JOURNAL OF VIROLOGY, Mar. 1977, p. 1019-1030

Vol. 21, No. 3
Printed in U.S.A.

Early Gene Expression of Adenovirus Type 2: R-Loop Mapping of mRNA and Time Course of Viral DNA, mRNA, and Protein Synthesis

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Received for publication 20 July 1976

Adenovirus type 2 DNA was hybridized to early mRNA isolated from the cytoplasm of infected cells prior to the initiation of viral DNA synthesis. Resulting R loops were visualized in the electron microscope, and their positions were oriented with the help of DNA fragments generated by digestion with the restriction endonuclease *Bam*HI. Early RNA was found to map (in order of relative R-loop frequency) with midpoints near positions 0.95, 0.80, 0.03, 0.65, and 0.09 on the conventional adenovirus map. The time of appearance of individual viral mRNA's was compared to the time course of viral protein and DNA synthesis. We present a refined map of adenovirus gene functions which is based on results documented in this and the accompanying study by Meyer et al. (1977), as well as on data published by other laboratories.

The intricate control system of initial adenovirus type 2 (Ad2) gene expression assigns a number of early viral functions to individual classes of mRNA, transcribed from distinct regions of the genome (5, 7) which comprise noncontiguous parts of either viral DNA strand (32, 38). At least six polypeptides of different electrophoretic mobilities, specified by these RNAs, have been observed in extracts of infected cells (4, 16, 19, 36, 37, 44) or in cell-free translations (2, 10, 26, 36, 37). Lewis et al. (26) allocated these early viral gene functions to specific regions of the Ad2 genome identified by restriction endonuclease cleavage.

Upon entering a permissive cell, the adenovirus needs to bring the host's regulatory system under its own control so that viral and cellular functions can cooperate in virus gene expression. The early Ad2 polypeptides observed by the various laboratories are integrated in this process in some as yet unknown manner. They are commonly listed according to molecular weight. The 72,000 molecular weight polypeptide, seen first by Anderson et al. (1) in Ad2-infected cells, is the polypeptide subunit (26) of a single-stranded DNA-binding protein (42) which is implicated in DNA replication (21, 43). At least five additional early virus-specific polypeptides (see Table 2) have been described in the literature, with molecular weights at or near 50,000, 19,000, 17,000, 14,000, and 11,000. Each of these polypeptides qualifies as an early viral gene product encoded in one of the early regions of the Ad2 genome (26). The 19,000 molecular weight component is a glycopolypeptide (19). Some of the early polypeptides are enriched in nuclear, others in cytoplasmic fractions of the infected cell (see Table 3).

A subset of early viral RNA found in Ad2-transformed cells (15) may code for early viral functions involved in the process of virus-induced cell transformation. This RNA represents several early species, of which those derived from the left end of the viral genome (12) are sufficient to elicit cell transformation (14). RNA transcribed from the left end of the genome during lytic infection gives rise in vitro to two polypeptides with molecular weights near 50,000 and 14,000 (26). Virus-specific polypeptides of corresponding molecular weights have not yet been detected within transformed cells, whereas, in several established lines of Ad2-transformed cells, the 72,000 molecular weight polypeptide has been unequivocally identified (22). The functions of early virus polypeptides during lytic infection or in the process of cell transformation remain to be elucidated.

In this report, we deal with some quantitative aspects of early Ad2 gene expression. Using the R-loop technique of White and Hogness (personal communication) we visualized indi-

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vidual classes of early mRNA hybridized with Ad2 DNA, established their map positions, and compared their relative frequencies at various times after infection. At the same time, we examined the time course of in vivo synthesis of early and late Ad2 polypeptides.

MATERIALS AND METHODS

Cells and virus stocks. The propagation and infection of KB cells with purified virus is described in the accompanying article (28).

Purification and electron microscopy of early Ad2 mRNA. Poly(A)-containing RNA was extracted at various times after infection from the cytoplasm of cells incubated in the presence of cycloheximide (10 μg/ml) as previously reported (9, 11, 28).

The hybridization of early Ad2 mRNA with Ad2 DNA and the visualization of R loops in the electron microscope are described in the accompanying article (28). In all of the hybridizations of this report, the DNA concentration was 10 μg/ml and the RNA concentration was 100 μg/ml. The use of higher RNA concentrations was impractical since extensive RNA background interfered with the visualization of R loops.

