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Electron Microscopy of Late Adenovirus Type 2 mRNA Hybridized to Double-Stranded Viral DNA

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R loops were generated with late adenovirus type 2 (Ad2) mRNA in double-stranded viral DNA, and visualized by electron microscopy. Unpaired DNA sequences in Ad2:Ad2$^+$ND$_4$ heteroduplex DNA served as a visual marker for the orientation of R loops with respect to the conventional DNA map. The most abundant classes of late Ad2 mRNA observed by this technique hybridized, in order of R-loop frequency, with midpoints near positions 0.57, 0.88, 0.77, and 0.40 to 0.50 of the DNA map. The R loop at position 0.57 corresponded to a region containing the hexon gene; the one at position 0.88 corresponded to a region containing the fiber gene. The relative frequencies of these two R loops paralleled those of the encoded gene products. The mRNA sizes, calculated from those of the respective R loops, were slightly larger than needed to code for these polypeptides. Using the R-loop technique, two locations at which adjacent mRNA's hybridized to different strands were accurately mapped at positions 0.61 and 0.91 of the DNA. The map positions of late Ad2 mRNA correlated well to published RNA and protein maps.

The first viral mRNA molecules appear in cytoplasmic polysomes shortly after lytic infection of human cells with adenovirus type 2 (Ad2) (17, 18, 26). Early RNA gradually accumulates throughout the infection (34). With the onset of viral DNA replication, a sudden burst of late RNA synthesis occurs which far outweighs early RNA synthesis and replaces most or all host RNA production (13). Early and late RNAs hybridize with distinct regions of the h and l strand of viral DNA (29, 31).

Individual classes of viral mRNA have been fractionated according to size (17, 26) and informational content (1, 37). Moreover, using preparative hybridization (11), mRNA's coding for individual virus polypeptides have been assigned to distinct segments of viral DNA generated by restriction endonuclease action (2, 15, 16). The relative frequencies of individual classes of viral RNA produced during lytic infection have yet to be determined.

Recently, loop structures that contain RNA displacing homologous sequences within double-stranded DNA have been observed in the electron microscope (4, 12, 25), and with the development of the R-loop technique, any RNA hybridizing in vitro to a particular region of the double-stranded DNA may be visualized (33; White and Hogness, personal communication). We were able to show that a physical map of mRNA locations along the Ad2 DNA contour can be obtained by this technique (38). In this report, we describe the details of R-loop formation by late Ad2 mRNA and show histograms of R loops properly aligned with respect to the conventional Ad2 DNA map. These histograms indicate the map positions of individual classes of late Ad2 mRNA and the relative frequencies of R-loop formation. In addition, we demonstrate that points at which the polarity of adjacent mRNA's are reversed by a strand switch can be mapped accurately by the R-loop technique. These results confirm the mapping data obtained by alternate methods and also make this technique applicable to other genetic systems where biochemical mapping analyses are not yet available. The locations of R loops generated with early mRNA will be discussed in an accompanying paper (24).

MATERIALS AND METHODS

Cells and virus. Suspension cultures of KB cells (North American Biologicals Co.) were propagated in Eagle Spinner medium (9) supplemented with 5% (vol/vol) horse serum. The preparation of adenovi-

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rus type 2 stocks was a modification of the method of Maizel et al. (19). Infected cells were disrupted by freezing and thawing three times in an equal volume of 0.01 M Tris-hydrochloride (pH 7.9), followed by sonic oscillation. Cell debris was removed by centrifugation, and the supernatant containing the virus was diluted with 1 volume of 0.01 M Tris-hydrochloride (pH 7.9) and extracted with 1 volume of Freon in a Sorvall Omnimixer (full power for 2 min on ice). The phases were separated by low-speed centrifugation, and the aqueous layer was subjected to a second centrifugation at 12,000 $\times$ g_{avg} for 10 min at 4°C. The supernatant was layered over 6 ml of 1.39 g of CsCl per ml, 0.5 M Tris-hydrochloride (pH 7.9) in cellulose nitrate tubes, and centrifuged at 26,000 rpm for 2 h at 4°C in an SW27 rotor. The viruses were banded once more in 1.39 g of CsCl per ml, 0.5 M Tris-hydrochloride (pH 7.9) at 37,000 rpm for 16 h at 4°C in a Spinco SW50 rotor. The virus band was removed and diluted to 1 A_{260} (absorbancy at 260 nm) unit/ml with 0.14 M NaCl, 0.1% (wt/vol) bovine serum albumin (BSA), 0.01 M Tris-hydrochloride (pH 7.5), and then sterilized by filtration through a Nalge 0.2-μm filter and stored at 2 to 4°C. The strain of adenovirus type 2, originally obtained from J. A. Rose (NIH, Bethesda, Md.) was free of adeno-associated virus.

