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Transcription and processing of adenoviral RNA by extracts from HeLa cells

(RNA polymerase/promoters/mRNA/RNA splicing)

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ABSTRACT **Concentrated extracts from HeLa cells prepared by the procedure of Sugden and Keller [Sugden, B. & Keller, W. (1973) *J. Biol. Chem.* 248, 3777–3788] can synthesize RNA molecules of discrete lengths in the presence of DNA templates carrying complete viral transcription units. Accurate initiation of transcription was shown to occur at the "major late" promoter and at the promoter for the "early region III" transcription unit of the adenovirus 2 genome. A cloned fragment of adenovirus 2 DNA containing the early region III transcription unit directs the synthesis of discrete RNA species, many of which correspond in length to spliced early region III mRNAs isolated from adenovirus 2-infected HeLa cells. As shown by hybridization/nuclease S1 analysis, RNA splicing and the formation of specific 3′ ends are also taking place *in vitro*.**

Since the discovery of spliced mRNAs in cells infected with adenovirus 2 (Ad2; refs. 1–3), it has become apparent that many other eukaryotic mRNAs are composed of sequences of nucleotides that are not contiguous with the DNA template. Mature mRNAs are derived from primary transcripts by several post-transcriptional processing steps, which include modification of the 5′ ends by a "cap" structure containing 7-methylguanosine, addition of a tract of poly(A) at the 3′ termini, and removal of intervening sequences by RNA splicing (for review, see ref. 4). Many of these processes can be demonstrated in isolated nuclei from virus-infected cells (5–9). However, to study the enzymes and other components involved in these reactions, soluble cell-free systems are required that carry out transcription and processing of RNA *in vitro*. Specific initiation of transcription of mRNA precursors has recently been observed *in vitro* in two different cell-free systems. One of these uses purified RNA polymerase II and a high-speed supernatant from a KB-cell lysate (10), and the other (11) uses a concentrated extract of HeLa cells prepared by a method originally devised for the isolation of RNA polymerase (12). Both of these systems have been used to examine the transcription of purified fragments of viral DNA that include known promoter sequences but do not contain natural transcriptional termination signals. Under these conditions, RNA molecules are generated that are initiated at the specific site known to be used *in vivo* and extend to the end of the DNA fragment used as template. As expected, such "run-off" transcripts are neither polyadenylylated nor spliced, although they do include "cap" structures at their 5′ ends (10, 11).

The transcription experiments described here use extracts of HeLa cells prepared by a modification of an earlier procedure (12). As templates, we used the DNA of either Ad2 or of a plasmid containing the DNA coding for a single Ad2 transcription unit. In each case, the synthesis of a number of discrete viral transcripts that appear to have counterparts in the cytoplasm of human cells infected with Ad2 was observed.

MATERIALS AND METHODS

Preparation of DNA. Ad2 and DNA were prepared as described (13). Restriction endonuclease *Sma* I fragment C was inserted by ligation into the *Sma* I site of the plasmid pACYC 177 (14) and cloned in *Escherichia coli* C 600 by standard procedures (15). The cloning and growth of the recombinant plasmid was carried out under P2/EK1 containment according to the National Institutes of Health guidelines for recombinant DNA research. The resulting recombinant plasmid was designated pAd2 SmaC. Plasmid DNA was prepared as described (16). *In vitro* labeling of DNA by nick translation was carried out as described (17).

Plasmids pAd2 BaL E and pAd2 HindIII H were gifts from J. Manley and S.-L. Hu, respectively.

Preparation of mRNA from Ad2-Infected HeLa Cells. Exponentially growing suspension cultures of HeLa cells were infected at a density of 2–4 × 10^5 cells per ml in 0.1 vol of culture medium with Ad2 at a multiplicity of infection of 50–100 plaque-forming units per cell. One hour after infection, fresh culture medium containing 10% calf serum and cycloheximide at 25 μg/ml was added up to the original volume. The infected cells were collected 15 hr later by centrifugation and washed three times with phosphate-buffered saline. Cytoplasmic poly(A) containing RNA was isolated as described (18).