In vivo labeling of proteins and preparation of cell extracts. The polypeptides of Ad2-infected cells were labeled with [^{35}S]methionine as follows. At various times after infection, aliquots of 6×10^6 cells were removed from both infected and mock cultures. The cells were sedimented by centrifugation, washed once with 10 ml of methionine-free medium, and resuspended in 10 ml of methionine-free medium to which was added 0.3 mCi of L [^{35}S]methionine (233 Ci/mmol, New England Nuclear). The cells were labeled for 1 h at 37°C, sedimented by centrifugation, washed once with 10 ml of phosphate-buffered saline (PBS), and suspended in 1 ml of 0.15 M KCl, 5 mM $MgCl_2$, 3 mM $CaCl_2$, 0.1 mM EDTA, 0.01 M Tris-hydrochloride (pH 7.5). The cells were lysed and fractionated as described previously (9), and the nuclei were resuspended in 1 ml of PBS. The supernatant cytoplasm was clarified by a second centrifugation at 12,000 × *g* for 5 min. Both the nuclear and cytoplasmic fractions were diluted with 1 volume of 2× electrophoresis sample buffer (1× sample buffer = 1% [wt/vol] sodium dodecyl sulfate [SDS], 1% [vol/vol] β-mercaptoethanol, 10% [vol/vol] glycerol, 0.01% [wt/vol] bromophenol blue, 0.05 M Tris-hydrochloride [pH 6.8]), boiled for 2 min, and applied to 13% SDS-polyacrylamide gels for electrophoresis (9, 11). Quantitation of individual proteins was obtained by cutting the bands out of the dried gel and counting the gel slices in 10 ml of Filter-Solv liquid scintillation fluid (Beckman). Radioactivity in the corresponding region of the mock-infected cell sample was subtracted from the counts in each protein band cut out of the infected cell sample. The use of long (26 cm) slab gels allowed superior separation of the individual protein bands and thus facilitated quantitation by this method.

Time course of DNA synthesis. The appearance of Ad2-specific DNA was determined by DNA:DNA hybridization on nitrocellulose membranes. KB cells were infected as described (28), and 10-ml aliquots containing 3×10^6 cells were removed every hour from 0 to 9 h after infection, and again at 25 h after infection, and labeled with 50 μCi of [*methyl*-^{3}H]thymidine (12 Ci/mmol, Schwarz-Mann) for 30 min at 37°C. The labeled cells were sedimented by centrifugation, suspended in 1 ml of 0.3 N NaOH, and placed in boiling water for 10 min. The lysate was cooled to 0°C and neutralized with 1 ml of 0.3 N HCl, 0.01 M Tris-hydrochloride (pH 7.5). DNA was extracted twice with an equal volume of phenol (pH 7.5), precipitated with 2.5 volumes of ethanol at −20°C, suspended in 200 μl of 1.5 mM NaCl, 0.15 mM sodium citrate (pH 6.8), heated for 15 min at 100°C, rapidly cooled to 0°C, and hybridized with 3 μg of purified Ad2 DNA bound to nitrocellulose filters (17). Radioactivity in hybrids, measured at given time points, was normalized to the total DNA in each sample to correct for minor variations in the amount of DNA added to each filter.

RESULTS

Visualization and mapping of R loops generated by early mRNA in Ad2 DNA molecules. In the first section of this report we described the visualization of early Ad2 mRNA in viral DNA:RNA hybrids. Figure 1 depicts full-sized Ad2 DNA molecules carrying several loops which were generated by Ad2 mRNA annealing to antiparallel regions of the double-stranded DNA and thereby displacing the homologous DNA sequences. One branch of each R loop thus formed contained double-stranded nucleic acid (thick contour); the other branch contained single-stranded nucleic acid (thin contour). RNA which did not participate in the hydridization reaction displayed a highly condensed secondary structure seen especially in the background of the upper electron micrograph.

The RNA used in this series of experiments was synthesized in human cells lytically infected with a high dose of Ad2. We found that, under our conditions of infection, the individual stages of the lytic cycle were both synchronized and condensed, as evidenced by the fact that DNA synthesis (see Fig. 8, top panel, and Table 4) occurred earlier and at a more rapid rate than was reported previously (40). This allowed for a more accurate assessment of the time

FIG. 1. *Whole Ad2 DNA containing R loops formed by hybridization to RNA isolated from Ad2-infected cells 8 h after infection. DNA (0.1 μg) and RNA (1.0 μg) were incubated in 10 μl of R-loop buffer at 52°C for 4 days, and examined in the electron microscope. The bars indicate 1 μm. Abbreviations: ds, double-stranded side of loop; ss, single-stranded side of loop.*


ds
ss

course of Ad2 genetic expression. Since early and late mRNA synthesis followed each other within a narrow time span in cells infected in this manner, we had to take proper precautions to obtain early RNA preparations that were free of contaminating late RNA. We chose cycloheximide as an inhibitor of late viral RNA synthesis (18) because no quantitative or qualitative influence of this drug on early viral RNA synthesis was noticed in previous experiments (6, 16, 26).

