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Splicing of adenovirus RNA in a cell-free transcription system

(mRNA processing/HeLa whole cell extract/RNA splicing)

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ABSTRACT **A soluble whole-cell extract prepared accurately from HeLa cells splices 2–3% of the RNA transcribed from a DNA template containing the first and second leader exons of late adenovirus RNA. The spliced RNA was detected by a sensitive technique using hybridization to a single-stranded phage M13 cDNA clone, followed by binding to nitrocellulose filters. The identity of the spliced RNA was established by RNase T1 and pancreatic RNase two-dimensional peptide mapping. The bond formed during the *in vitro* splicing reaction appears to be a typical 3′,5′-phosphodiester bond as judged by its sensitivity to RNase T1. The splicing reaction is specifically inhibited by KCl at concentrations greater than 50 mM and by the addition of cellular RNA. Three features of this system may account for the detection of splicing in a soluble extract: (*i*) the sensitive and unambiguous hybridization assay, (*ii*) the high transcriptional activity of the major late promoter of adenovirus, and (*iii*) the use of the first and second leader exon splice of adenovirus, which may be unusually rapid.**

The evolutionary significance of intervening sequences and their possible role(s) in the control of gene expression have been a source of much speculation. However, our understanding of the process of splicing of precursors of mRNAs has not significantly advanced since the discovery of splicing (1). Progress has been made in determining the substrate requirements and mechanisms of the splicing reactions that remove intervening sequences from yeast tRNA precursors (2, 3) and the precursor to *Tetrahymena* ribosomal RNA (4, 5). These studies have been facilitated by the development of soluble systems that carry out the reactions *in vitro*. In contrast, the elucidation of the details of mRNA splicing has been slow because of the difficulty in developing such a system.

Here we present evidence that an adenovirus mRNA precursor can be spliced in a soluble *in vitro* system derived from HeLa cells. The splicing reaction is precise, and the product RNA can be recovered for structural and mechanistic studies.

MATERIALS AND METHODS

Extract. Whole cell extract from HeLa S3 cells was prepared according to Manley *et al.* (6). The final pellet was resuspended in half the original packed cell volume in a buffer of 17% (vol/vol) glycerol, 0.1 M KCl, 12.5 mM $MgCl_2$, 20 mM Hepes (pH 7.9), 0.1 mM EDTA, and 1 mM dithiothreitol. This extract was dialyzed against two changes of buffer for a total of 14 hr and then frozen in liquid nitrogen and stored at −70°C. The transcription and splicing activities are stable for at least 9 months.

Transcription Reactions. Analytical reactions were carried out in a total volume of 25 μl containing template DNA (30 μg/ml), 500 μM ATP, 500 μM GTP, 50 μM CTP, 5 μM UTP, 10 μCi of [α-^{32}P]UTP, 4 mM creatine phosphate, and 10 μl of extract. KCl was added to a final concentration of 50 mM, including the contribution from the extract. The reactions were incubated at 30°C for 3 hr, stopped by the addition of 300 μl of 7 M urea/100 mM LiCl/10 mM Tris·HCl, pH 7.7/10 mM EDTA/0.5% $NaDodSO_4$, and extracted once with phenol/chloroform, 1:1 (vol/vol), and once with chloroform. After precipitation in ethanol, the samples were dissolved in 15 μl of 0.75 M NaCl/50 mM Hepes, pH 7.0/1 mM EDTA/0.2 μg of M13-pJAW 43 DNA. Hybridization involved heating samples to 80°C for 10 min followed by incubation at 50°C for 3 hr. After hybridization, the samples were diluted with 200 μl of filtering buffer (0.2 M NaCl/10 mM Hepes, pH 7.6/1 mM EDTA), and 5 units of RNase T1 (Calbiochem) were added. After incubation for 30 min at room temperature, proteinase K was added to a final concentration of 200 μg/ml, and the samples were further incubated for 1 hr at 30°C. RNA·DNA hybrids were selected by filtering samples through nitrocellulose filters (Schleicher & Schuell, BA85) equilibrated with filter buffer at a flow rate of 1 ml/min. The filters were then washed twice with 2 ml of filter buffer, blotted dry, and transferred to capped plastic tubes. The RNA was eluted from the hybrid by heating the filters to 100°C for 5 min in 1.5 ml of 2 mM EDTA (pH 7.4). The eluted RNA was precipitated in ethanol with 30 μg of tRNA carrier and was electrophoresed in a 10% polyacrylamide gel containing 8.3 M urea in 90 mM Tris borate, pH 8.3/2.5 mM EDTA. The gel was dried and autoradiographed using Kodak XAR-5 film and Dupont Cronex intensifying screens at −70°C.