KB cells in Spinner culture were grown to a density of 4 $\times$ 10^5 cells/ml, concentrated 10-fold in serum-free medium, and infected with 10,000 particles (100 to 200 PFU) per cell. The viruses were allowed to adsorb for 1 h at 37°C with stirring. The cells were then sedimented and resuspended in fresh medium at 4 $\times$ 10^5 cells/ml. The time of resuspension was taken as time zero, and the infected cells were thereafter incubated at 37°C. Mock-infected cultures were prepared by the same method except that 0.14 M NaCl, 0.1% (wt/vol) BSA, 0.01 M Tris-hydrochloride (pH 7.5) was added in place of the virus stock.

Nucleic acids. Ad2 DNA was isolated from purified virus as described by Pettersson and Sambrook (28). Late poly(A)-containing mRNA used in all experiments (except those described in Fig. 3 and 4) was derived from cytoplasmic fractions of cells harvested 24 h after infection. Preparation of this RNA followed essentially a previously published procedure (10), except that the cytoplasmic supernatant was directly diluted five-fold in sodium dodecyl sulfate-Pronase buffer, thus sidestepping polysome isolation.

R-loop formation. Native DNA (10 μg/ml) and RNA (100 μg/ml) in 5 to 10 μl of R-loop buffer (70% [vol/vol] formamide [analytical grade], 0.5 M NaCl, 0.01 M EDTA, 0.1 M *N*-[tris(hydroxymethyl)-methyl]glycine ([Tricine]NaOH, pH 8)) were sealed in disposable 25-μl micropipettes and incubated at 52°C.

Electron microscopy. The hybridization mixtures were diluted 1:10 to 1:30 into R-loop buffer at 52°C, cytochrome *c* was added to 100 μg/ml, and samples were spread onto a hypophase of 10% (vol/vol) formamide, 0.1 mM EDTA, 0.01 M Tris-hydrochloride (pH 8.5), or onto deionized water. Parlodion-coated microscope grids were touched to the cytochrome film, stained with uranyl acetate (6), and rotary-shadowed with platinum-palladium. Pictures were taken in a Philips 300 electron microscope at 40 kV and 4,000- to 10,000-fold magnification. The magnification was calibrated with a carbon replica of a diffraction grating (E. Fulham no. 1002X). In many preparations SV40 DNA and ϕX174 DNA were included as internal length standards for double-stranded and single-stranded DNA.

Measurement and analysis of molecules. Molecules were photographed, measured from 20-fold-enlarged negatives with a graphics calculator (Numonics), and displayed initially in terms of their lengths in micrometers. Length measurements of full-sized molecules fell within the range reported previously for Ad2 DNA (38). Measurements began at the end of a duplex molecule and followed the double-stranded contour within R-loop regions. The ability to recognize differences in strand thickness along with an evaluation of the angles of intersection were used to trace molecules that displayed "crossovers" or partial entanglement during spreading.

The mean length of DNA molecules carrying R loops was not significantly different from that of DNA molecules without R loops. Thus, the linear density of an RNA:DNA hybrid was close to that of a DNA duplex under our experimental conditions. However, the linear density of single-stranded DNA within R loops varied considerably. This was concluded from the fact that the length ratio of single-stranded to double-stranded branches of the R loops was 0.97 $\pm$ 0.21, for 60 R loops longer than 0.5 μm.

In some instances, small, variable tails were observed at the ends of loop regions. Although it might be argued that these were unduplexed RNA segments, including polyadenylate sequences, we could not reliably orient or measure them. The spreading conditions did not result in good extension of unduplexed RNA, as evidenced by the partially collapsed RNA in the background of Fig. 2, 3, and 7, which often was superpositioned on extended DNA molecules. Therefore, tails were not included in the R-loop measurements presented here. Obviously fragmented and immeasurably entangled molecules were not photographed. They constituted only a minor fraction (<5%) of the molecules in any one spreading.

Galleries of R-loop patterns (such as those shown in Fig. 4 and 6) were redrawn using a Hewlett-Packard programmable calculator to normalize each molecule to its own length. Orientations were selected subjectively by the operators, and final patterns and histograms were thus produced. The "position" of an R loop is generally designated as the fractional genome length estimated by eye to be the midpoint of a given histogram peak.