RNA was extracted from the cytoplasm at different times after infection, and purified on oligo(dT)-cellulose (3) to select for poly(A)-containing Ad2 mRNA (33). R loops were generated under conditions defined previously (28, 46) and examined in the electron microscope. Figure 2 summarizes observations of Ad2 DNA molecules carrying early Ad2 mRNA extracted at 2, 4, 6, or 8 h after infection. The histograms show the average size, position, and frequency of R loops. Molecules with multiple loops facilitated the orientation of the various R loops with respect to each other. Molecules containing a single loop were aligned such that the R loop was coincident with one of the positions on the multilooped molecules. For example, the left-most R loop (midpoint near position 0.03) was closer to the end of the DNA molecule than was the right-most loop (midpoint near position 0.95). The orientation with respect to the conventional Ad2 DNA map was based upon experiments which will be described below, and which confirmed the results obtained in Fig. 2. Equal amounts of cytoplasmic RNA were used for each of the four hybridizations. Thus, the histograms show the relative amounts of each species of early Ad2 mRNA contained in the cell at the indicated times after infection. As expected, the R loops occurred in the early regions (32, 38) of the Ad2 DNA, and mapped, in order of relative frequency, near positions 0.95, 0.80, 0.03, 0.65, and 0.09. These positions refer to the midpoints of each histogram peak displayed in Fig. 2. At very early times (2 h after infection), R loops were infrequently observed between positions 0.5 and 0.6 (e.g., Fig. 2, top panel). Since these loops are located in a region coding for hexon mRNA (28), the significance of this finding is unknown.

Within the areas of the major loops, i.e., at positions 0.95, 0.80, and 0.03, the quantity of early RNA appeared to increase steadily during the first 6 h of infection, whereas surprisingly few loops formed at position 0.65, which corresponds to the RNA coding for the 72,000 molecular weight polypeptide (26), an early Ad2 polypeptide giving rise to a very prominent band on

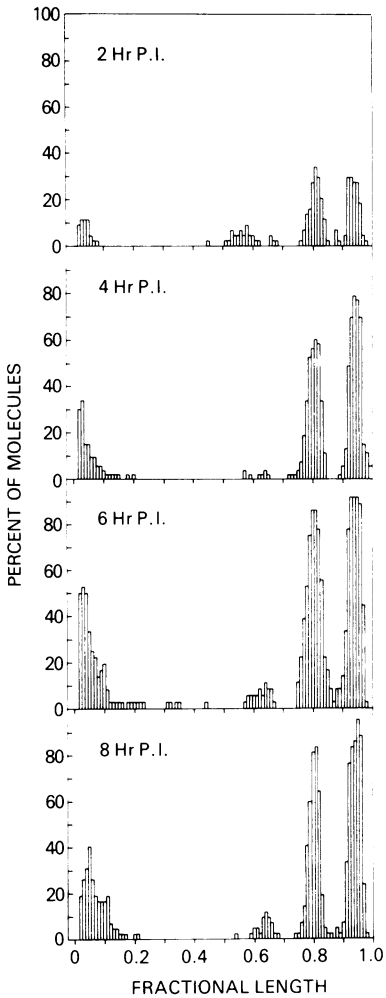

FIG. 2. *Time course of Ad2 mRNA production in infected cells as determined by R-loop analysis. Poly(A)-containing RNA was isolated from infected cells at various times after infection. Each RNA preparation was hybridized to whole Ad2 DNA for 16 h at 52°C. Hybrid molecules were examined in the electron microscope, and the positions of R loops were determined. The combined data for 50 molecules at each time point are presented in the form of a histogram. The abscissa shows the fractional length of the adenovirus genome, and the ordinate gives the percentage of DNA molecules containing an R loop at that position.*

SDS-polyacrylamide gels (i.e., Fig. 7). This finding prompted us to repeat part of the experiment, allowing more time for RNA:DNA hybridization to occur. And indeed, Fig. 3 shows that, under these conditions, the 8-h RNA, but not the 2-h RNA, formed a prominent peak of R loops at position 0.65. We cannot decide, on the

basis of this experiment, whether the RNA specific for position 0.65 was in short supply even 8 h after infection or whether the higher G+C content of DNA in that region of the genome (8, 29) caused a delay (28, 41) in the rate of DNA:RNA hybridization. The experiment did show, however, that significantly more of this RNA was present at 8 h than at 2 h after infection.

Orientation of R-loop positions to the conventional Ad2 map. Cuts introduced by the restriction endonuclease *Bam*HI into Ad2 DNA were utilized as markers for the orientation of R loops. The enzyme cleaves at positions 0.3, 0.43, and 0.6 of the conventional Ad2 DNA map (Mulder and Greene, personal communication), thereby generating four fragments in the order B, D, C, A. The length distribution of all fragments contained in the complete Ad2 DNA digest is shown in Fig. 4. The two larger fragments, A and B, corresponding to the ends of the molecule, showed distinct length distributions and thus could easily be identified in electron micrographs of a mixture of fragments. This task was more difficult with the small internal fragments, C and D, which displayed overlapping length distributions. We were nevertheless able to arrive at a correctly oriented map of R loops generated by early mRNA, since we could correlate R loops in distinct fragments to whole Ad2 DNA molecules carrying multiple loops. The electron micrograph of Fig. 5 depicts a *Bam*HI A-fragment containing two R loops. In Fig. 6, line drawings of fragmented DNA carrying R loops have been arranged in such a way that they reflect the distribution of loops in intact DNA. This method of R-loop orientation was used to determine the accuracy of subjective orientation based upon the position of a single loop in relation to one of the positions on a multilooped molecule (Table 1). Maps of R loops were established either with the help of specific fragments of DNA carrying R loops (Fig. 6) or with the help of intact molecules containing multiple R loops (Fig. 2). In either case, the ratio of R loops occurring to the left of position 0.07 and to the right of position 0.9 was close to 1:2 in hybridizations using RNA extracted 2 h after infection, and 1:1 in those using RNA extracted 8 h after infection.

