Proc. Natl. Acad. Sci. USA
Vol. 73, No. 6, pp. 2069–2071, June 1976
Genetics

Mapping of adenovirus messenger RNA by electron microscopy

(DNA·RNA hybridization/physical gene mapping)

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Communicated by Bernhard Witkop, March 15, 1976

ABSTRACT Late adenovirus messenger RNA was annealed to complementary regions of partially melted viral double-stranded DNA. The RNA·DNA hybrid regions within the DNA molecules were visualized as loops in the electron microscope. Loops occurred at several regions of the DNA, most frequently, however, at a location near the center of the molecule. This hybridization technique appears well suited for an accurate mapping of messenger RNA, as well as for studies of RNA processing.

Adenovirus type 2 (Ad_2) messenger RNA extracted from human cell cultures at late stages of productive infection can be fractionated into several distinct classes of molecules coding for individual virus polypeptides (1–3). By decoding the message of RNA moieties hybridizing to specific fragments of the viral genome, it has become possible to arrive at a crude physical map of some of the known viral functions (4, 5).

Electron microscopy holds the promise for a more accurate mapping of individual viral transcripts, provided regions of RNA·DNA duplex can be visualized on the viral DNA strand. Recently, White and Hogness, and Thomas, White, and Davis (personal communications) have developed an elegant technique fulfilling this promise. The technique is based on the observation that at high salt an RNA·DNA duplex has a higher melting point than a DNA·DNA duplex of identical base sequence. If hybridization conditions are chosen that result in partial melting of DNA·DNA duplex, RNA complementary to a certain region of the DNA molecule will replace the homologous DNA region and hybridize with the antiparallel region of the DNA. Loops are thus generated in the double-stranded DNA molecule that can be visualized in the electron microscope. One side of the loop is double-stranded RNA·DNA, the other side is single-stranded DNA. We have made use of this technique in analyzing hybrids of adenovirus DNA and late polysomal viral RNA.

MATERIALS AND METHODS

Nucleic Acids. Ad_2 DNA was prepared from purified virions (6). Late poly(A)-containing polysomal RNA was extracted from infected or mock-infected KB spinner cell cultures (7). The biological activities of these RNAs in cell-free translation assays have previously been reported (see ref. 8 for review).

Molecular Hybridization. Native DNA (100 μg/ml) and RNA (500 μg/ml) were incubated at 47° for 20 hr in 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 8.0.

RNase H Assay (9). Hybridization assays (see above) were diluted 25-fold with enzyme buffer (0.05 M KCl, 1 mM 2-mercaptoethanol, 0.01 M magnesium acetate, 0.01 M Tris·HCl, pH 7.2), and incubated 15 min at 37° with or without 0.002 volumes of enzyme. Phage ϕX174 [^{3}H]RNA·DNA hybrids were added to monitor the enzyme action. At the end of the incubation, >95% of the radioactivity of this substrate was found to be Cl_3CCOOH-soluble. No degradation of single-stranded RNA was observed under these assay conditions.

Electron Microscopy. Hybridization mixtures and enzyme assays were adjusted to 50% (vol/vol) formamide (repurified, ref. 10), 0.5–1 μg/ml of DNA, 5 mM NaCl, 10 mM EDTA, 0.1 M Tricine·NaOH, pH 8.0, 100 μg/ml of cytochrome *c* (11); spread on a hypophase of 10% (vol/vol) formamide, 0.1 mM EDTA, 0.01 M Tris·HCl, pH 8.5; picked up on parlodion-coated grids; stained with uranyl acetate; and shadowed with platinum–paladium (12). Pictures were taken with a Philips 300 electron microscope at 40 kV and 10,000-fold magnification. Using a grating replica (12) for calibration, length measurements were made on an XY stage (Numonics Corp. North Wales, Pa.) from 20-fold enlarged negatives. Length measurements of DNA carrying loops followed the double-stranded contour throughout the molecule. The data were processed by a Hewlett-Packard calculator. Simian virus 40 (SV40) circular DNA was included in some spreadings as an internal standard for DNA length (13).