RESULTS

Visualization and orientation of R loops on Ad2 DNA. An RNA molecule which is complementary to a region within partially melted, double-stranded DNA can anneal to the antiparallel region of that DNA, thereby displacing the homologous DNA sequence and forming an

R loop (White and Hogness, personal communication). Optimal kinetics of this reaction were obtained at a temperature near the T_m of the double-stranded DNA within the R-loop region (33). We determined the melting temperature of Ad2 DNA under our reaction conditions (Fig. 1), and chose the temperature of 52°C, slightly below T_m, for all hybridizations shown. Incubation of Ad2 DNA with late cytoplasmic mRNA resulted in the formation of a number of R loops at various locations along the DNA, as exemplified in Fig. 2. Each R loop contained a double-stranded branch (thick contour) and a single-stranded branch (thin contour). Most RNA molecules which did not participate in the hybridization reaction displayed a highly condensed structure seen in the background of the electron micrograph.

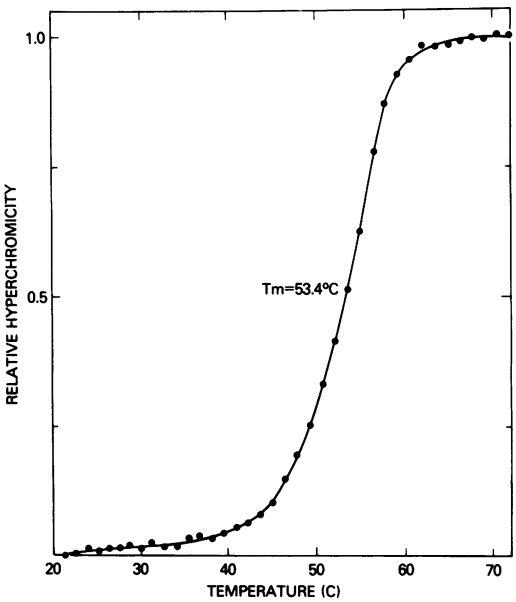

FIG. 1. *Melting profile of Ad2 DNA (20 μg/ml) in R-loop buffer. Hyperchromicity was monitored automatically in a Beckman Acta III spectrophotometer. The wave length of 270 nm was chosen to minimize UV light absorption by impurities contained in the formamide buffer. The cell was heated at a constant rate of 1°C per 1.5 min. Total increment of absorption was 0.20 OD_{270} per ml.*

Occasionally, we observed small, irregular tails protruding from the forks of R loops (Fig. 2). Most of these appeared to represent partially displaced RNA. Displacement of RNA by reannealing DNA seemed to occur while preparations were spread for electron microscopy, for we were able to reduce significantly the number and length of tails by keeping the samples in R-loop buffer at 52°C until the moment of spreading. Tails of displaced RNA have not been included in our length measurements. Therefore, mRNA molecules may actually be somewhat longer than indicated by the R-loop size. Some tails, most of them probably too small to be visualized directly, are likely to represent the poly(A) moieties attached to the 3′ ends (30) of Ad2 mRNA's. It will be interesting to see whether tails of this type, which would indicate the direction of transcription, may be visualized with the help of electron optical markers (3).

To orient the molecules with respect to the conventional Ad2 DNA map, we used Ad2:Ad2$^+$ND$_4$ heteroduplex DNA as a visual marker. Ad2$^+$ND$_4$ is a nondefective Ad2:SV40 hybrid virus belonging to a group of closely related particles which contain various