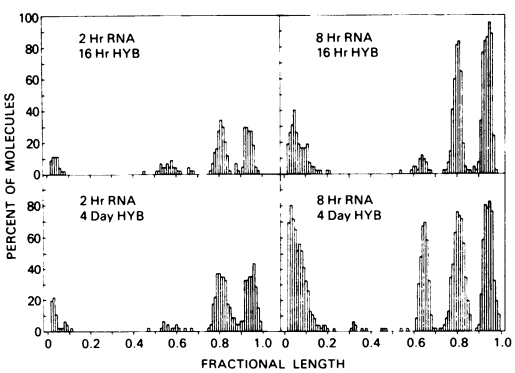

FIG. 3. *R loops generated by Ad2 early mRNA after different periods of hybridization. Poly(A)-containing RNA was prepared from infected cells at 2 and 8 h after infection, and hybridized to Ad2 DNA at 52°C for 16 h or 4 days (96 h).*

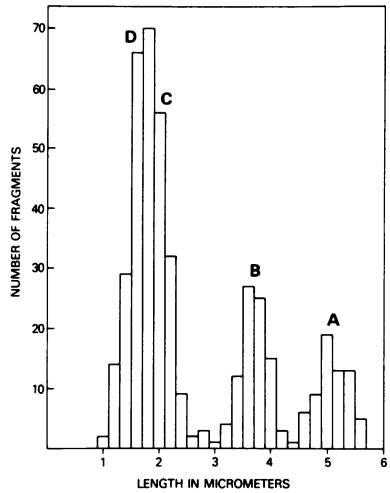

FIG. 4. *Length distribution of fragments generated by digestion of whole Ad2 DNA with BamIII restriction endonuclease. BamHI (0.4 unit, Bethesda Research Laboratories, Inc.) was added to 3 μg of Ad2 DNA in 10 μl of 6 mM $MgCl_2$, 6 mM β-mercaptoethanol, 6 mM Tris-hydrochloride (pH 7.5), and incubated for 2 h at 37°C (20). The digested DNA was diluted into R-loop buffer and examined in the electron microscope. Electron micrographs of 437 BamHI fragments were measured and the distribution was presented as a histogram. Since the probability of larger fragments extending off the electron micrograph, and thus not being scored, was greater than that of smaller fragments, the number counted was inversely proportional to the size of fragments.*

Time course of Ad2 polypeptide synthesis in vivo. In an effort to correlate the time course of early Ad2 RNA synthesis to that of the corresponding viral gene products, cells were labeled with [^{35}S]methionine at various times after infection, and the polypeptides contained in fractionated cell extracts were examined. Autoradiograms of ^{35}S-labeled polypeptides resolved by SDS-polyacrylamide gel electrophoresis are shown in Fig. 7. Five of the early Ad2-specific polypeptides—of 72,000, 19,000, 17,000, 14,000 and 11,000 molecular weight—could clearly be discerned from the background of host cell polypeptides present at the initial stages of infec-

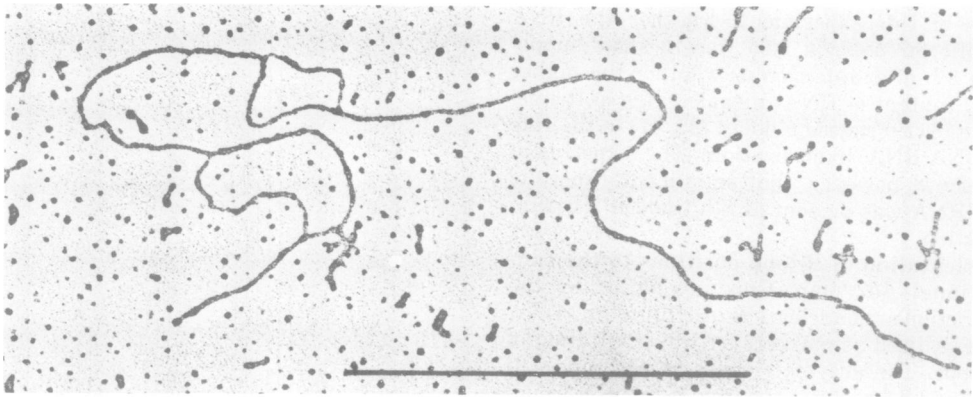

FIG. 5. *BamHI A-fragment containing two R loops formed by hybridization with RNA isolated from cells 2 h after infection with Ad2. The DNA was digested with BamHI enzyme as described in the legend to Fig. 4, and hybridized to the RNA for 16 h at 52°C. The bar indicates 1 μm.*

