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Adenovirus-2 mRNA is transcribed as part of a high-molecular-weight precursor RNA

(transcription unit/adenovirus-2 DNA/mRNA formation)

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ABSTRACT **The order of transcription and the length of nascent RNA transcripts from adenovirus-2 (Ad-2) DNA in the nucleus of infected cells has been deduced by labeling the growing RNA chains *in vivo* for a very brief period, separating the RNA on the basis of size, and hybridizing to the ordered *Eco*RI restriction endonuclease fragments derived from Ad-2 DNA. The majority of the virus-specific RNA molecules are synthesized as very high-molecular-weight units beginning at a common point at least 25–30,000 base pairs from one end of the Ad-2 DNA. These molecules can be reduced in size without further RNA synthesis. The experiments indicate the obligatory origin of Ad-2 mRNA from a high-molecular-weight precursor molecule.**

After the finding that rRNA was derived from a higher-molecular-weight precursor molecule in the nucleus, it was suggested that mRNA might similarly be derived from a higher-molecular-weight class of extranucleolar nuclear RNA, the heterogeneous nuclear RNA (hnRNA) (for review, ref. 1). Support for this idea has come from identifying sequences in the hnRNA that are also present in mRNA. For example, cells transformed by DNA viruses contain high molecular-weight molecules and lower-molecular-weight cytoplasmic molecules which are virus-specific (2, 4). Likewise cells making hemoglobin mRNA contain hemoglobin sequences in nuclear RNA molecules larger than cytoplasmic hemoglobin mRNA (4–6). Finally, poly(adenylic acid), a post-transcriptional addition product of 200 adenylic acid residues, is found at the 3′ terminus of both hnRNA and mRNA (for review, see ref. 1). Most recently methyl groups, particularly N^6-MeAp, have been found in both mRNA and high-molecular-weight hnRNA (ref. 7 and Salditt-Georgieff, Jelinek, Darnell, Furuichi, Morgan, and Shatkin, in preparation).

The sharing of sequences between hnRNA and mRNA is only suggestive that the smaller mRNA derives by cleavage of the larger, supposed precursor molecule; proof of such an mRNA precursor role for hnRNA would lie in the demonstration that the precursor is the obligatory original product of transcription. Such evidence could best be obtained by labeling cells for a short period (shorter than that necessary to completely synthesize the putative precursor) and determining whether all the labeled mRNA-like sequences are part of molecules larger than mRNA.

Such a pulse-label experiment is not feasible with the potential precursors of most specific cellular mRNAs because the fraction of labeled nuclear RNA represented by any single gene product is too small (5). However, cells infected with adenovirus contain 10% of total labeled nuclear RNA as adenovirus-specific RNA, including some molecules much larger than mRNA (8, 9). Moreover, adenovirus mRNA formation includes the same post-transcriptional modifications [poly(A) addition (10) and methylation (ref. 7 and Sommer, Salditt-Georgieff, Bachenheimer, Darnell, Furuichi, Morgan, and Shatkin, in preparation)] that cell mRNA does. This induction of the formation of large amounts of virus-specific RNA in each infected cell has allowed us to obtain sufficient RNA precursor incorporation in 1–2 min so that the nature of the original RNA transcripts from the adenovirus DNA genome could be examined. It appears that the great majority of the newly synthesized virus-specific RNA represents a transcript of all (or a very large segment) of the adenovirus genome. This high-molecular-weight RNA molecule is then processed within a few minutes to give rise to mRNA.

Abbreviations: Ad-2 and adeno-2, adenovirus-2; *Eco*RI, restriction endonuclease I from *Escherichia coli*; hnRNA, heterogeneous nuclear RNA; kb, kilo bases, thousands of base pairs in double-stranded nucleic acids or thousands of nucleotides in single-stranded nucleic acids; NETS, NaCl/EDTA/Tris buffer.