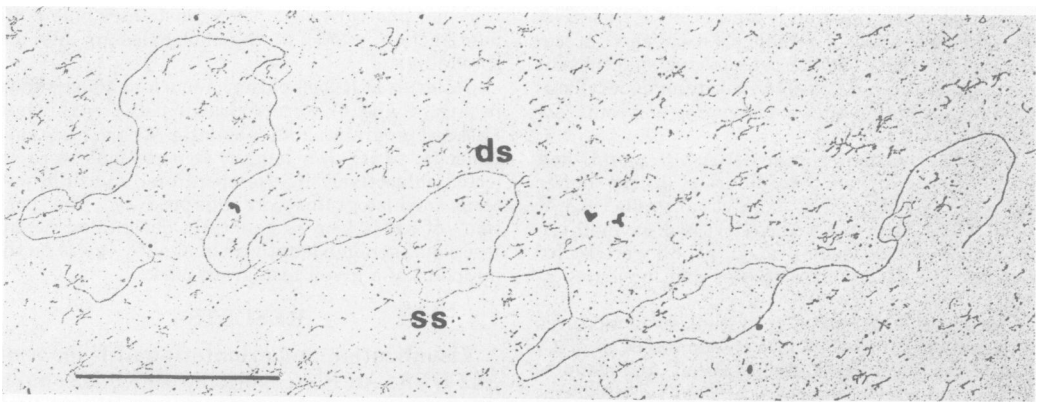

FIG. 2. *Electron micrograph of an Ad2 DNA molecule carrying seven R loops. The molecule was contained in the hybridization assay of Fig. 6, panel 3. R loops were generated by late Ad2 mRNA. RNA molecules which did not participate in the hybridization reaction are seen in the background. The bar in this electron micrograph indicates 1 μm. Abbreviations: ds, double-stranded nucleic acid; ss. single-stranded nucleic acid.*

amounts of SV40 DNA at the site of a deletion of about 4% of the Ad2 DNA sequences (14), located near the right-hand end (23) of the Ad2 genome. Heteroduplexes between Ad2 DNA and $Ad2^{+}ND_4$ DNA thus displayed a DNA loop carrying SV40 DNA sequences in one branch and Ad2 DNA sequences in the other (14). Since a DNA loop of this type contained only single-stranded DNA, it was easily distinguished from an R loop. The DNA loop had a thin contour in each strand, the R loop had a thin contour in one and a thick contour in the other strand. We hybridized heteroduplex $Ad2:Ad2^{+}ND_4$ DNA with an mRNA fraction which produced predominantly the most frequently observed R loop made by total Ad2 late mRNA (see legend to Fig. 3 for details), and obtained electron micrographs of the type shown in Fig. 3. Both the DNA loop and the R loop were located toward the same end of the DNA; their map positions are defined in the histogram of Fig. 4 which represents a larger number of molecules of this type. Based on this histogram, along with published genetic (21) and biochemical (2, 15) analyses, we will refer to the R loop located near position 0.57 of the DNA map as the "hexon" loop.

Kinetics of R-loop formation. The R-loop technique allowed us to examine the kinetics of hybrid formation of individual classes of late Ad2 mRNA with Ad2 DNA. When total cytoplasmic mRNA was incubated with Ad2 DNA under the standard conditions defined in Materials and Methods, hybridization of RNA with DNA, monitored by the percentage of each DNA molecule contained in R loops, was directly proportional to the logarithm of hybridization time (Fig. 5). However, when the number of R loops per molecule was plotted as a function of time, there appeared to be a disproportionate increase in number at longer times. This was probably due to fragmentation of RNA molecules. "Hexon" R loops were observed most frequently in these samples and constituted a convenient means of orienting the molecules with respect to the conventional Ad2 DNA map. Histograms depicting R-loop distribution after various times of hybridization are shown in Fig. 6. R loops appeared in certain regions of the DNA, notably (in order of frequency) near positions 0.57, 0.88, 0.77, 0.40 to 0.50, and 0.05 to 0.15, the latter corresponding in part to early Ad2 mRNA (24) present late in infection (5, 18, 34). Judged by their relative frequencies, R loops at position 0.57 and 0.88 seemed to appear with comparable rates. However, R-loop formation in the other three regions was delayed and appeared to follow different kinetics. We will comment on this observation in the discussion of our results.