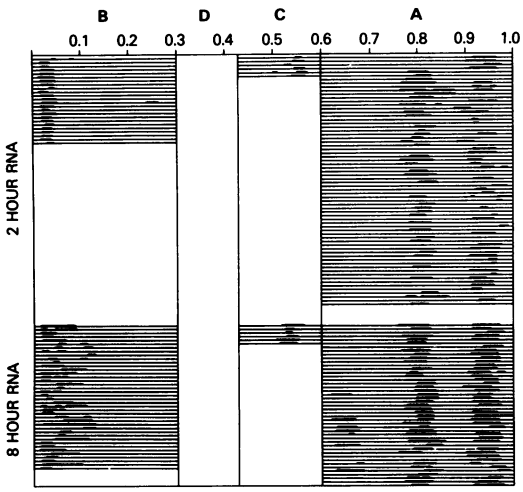

FIG. 6. *Positions of R loops on BamHI fragments of Ad2 DNA as described in Fig. 5. All fragments containing one or more loops were photographed, and the positions of the R loops were determined. The fractional length of the conventional Ad2 map covered by each fragment is given at the top of each column. Upper set: R loops formed by hybridization to RNA isolated 2 h after infection. Lower set: R loops formed by hybridization to RNA isolated 8 h after infection.*

TABLE 1. *Analysis of end loops*

Time after infection (h)	Position of loop	*Bam* fragments[a]		Whole DNA[b]	
		No.	Frequency	No.	Frequency
2	0–0.07	24	0.36	22	0.37
	0.9–1.0	42	0.64	38	0.63
8	0–0.07	38	0.49	23	0.48
	0.9–1.0	40	0.51	25	0.52

[a] As described in Fig. 6.

[b] Whole Ad2 DNA hybridized to cytoplasmic mRNA at 52°C for 16 h and oriented according to the relative positions of multiple loops occurring within individual molecules (Fig. 2).

tion. Some of these virus-specific polypeptides were abundant in the nucleus, others in the cytoplasm. A survey of early virus-specific polypeptides described in the literature (Table 2) shows that, in addition to the early products seen in Fig. 7, one additional polypeptide, around 40,000 to 50,000 molecular weight, was commonly observed by other laboratories. The high background of host cell polypeptide bands in the area of the gel corresponding to a product of this size may be responsible for our failure to detect the 40,000 to 50,000 molecular weight polypeptide. Polypeptides of comparable electrophoretic mobilities were observed in infected cells pretreated with 25 μg of cycloheximide per ml (16). The distribution of early virus-specific polypeptides among subcellular fractions (Fig. 7) compared well with similar data in the literature (Table 3). Minor variations were most likely due to differences in the methods of cell fractionation used by the individual groups of investigators.

To quantitate our observations, we determined the radioactivity contained in individual polypeptide bands, subtracted the corresponding mock values, and related these data to the time course of Ad2 DNA synthesis in the infected cells. Time points between 7 and 25 h after infection were taken from a gel (not shown) similar to that of Fig. 7. Only virus-specific polypeptides that could be cleanly cut from the gels were utilized in this analysis. The results (Fig. 8 and Table 4), indicated that 19,000 and 11,000 molecular weight polypeptides were among the earliest of the virus-spe-