METHODS AND MATERIALS

HeLa cell growth and adenovirus propagation have been described, as have procedures for preparing labeled RNA from infected cells (8, 10). Cells to be labeled with [^{3}H]uridine for short times (1 or 2 min) were concentrated after 18 hr of infection to 3×10^6 cells per ml, label was added (20 mCi to 100 ml; 30 Ci/mmol) for the appropriate time, and the culture was then poured over an equal volume of frozen phosphate-buffered saline (PBS; 0.14 M NaCl, 0.01 M phosphate, pH 7.0). The cells were disrupted by Dounce homogenization in hypotonic buffer (11), nuclei were detergent washed, and RNA was extracted as described (18). The phenol-extracted, ethanol-precipitated RNA was dissolved in 0.05 NETS buffer (0.05 M NaCl; 0.01 M Tris·HCl, pH 7.4; 0.01 M EDTA; 0.2% sodium dodecyl sulfate) at room temperature, layered on sucrose gradients (15–30% weight/weight sucrose in 0.05 M NETS), and centrifuged as described in figure legends.

Adenovirus DNA was purified as described (8) and fragments resulting from *Eco*RI restriction endonuclease treatment were prepared by electrophoresis through agarose according to Lewis *et al.* (12). The recovery of DNA from agarose was efficiently accomplished by dissolving the agarose in 5 M sodium perchlorate and passing the mixture over hydroxyapatite. After the column had been washed with 5 M $NaClO_4$ and 0.05 M phosphate buffer (pH 7.0), the bound DNA was eluted with 0.4 M phosphate buffer as described by Lewis *et al.* (12). DNA filters were prepared according to Gillespie and Spiegelman (13) and contained either 2 μg of adenovirus-2 (Ad-2) DNA or an amount of an *Eco*RI fragment which would be contained in 2 μg of total DNA.

RNA from sucrose gradient fractions was treated for 5 min with alkali (14) to reduce the chain size to about 500 nucleotides or less. The RNA solution was neutralized and adjusted to a final NaCl concentration of 0.3 M and hybridized

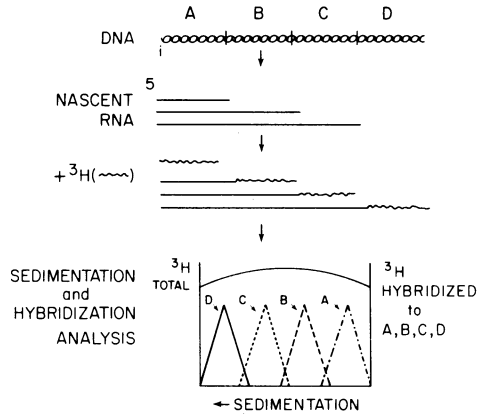

FIG. 1. Diagram of pulse-label experiment to detect, by sucrose gradient analysis, nascent RNA complementary to restriction endonuclease fragments A, B, C, and D of DNA.

to DNA filters for 40 hr at 65°. Hybridized RNA resistant to RNases A and T1 was then measured as described (8).

RESULTS

A successful experiment with intact cells which determined the direction of synthesis of biological polymers, as well as providing proof of stepwise chain growth and the nature of nascent (not yet completed) molecules, was made in 1960–61 by Howard Dintzis in his studies on hemoglobin synthesis (15). His ideas have recently been reused to determine the direction and rate of chain growth of simian virus 40 DNA (16). It is also possible, using the Dintzis principles, to design experiments on RNA chain growth and the nature of the newly produced product as outlined in Fig. 1.

DNA can be divided by restriction enzymes into reproducible segments the order of which can be determined in a variety of ways. Suppose such a hypothetical DNA molecule comprising four equal restriction segments—ABCD—were being transcribed from A → D and radioactive RNA precursor were added to cells for a period of time sufficient to synthesize only one fourth of the total region A–D. The resulting labeled RNA molecules would contain some labeled RNA regions complementary to each region of the DNA, but the RNA complementary to A would be in the shortest chains and the RNA complementary to B, C, and D would be in successively longer chains.