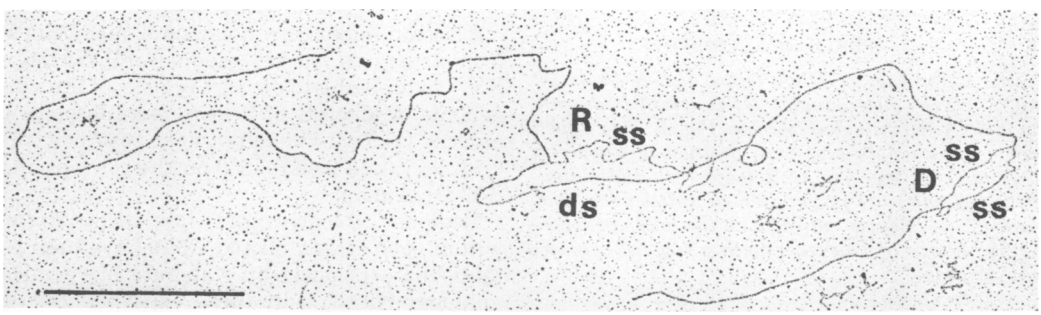

FIG. 3. *Electron micrograph of an $Ad2:Ad2^{+}ND_4$ heteroduplex DNA carrying a hexon R loop. Equal amounts of native Ad2 and $Ad2^{+}ND_4$ DNA were denatured at 34 μg/ml in 0.1 N NaOH for 30 min at room temperature. The DNA was reannealed at 3.4 μg/ml in 30% (vol/vol) formamide, 1.5 M urea, 30 mM NaCl, 10 mM EDTA, 0.1 M Tricine-NaOH (pH 8), for 20 h at 37°C (7, 36). For R-loop formation, hybridization assays containing 1.1 μg of DNA per ml and 2 μg of RNA per ml were incubated in R-loop buffer for 20 h at 52°C; assays were diluted with 1 volume of R-loop buffer in preparation for electron microscopy. The mRNA fraction used in this experiment was prepared as follows: poly(A)-containing RNA was isolated from polysomes (10) of 8×10^8 cells that had been labeled with 10 mCi of $H_3{}^{32}PO_4$ in 200 μl of phosphate-free medium from 16 to 18 h after infection; 30 μg of this RNA (specific radioactivity = 20,000 cpm/μg) in 80 μl of water was heated for 1 min at 100°C, chilled in ice, layered onto a gradient of 15 to 30% (wt/vol) sucrose in 0.1 M NaCl, 0.1 mM EDTA, 0.01 M Tris-hydrochloride (pH 7), and centrifuged at 33,000 rpm for 21 h at 4°C in a Spinco SW41 rotor. RNA contained within the fraction sedimenting at 23 to 24S was regained by ethanol precipitation and redissolved in water at a concentration of 50 to 100 μg/ml. In vitro translation of this fraction produced primarily hexon polypeptide (1; Lai and Westphal, unpublished results), and when hybridized to intact Ad2 DNA gave predominantly one loop near the center of the molecule. The use of this mRNA fraction avoided interference from R loops near the region of the SV40 insertion within $Ad2^{+}ND_4$ DNA. Abbreviations: D, loop formed by heterologous DNA; R, R loop. Bar indicates 1 μm.*

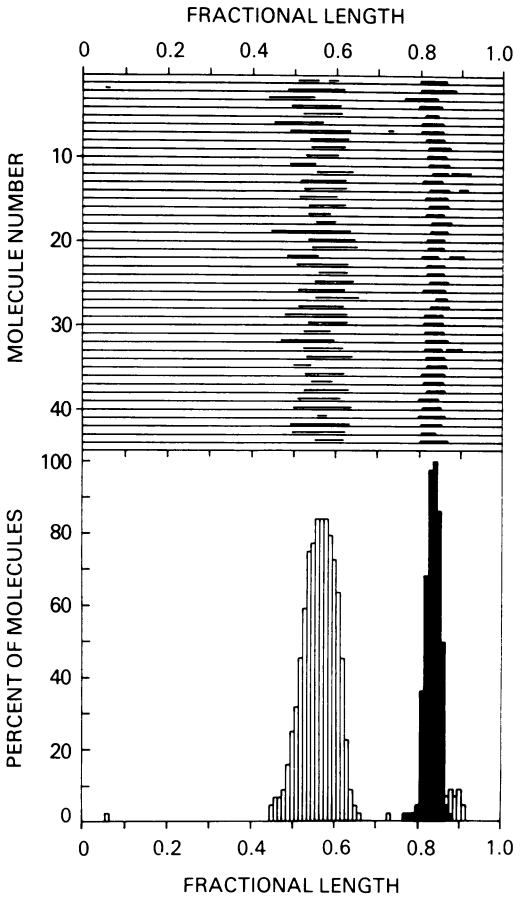

FIG. 4. *Position of DNA loops and R loops in Ad2:Ad2+ND$_4$ heteroduplex DNA hybridized with the mRNA fraction described in Fig. 3. The upper panel shows length and map position of unpaired DNA regions (■), and of R loops (▭). Individual DNA molecules are normalized to unit length. The frequency of loops is indicated in the lower panel as the percentage of molecules with a loop at a given map position.*

Besides hexon, one other Ad2 gene has been mapped by both genetic (21) and biochemical (2, 15) methods. These studies have shown the fiber gene to lie between positions 0.85 and 0.98 on the Ad2 map, corresponding to the R loop observed at position 0.88. We will refer to this R loop as the "fiber" loop.

When mock-infected cytoplasmic mRNA was hybridized with Ad2 DNA under identical conditions, R loops were never observed, even after long periods of incubation (Fig. 5). Thus, R-loop formation, even at positions where it rarely occurred, indicated the presence of a viral RNA homologous to that particular region of the genome.