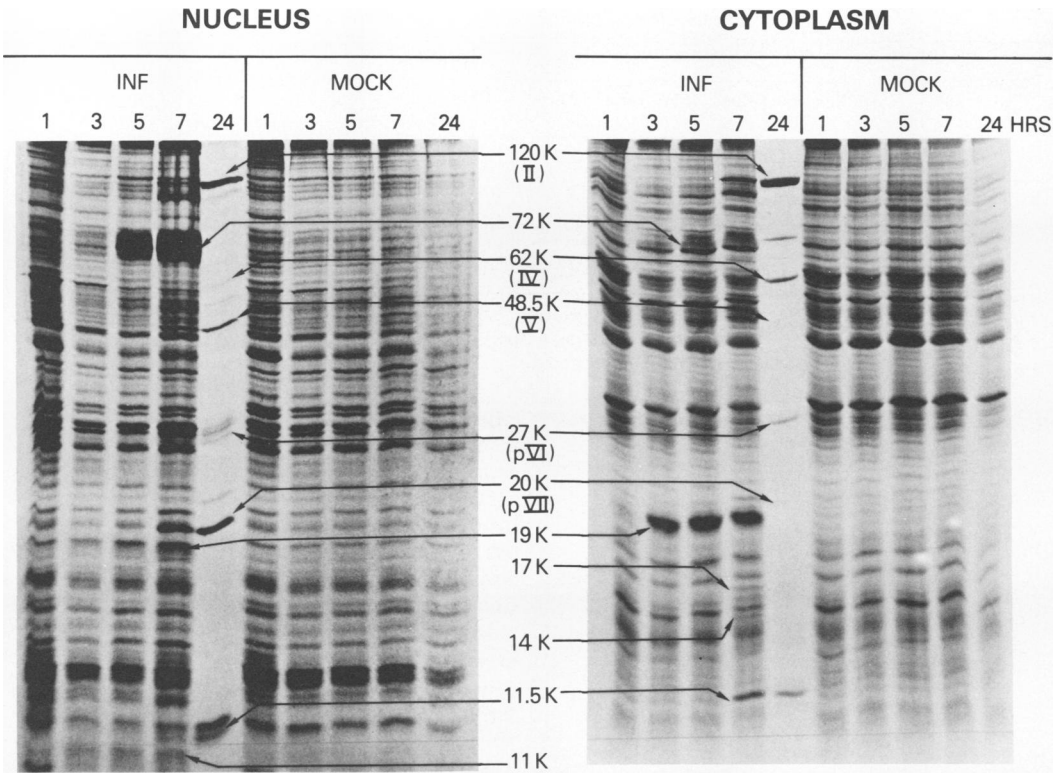

FIG. 7. *SDS-polyacrylamide gel electrophoresis of nuclear and cytoplasmic polypeptides synthesized at various times after infection. Ad2-infected or mock-infected KB cells were pulse-labeled with [^{35}S]methionine for 1 h prior to harvesting. Cells were fractionated into nuclear and cytoplasmic fractions and analyzed on 13% polyacrylamide slab gels. Sampling times (hours after infection) are shown at the top of each column. Apparent molecular weights (calibrated by co-electrophoresis of the 24-h INF sample with known marker proteins) are given in the center, with virion polypeptides (24) shown in parentheses. Abbreivations: INF, samples prepared from Ad2-infected cells; MOCK, samples prepared from mock-infected cells.*

TABLE 2. *Comparison of early virus-specific polypeptides observed by various groups of investigators*

Results of this study (in vivo)	Walter and Maizel (44) (in vivo)	Chin and Maizel (4) (in vivo)	Saborio et al. (37)		Saborio and Öberg (36)		Atkins et al. (2) (in vitro)
			In vivo	In vitro	In vivo	In vitro	
72[a]	70	70	70	70	74	74	72[b]
					67	67	
					60	60	
			45			45	44–55[b]
		42			42–50	42	
						40	
					35	35	
					26.5		
19	19	19	19	19	19	19	19[b]
					18.5	18.5	
17		17	17	17	17.5	17.5	
					14.5–16	15.5	15.5[b]
						15	15[b]
14		14			14.5	14.5	
					12.5	12.5	
11	10	11	10	10	10.5	10.5	11[b]

[a] Molecular weight $\times 10^3$.

[b] Shown to be virus-coded by Lewis et al. (26).

TABLE 3. *Intracellular location of early viral-specific polypeptides*

Polypeptide (mol wt × 10^3)[a]	Location				
	Results of this study	Walter and Maizel (44)	Chin and Maizel (4)	Saborio et al. (37)	Saborio and Öberg (36)
72	N(C)[b]	C	N,C	C	C
40–50			C	C	C
19	C(N)	C	C	C(N)	C(N)
17	C		C		C
14	C		C		C
11	N	N	N	N	N

[a] Apparent molecular weights vary slightly among observations by different laboratories.

[b] Abbreviations: N, polypeptide found in nucleus; C, polypeptide found in cytoplasm. Letters in parentheses indicate location of a minor fraction of polypeptide.

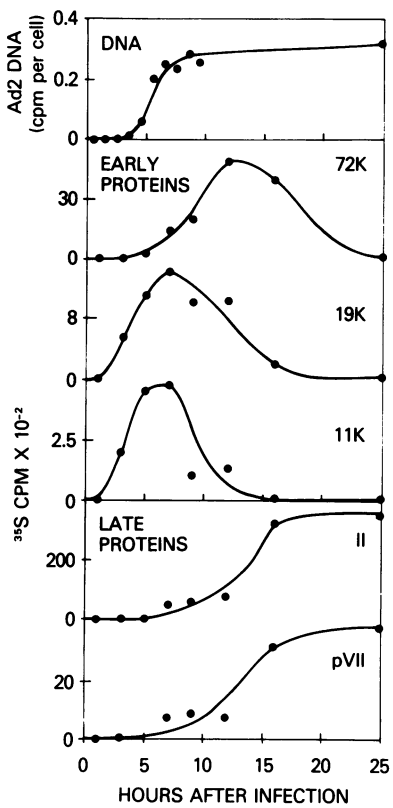

FIG. 8. *Time of appearance of Ad2-specific proteins and of viral DNA in infected cells. DNA synthesis was measured by DNA:DNA hybridization on nitrocellulose filters as described in Materials and Methods. Levels of both early and late polypeptides synthesized at each time point were determined by scintillation counting of ^{35}S radioactivity contained in individual polypeptide bands of the SDS-polyacrylamide gels. Radioactivity present in the same regions of the corresponding mock sample was subtracted from each value and the net amount was plotted. Total radioactivity recovered from the appropriate regions of each gel was as shown in Table 4.*

cific polypeptides, followed by the 72,000 molecular weight polypeptide, which appeared concomitant with the start of Ad2 DNA replication. Polypeptides II (hexon) and pVII, both late Ad2 gene products located in the virion particle, appeared within a few hours after the onset of Ad2 DNA synthesis, and continued to be produced while the rate of early polypeptide synthesis decreased. The high background of host cell polypeptides present in the 17,000 and 14,000 molecular weight regions of the gels prevented quantitation of these early, virus-specific polypeptides. Distinct bands were not observed at these locations in the autoradiograms until 5 to 7 h after infection. However, these polypeptides may exist prior to that time at levels undetectable by our assay method.