Preparative reactions were carried out in the same way by using 10 times the volume at each step, except that no unlabeled UTP was added and 1 mCi (1 Ci = 37 GBq) of [α-^{32}P]-UTP (New England Nuclear; 3,000 Ci/mmol) was used. After electrophoresis, the gel was autoradiographed, the bands to be mapped were excised and ground up, and the RNA was eluted with 0.5 M ammonium acetate/0.1% $NaDodSO_4$/1 mM EDTA.

Two-Dimensional Chromatographic Analysis. RNA was dissolved in 20 μl of 10 mM Tris·HCl, pH 7.5/1 mM EDTA and digested with 1 unit of RNase T1 for 4 hr at 37°C or with 0.6 μg of pancreatic RNase for 1 hr at 37°C. The sample was lyophilized to dryness, resuspended in 3 μl of H_2O, and the oligonucleotides were separated by electrophoresis on cellulose acetate followed by homochromatography as described (7, 8). Nucleotide composition of spots was determined by elution with 1 M triethanolamine bicarbonate, RNase T2 digestion, and one-dimensional chromatography (7).

RESULTS

Detection of Spliced RNA. During the late stage of adenovirus infection, three leader segments are spliced to all transcripts from the late transcription unit (1, 9). The first (L1) and

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Abbreviations: Ad2, adenovirus 2; WCE, whole-cell extract; L1, first leader exon; L2, second leader exon; L3, third leader exon; L1-2, spliced form of first and second leader exons; L1-2-3, spliced form of first, second, and third leader exons.

second (L2) leaders, mapping at 16.55 and 19.5 map units, are 41 and 72 nucleotides in length, respectively. Sequences for these two leaders, separated by an intron of 1,020 nucleotides, are present in the *Bal* I E fragment of adenovirus 2 (Ad2) (Fig. 1*A*). The major late adenovirus promoter site in this fragment is the most active known substrate for *in vitro* transcription in the HeLa whole-cell extract (WCE) system (6). To assay for splicing of ^{32}P-labeled RNA transcribed from this fragment, RNA was hybridized to a single-stranded DNA from a recombinant M13 phage that contains a cDNA clone of part of the tripartite leader. This 172-nucleotide cDNA segment contains the entirety of the L1 and L2 exons and 59 nucleotides of the third leader (L3) exon joined in their spliced configuration (L1-2-3) (Fig. 1*B*). Hybrids formed between the ^{32}P-labeled RNA and the single-stranded M13 DNA were digested with RNase T1 to degrade single-stranded RNA. The RNase T1-resistant RNA·DNA duplex was selected by filtering through nitrocellulose under conditions where only the single-stranded DNA will bind. The RNA was then released from the hybrid and resolved by electrophoresis in a polyacrylamide gel containing 8.3 M urea. Labeled unspliced RNA transcribed *in vitro* from the *Bal* I E fragment would be expected to produce protected RNA species of 41 and 72 nucleotides from the L1 and L2 exons, respectively. A spliced RNA (spliced form of the first and second leader exons, L1-2) would generate a protected RNA of 113 nucleotides (see Fig. 1).

Transcription of the *Bal* I E fragment of Ad2 in a HeLa WCE produced an intense run-off band of 1,800 nucleotides. When the [α-^{32}P]UTP-labeled RNA from such a reaction was assayed for L1-2-spliced RNA by the M13 phage hybridization technique, a low level of spliced product was observed (Fig. 2). As expected, the two most abundant RNAs protected by M13 hybridization were derived from the unspliced L1 and L2 exons. The multiple bands migrating as approximately 41 and 72 nucleotides, respectively, are due to nibbling by RNase T1 at the ends of the RNA·DNA duplex, as the ratio of these bands changed after digestion with increasing concentrations of RNase T1 (Fig. 2, lanes 1–3). The faint doublet migrating as a 113-nucleotide RNA (marked L1-2) was the correct length to have been generated by the precise splicing of the L1 and L2 exons. These bands comigrated with bands generated by hybridization to ^{32}P-labeled *in vivo* RNA extracted from the late stage of Ad2-infected cells (Fig. 2, lane 4). Previous studies have shown that spliced intermediates with only the L1 and L2 leaders joined accumulate in the nucleus (13, 14). RNA labeled *in vivo* for 1 hr yielded an approximately equal amount of L1-2 and L1-2-3 RNAs (113 and 172 nucleotides, respectively; Fig. 2, lane 4). It was reassuring that both the *in vitro* (lane 3) and *in vivo* (lane 4) labeled RNAs gave the same doublet pattern migrating at 113 nucleotides after digestion with RNase T1.