Ad-2 DNA can be cleaved by *Eco*RI restriction endonuclease into six fragments, A–F, in decreasing size whose arrangement in the whole Ad-2 DNA molecules has been determined by Petersson *et al.* to be ABFDEC (17) (Fig. 2). Late in adenovirus infection the vast majority of RNA formed is reported to come from only one of the two DNA strands (18) and a great deal of the mRNA that is synthesized late is transcribed in the left to right direction as indicated in Fig. 2 (19). Finally, a number of workers have reported high-molecular-weight nuclear virus-specific RNA to be present in cells after 15 hr of infection (8, 9, 20). These high-molecular-weight virus-specific molecules might represent the only (or at least the major) method of transcription of Ad-2 DNA. If so, when infected cells are exposed very briefly to [^{3}H]uridine, the shortest labeled virus-specific molecules should hybridize to fragment A; RNA complementary to fragment B would be contained in molecules 20–30 kb and RNA complementary to fragments F, D, E, or C would be in increasingly longer molecules (Fig. 2).

Accordingly, nuclear RNA was prepared from cells labeled for either 1 or 2 min, times comparable to the synthesis time in HeLa cells of poliovirus RNA [8 kb in 1–1.5 min (21)], and for pre-rRNA [14 kb in 2 min (22)]. The labeled RNA was sedimented through sucrose-sodium dodecyl sulfate gradients and fractions were hybridized to fragments A, B, C, and E (fragments D and F were not used in this experiment because not enough labeled RNA was available to test every fragment). In the sample labeled for 1 min (Fig. 3), RNA complementary to fragment A, which is 23 kb long itself, was found in virtually every fraction from <18 S to >45 S, with somewhat more hybridized RNA in the slowly sedimenting than in the rapidly sedimenting regions. RNA complementary to fragment B was found predominantly as a

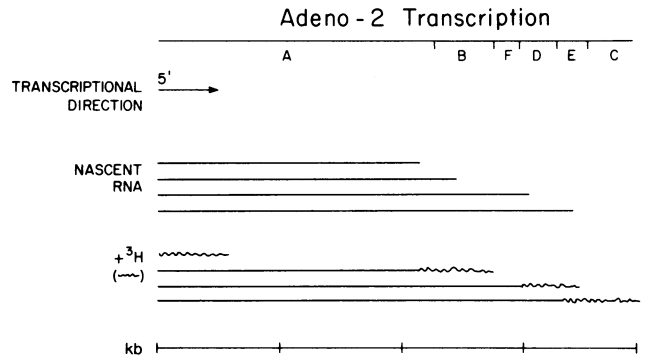

FIG. 2. Model of Ad-2 DNA transcription. Ad-2 DNA restriction fragment map A, B, F, D, E, C, from Petersson *et al.* (17) is known to be at least partly transcribed in direction shown [Sharp *et al.* (19)]. If transcription of nascent RNA proceeded as in model, then labeled portions (sum) of nascent RNA of increasing size would be complementary to regions A, B, F, D, E, C, in order. kb = thousands of bases.

