

Replication of Mammalian Parvoviruses

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Cover: Particles of minute virus of mice, approximately 250 Å in diameter
(electron micrograph courtesy of Peter Tattersall).



DNA Structure of Incomplete Adeno-Associated-Virus Particles

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When lysates of cells infected with adeno-associated virus (AAV) and a helper adenovirus are banded to equilibrium in CsCl buoyant density gradients, a number of virus bands can be detected visually (Hoggan 1971). The major and minor infectious AAV components band at densities of 1.41 and 1.45 g/cm³, respectively. In the electron microscope, these virions appear to be "full" particles. Infected-cell lysates also contain a heterogeneous population of "light," noninfectious AAV particles which band at densities of less than 1.41 g/cm³. These light particles appear "empty" in the electron microscope and have been variously reported to contain either no DNA (Johnson et al. 1975) or DNA of less than genome length (Torikai et al. 1970).

We have examined the AAV DNA present in both "full" and "empty" particles. These studies revealed that all density classes of AAV virions appear to contain varying proportions of incomplete genomes. It is not clear whether there are any truly empty virions. Analysis of the physical and biological properties of these incomplete DNA molecules is a useful approach for studying the multiplication mechanism of AAV and the nature of its interaction with its helper virus or host cell.

We summarize here the physical properties of the incomplete AAV genomes. A more detailed description of their physical and biological

properties will be reported elsewhere (L. M. de la Maza, F. T. Jay, and B. J. Carter, manuscript in preparation).

MATERIALS AND METHODS

VIRAL DNA. AAV2 was grown in KB3 spinner cells with Ad2 as the helper (Carter et al. 1973), and DNA was prepared as described previously (de la Maza and Carter 1977).

CLEAVAGE WITH RESTRICTION ENZYMES AND ELECTROPHORESIS. Restriction-endonuclease-digestion reactions and electrophoresis of the digested DNA in polyacrylamide gels were carried out as described previously (de la Maza and Carter 1977).

SUCROSE GRADIENTS. Neutral and alkaline sucrose sedimentation have been described previously (Carter and Khoury 1975).

RESULTS

Fractionation of Virus Particles in CsCl Gradients

Virus particles present in lysates of KB cells infected with AAV2 and Ad2 were fractionated by equilibrium density centrifugation in CsCl. The regions containing visible bands at densities of 1.45, 1.41, 1.35, and 1.32 g/cm³ were individually pooled and centrifuged to equilibrium in CsCl two or three more times. Electron micrographs of negatively stained preparations showed that 95–98% of the AAV particles in the two heaviest bands appeared "full," whereas a large proportion of those present in the 1.35- and 1.32-g/cm³ bands were "empty." Full adenovirus was found in the 1.35 band, and amorphous aggregates probably corresponding to disrupted adenovirus particles were observed in the 1.32-g/cm³ band. When the areas between the visible bands were pooled and concentrated from large-volume preparations and then rebanded in CsCl, visible virus was observed, which indicated the presence of "incomplete" particles of different densities spread throughout the gradient.

Fractionation of Incomplete DNA from Purified Virions

DNA was released from virions by sedimentation in alkaline sucrose gradients, then reannealed in formamide and fractionated further in neutral sucrose gradients. When DNA was prepared from AAV virions of different densities, several classes of incomplete molecules were obtained (as summarized in Table 1). Specific classes of incomplete AAV DNA molecules were isolated in the following way: When virions from the

TABLE 1
Sedimentation Properties of Incomplete AAV-DNA Molecules

DNA	Density of virion ^a (g/cm ³)	Sedimentation coefficient and molecular weight of isolated DNA ^b			
		alkaline sucrose		neutral sucrose	
		S	m.w. × 10 ⁻⁶	S	m.w. × 10 ⁻⁶
Intact	1.41	15.5	1.4	14.5	2.8
Type I	1.41	15.5	1.4	11.5	1.4
Type II	1.41	12.0	0.8	10.0	0.8
Type III (oligomer)	1.35	8.5	0.3	8–15	~1.4
Type III	1.35	8.5	0.3	(1) 8.2 (2) 7.0	0.6 0.3
Type IV	1.32	5.5	~0.13	5.0	~0.13

^a The value listed indicates the density in a CsCl buoyant density gradient of the visible band of virus from which the DNA was obtained.