Mapping of transcriptional strand switch points. Hybridization of Ad2 DNA with excess RNA has shown that both strands serve as templates for viral RNA. Points at which the polarity of transcription is reversed by strand switch are not infrequent (29, 31). We have incubated Ad2 DNA with early and late virus-specific RNA in an effort to map one or more of these switch points. Examples of R loops displaying strand switches are shown in Fig. 7. On opposite points of each strand of the loop, the contour changed from thick to thin, and vice versa. We interpret this to mean that two RNA molecules hybridizing in tandem, albeit with opposite polarity and therefore on opposite strands, formed a common R loop. The two main switch points which we observed mapped quite accurately at positions 0.61 ± 0.02 and 0.91 ± 0.02, respectively, of the DNA (Fig. 8). The published results (29, 31) indicated that, at these two regions of the DNA, the 3′ ends of early and late RNA were juxtaposed. Since the RNA used in our experiments was selected for poly(A) content, and therefore presumably contained well preserved 3′ ends, switch loops at these two locations were expectedly more frequent than at any other. A less frequently observed strand switch was also seen near position 0.68, corresponding to the 5′ ends of early and late mRNA (29, 31).

DISCUSSION

Electron microscopy of late Ad2 mRNA in R-loop structures has revealed several classes of RNA hybridizing to distinct regions of the viral genome. Midpoints of R loops were located, in order of relative frequency, near positions 0.57, 0.88, 0.77, and 0.40 to 0.50 on the conventional Ad2 DNA map.

Regions of early and late transcription within each strand of Ad2 DNA have been established previously by elaborate biochemical analyses (29, 31). In these experiments, the separated strands of Ad2 DNA fragments generated by site-specific endonucleolytic cleavage were hybridized to saturating amounts of RNA isolated from infected cells. The results established the orientation and extent of transcription within the individual DNA fragments. The experiments were not designed to measure the relative frequencies of individual classes of mRNA, nor to accurately map the various viral transcripts.

The R-loop technique complements these mapping studies and supplies additional information. In its present stage of development, this method allows for the determination of the size and frequency of R loops and of their loca-

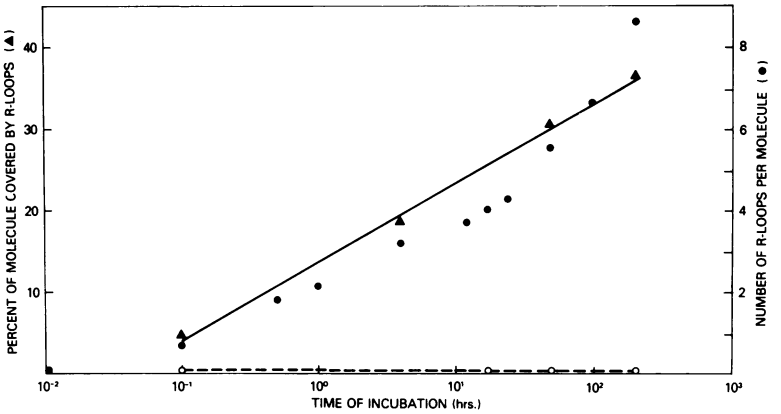

FIG. 5. *Kinetics of R-loop formation. Assays containing 10 μg of Ad2 DNA per ml and 100 μg of RNA per ml extracted from Ad2-infected cells 24 h after infection (●) or from mock-infected cells (○) were incubated at 52°C. At various times, samples were mounted for electron microscopy. The average number of R loops per molecule is based on the examination of 100 full-sized DNA molecules. The proportion of DNA covered by R loops (▲) was calculated from the experiments of Fig. 6.*

tions with respect to the conventional Ad2 genetic map. The procedure is fast and reproducible, and requires very limited quantities of unlabeled RNA and DNA. However, the R-loop technique presents two problems which make the interpretation of these results incomplete.