DISCUSSION

A genetic map of adenovirus type 2 may be constructed from the data contained in this study, in the accompanying report (28), and in the published accounts of other laboratories. In Fig. 9, we present two histograms which are representative for the spectrum of R loops observed in hybridizations of Ad2 DNA with early or late Ad2 mRNA. Below these histograms we have drawn a map of the genetic elements of Ad2 which, in our opinion, represents the best fit of all available data. Transcriptional units are drawn as arrows. Their extent and positions are defined by the sizes and positions of R loops, by the map positions of strand switches of transcriptional units (28, 32, 38), by the map positions of certain size classes of early RNA (5, 7), and by the map positions of the special markers VA and ND, i.e., the virus-associated RNAs (27, 30, 31, 34, 35, 39, 45), and the Ad2 DNA sequences deleted in nondefective (ND) Ad2-SV40 hybrid viruses (see reference 28 for details). Bars within the arrows indicate the minimum sequences needed to code for the var-

TABLE 4. *Total radioactivity recovered from the appropriate regions of each gel mentioned in the legend to Fig. 8*

Protein band	Cells[a]	^{35}S radioactivity (counts/min)							
		1[b]	3	5	7	9	12	16	25
72,000	INF	277	393	586	1,716	2,722	5,573	4,908	595
(nuc)	MOCK	402	414	353	336	850	733	908	492
19,000	INF	585	1,265	1,837	1,989	1,978	2,119	1,239	199
(cyto)	MOCK	621	724	728	583	967	1,064	1,049	629
11,000	INF	196	393	706	691	276	271	185	118
(nuc)	MOCK	222	200	250	212	178	143	176	206
II	INF	5,300	4,985	5,093	9,361	9,986	10,646	36,022	37,832
(cyto)	MOCK	5,679	5,911	5,749	4,994	4,492	4,031	4,092	3,731
pVII	INF	385	333	368	971	1,030	851	3,299	3,961
(nuc)	MOCK	336	317	282	264	216	169	206	240

[a] Abbreviations: INF, samples prepared from Ad2-infected cells; MOCK, samples prepared from mock-infected cells.
[b] Hours after infection.

ious early and late polypeptides listed on the map. These polypeptides have previously been allocated to restriction fragments of Ad2 DNA (2, 25, 26), and thus we were able to align them with the individual R loops.

Late Ad2 gene expression is discussed in the accompanying report (28). Here we will touch on some features of early Ad2 gene expression. The early region to the left of position 0.1 is of chief interest because of its primary involvement in cell transformation (14). The polypeptide most likely specified by the mRNA forming the left-most R loop is of uncertain size. According to cell-free translation, its size is near 50,000 molecular weight (26). However, the coding capacity of the corresponding mRNA, judged, by the R-loop size, is certainly greater. It is tempting to speculate that a 58,000 molecular weight polypeptide which is specific for Ad2-transformed hamster cells (Chin, Lewis, and Maizel, personal communication) is identical to the polypeptide seen by Lewis et al. (26), and thus specified by the left-most early Ad2 mRNA.

According to Atkins et al. (2), the 14,000 molecular weight polypeptide maps to the right of the 50,000 molecular weight polypeptide. This corresponds to the R-loop peak observed at position 0.09 in the upper histogram of Fig. 9. Although R loops were not observed in this region of the DNA when hybridized with RNA isolated 2 h after infection (Fig. 2 and 6), the limitations of the R-loop technique in regard to quantitation of individual mRNA species do not allow us to rule out the presence of this message at very early times after infection.

The R loop corresponding to the well characterized 72,000 molecular weight polypeptide appeared in the region between position 0.61 and 0.68, an area matching its minimal codon size and flanked by strand switch points (28, 32, 38). In keeping with the proposed function of this early Ad2 gene product during Ad2 DNA replication (43), it may be interesting to note, from the results of our study, that both the 72,000 molecular weight product and its mRNA appeared concomitant with Ad2 DNA synthesis, i.e., later than the earliest Ad2 polypeptides. A similar result was obtained by Gilead et al. (13).

The third early region of Ad2 mapped within positions 0.77 to 0.84 and included the ND deletion. Judging from the size of the corresponding R loop, a polypeptide of up to 80,000 molecular weight could be encoded here. Yet, the only product known to map in this area of the genome is the 15,500 molecular weight polypeptide of Lewis et al. (26), which probably corresponds to our 17,000 molecular weight polypeptide. In our Ad2 map, we have tentatively positioned this product to the left of the ND deletion because it seemed unlikely to us that the 15,500 to 17,000 molecular weight product is encoded by an area of the genome which is not required for lytic infection of human cells in culture (23). As yet, the presence of the 15,500 to 17,000 molecular weight polypeptide in Ad2^{+}ND4 infected cells has not been reported.