^b The molecular weights and sedimentation coefficients are computed from sucrose gradient profiles using the molecular-weight markers indicated in Fig. 1. The molecular weights and S values of these markers have been described previously (Carter and Khoury 1975; Carter et al. 1976). Intact DNA is the full-length infectious DNA of AAV2 which is the DNA component of the majority (90%) of the virions banding at a density of 1.4 g/cm³. The numbers in parentheses for the neutral sucrose components of type-III DNA refer to the peaks 1 and 2 indicated in Fig. 1e. The data for DNA types I, II, and III were calculated from Fig. 1. The data for type IV were from gradients to be described elsewhere (L. de la Maza, F. Jay, and B. Carter, manuscript in preparation).

1.41- or the 1.45-g/cm³ band of a CsCl gradient were sedimented in alkaline sucrose, about 90% of the DNA formed a single 15.5S peak and the remainder sedimented as a shoulder at about 12S. This 12S shoulder was pooled, neutralized, and reassociated, and is designated as incomplete DNA type II (see Table 1). The 15.5S AAV DNA component from the alkaline sucrose gradient was reassociated and sedimented in a neutral sucrose gradient to yield a major 14.5S component of intact AAV duplex. In the neutral sucrose gradient there was also a small amount of an 11.5S component, which was collected and designated as incomplete DNA type I (Table 1).

When the virions that banded in CsCl at a density of 1.35 g/cm³ were sedimented in alkaline sucrose, all of the AAV DNA sedimented at 8.5S. This DNA was pooled and reassociated, and was designated as incomplete DNA type III. All of the adenovirus DNA from the 1.35-g/cm³ virus band sedimented to the bottom of the alkaline sucrose gradient. Sedimentation of the reassociated type-III AAV DNA in neutral sucrose

resulted in a broad distribution of DNA from 8S to 15.5S. This peak was divided into leading and trailing edges and designated as type-III oligomers and type III, respectively.

Alkaline sedimentation of the AAV virus that had banded at 1.32 g/cm^3 in CsCl released a peak of DNA that sedimented at approximately 5S. This DNA component was reassociated and designated as DNA type IV.

It must be noted that the nomenclature of these incomplete AAV DNA preparations as listed in Table 1 is intended solely to facilitate discussion and does not indicate that these are the only size classes of incomplete molecules produced. There is in fact a continuous spectrum of AAV particles banding in a broad density range from 1.45 to 1.30 g/cm^3 .

Physical Properties of Incomplete AAV DNA

The physical properties and molecular weights of type-I, -II, and -III DNA molecules were examined by sedimentation in alkaline and neutral sucrose gradients (Fig. 1) and are summarized in Table 1. Type-I DNA

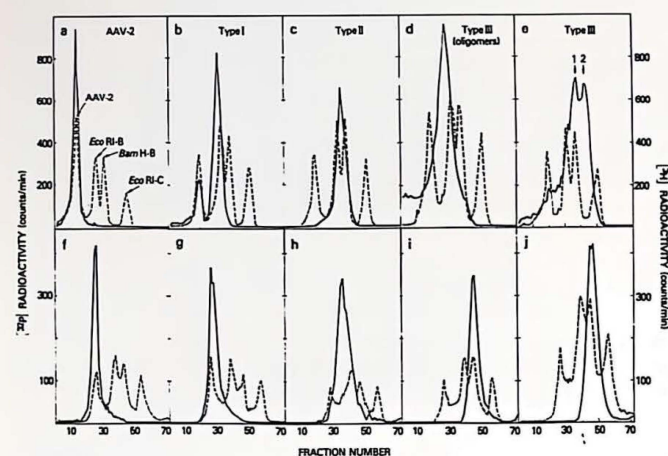


FIGURE 1

^{32}P -labeled DNA preparations were analyzed on neutral (a-e) or alkaline (f-j) sucrose gradients as described previously (Carter and Khoury 1975). Gradients contained ^{32}P -labeled AAV DNA (a, f), type-I incomplete DNA (b, g), type-II incomplete DNA (c, h), type-III oligomer fraction (d, i), or type-III incomplete DNA (e, j). All gradients contained as molecular-weight markers purified ^3H -labeled duplex monomer AAV2 DNA or restriction fragments EcoRI B (38% of genome), Bam B (22%), or EcoRI C (4.5%) as indicated in (a). Sedimentation is from right to left. —: ^{32}P -labeled DNA; ----: ^3H -labeled DNA.

sedimented in both neutral (Fig. 1b) and alkaline (Fig. 1g) sucrose gradients as a single component with a molecular weight of 1.4×10^6 . (However, type I is contaminated with some intact AAV DNA.) Similarly, the type-II DNA sedimented in both neutral (Fig. 1c) and alkaline (Fig. 1h) gradients as a single component with an apparent molecular weight of 0.8×10^6 . These properties indicate that both type-I and type-II DNA are "snapback" or "hairpin" structures.