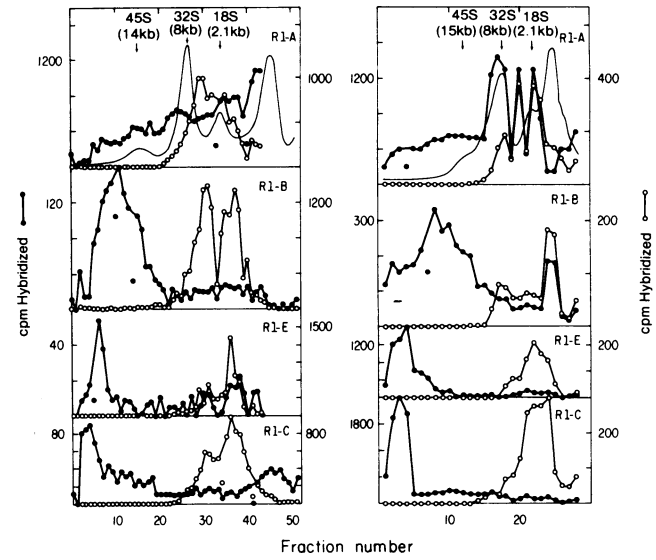

FIG. 3. Hybridization of pulse-labeled nuclear RNA with restriction fragments of Ad-2 DNA. Cells were labeled for 1 min (left) or 2 min (right) with [^{3}H]uridine (30 Ci/mmol, 200 Ci/ml) at 18 hr after infection, and nuclear RNA was isolated. [^{3}H]RNA was mixed with ^{32}P-labeled poly(A)-containing cytoplasmic RNA, isolated late in Ad-2 infection, and centrifuged through 15–30% sucrose, 0.05 M NETS buffer for 19 hr at 19,000 rpm (left) or 18,000 rpm (right) in a Spinco SW 27 rotor. Aliquots of individual fractions were hybridized with *Eco*RI-generated Ad-2 DNA fragments as described in *Methods and Materials*. Solid circles—pulse-labeled RNA; open circles—^{32}P-labeled cytoplasmic RNA; solid line—A_{260} absorbance profile.

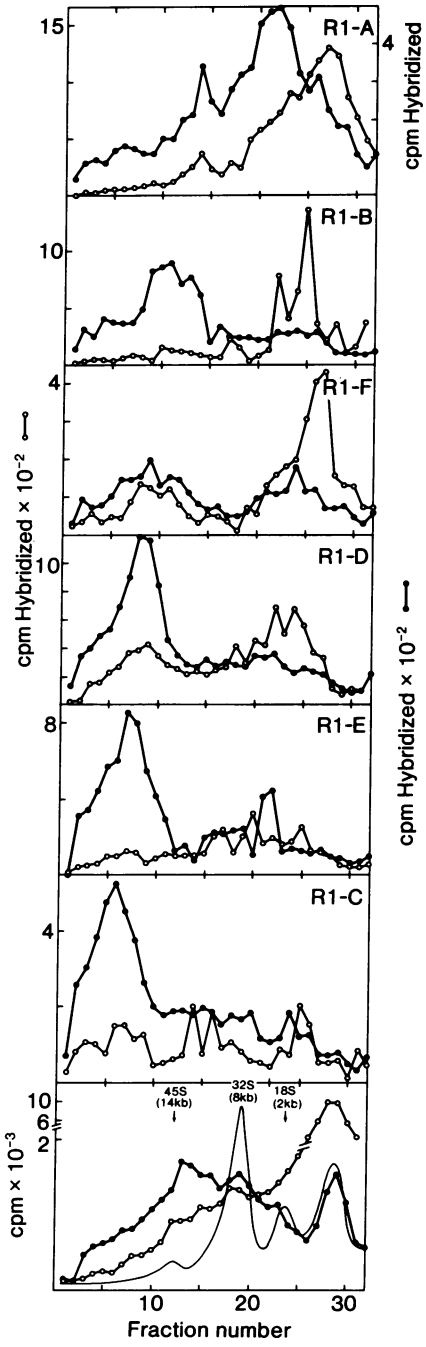

FIG. 4. Effect of actinomycin D on size distribution of pulse-labeled nuclear RNA. Cells were labeled for 2 min with [^{3}H]uridine at 18 hr after infection, and then "chased" for either 2 min or 20 min with actinomycin D, 7.5 μg/ml. Nuclear RNA was isolated and centrifuged through 15–30% sucrose, 0.05 M NETS buffer for 19 hr at 18,000 rpm in an SW 27 rotor. Aliquots of individual fractions were hybridized with *Eco*RI-generated Ad-2 DNA fragments. Panels R1-A through R1-F represent composites of the corresponding 2 min and 20 min hybridization profiles; lowest panel, total count profiles and A_{260} absorbance profile. Solid circles—2 min chase; open circles—20 min chase.

peak in fractions 5 through 16 which sedimented faster than the 45S pre-rRNA observed in the UV absorption profile of the nuclear RNA. RNA complementary to fragment C sedimented even faster than the RNA complementary to B and was contained predominantly in 4 to 5 fractions (3 through 6). The patterns were similar for the RNA labeled for 2 min with predominant peaks for B, C, and E in the fastest-sedimenting RNA.

