced, and the positions of exons are shown, with regions encoding known polypeptides boxed. Exons found in some, but not all, of the RNA molecules within one class of mRNA are in parentheses. Vertical lines mark exons from the regions of the Ad2 genome that are not included in the map shown here (see text).*

RNAs at a putative Ad2 acceptor sequence nearest the SV40 insert, but there are other possible acceptor sites to the left of this one. Downstream from this splice, RNA B is drawn as a colinear transcript of Ad2$^+$ND$_{4del}$. RNA C has a second splice, drawn here from a possible donor sequence near SV40 map position 0.49 to the established acceptor at 0.46. RNAs D and E are Ad2$^+$ND$_4$ transcripts likely to utilize the regular splice signals of early SV40 mRNA plus the acceptor signal at 0.46. All chimeric RNAs terminate at the polyadenylate addition site of early SV40 RNA.

The translation products of the various chimeric RNAs are T-antigen-related polypeptides. These proteins have a common C terminus, but their size varies as several internal AUG positions of the truncated T-antigen sequence appear to be used as initiation signals in individual mRNA's (14, 18). The possibility that some of the polypeptides may be chimeric cannot be excluded. Fused Ad2-SV40 translation products have been observed with the Ad2$^+$ND derivative Ad2$^+$ND$_1$ dp2 (6). Analysis of the Ad2$^+$ND$_{4del}$ sequence, however, excludes translation across either of the two recombination sites analyzed in this study because stop signals would block translation in all three frames. Sequences identical to the ones reported here have been established by using viral DNA from another stock of Ad2$^+$ND$_{4del}$ (S. Zain, personal communication), indicating that the sites of the Ad2-SV40 junction and of the SV40 deletion are genetically stable and that the conclusions presented here with regard to translation across the recombination sites are valid for more than one Ad2$^+$ND$_{4del}$ isolate. Not excluded is, of course, the fusion of Ad2 and SV40 coding sequences via RNA splicing. In the one example analyzed so far, namely, RNA A (Dhar, personal communication), the mRNA sequence again precluded translation of a chimeric polypeptide.

The deletion of SV40 splice sites and the selection of an acceptor not used by SV40 itself focus on the splicing mechanisms that control Ad2$^+$ND$_{4del}$ gene expression. Ad2$^+$ND$_4$ was originally isolated in primary monkey kidney cells (16, 17). When propagated in human cells, the majority of the genomes observed are of the Ad2$^+$ND$_{4del}$ type (11, 21, 31). Therefore, the dele-

tion appears to be favored in human cells. It is unlikely that the deletion is made to produce a functional part of T antigen, since the SV40 protein is not needed for the growth of the virus in its natural host. Rather, the synopsis of RNA structures in Fig. 7 may provide a rationale for deletion generation. The chimeric mRNA's and the fiber mRNA's have a strikingly similar leader composition, indicating common pathways of maturation. Is the location of SV40 splice sites immediately upstream from the fiber codons detrimental to fiber expression in human cells and is this one reason why these sites are removed? If so, why is fiber expression not impeded in monkey cells infected with Ad2+ND4? There is a clear need for a much more detailed analysis of the regulatory mechanisms that govern the growth of these hybrid viruses in the respective host cells.

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