The first problem concerns the interrelationship of the relative frequencies of individual classes of viral RNA with those of the corresponding R loops. At present, one cannot be directly correlated with the other. The rate of R-loop formation is not equal at any given location along the viral DNA, but is determined by the melting temperature of the double-stranded DNA at the site of each R loop (33). Partial denaturation maps of Ad2 DNA (8, 22) showed fast melting areas interspersed within regions that were more resistant to denaturation, a fact not indicated by the smooth melting profile of Ad2 DNA seen in Fig. 1. It would thus appear likely that RNA complementary to a fast melting region of the DNA will be more readily registered by R-loop analysis than RNA hybridizing to a DNA region that is more resistant to denaturation. For example, the "hexon" loop (position 0.57) and the "fiber" loop (position 0.88) were located within regions of the DNA that melted readily, whereas the R loops at positions 0.77 and 0.40 to 0.50 mapped in regions which were more resistant to partial denaturation (8, 22). Consequently, we found that "hexon" and "fiber" loops formed faster than the R loops at positions 0.77 and 0.40 to 0.50 (Fig. 6). We are studying modifications of the R-loop technique designed to eliminate this problem. However, this limitation may not prove too serious, since we found no region of the DNA to be totally refractive to R-loop formation, and, as discussed below, some of the most frequently occurring R loops mapped within regions of the genome which code for the most abundant late viral gene products. Further studies are needed to determine the accuracy of RNA quantitation by the R-loop technique.

The second disadvantage of the R-loop technique in its present form lies in the fact that the orientation of transcription within a given loop cannot be determined. Recently, a technique was developed which allows for the visualization of poly(A) locations at the 3′ ends of RNA molecules (3). The application of this method to the R-loop technique would be an advantage for experiments in genetic systems where transcripts have not yet been oriented with respect to polarity.

As stated above, the differential kinetics of R-loop formation may make it difficult to correlate the frequency of a particular R loop to the frequency of the mRNA responsible for its formation. Nevertheless, we feel that the relative proportions of hexon mRNA and fiber mRNA are reflected by the R-loop frequencies at positions 0.57 and 0.88, respectively. This conclusion is supported by the facts that similar proportions are found for the polypeptide products of these mRNA's in vivo (39), and that both genes are contained within fast-melting regions of the DNA (8, 22), which produce R loops readily at 52°C (Fig. 6, top panel).

In the case of the "hexon" and "fiber" loops, there appears to be a reasonable correlation between the size of each R loop and the amount of nucleic acid required to code for the correspond-

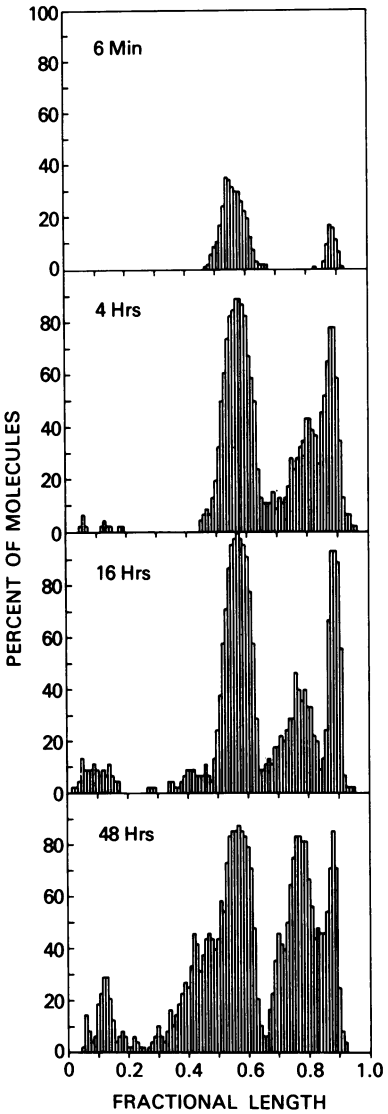

FIG. 6. *Position and frequency of R loops generated by late Ad2 mRNA. Each histogram is based on the examination of 45 to 100 full-sized DNA molecules which had been hybridized with RNA (see Fig. 5) for the indicated period.*

ing polypeptide. Assuming a molecular weight of 24.2×10^6 for Ad2 DNA (38), the "hexon" loop contains approximately 1.45×10^6 daltons and the "fiber" loop contains 0.65×10^6 daltons of mRNA. Using the conversion factor of 0.1 between the size (molecular weight) of RNA and its coding capacity, these values correspond reasonably well with the polypeptide molecular weights of 120,000 and 62,000, respectively. The "hexon" loop contains slightly more information than is necessary to code for hexon; but whether another Ad2 polypeptide is encoded within this message is presently unknown.