Two-Dimensional Chromatographic Analysis of Spliced RNA. Definitive identification of the 113-nucleotide band as the L1-2-spliced RNA required two-dimensional chromatographic analysis of the *in vitro* product. Preparative reactions were la-

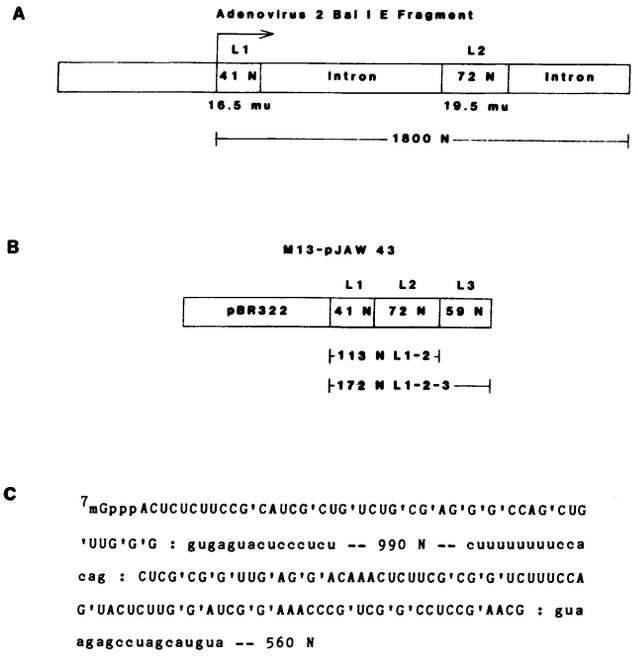

FIG. 1. Structure of DNA template, probe, and RNA transcript. (*A*) Structure of the Ad2 *Bal* I fragment E transcription template cloned into the *Bam*HI site of pBR322. The clone contains 679 bases of 5′ noncoding sequences, the major late promoter, the L1 exon at 16.5 map units (mu), a 1,020 base intron, the L2 exon at 19.5 map units, and 578 bases of the second intron. (*B*) Structure of the M13 cDNA clone. The *Eco*RI–*Xho* I fragment of pJAW 43 (10) containing the *Pst* I–*Eco*RI fragment of pBR322 and the L1-2-3 of Ad2 was inserted between the *Eco*RI and *Sal* I sites of M13 mp9. (*C*) Sequence of L1 and L2 exons. The upper case nucleotide sequences are of the leader exons (10, 11) and the lower case sequences are of portions of the introns proximal to the splice sites (12). The exon sequences are separated into the oligonucleotides produced by the action of RNase T1.

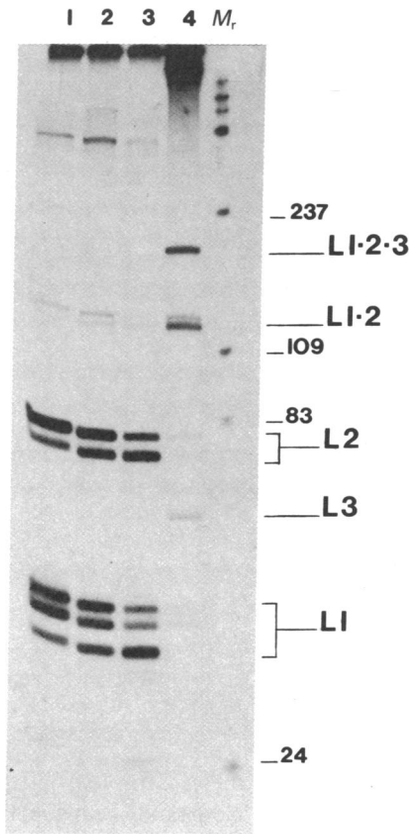

FIG. 2. Analysis of *in vitro* and *in vivo* RNAs complementary to M13 pJAW 43. Lanes: 1–3, *in vitro* reactions in which the RNA was hybridized to M13 pJAW 43 DNA and digested with 5 (lane 1), 10 (lane 2), and 20 (lane 3) units of RNase T1; 4, total cellular RNA (from Ad2-infected HeLa cells labeled for 1 hr with ^{32}P phosphate at 20 hr after infection) which was hybridized to M13 pJAW 43 and treated with 20 units of RNase T1. The RNase-resistant RNA·DNA hybrids were selected on nitrocellulose filters. The RNA was released and run on a 10% polyacrylamide gel containing 8.3 M urea. The M_r markers (shown $\times 10^{-3}$) are simian virus 40 DNA cleaved with *Hin*fI.