Preparation of HeLa Cell Extracts. Extracts from exponentially growing uninfected HeLa cells were prepared and concentrated by $(NH_4)_2SO_4$ precipitation and dialysis as described (12). The heat treatment after lysis of the nuclei and the treatment with phenylmethylsulfonyl fluoride were omitted. Sixteen milliliters of packed cells gave 11 ml of dialyzed extract containing ≈30 mg of protein/ml.

Synthesis and Purification of RNA. RNA was synthesized for 40 min at 30°C (unless otherwise specified) in 50-μl reaction mixtures of (final concentrations) 25 mM Tris·HCl, pH 7.9/20 mM $(NH_4)_2SO_4$/0.5 mM EDTA/6 mM $MgCl_2$/0.5 mM dithiothreitol/7.5% (vol/vol) glycerol/0.5 mM each of ATP, CTP, and GTP/0.05 mM [α-^{32}P]UTP (≈10,000 cpm pmol^{-1})/5 mM creatine phosphate/25 μl of HeLa cell extract Ad2 DNA or pAd2 SmaC DNA as indicated. After synthesis, the reaction mixtures were diluted with 2.4 ml of 50 mM NaOAc, pH 5.4/1% $NaDodSO_4$/10 mM EDTA; 2.4 g of CsCl was added and dissolved by heating to 60°C for 2 min, and the solution was

Abbreviations: Ad2, adenovirus serotype 2; E III, early region III of the Ad2 genome.

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layered onto 1.2 ml of 5.7 M CsCl/0.1 M EDTA in nitrocellulose centrifuge tubes. The tubes were centrifuged for ≈14 hr at 36,000 rpm and 20°C in an SW 60 rotor (19). The pelleted RNA was dissolved in 0.3 ml of 0.3 M NaOAc, pH 5.4, and precipitated with 2 vol of EtOH. The precipitate was dissolved in H_2O and stored at −20°C.

RNA Analysis. DNA–RNA hybridizations and treatment with nuclease S1 (Sigma; 300 units/ml) were carried out as described (20). DNA–RNA hybrids were separated by electrophoresis through 1.4% (wt/vol) agarose gels in the buffer described by Bailey and Davidson (21). When denaturing conditions were required, 6 mM MeMgOH was included in the agarose gel (21). Blotting of DNA onto nitrocellulose was carried out by a modification (22) of Southern's procedure (23). Hybridization of nick-translated DNA probes with immobilized DNA on Southern blots was carried out for 24 hr at 42°C in 50% (vol/vol) formamide/0.60 M NaCl/0.060 M Na citrate/Denhardt's solution (24)/0.1% $NaDodSO_4$ containing 2–5 × 10^6 cpm of [^{32}P]DNA. Afterward, the blots were washed extensively with 50% formamide/0.60 M NaCl/0.060 M Na citrate at 42°C, followed by several washes with 0.20 M NaCl/0.02 M Na citrate at room temperature, air dried, and prepared as autoradiographs.

RESULTS

***In Vitro* RNA Synthesis.** The total amount of RNA made in a standard reaction is 5–40 pmol (calculated by multiplying the number of [α-^{32}P]UMP incorporated by four), depending on the batch of extract used. In the absence of added DNA, the level of *in vitro*-labeled RNA is ≈5% of that made in the complete reaction. Under standard reaction conditions, RNA synthesis is linear for 30–40 min, then gradually levels off, and reaches a plateau after ≈1 hr.

A preliminary characterization of the ^{32}P-labeled RNA synthesized *in vitro* on Ad2 DNA was carried out by electrophoresis through agarose gels containing the denaturant MeHgOH (21). Under these conditions, we consistently observed the presence of multiple discrete size classes of RNA. Because RNA synthesis was highly dependent on added DNA, we considered the possibility that these discrete RNAs might be viral transcripts. To establish this, we hybridized total *in vitro* RNA to Ad2 DNA, digested the DNA–RNA hybrids with nuclease S1 (20), and separated the products by electrophoresis in neutral agarose gels. By using two concentrations of template, we observed a large number (>20) of discrete S1-resistant viral DNA–RNA hybrids of various lengths. The synthesis of these RNAs is completely inhibited by α-amanitin at 1 μg/ml, indicating that the viral transcripts are generated by RNA polymerase II (10) (results not shown). Depending on the particular batch of extract used for the transcription experiments, we have also found variable amounts (5–20% of the total) of small molecular weight RNA having an electrophoretic mobility in denaturing agarose gels slightly less than that of tRNA. The synthesis of this RNA was dependent on Ad2–DNA and could be inhibited by high concentrations (>50 μg/ml) of α-amanitin (results not shown). Presumably, this represents virus-associated RNA (25) transcribed by RNA polymerase III, as has been observed previously (26, 27).