The R loop observed at position 0.77 probably corresponds in part to the gene for the 100,000 molecular weight polypeptide, a very abundant nonvirion Ad2 protein which maps in that region of the DNA (2, 15). A number of prominent late Ad2 gene products, such as penton base and the major core components of the virion, map in the left-hand region of the Ad2 DNA map (2, 15). The RNAs coding for these polypeptides are probably transcribed from the

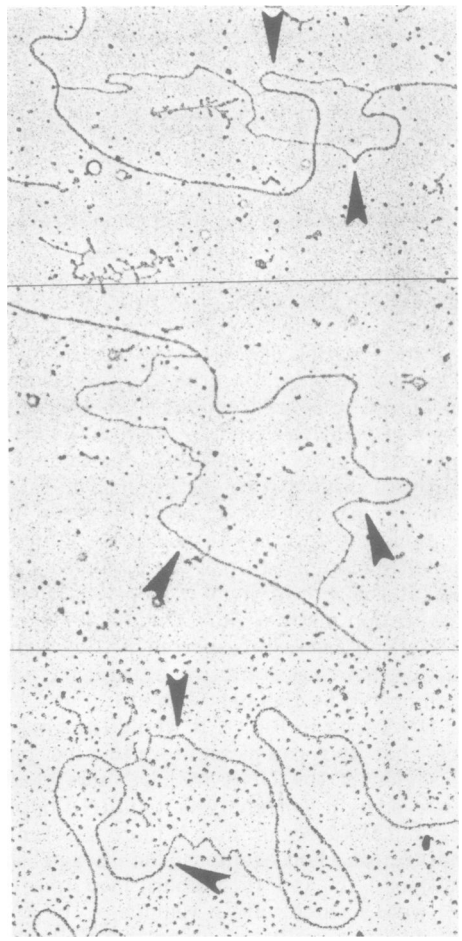

FIG. 7. *Electron micrographs of strand switch R loops. Hybridization mixtures containing 10 μg of Ad2 DNA per ml, 25 μg of late (24 h after infection) mRNA per ml, and 75 μg of early (8 h after infection) mRNA per ml (24), were incubated up to 4 days at 52°C. The photographs depict three R loops which contain a point of transition from single-stranded to double-stranded contour (see arrows) in each branch.*

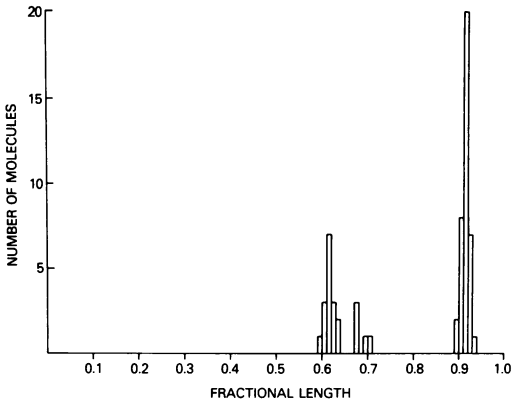

FIG. 8. *Frequency and map position of strand switches. DNA of the assays described in Fig. 7 was screened for molecules containing, in addition to the regular R loops, at least one strand switch R loop. Orientation of the molecules with respect to the conventional Ad2 DNA map posed no problems because each molecule carried multiple loops. The histogram depicts frequency and location of strand switches observed in a total of 50 DNA molecules carrying one or two strand switch R loops.*

area flanked by positions 0.3 (virus-associated RNA; see below) and 0.5 (hexon). We saw a substantial number of R loops which were too closely clustered to be discerned into individual peaks. The high G+C content of the DNA in this region of the genome (8, 22) may be responsible for the delayed kinetics of R-loop formation, as seen in Fig. 6.

Early Ad2 RNA present in preparations of late Ad2 mRNA (5, 13, 18, 34) was responsible for the formation of R loops which we observed at the extreme left-hand side of the map and at some locations interspersed between the loops in the hexon, 100,000 molecular weight polypeptide, and fiber areas. We will discuss these R loops in an accompanying paper (24). The area between positions 0.20 and 0.30 was essentially free of R loops. Published accounts from a number of laboratories (20, 27, 32, 35) described several Ad2 RNAs of low molecular weight that are derived from a region near position 0.3 of the DNA map. These virus-associated RNAs lack poly(A) tails, and were therefore probably not present in our mRNA preparations. Moreover, minute R loops formed by an RNA of this size may be hard to see. Therefore it comes as no surprise that very few R loops appear in that part of the genome in Fig. 6.

The mapping of genes by displacement hybridization should prove very useful in the study of eucaryotic gene organization and expression, especially in light of the paucity of genetic techniques available as compared to procaryotic systems. Further improvements of this method could be most easily accomplished in a well defined system such as ours, but once refined, should be applicable to any genetic system. In future studies of adenovirus gene expression, R-loop analysis should be useful in detecting less abundant transcripts in lytically infected cells as well as in Ad2-transformed cells. This method should also be of value in examining the precursor-product relationship of nuclear and cytoplasmic RNA.

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