beled with [α-^{32}P]UTP, and the RNA was processed by hybridization to the M13 cDNA clone, RNase T1 digestion, and polyacrylamide gel electrophoresis. Labeled RNAs migrating in bands of 41, 72, and 113 nucleotides were eluted and either purified further by electrophoresis in a 12% polyacrylamide gel containing 4 M urea before analysis or subjected directly to RNase T1 and pancreatic RNase analysis.

Because the site of joining of the two leaders is a guanosine residue, an RNase T1 digest of the spliced L1-2 RNA would be expected to contain only those oligonucleotides present in the isolated leaders (Fig. 1*C*). Fig. 3 shows the results of the two-dimensional chromatographic analysis of the isolated 41- and 72-nucleotide leader bands and the spliced 113-nucleotide band from [α-^{32}P]UTP-labeled RNA. The 41-nucleotide L1 contains a RNase T1-resistant undecanucleotide that is derived from the 5′ end of the mRNA molecule and contains the 5′ cap structure (Fig. 3*A*) (15). The 72-nucleotide L2 contains three large internal RNase T1-resistant oligonucleotides of 12, 9, and 8 nucleotides (Fig. 3*B*). In addition to these large marker oligonucleotides, the 41- and 72-nucleotide bands yielded several smaller oligonucleotides that corresponded exactly to the pattern predicted from the L1 and L2 sequences. Fig. 3*C* shows a mixture of RNase T1 digests of the 41- and 72-nucleotide bands. The sequence of the various spots was determined by comparing relative mobilities and nucleotide compositions with the known sequence (see Fig. 1*C*). Fig. 3*D* shows the same analysis performed on the 113-nucleotide spliced band. All of the oligonucleotides contained in the two leaders were present in this RNA. This establishes the 113-nucleotide band as the product of splicing the two leader exons.

In experiments not shown, an equivalent set of two-dimensional chromatographs was done with the 41-, 72- and 113-nucleotide bands prepared from RNA labeled with [α-^{32}P]CTP. Again, RNase T1 digestion of the 113-nucleotide band produced a set of oligonucleotides identical to those seen in a mixture of oligonucleotides from the 41- and 72-nucleotide bands.

RNase T1 cleaves the L1-2 RNA at the splice junction and, thus, does not release a unique oligonucleotide formed by the splicing reaction. However, two-dimensional chromatographic analysis after a pancreatic RNase digestion of the L1-2 RNA should resolve the tetranucleotide G-G-G-Cp, which spans the splice junction. Pancreatic RNase digestion of either L1 or L2 RNA will not produce an oligonucleotide of this sequence (see Fig. 1*C*). The G-G-G-Cp oligonucleotide will be labeled in RNA made with [α-^{32}P]UTP by transfer of the 3′ phosphate to the terminal C residue.

unspliced ...U-U-G-G-G:G-U-G...C-A-G:C$\overset{*}{p}$U-C-G...
spliced ...U-U-G-G-G-C$\overset{*}{p}$U-C-G...

Results of two-dimensional chromatographic analysis of pancreatic RNase digests of a mixture of L1 and L2 RNAs and of the L1-2 spliced RNA are seen in Fig. 3 *E* and *F*, respectively. The spliced material produced a spot with the expected migration of G-G-G-Cp that was not present in the unspliced leaders. This spot migrated identically with a spot found in L1-2 RNA labeled *in vivo* and digested with pancreatic RNase (data not shown). The sequence of the oligonucleotides resolved after pancreatic RNase digestion of *in vivo* labeled spliced RNA was determined by comparing relative mobilities and nucleotide compositions with the known sequence. The presence of G-G-G-Cp in the *in vitro* spliced L1-2 RNA demonstrates that the *in vitro* reaction produces an RNA product identical in sequence with that found *in vivo*.