To simplify analysis of the *in vitro*-synthesized RNA, we restricted our subsequent studies to the "early region III" (E III) transcription unit of the Ad2 genome. To this end, the viral DNA was cut with *Sma* I and the fragment (*Sma* C) carrying the E III transcription unit was inserted into a plasmid vector and cloned in *E. coli*. The DNA of the recombinant plasmid "pAd2 SmaC" was then used as a template for *in vitro* transcription, as well as for hybridization experiments with *in vivo* Ad2 mRNA. The segment of viral DNA in pAd2 SmaC is ≈5000 nucleotide pairs long and spans the map coordinates 76.5–91.9 on the Ad2 genome (ref. 28; Fig. 1). The sequence T-A-T-A-A, which is located near the start site of E III RNA, is present in pAd2 SmaC eight nucleotides distal from the *Sma* I site at map coordinate 76.5 (29).

Analysis of E III mRNAs. The E III region of the Ad2 genome is transcribed *in vivo* into a complex set of mRNAs (Fig. 1). A detailed electron microscopic analysis (30) shows eight types of E III mRNAs. These different forms of RNA have a common leader segment of 372 nucleotides at their 5′ ends, followed by a splice deletion corresponding to 385 nucleotides (29). They differ from each other by the positions of their 3′ ends, which map at three alternative locations. In addition, five of the eight types of E III mRNA carry a second splice deletion beginning at coordinate 79.2 and ending at four alternative sites. The sequence of the 5′ end of the E III mRNAs and the corresponding sequence of Ad2 DNA has been determined by Baker and Ziff (31), who have also discovered the sequence T-A-T-A-A at position −29 to −25 upstream of the E III mRNA initiation site. This sequence was first noted by Hogness and Goldberg (for discussion, see ref. 32) and is present near position −20 to −30 upstream from the initiation site of many eukaryotic mRNA coding genes.

The analysis of *in vivo* E III mRNAs by hybridization/nuclease S1 digestion, followed by agarose gel electrophoresis of the DNA–RNA hybrids is shown in Fig. 2A. As hybridization probe, we used pAd2 SmaC DNA that had been linearized by cutting with *Bam*HI, which leaves the inserted Ad2 sequences intact. The lengths of the different molecules were estimated by comparing their electrophoretic mobilities with those of DNA marker fragments. Our nuclease S1 mapping results are in general agreement with the electron microscopic measure-

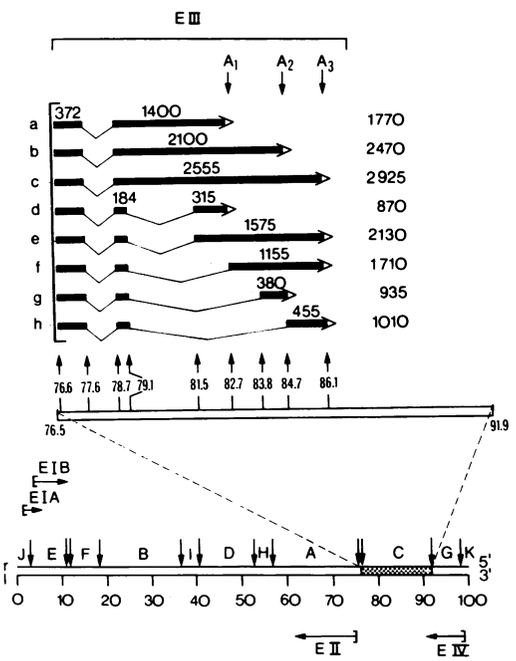

FIG. 1. Map of Ad2 genome showing early transcription units I–IV. The fragments of Ad2 DNA generated by *Sma* I (28) are indicated by capital letters. Numbers above and to the right of the enlarged E III transcripts a–g indicate length in nucleotides as compiled from electron microscopic measurements (29) and DNA sequence analysis data (30). The direction of transcription is indicated by arrows and spliced-out RNA segments are indicated by carets. A_1, A_2, and A_3 correspond to the three alternative poly(A) addition sites in the E III transcription unit.