A rather large and quite abundant R loop generated by early Ad2 mRNA was located to the right of position 0.9. The corresponding 19,000 and 11,000 molecular weight polypeptides (26) require only a fraction of the coding capacity of an RNA of this size. We have no evidence that more than one RNA is responsible for their synthesis. Both polypeptides may, in fact, be breakdown products of some larger, yet unstable, Ad2 gene product. The 19,000 and 11,000 molecular weight polypeptides appear to be the earliest viral gene products synthesized after infection.

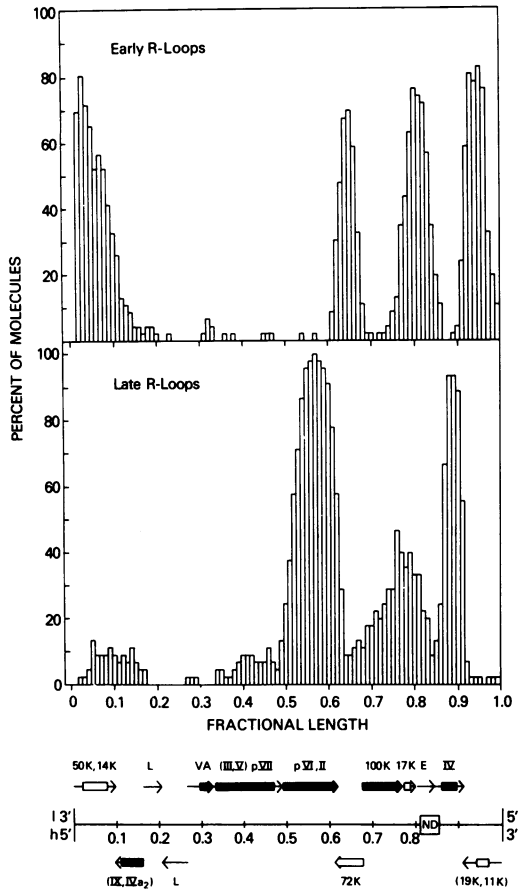

FIG. 9. *Comparison of the R-loop patterns generated by early and late Ad2 RNA and proposed genetic map of adenovirus type 2. The upper histogram is representative of early R loops and is derived from Fig. 3 (lower right panel). The lower histogram is representative of most late R loops and is derived from Fig. 6, panel three, of Meyer et al. (28). The map shown below the histograms is based upon the accumulated data of several laboratories, and is discussed in detail in the text. The polarity of both the heavy (h) and light (l) strands of the DNA molecule is indicated (38). ND refers to the region of deletion of Ad2 DNA sequences in nondefective Ad2-SV40 hybrids. The arrows give the direction of transcription and size of mRNA species produced both early and late in infection. Boxes inserted in these arrows indicate the minimal length of mRNA required to code for each polypeptide. The locations of boxes within arrows is arbitrary. Early (open boxes) and late (closed boxes) polypeptides most likely encoded by these messages are indicated. The gene order is unknown for those polypeptides within parentheses. Abbreviations: L, unknown late products; E, unknown early products. VA shows the map position of virus-associated, low-molecular-weight RNA.*

Because of the differential kinetics of loop formation in various regions of the Ad2 genome, it is difficult to correlate the relative frequencies of R loops with the proportions of mRNA's present in infected cells (28). However, in this report we have compared the frequencies of R loops generated by mRNA's isolated at various times after infection. In this case, we feel it is valid to correlate R-loop frequency at any one position to the relative amount of mRNA present at different times of infection, since dissociation of the DNA duplex in this region would remain constant for each hybridization. Thus, although one cannot accurately compare the frequencies of mRNA's producing R loops at, for example, positions 0.65 and 0.95, one can say that the RNA which generates the R loop at position 0.65 is considerably more abundant at 8 h than at 2 h after infection.

The R-loop technique certainly has not yet been fully exploited by the experiments discussed here. Further studies are expected to detect additional, less abundant classes of Ad2 mRNA. It should also be interesting to map the Ad2 mRNA's present in various Ad2-transformed cells, as well as the viral transcripts isolated from nuclei of both virus-infected and transformed cells. Since, at the same time, work on the Ad2 polypeptides is expected to reveal further details of the various viral gene functions, all the pieces of the puzzle may eventually be elucidated so as to construct a gratifying picture of the molecular genetics of Ad2 gene expression.

ACKNOWLEDGMENTS

The expert technical assistance of Sing Ping Lai and Margery Sullivan and the editorial assistance of Cecilia Levi are gratefully acknowledged. We wish to thank William Chin and Andrew Lewis, Jr., Raymond White and David Hogness, and Carel Mulder and Ronni Greene for transmitting their results prior to publication. We are also grateful to José Saborio and Bo Öberg, and to Marjorie Thomas, Raymond White, and Ronald Davis for making their manuscripts available before publication. Jürg Meyer was supported by a fellowship from the Swiss National Science Foundation and by a Public Health Service International Research Fellowship (no. F05TW2256-01).

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