The Splice Junction Contains a Typical 3′,5′-Phosphodiester Bond. From the analysis of the spliced RNA, some information about the structure of the newly formed internucleotide bond can be deduced. In particular, the presence of the C-U-C-Gp oligonucleotide in the two-dimensional chromatographic analysis of the spliced band shows that the phosphodiester bond 5′ of the C was not modified to a RNase T1-resistant state during the reaction. This bond is formed during the splicing reaction if it occurs by cleavage at the :G-U and A-G: sites (16). The splicing of tRNAs in yeast has been shown to form a RNase T1-resistant bond with a 2′-phosphate (3). Methylation of the 2′ position also would yield a T1-resistant bond. The actual phosphodiester bond formed during the splicing reaction was ambiguous because of a duplication of sequence at the 5′ and 3′ splice sites (see Fig. 1*C*). Thus, splicing could have formed either the phosphodiester bond between the G and C residues discussed above or between the two adjacent G residues. In the latter case, failure to cleave the newly formed phosphodiester bond of GpG with RNase T1 would result in a novel labeled spot in splicing RNA labeled with [α-^{32}P]CTP. As no new spot was observed (data not shown), it is likely that the newly formed bond in the spliced 113-nucleotide RNA is a typical 3′,5′-phosphodiester bond.

Effects of Reaction Conditions on Transcription and Splicing. In order to further characterize and optimize the *in vitro* splicing reaction, we examined the effects of modifications of the reaction conditions on the production of the L1-2 spliced product. Fig. 4 shows the effects of increasing $MgCl_2$, KCl, and total HeLa cell RNA concentrations on the reactions. Adding increasing amounts of total HeLa cell RNA to the reaction inhibited splicing more than transcription (Fig. 4, lanes 1–4). At 10 μg of added RNA, splicing was inhibited by 97% while transcription was inhibited by only 42%. Increasing concentrations of $MgCl_2$ had a similar effect (Fig. 4, lanes 5–8); the effect of raising the $MgCl_2$ concentration from 5 to 7 mM inhibited splicing by 95% but transcription by 48%. An even more specific effect was seen with increasing KCl concentration (Fig. 4, lanes 9–11). As the KCl concentration was raised from 50 to 70 mM, the splicing reaction was reduced by 83%, with essentially no effect on the level of transcription. Under the optimal conditions, the spliced band contained 2–3% of the radioactivity in the combined unspliced leader bands.

DISCUSSION

An important step in the study of a complex biochemical process is the development of an *in vitro* reaction where enzymes, cofactors, and substrates can be manipulated. We have shown that a soluble cell-free extract is capable of both transcribing a DNA template containing the L1 and L2 of adenovirus and splicing 2–3% of the newly synthesized RNA.

WCEs are prepared by lysing HeLa cells grown in culture and removing chromatin and membrane fragments by sedimentation. The extract should contain many cellular activities and has been used widely to transcribe exogenously added DNA templates; however, it has been difficult to document the splicing of mRNA precursors in this extract (17). Perhaps three features of the system described here account for the successful detection of splicing: (*i*) the major late promoter of Ad2 is the most active template known in the WCE system and, thus, generates large amounts of a radioactive substrate; (*ii*) the splicing of L1 and L2 of Ad2 occurs very rapidly *in vivo*, probably while the RNA is still nascent, suggesting an unusually high efficiency (14, 18); (*iii*) the assay system of hybridization of radioactive RNA to cDNA sequences cloned into a single-stranded M13 phage followed by selection of hybrids (unpublished data) is more sensitive than other techniques. In addition, the assay permits direct RNase T1 and pancreatic RNase two-dimen-