In the experiments just described, a sample of cytoplasmic RNA from cells infected with Ad-2 and labeled with ^{32}P between 15 and 18 hr after infection was precipitated together with the briefly labeled [^{3}H]RNA. Adenovirus-specific cytoplasmic RNA is known to be of lower molecular weight, approximately 1–4 kb (23). ^{32}P-Labeled RNA complementary to fragment A, B, C, and E was observed only in fractions sedimenting more slowly than 32S pre-rRNA, which is 8 kb. The [^{32}P]RNA serves as a control to argue strongly that the specific pattern of hybridization of [^{3}H]RNA to specific DNA fragments is not likely due to any type of RNA aggregation.

The model shown in Fig. 2 would appear to describe the actual mode of transcription of Ad-2 DNA. Because only RNA complementary to the A fragment was present in short molecules, the only starting point(s) for transcription appear to be in the A fragment, conceivably the beginning of the A fragment. However, because the size of the A fragment is so large (17), it is not possible on the basis of hybridization to *Eco*RI fragments to decide whether all of A is transcribed into one RNA molecule. After a 1 or 2 min label, the B region is only represented in molecules longer than 15 kb; E and C are in still longer molecules, suggesting that the RNA synthesis continues to the end (or close to the end) of the Ad-2 genome. Thus the briefly labeled molecules complementary to A, B, and E are in fact nascent RNA, while those complementary to C might contain newly terminated molecules as well. The direction of synthesis A → B → E → C would appear correct.

"Chase" of Ad-2 RNA from high-molecular-weight to mRNA-sized molecules

If the only (or major) RNA product from Ad-2 DNA is longer than 20 kb and the cytoplasmic mRNA is smaller than 4 kb a size reduction by specific cleavage to form mRNA can be inferred. The experiment outlined in Fig. 4 is an attempt to show that the radioactivity in the large molecule is actually transferred to smaller molecules. An infected culture was labeled for 2 min with [^{3}H]uridine and then treated with a high dose (7.5 μg/ml) of actinomycin D to stop further RNA synthesis as soon as possible (24). Samples were removed 2 min and 20 min later. Nuclear RNA was prepared, sedimented through sucrose gradients, and analyzed as before for RNA complementary to Ad-2 DNA fragments.

The pattern of hybridization in the sample from cells exposed to a 2 min label plus 2 min actinomycin chase confirmed the results of Fig. 3. The successive genomic fragments B, F, D, E, and C hybridized mainly to RNA molecules which sedimented successively faster and were all larger than 45 S. Hybridization to the A fragment again showed molecules in all size classes, with RNA sedimenting slower than 32 S predominating.

After 20 min of actinomycin treatment almost all the virus-specific RNA larger than 45 S had disappeared and nuclear RNA less than 32 S was found complementary to most fragments.

After 2 min of label very little cytoplasmic virus-specific RNA could be detected compared to nuclear virus-specific RNA. An increase in cytoplasmic virus-specific RNA was noted by 2 min of actinomycin treatment and a much larger increase after 20 min. Even after 20 min, however, only about 1/8 of the total adenovirus-specific RNA was cytoplasmic (Table 1). Most (>80%) of the cytoplasmic virus-

Table 1. Incorporation into virus-specific RNA*

RNA fraction	2 min [^{3}H]Urd	2 min [^{3}H]Urd + 2 min Act. D	2 min [^{3}H]Urd + 20 min Act. D
Nuclear	94,000	676,000	498,000
Cytoplasmic	2,400	19,300	69,600†

Urd, uridine; Act. D, actinomycin D.
* Figures are radioactivity in cpm.
† >4 S = 80%.

specific RNA however was larger than 4–6 S and was in the size range of mRNA.