The type-III DNA sedimented in alkaline sucrose with a mean molecular weight of 0.3×10^6 . In neutral sucrose the type-III DNA yielded linear duplexes of molecular weight 0.6×10^6 (peak 1, Fig. 1e), "snapback" duplexes of molecular weight 0.3×10^6 (peak 2, Fig. 1e), and also a variety of faster-sedimenting oligomeric structures (Fig. 1d,e). It is not clear whether the linear duplexes in peak 1 (Fig. 1e) are composed of individual strands which might form "snapback" hairpin structures if the annealing was done at lower concentration. The type-IV AAV-DNA molecules (data not shown) also reassociate to form a variety of structures analogous to those exhibited by type-III DNA.

Other experiments (L. de la Maza, F. Jay, and B. Carter, manuscript in preparation) have confirmed this interpretation of the data in Figure 1. After denaturation and rapid chilling, most DNA molecules of types I and II and about 20% of type III, but none of the complete AAV-DNA molecules, reassociate into structures resistant to digestion by S1 nuclease. The type-III oligomeric molecules appear to be joined largely by the "cohesive ends" of AAV DNA (de la Maza and Carter 1977; Berns, this volume) and can be converted to duplex molecules of molecular weight 0.6×10^6 by melting at 80°C in 0.165 M NaCl (Carter and Khoury 1975). Hybridization experiments and analysis of reassociation kinetics have shown that nearly all of the incomplete AAV DNA is AAV-specific.

Restriction-Endonuclease Cleavage of Incomplete Genomes

The DNA preparations used for the sedimentation analysis (Fig. 1) were cleaved with the restriction endonuclease *Hha*I and electrophoresed in an acrylamide slab gel. The densitometer scans of the gel autoradiogram are shown in Figure 2. Additional *Hha* digests of incomplete DNA preparations are shown in the gel autoradiograms of Figure 3. The sizes of the uncleaved incomplete DNA molecules estimated by gel electrophoresis agree with those derived from neutral sucrose gradients. Cleavage with *Hha* clearly shows that as the sizes of the incomplete genomes decrease there is a progressive enrichment for the terminal regions of the AAV genome and a progressive loss of the internal regions. For example, all the *Hha* fragments from B to F are present in a low molar ratio in type-II DNA and are not seen at all in type III or IV. The fragments H, K*, and K, which map near the ends of the genome and contain the inverted repeated