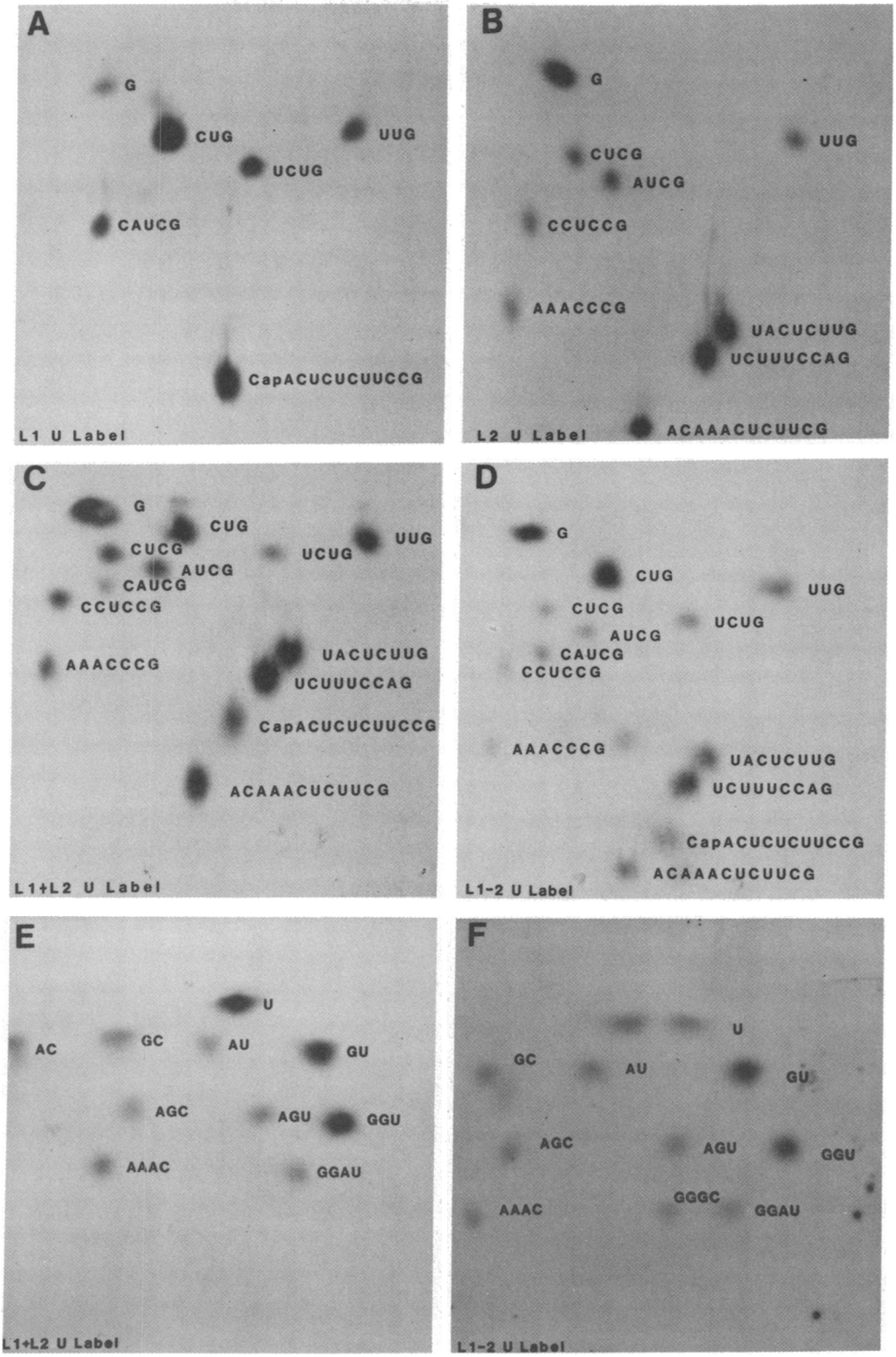

FIG. 3. Two-dimensional chromatography of spliced and unspliced RNAs. RNA labeled *in vitro* with [α-^{32}P]UTP was eluted from gel bands, digested to completion with RNase T1 or pancreatic RNase, and fractionated by electrophoresis on cellulose acetate at pH 3.5 from left to right and homochromatography on DEAE-cellulose thin-layer plates from bottom to top. (*A–D*) RNA digested with RNase T1. (*E* and *F*) RNA digested with pancreatic RNase. RNAs: 41-nucleotide L1 RNA (*A*), 72-nucleotide L2 RNA (*B*), L1/L2 RNAs (*C* and *E*), and 113-nucleotide L1-2 spliced RNA (*D* and *F*).

sional chromatographic analysis of the spliced RNA product, providing unambiguous results. Others have reported detection of RNA splicing in similar extracts (19, 20). More recently, Kole and Weissman (21) determined the sequence of reverse transcription products of *in vitro* transcribed RNA and detected spliced product. This sequence analysis also unambiguously identifies spliced RNA but is more burdensome than the assay described here.