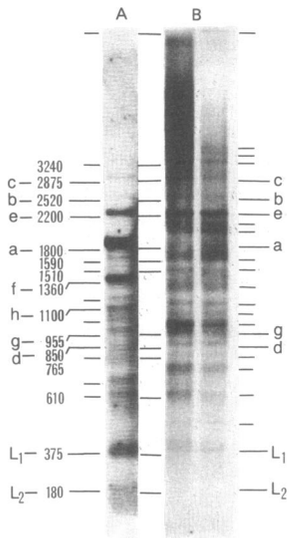

FIG. 2. Comparison of *in vivo* early Ad2 mRNAs and RNAs transcribed *in vitro* on pAd2 SmaC as template. (*A*) Poly(A)$^+$ RNA (≈2 μg) isolated from HeLa cells 14 hr after infection with Ad2 in the presence of cycloheximide ("early RNA") was hybridized with 1 μg of pAd2 SmaC DNA that had been linearized by digestion with *Bam*HI, and the hybrids were treated with nuclease S1 and separated by electrophoresis as described in *Materials and Methods*. The positions of the hybrids were visualized after blotting of the gel by hybridization with Ad2[^{32}P]DNA and autoradiography. (*B*) [^{32}P]RNA was synthesized *in vitro* for 15 min (right-hand lane) and 30 min (left-hand lane) on intact pAd2 SmaC DNA (5 μg per reaction) and purified as described in *Materials and Methods*. Aliquots of ≈10,000 cpm were hybridized with 1 μg of Ad2 DNA, and the hybrids were treated with nuclease S1 and run on the same gel as the DNA–RNA hybrids formed with *in vivo* viral mRNAs. Letters a–g refer to the different E III mRNAs shown in Fig. 1. L_1 and L_2 represent fragments possibly generated by nuclease S1 cutting the hybrid molecules at splice points. The numbers indicate lengths in nucleotides. Connecting lines between *A* and *B* indicate bands that have the same electrophoretic mobilities.

ments of Chow *et al.* (30): for all the E III RNA species described by those workers, DNA–RNA hybrids of corresponding lengths could be seen. However, the hybridization/nuclease S1 analysis showed many additional RNAs that have not been detected by electron microscopy or by the earlier nuclease S1 mapping experiments of Berk and Sharp (33). Some of these fragments could originate from an artifact occurring during the nuclease S1 digestion of DNA–RNA hybrids: nuclease S1 is known to occasionally cut the RNA strand opposite a nick or gap in the DNA strand generated by the nucleolytic digestion of an intervening DNA sequence (34), causing the original hybrid to break into smaller fragments.

Comparison of *in Vitro* RNA with E III *in Vivo* RNA. RNA was synthesized *in vitro* on pAd2 SmaC DNA and hybridized to Ad2 DNA, and the DNA–RNA hybrids were treated with nuclease S1 and separated by agarose gel electrophoresis. The resultant pattern is shown in Fig. 2*B*. Comparison with the bands generated by *in vivo* mRNA and subjected to electrophoresis through the same gel (Fig. 2*A*) shows that a number of RNA species from the *in vitro* reaction have counterparts in the *in vivo* channels of the gel. The strongest *in vitro* RNA band comigrates with a prominent *in vivo* species 2200 nucleotides long. This most likely represents RNA species e with two splice deletions, as shown in Fig. 1. Other *in vitro* RNA bands that appear to correspond to known *in vivo* RNAs are types a–d, g, and probably h. In addition, there are two low molecular weight RNAs, 375 and 180 nucleotides long, present in both the *in vivo* and the *in vitro* part of the autoradiograph. These bands probably originate from cutting of the RNA by nuclease S1 at splice junctions (see above) and could represent the first leader segment (372 nucleotides) common to all E III mRNAs and the second leader segment (184 nucleotides) present in E III RNA types d–h. This assumption is supported by our finding that the intensity of these two species was reduced by carrying out the S1 digestion at 15°C instead of the standard 42°C, thus minimizing the cutting of the RNA strand at splice points (ref. 34; results not shown). In addition to the RNA bands that have *in vivo* counterparts, there are a number of species from the *in vitro* synthesis for which no corresponding *in vivo* correlate can be found. The nature of these RNAs is at present unknown. Because all viral *in vitro* RNAs originate from the same promoter at coordinate 76.6 (see below), the additional RNAs could represent molecules whose synthesis was terminated at sites on the DNA template not used *in vivo* or molecules whose intervening sequences have not been completely removed by RNA splicing.