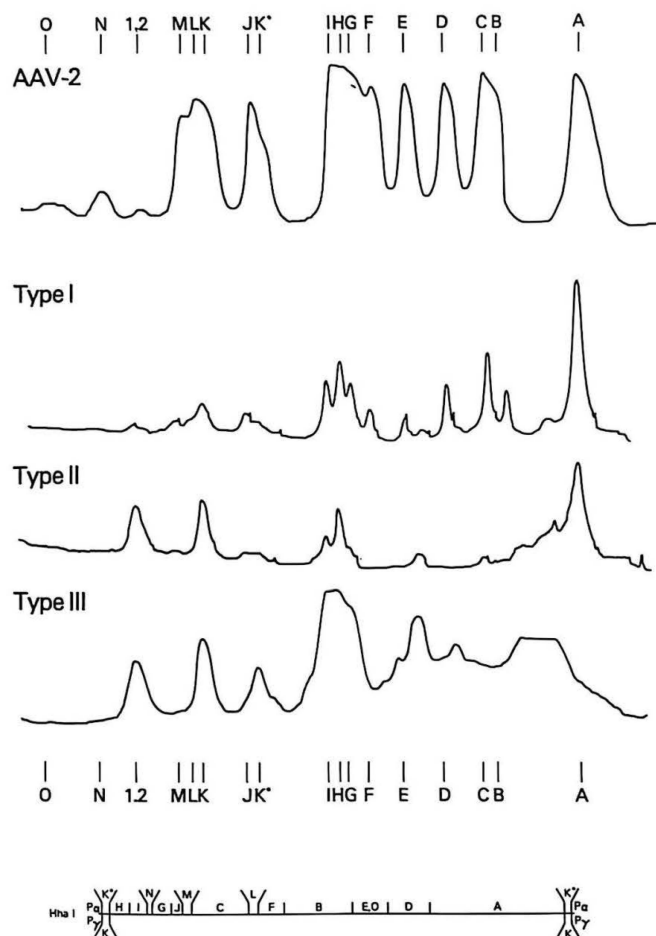


FIGURE 2

Restriction-endonuclease cleavage of incomplete AAV genomes. Samples of the ^{32}P -labeled DNA preparations that were analyzed in the sucrose gradients shown in Fig. 1 were also digested with restriction endonuclease *Hha*I. The digests were electrophoresed in an acrylamide slab gel and the gel was subsequently autoradiographed using X-ray film. The autoradiogram was then scanned in a Beckman densitometer. The peaks corresponding to individual *Hha* fragments are indicated by the letters at top and bottom. The terminal *Hha* fragment P_aP_y migrated off the gel. The map of the *Hha* cleavage sites on AAV DNA is shown at the bottom.

INCOMPLETE

H H A ~ I

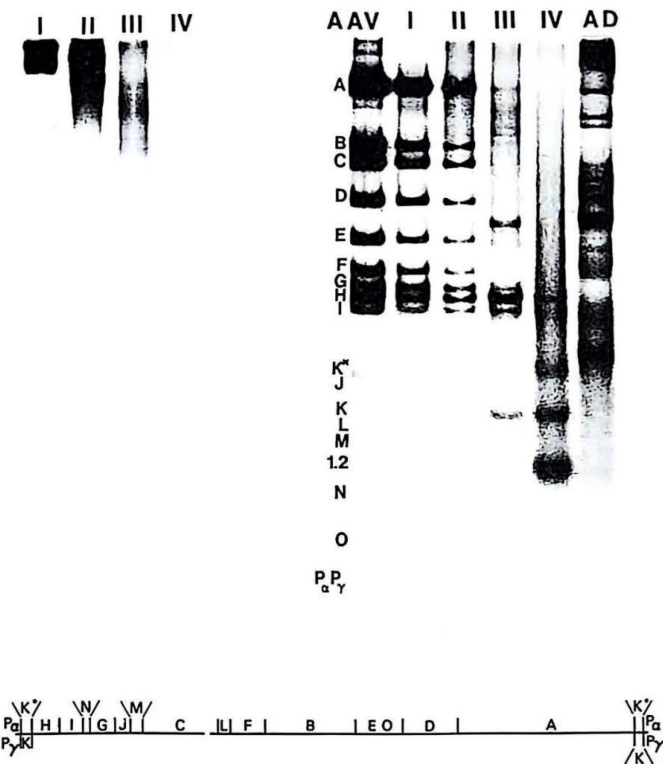


FIGURE 3

Cleavage of incomplete AAV genomes with restriction endonuclease *Hha*I. ^{32}P -labeled DNA was electrophoresed in acrylamide slab gels and autoradiographed. The tracks on the left gel contain uncleaved, incomplete genomes of types I-IV, as indicated. The predominant component of type-IV DNA migrated at the same rate as the *Hha* J fragment (about 2.5% of the genome), and some additional, more heterogeneous material migrated at the rate of *Hha* fragments D-H (9-4.3% of the genome). The tracks in the right gel contain *Hha* digests of the ^{32}P -labeled DNA preparations indicated. The *Hha* cleavage map of AAV2 DNA is shown at the bottom. The fragment designated 1.2 in the right gel is discussed in the text.

sequences of AAV-DNA, are present in all the virions. Thus, type-IV DNA contains little other than the inverted terminal repetitions which comprise 147 nucleotides at each terminus (de la Maza and Carter 1977; Berns, this volume).

The terminal *Hha* fragments P_a and P_r are present in all the incomplete AAV-DNA preparations, but their enrichment is significantly less than for H, K^* , and K (see, e.g., Fig. 4, track IV, right panel). Concomitant with the lesser enrichment for P_a and P_r is the appearance in progressively larger molar amounts of a new 1.2 fragment (equivalent in size to 1.2% of the AAV genome). This is seen in both the densitometer scans (Fig. 2) and the autoradiogram (Fig. 3). The 1.2 fragment is not seen in cleavages of complete AAV2 DNA, but in the smallest molecules (such as type IV) this fragment has the highest molar concentration. This suggests that the incomplete DNA, although preferentially enriched for the ends of the AAV genome, may have a rearrangement of the region at or very close to the terminus within the inverted terminal repeat.