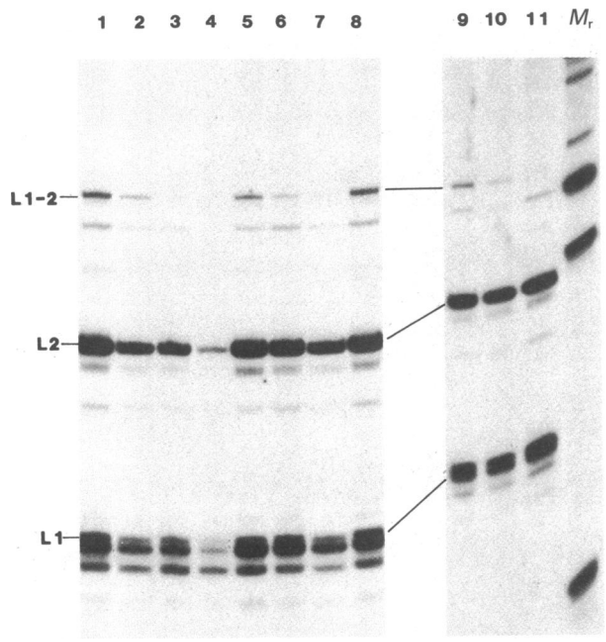

FIG. 4. Effects of adding total HeLa cell RNA, $MgCl_2$, or KCl on the splicing reaction. Lanes: 1–4, standard analytical reactions containing 50 mM KCl and 5 mM $MgCl_2$ were modified by addition of total HeLa cell RNA as follows: 2 μg (lane 1), 5 μg (lane 2), 10 μg (lane 3), and 15 μg (lane 4); 5–8, standard analytical reactions containing 50 mM KCl were modified by the addition of $MgCl_2$ to final concentrations of 6 mM (lane 5), 7 mM (lane 6), 9 mM (lane 7), and 5 mM (lane 8); 9–11, standard analytical reactions containing 5 mM $MgCl_2$ were modified by the addition of KCl to final concentrations of 50 mM (lane 9), 60 mM (lane 10), and 70 mM (lane 11).

Approximately 2–3% of the *in vitro* transcribed RNA is spliced under current conditions. The extracts have been prepared as previously published and have been shown to contain a high level of small nuclear RNAs (unpublished data). The reaction conditions have been optimized for splicing with the constraint that the transcription reaction is also necessary. The fraction of spliced RNA product (*i*) is optimal at 50 mM KCl and 5 mM $MgCl_2$, (*ii*) decreases as exogenous total cellular RNA is added, and (*iii*) has been optimized with respect to temperature, time, extract concentration, and nucleotide triphosphate concentration. It is perhaps significant that the detergent Nonidet P-40 at a concentration of 0.1% does not inhibit the splicing reaction, whereas a higher concentration of 0.5% partially inhibits both transcription and splicing (data not shown). This suggests that intact membrane components are not necessary for the splicing reaction.

Two-dimensional chromatographic analysis of the RNase T1 digests of the *in vitro* spliced RNA shows that the phosphodiester bond formed between the two leader exons is RNase T1 sensitive. Recent studies of the splicing of tRNAs have detected the formation of a RNase T1-resistant bond that has a 2′-phosphate (3). If a similar reaction is responsible for the splicing of mRNA precursors in the *in vitro* system, the 2′-phosphate must be completely removed. The 5′ oligonucleotides from both the spliced and unspliced RNAs were predominantly capped (data not shown); thus, we cannot conclude whether capping is necessary for splicing. Polyadenylylation is probably not required for *in vitro* splicing, as the truncated template used in these studies is not thought to contain signals for polyadenylylation. However, the 3′ end of the *in vitro* spliced RNA has not been analyzed directly.

The development of a soluble *in vitro* splicing system provides a powerful method with which to explore the biochemistry of the reaction. Use of such radioactive substrates may permit analysis of intermediates in the splicing reaction. It is now possible to test several of the suggestions made about the requirements of the reaction, such as the need for certain structures in the RNA, the role of small nuclear RNPs (22, 23), and the relationship of mRNA splicing to tRNA and rRNA splicing.

Recently, we have also found that exogenous RNA substrates are spliced in the WCE reaction mixture. Labeled unspliced substrates were prepared by transcription of *Bal* I E fragment and purified by electrophoresis in a polyacrylamide gel. The extent of splicing of the exogenous substrate was similar to that observed in the coupled system described here. The uncoupling of transcription and splicing will allow determination of the substrate and cofactor requirements of splicing *in vitro*.

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