RESULTS

Late poly(A)-containing RNA, extracted from polysomes of Ad_2-infected KB cells, was incubated at 47° for 20 hr with double-stranded virion DNA in the presence of 0.5 M NaCl and 70% formamide. This resulted in the appearance of loops at various locations of the DNA molecules, as visualized by electron microscopy. Fig. 1 depicts a molecule carrying several loops, of various sizes. Each loop appears to consist of a single-stranded (thin contour) and a double-stranded (thick contour) branch.

We performed a number of controls (Table 1) to show that the loops were generated by RNA·DNA hybridization. First, loops formed only in the presence of RNA from infected cells. DNA incubated without RNA or with RNA extracted under the same conditions from mock-infected cells showed no loops. Second, RNase H, an enzyme that specifically degrades RNA contained in RNA·DNA hybrids (14), removed the loops. DNA was not noticeably degraded during the incubations. However, the overall double-stranded contour length of molecules carrying loops was slightly smaller than that of the controls, probably due to the tighter base stacking in the RNA·DNA portions of the loops.

A histogram of 125 Ad_2 DNA molecules carrying one or more loops (Fig. 2) revealed a nonrandom pattern of RNA·DNA hybrid regions. We chose to orient the molecules with the most frequently observed loop located to the right of the center. With the help of molecules carrying multiple loops, the DNA molecules could be arranged with little ambiguity relative to the major loop, except for molecules 14, 15, and 83.

The diagram at the bottom of Fig. 2 represents a summary of the data. The most frequently hybridizing class of late Ad_2

Abbreviations: Ad_2, adenovirus type 2; SV40, simian virus 40.

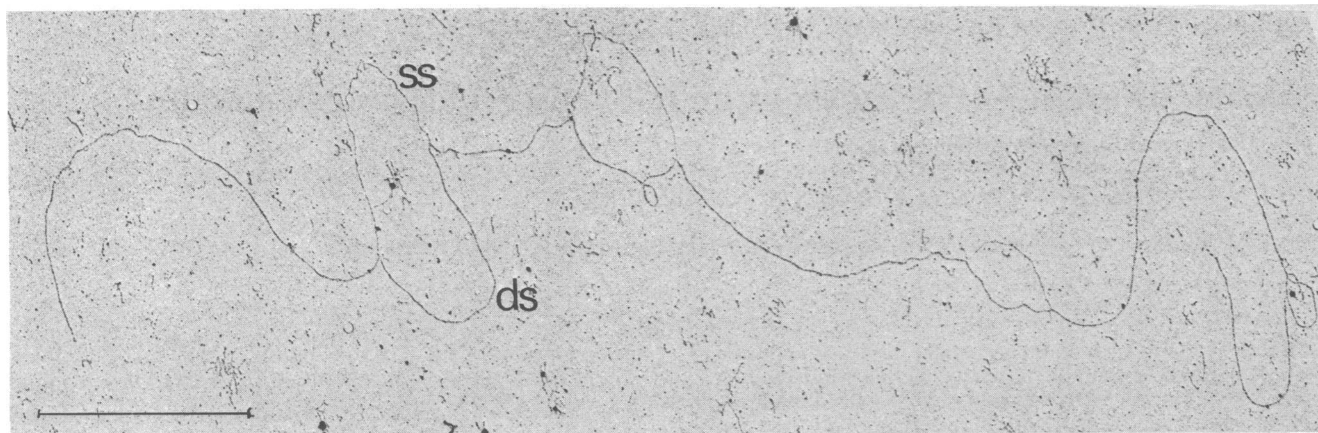

FIG. 1. Electron micrography of Ad_2 DNA carrying loops. See text for details. The bar indicates 1 μm; ss = single stranded, ds = double stranded nucleic acid.

mRNA molecules annealed to a DNA segment located between 0.5 and 0.6 fractional length. Most of the remainder of the loops were located to the right of this position. However, a small number of loops appeared to the left, notably at a position near 0.1 fractional length.