Mapping of the 5′ End of *in Vitro* RNAs. To test whether the viral transcripts generated *in vitro* originate by the interaction of RNA polymerase II at the correct promoter sites, RNA was synthesized in the presence of "truncated" templates (Fig. 3). Two promoter-containing DNA fragments were used: pAd2 Bal E (11) carrying a viral insert with the major late promoter at map coordinate 16.5 and pAd2 SmaC with the E III promoter at coordinate 76.6. As shown in Fig. 3*A*, digestion of the plasmid template pAd2Bal E with *Bam*HI gave rise to a "runoff" *in vitro* transcript of 1750 nucleotides; digestion of the plasmid pAd2 SmaC with *Bgl* II resulted in the synthesis of a RNA of 565 nucleotides (Fig. 3*B*). In each case, the length of the *in vitro* transcripts corresponded to the distance between the position of the promoter and the end of the DNA template. These results demonstrate that the initiation of RNA synthesis in our *in vitro* system is guided by the promoters operating *in vivo*.

***In Vitro* RNA Splicing.** To demonstrate that RNA splicing takes place in the *in vitro* system, a modified hybridization/nuclease S1 experiment (35) was carried out as outlined schematically in Fig. 4*D*. Unlabeled RNA was synthesized in a standard reaction containing pAd2 SmaC DNA template and purified as described in *Materials and Methods*. A plasmid clone containing the Ad2 HindIII H fragment (coordinates 73.6–79.9) was digested with *Hin*dIII, and the 5′ ends of the resulting fragments were labeled with [γ-^{32}P]ATP and DNA kinase. The end-labeled DNA fragments were hybridized to the *in vitro* RNA, and the resulting hybrids were digested with nuclease S1 and analyzed on a denaturing agarose gel. The same experiment was also carried out with mRNA from Ad2-infected HeLa cells as a control. As shown in Fig. 4*D*, unspliced E III RNA would protect a DNA fragment of 1044 nucleotides from nuclease S1 digestion. E III RNAs that have a splice deletion (Fig. 1) after the common leader segment will lead to the formation of two protected DNA segments, only one of which will be radiolabeled. The results of this experiment are shown in Fig. 4. The *in vivo* RNA protected two radiolabeled DNA bands whose lengths corresponded to those predicted from the known nucleotide sequence of this region of the Ad2 genome (ref. 30; Fig. 4*A*). The fragment of ≈280 nucleotides was much more intense than that of ≈1040 nucleotides, showing that most *in vivo* E III RNAs contain a splice junction. The presence of a weak DNA band of 1040 nucleotides shows that a small fraction of the *in vivo* E III RNA consists of unspliced molecules. The RNA synthesized *in vitro* also protected two labeled DNA fragments from nuclease digestion whose sizes corresponded to those found in the *in vivo* RNA experiment (Fig. 4*B*). The presence of a DNA band of ≈280 nucleotides indicates that RNA synthesized *in vitro* on DNA carrying the E III transcription unit