Cleavage with a variety of other restriction enzymes (see, e.g., Fig. 4) showed that the enrichment is for left or right terminal regions of the

genome. These digestions by enzymes such as *Hae*II or *Kpn*I that do not cleave within the inverted repetition also indicate that there is a rearrangement of the normal termini in the incomplete genomes as indicated for type-I and type-II DNA in Figure 4. In the *Hae*II digest (Fig. 4) the left terminal doublet component C_a is clearly reduced in amount and a new fragment appears which is about 50–100 nucleotides shorter than C_r . A similar phenomenon is seen in the right end for *Kpn*I C_a and C_r . This appears to correspond to the relatively greater loss of the *Hha* P_a and P_r fragments and the appearance of the *Hha* 1.2 fragment (Figs. 2 and 3).

DISCUSSION

The DNA from incomplete AAV particles that arises during the growth of this virus in the presence of helper adenovirus has been analyzed. CsCl density gradients, alkaline and neutral sucrose gradients, and restriction-endonuclease cleavage show that these molecules vary in length and genome-sequence representation. The largest molecules analyzed (type I) have a molecular weight of 1.4×10^6 in both alkaline and neutral sucrose gradients and appear to be hairpin duplexes. Restriction-endonuclease cleavage indicates that both the right and left ends of the molecule are present and that only 10–20% of the internal portion of the genome is missing from this population of molecules. Individual molecules therefore appear to be hairpin duplexes containing either the left or the right terminal 30–50% of the genome. The type-II molecules have a similar structure but contain only 20–30% of the left or the right terminal region. Type-III molecules have a more heterogeneous structural configuration. These molecules contain only the left or right 10–20% of the genome and form either hairpin molecules or non-cross-linked duplexes. Some of these molecules may form oligomeric species held by the "cohesive ends" of the AAV-DNA termini (Gerry et al. 1973; Carter and Khoury 1975; de la Maza and Carter 1977; Berns, this volume).

Analyses of the restriction cleavage maps of the incomplete DNA molecules suggest that they may have a rearrangement or a deletion involving 50–100 nucleotides within the inverted repetitious terminal sequence. For instance, cleavage with *Hae*II or *Kpn*I (Fig. 4) gave rise to a fragment that was about 50–100 nucleotides smaller than the terminal doublet obtained from the complete genome. The relative amount of this new component was inversely related to the amount of the a component of the regular terminal doublet, and the molar amount of this new fragment increased as the size of the incomplete particles decreased. This phenomenon was also reflected by the new 1.2% fragment present in the *Hha*I cleavage, which may arise by rearrangement or deletion of the two

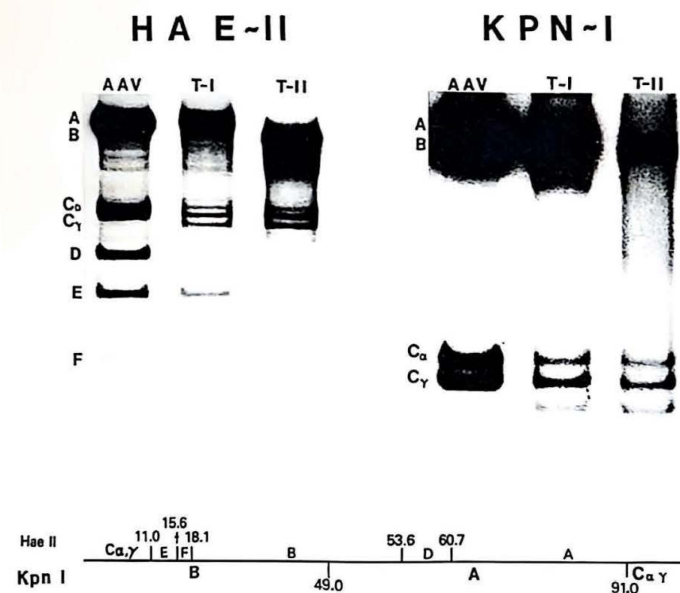


FIGURE 4
Electrophoresis of *Hae*II and *Kpn*I digests of AAV2, type-I, and type-II DNA. The *Hae*II and *Kpn*I cleavage maps for AAV2 DNA are shown.

regular terminal fragments P_a and P_r and part of K or K^* . It should be noted that it has not been proved rigorously that this 1.2% fragment is actually AAV DNA and the possibility remains that it might be adenovirus-specific or cell-specific.

The structural configuration of the incomplete AAV-DNA molecules suggests that they arise during replication, since the regions enriched for include the inverted repetitions which are believed to contain the replication origin (Hauswirth and Berns, this volume).