DISCUSSION

Loops containing late polysomal Ad_2 mRNA appear at various locations of the viral DNA. The most frequently observed loop, located near the center of the DNA molecule, is probably generated by RNA coding for the most ubiquitous of the adenovirus gene products, namely, the hexon polypeptide. Hexon mRNA hybridizes to a DNA segment located to the right of the center of the conventional DNA map (4), and we oriented our map accordingly. There is some variation in the size of this major loop. The largest loops comprise about 10% of the DNA length, or slightly more than the region needed to code for hexon (molecular weight, 120,000). Smaller loops in that area of the genome most likely reflect mRNA degradation which, in turn, may account for the relatively small amount of hexon synthesized *in vitro* (8).

Occasionally, we found loops far exceeding the size of any known polysomal Ad_2 mRNA. These loops may have been generated by two or more RNA molecules hybridizing in tandem on the same or on opposite DNA strands.

Small tails protruding from one of the forks of a loop were frequently observed, although not yet mapped. They may represent poly(A) moieties located at the 3′ end of Ad_2 mRNA (15). If this can be verified, the tails may serve as markers to determine the polarity of transcription within a given loop. Alternatively or in addition, tails may represent RNA sequences that were unable to displace the homologous DNA sequence under our hybridization conditions.

Besides the presumptive hexon loop, Fig. 2 depicts the location of several less frequently hybridizing classes of Ad_2 mRNA. Under improved hybridization conditions and at great RNA excess, more loop locations may become apparent. Ad_2 mRNA can be fractionated into classes coding for individual viral polypeptides (1–5). Utilizing the loop technique, mapping of these classes of viral RNA should pose no major experimental difficulties.

It will be quite interesting to compare the size of a given Ad_2 mRNA (as determined from the extent of the loop) with the number and size of the encoded gene products. While a study of this kind may reveal details of the regulation of adenovirus gene expression, a comparison of loops obtained with nuclear versus polysomal Ad_2 RNA may help to elucidate some of the regulatory processes that accompany the degradative pathway of primary adenovirus transcripts.

Table 1. Specificity of Ad_2 RNA·DNA hybridization

	% Molecules with loops	Length of DNA (μm)
1. DNA alone	0	13.64 ± 0.81
2. DNA + MOCK RNA	0	13.19 ± 0.71
3. DNA + INF RNA	>98	12.02 ± 0.93
4. DNA + INF RNA + RNase H	0	13.27 ± 0.52
5. DNA + INF RNA − RNase H	>98	Not determined

INF and MOCK RNA refer to late poly(A)-containing polysomal RNA from infected or mock-infected KB cells, respectively. Nucleic acids were hybridized (lines 1–3), subsequently incubated in the presence or in the absence of RNase H (lines 4 + 5), and prepared for electron microscopy. In each sample, >100 molecules were observed to determine the fraction carrying loops, and 40–50 molecules were measured to determine the average length. Using SV40 DNA as an internal standard (molecular weight 3.28×10^6, ref. 13), we calculated an average Ad_2 genome mass of 24.2×10^6 daltons.

We thank Dr. P. Wellauer for introducing us to the hybridization technique; Drs. R. White, D. Hogness, M. Thomas, and R. Davis for communication of unpublished results; Dr. R. Crouch for a gift of RNase H and of φX174 RNA·DNA hybrids; and Mrs. M. Sullivan and Mrs. S. P. Lai for superb technical assistance. J. M. was supported by a fellowship from the Swiss National Science Foundation and by a US PHS International Research Fellowship.