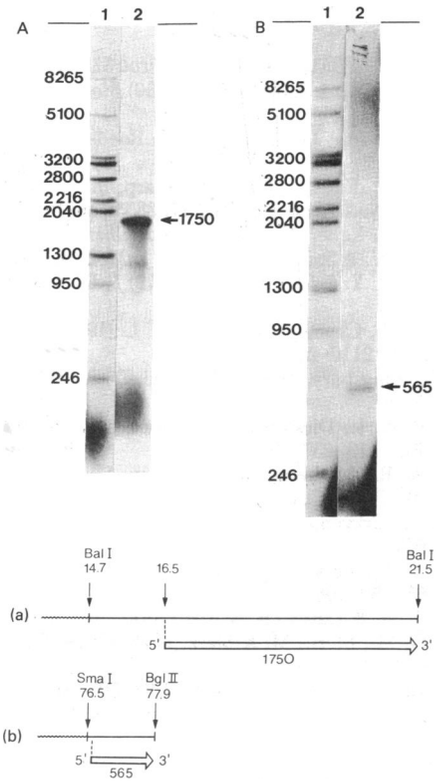

FIG. 3. Runoff transcription from the major late Ad2 promoter and the E III promoter. (*Upper*) RNA was synthesized in standard reaction mixtures. DNA templates were 2.5 μg of pBal E/pBR322 (II) digested with *Bam*HI (*A*) and 2.5 μg of pAd2 SmaC/pACYC 177 digested with *Bgl* II (*B*). [^{32}P]RNA was analyzed in 1.4% agarose gels containing MeHgOH after hybridization to Ad2 DNA and treatment with nuclease S1 along with Ad2 [^{32}P]DNA digested with *Hin*dIII as marker. Numbers adjacent to the gel tracks indicate lengths in nucleotides. (*Lower*) Positions of the major late Ad2 promoter at map coordinate 16.5, and the *Bal* I restriction sites (*a*) and the E III promoter at map coordinate 76.6 and the *Sma* I and *Bgl* II restriction sites (*b*). The expected runoff transcripts are indicated by arrows.

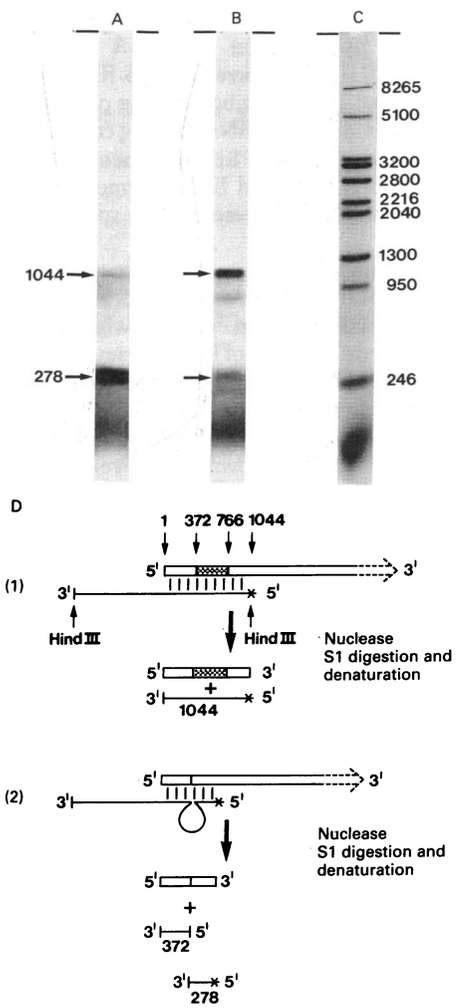

FIG. 4. Detection of splice junctions in E III RNA. Unlabeled RNA was synthesized in a standard reaction mixture with 5 μg of pAd2 SmaC DNA as template and purified as described in *Materials and Methods*. Plasmid pAd2 HindIII H was digested with *Hin*dIII, and the resulting fragments were end labeled with [γ-^{32}P]ATP and DNA kinase (35). Approximately 100,000 cpm of end-labeled DNA was hybridized to $\approx$5 μg of poly(A)$^+$RNA from Ad2-infected HeLa cells (*A*) or to *in vitro* RNA from a standard reaction mixture (*B*). After hybridization, the mixtures were digested with nuclease S1 at 30°C as described in *Materials and Methods* and then analyzed by electrophoresis through a 1.4% agarose gel containing 6 mM MeHgOH, dried, and autoradiographed. (*C*) End-labeled Ad2 HindIII fragments as size markers. (*D*) Rationale of the experiment. Numbers over the arrows above the RNA in (*D*1) indicate numbers of nucleotides, starting with the 5′ end as number 1, and are derived from the DNA sequence data (29). ▭, intervening region common to all E III mRNA precursors; *, ^{32}P-labeled 5′ ends of DNA fragments.