Incomplete AAV2 genomes with properties similar to the encapsidated molecules described here have been detected by pulse-labeling infected cells with [^{3} H]thymidine and analyzing the AAV DNA in the Hirt supernatant (Hauswirth and Berns, this volume; B. Carter and L. de la Maza, unpublished). These studies show clearly that the incomplete genomes arise directly by replication rather than by subsequent degradation or recombination. The incomplete AAV genomes contained in the Hirt supernatant also yielded the 1.2 fragment when cleaved with *Hha* (B. Carter and L. de la Maza, unpublished).

We do not yet know whether the hairpin or cross-link in the incomplete DNA corresponds to an "internal" or an "external" region of the AAV genome. Several possibilities that can be suggested for the origin of these incomplete molecules are illustrated in Figure 5. The scheme for normal replication of AAV is based on those described for self-primed 3' hairpin replication (Cavalier-Smith 1974; Straus et al. 1976; Tattersall and Ward 1976). In this scheme, any nicks that occur on the template strand result in production of an incomplete molecule with an "external" hairpin or cross-link. Two models would at least give rise to "internal hairpins." In one, the strand being displaced would be nicked and the 3' end generated would fold back via some "illegitimate" base pairing and provide a primer for DNA replication. This model was proposed for adenovirus incomplete DNA molecules (Daniell 1976). Alternatively, any event that causes the DNA polymerase to turn the fork at the growing point and proceed with synthesis along the displaced strand provides the possibility to excise an internal hairpin molecule. This type of model has been proposed for inverted duplications of λ dv and polyoma molecules (Chow et al. 1974; Robberson and Fried 1974).

The apparent structural alteration at the termini of the incomplete genomes as exemplified by the 1.2 fragment in the *Hha* cleavages may be explained by a decrease in length relative to the complete duplex DNA. This might arise either by failure to duplicate the terminal palindrome or by its complete loss, as illustrated in Figure 5 for the external-hairpin model. An analogous process could occur for the open end of an internal hairpin molecule. This suggests that the incomplete genomes

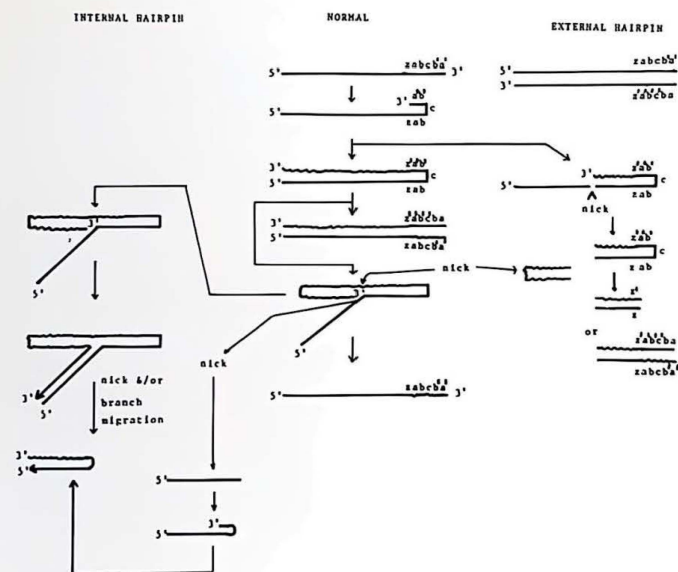


FIGURE 5

Possible models for derivation of incomplete genomes of AAV during replication. The models are explained in the text.

may have an altered or inoperative replication origin. Consistent with this model are preliminary experiments which indicate that type-III molecules cannot replicate and do not interfere with either AAV- or adenovirus-DNA replication (B. Carter and L. de la Maza, unpublished).

The density of the incomplete virus particles appears to be correlated with the size of the incomplete DNA, which suggests that each particle contains only one DNA molecule. This implies that packaging of AAV DNA does not occur by a "headful" mechanism. It also implies that virus assembly occurs by encapsidation of DNA into preformed capsids, since any model for crystallization of viral capsids around the DNA might be expected to yield particles with aberrant morphology with smaller DNA. It was noted by Daniell (1976) that the DNA strands in the hairpin molecules could have a total length up to twice that of a normal genome. We have observed no encapsidated AAV-DNA hairpin strand longer than a normal AAV molecule. This suggests that 1.4×10^6 daltons of DNA is the most that can be packaged in an AAV particle.

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