DISCUSSION

For some years now it has been clear that nuclear virus-specific RNA molecules exist which are as large or nearly as large as the DNA genome from which they are transcribed (8, 9, 20). Furthermore, McQuire *et al.* (20) have shown that in the presence of toyocamycin, an adenosine analogue which is incorporated into RNA, the appearance of virus-specific mRNA is inhibited and the nuclear virus-specific RNA remains larger than 32 S for many minutes. The present experiments strongly suggest that the very high-molecular-weight Ad-2-specific molecules are the major, if not the only, RNA transcripts formed from Ad-2 DNA. Furthermore, the high-molecular-weight molecules can in the absence of further RNA synthesis be cleaved to yield smaller RNA regions complementary to various regions of the DNA. Coincident with this size reduction of nuclear virus-specific RNA is the appearance of labeled cytoplasmic RNA. Thus "processing" to form Ad-2 mRNA is strongly suggested. Many analogies could be drawn with other RNA transcription and processing pathways, e.g., 45S pre-rRNA in animal cells (1) and pre-tRNA in animal (26, 27) and bacterial systems (28). A particularly interesting and possibly close parallel would be the processing of phage T7 early RNA transcripts (29). RNase III, which is active against double-stranded RNA, cleaves the T7-specific RNA at presumed double-stranded looped regions of the RNA to give precisely terminated mRNA molecules (30). Attempts to determine if such a cleavage mechanism exists for the Ad-2 nuclear RNA are obviously in order.

While the present experiments provide strong evidence that RNA complementary to fragments B, F, D, E, and C are present on a set of increasingly long transcripts, the nature of original transcripts from the A region is less clear. Because the A region is itself so large, 23 kb, it might very well be transcribed into a molecule, say 10–12 kb, plus another unit of 10–12 kb which was continued through the BFDEC region. This can be determined with restriction fragments of the A region by the same type of short label experiments described here.

A final point raised by these experiments concerns the speed with which processing occurs and how this affects the chances of observing original RNA transcripts containing an mRNA. For example, pre-rRNA, 14 kb, requires about 2–2.5 min to be synthesized (22); in approximately 10 min, finished 45S molecules are processed (1, 22). Thus, it is possible to observe radioactivity flowing first into nascent 45S pre-rRNA, then into finished unit-sized 45S pre-rRNA, before radioactive processed products, also discrete in size, are observed. Thus only if the processing time is slow relative to synthesis time and only if some means of observing discrete finished and/or processed products is available, can nascent RNA be distinguished from processed RNA. It appears that in the majority, the long adenovirus transcripts do survive at least through transcription, so that specifically sized, newly synthesized molecules complementary to specific DNA fragments can be observed. Thus in Fig. 4 the peaks of radioactivity for the successive fragments B, F, D, E, and C are in tubes 11, 9, 8, 7, and 6, respectively. Since an entire adenovirus transcript might require at least 400 sec (40,000 nucleotides at 100 nucleotides/sec) (21, 22) for synthesis, it would appear that processing of adenovirus "hnRNA" begins at least several minutes after the first nucleotides are polymerized. That some processing may occur within a few minutes is suggested, however, because in the experiment where a 2 min label time was followed by 2 min exposure to actinomycin as well as in the 2 min label of Fig. 3, some small RNA complementary to B, E, and C was observed in the slower sedimenting RNA.

The possibility of very rapid processing must be borne in mind when attempts are made to measure "transcripts" of cell genomes containing specific gene sequences. Experiments in which the most newly synthesized RNA is examined are necessary. Experiments which examine the total accumulated RNA may not reveal the true transcript due to the large accumulation of processed products.

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