contains a splice junction at the same position as *in vivo* E III RNA. This conclusion was corroborated by the finding that, on electrophoresis of the same samples in a nondenaturing agarose gel, in which the two DNA fragments resulting from nuclease S1 digestion remain hybridized to RNA, no band of $\approx$280 base pairs was observed; instead, a band of $\approx$650 base pairs appeared, corresponding to the distance between the 5′ end of the DNA probe and the 5′ end of spliced EIII RNA (results not shown). The relative intensities of the bands of 1040 and 280 nucleotides do not reflect the ratio of spliced versus unspliced *in vitro* RNA. As the end-labeled DNA had its 5′ end within the second intervening sequence of E III RNA species d–h (Fig. 1), only RNAs of type a, b, or c are scored by this analysis. Further experiments with different DNA probes should allow us to estimate the efficiency of *in vitro* RNA splicing. In addition to the two DNA fragments described above, the *in vitro* RNA protected two additional labeled fragments from nuclease digestion (Fig. 4*B*). These fragments, which have no counterparts in the *in vivo* part of the experiment, result from the protection of end-labeled pBR322 *Hin*dIII DNA fragments, as shown by a control experiment in which end-labeled pBR322 *Hin*dIII fragments were used as hybridization probes (result not shown). This indicates that the *in vitro* RNA must contain a certain number of molecules that originated from the transcription of plasmid sequences.

DISCUSSION

We have presented evidence that a concentrated extract from uninfected HeLa cells contains the components necessary to synthesize RNA molecules of discrete lengths *in vitro*. Plasmid DNA carrying the Ad2 E III transcription unit directs the synthesis of a number of transcripts that, on electrophoresis, comigrate with authentic E III mRNAs from infected HeLa cells.

The length of RNA bands generated on truncated templates suggests that all viral RNAs made *in vitro* start at the correct *in vivo* initiation sites. The formation of E III RNAs of different lengths starting from a common initiation point indicates that they must have different 3′ end points or internal splice deletions of different sizes or both.

The finding that the majority of DNA–RNA hybrid bands generated after hybridization of mRNA from Ad2-infected HeLa cells to E III DNA correspond to RNAs synthesized *in vitro* suggests that RNA splicing is taking place in the cell-free system. Direct evidence for RNA splicing comes from the modified hybridization/nuclease S1 experiment (Fig. 4), in which the generation of a protected DNA fragment of ≈280 nucleotides is diagnostic for the presence of a splice junction in the *in vitro* RNA.

From our experiments, it is not possible to determine whether the 3′ ends of the *in vitro* RNAs are the result of specific transcription termination events or whether they are made by endonucleolytic cleavage of longer primary transcripts. The latter mechanism has been found to operate *in vivo* (36, 37).

In agreement with earlier work (10, 11), we find that the RNAs synthesized *in vitro* carry methylated cap structures at their 5′ ends (results not shown). However, we are at present not certain whether polyadenylylation of the 3′ ends also takes place. Variable amounts of the total RNA synthesized *in vitro* (5–20%) could be retained on a column of oligo(dT)-cellulose and eluted with low-salt buffer (38). This low efficiency of binding may be due to the presence of poly(A) tails too short to form stable hybrids with oligo(dT). Moreover, we cannot exclude the possibility of unspecific binding to oligo(dT)-cellulose of RNA molecules containing no poly(A) sequences.

Transcription and processing of RNA to generate mature mRNAs requires the concerted action of many enzymes and other components. Particularly interesting are protein factors that promote the initiation of RNA synthesis at specific sites (39) and the enzyme(s) and cofactors such as ribonucleoprotein particles containing small nuclear RNA that have been implicated as possible participants in RNA splicing (40, 41). Perhaps, by fractionating the HeLa cell extract, it will be possible to identify some of the components necessary for the expression of eukaryotic genes.

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