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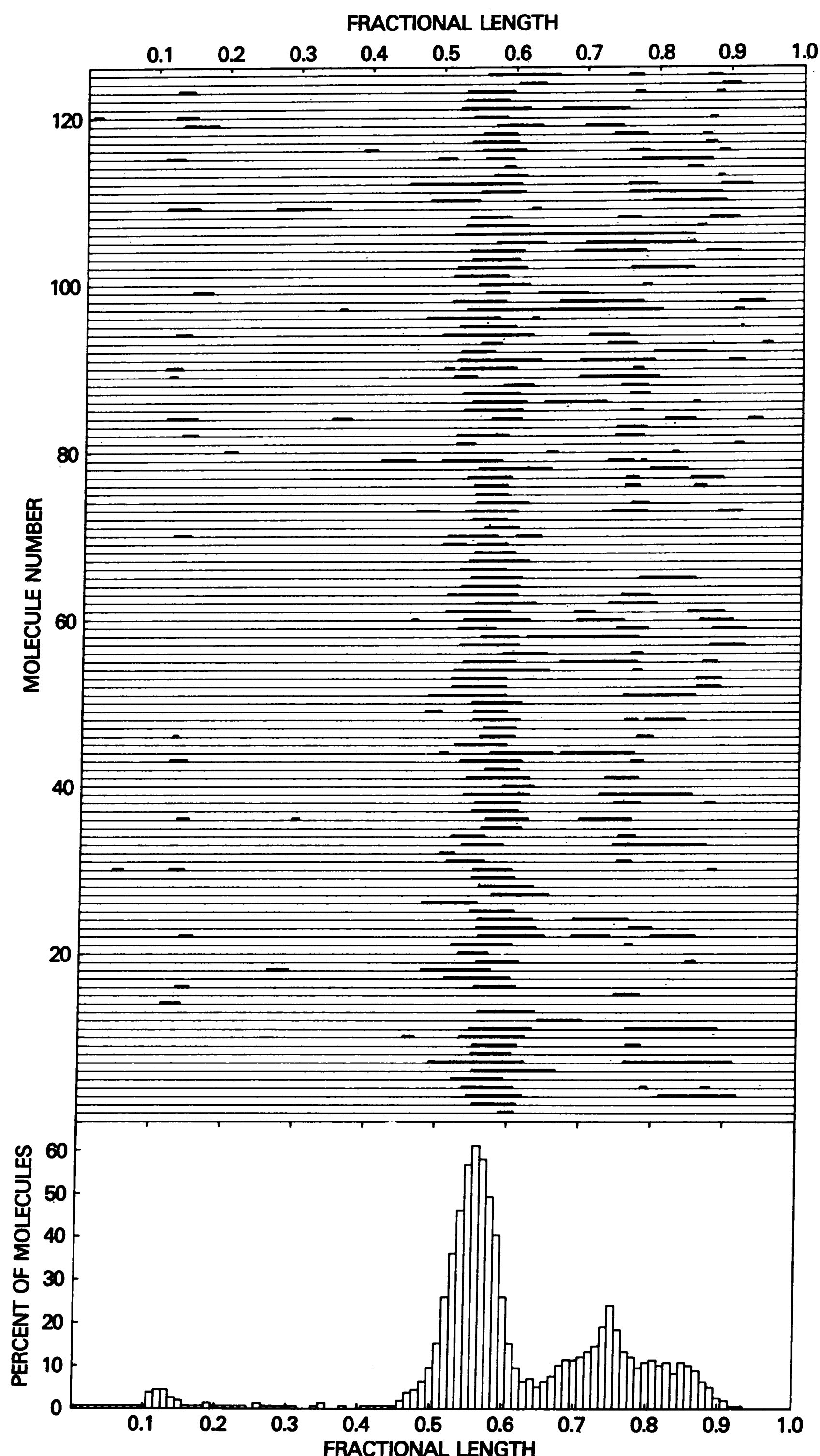

FIG. 2. Histogram of 125 Ad_2 DNA molecules with one or more loops. The computer printout shows the length and location of loops (upper diagram) and the frequency of occurrence along the DNA (lower diagram). Molecules, selected at random from the hybridization assay, were normalized to standard size, expressed as fractional length = 1.

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