Intracellular Site of U1 Small Nuclear RNA Processing and Ribonucleoprotein Assembly

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ABSTRACT We have investigated the intracellular site and posttranscriptional immediacy of U1 small nuclear RNA processing and ribonucleoprotein (RNP) assembly in HeLa cells. After 30 or 45 min of labeling with [^{3}H]uridine, a large amount of U1-related RNA radioactivity in the cytoplasm was found by using either hypotonic or isotonic homogenization buffers. The pulse-labeled cytoplasmic U1 RNA was resolved as a ladder of closely spaced bands running just behind mature-size U1 (165 nucleotides) on RNA sequencing gels, corresponding to a series of molecules between one and at least eight nucleotides longer than mature U1. They were further identified as U1 RNA sequences by gel blot hybridization with cloned U1 DNA. The ladder of cytoplasmic U1 RNA bands reacted with both RNP and Sm autoimmune sera and with a monoclonal Sm antibody, indicating a cytoplasmic assembly of these U1 RNA-related molecules into complexes containing the same antigens as nuclear U1 RNP particles. The cytoplasmic molecules behave as precursors to mature nuclear U1 RNA in both pulse-chase and continuous labeling experiments. While not excluding earlier or subsequent nuclear stages, these results suggest that the cytoplasm is a site of significant U1 RNA processing and RNP assembly. This raises the possibility that nuclear-transcribed eucaryotic RNAs are always processed in the cell compartment other than that in which they ultimately function, which suggests a set of precise signals regulating RNA and ribonucleoprotein traffic between nucleus and cytoplasm.

Eucaryotes contain a set of abundant, low molecular weight nuclear RNAs, 100–200 nucleotides in length (1). One of these, called U1 RNA, has been implicated in messenger RNA splicing (2–8). There has also been recent progress on the ribonucleoprotein structure of U1 RNA (9–12; B. Setyono and T. Pederson, manuscript submitted for publication). However, much less is known about U1 RNA transcription and processing. U1 RNA is transcribed by RNA polymerase II (13–15), and human genomic DNA containing sequences colinear with U1 RNA has been cloned (16). However, the initiation and termination sites for U1 transcription have not been defined.

Previous studies have indicated the existence of short-lived precursors of U1 RNA that can be detected in the *cytoplasm* as pulse-labeled molecules one to three nucleotides longer than mature U1 (17–21). In the present investigation, we explored this intriguing situation further, with particular reference to the stages of U1 maturation at which the RNA associates with proteins, as probed with human autoantibodies specific for U1 RNA-ribonucleoprotein complexes (22–24).

MATERIALS AND METHODS

HeLa cells were maintained in suspension culture as previously described (25). [^{3}H]Uridine labeling and pulse-chase conditions are given in the figure legends. Unless noted otherwise, cell fractionation was carried out as detailed previously (25). All the procedures used for preparing nuclear and cytoplasmic extracts, reaction autoantibodies electrophoresis of RNA in 7 M urea–10% polyacrylamide gels, RNA gel blot hybridization and preparation of IgG from autoimmune disease patient sera have been described in detail (9, 22–24). The Sm antibody used in this study was obtained from a patient with systemic lupus erythematosus and, the ribonucleoprotein (RNP)[1] antibody was obtained from a patient with mixed connective tissue disease. The small nuclear RNAs that are specifically precipitated by these two particular patient sera have been described previously (24). Another antibody used in this study, designated "anti-U1/U2," was obtained from the same mixed connective tissue disease patient but from a venipuncture performed ~1 yr later than that in which the anti-RNP antibody (23, 24) was obtained. IgG from this latter serum was found to specifically precipitate U1 RNP and also some U2 RNP, hence the designation "anti-U1/U2" (see also Results). A mouse monoclonal Sm antibody

[1] *Abbreviations used in this paper:* RNP and Sm, standard terms for autoimmune sera as defined by immunological and biochemical criteria (see references 22, 24, 26, 27); snRNP, small nuclear ribonucleoprotein; RSB, reticulocyte standard buffer (see legend to Fig. 1).

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(26) was also used for some experiments. Control, nonimmune IgG was obtained from sera of healthy laboratory personnel.

RESULTS

The experiments reported here combine [^{3}H]uridine pulse-labeling, cell fractionation and the use of autoantibodies to define the intracellular site and posttranscriptional immediacy of U1 RNA processing and RNP assembly. When HeLa cells are fractionated in hypotonic buffer, the great majority of U1 RNA is recovered in the nuclear fraction (e.g., see Fig. 7 in reference 6). However, when nuclear and cytoplasmic extracts from cells pulse-labeled with [^{3}H]uridine for 45 min are precipitated with U1 RNA-reactive autoantibodies, the results are quite different (Fig. 1). Lanes *4* and *5* in Fig. 1 are equivalent amounts of nuclear and cytoplasmic fractions (each derived from 1.5×10^7 HeLa cells) reacted with anti-U1/U2. While there is a small amount of radioactivity in mature-size U1 and U2 in the nuclear fraction (Fig. 1, lane *4*), we found the majority of antibody-reactive RNA in the cytoplasm (Fig. 1, lane *5*) as ladders of closely spaced bands running behind the positions of U1 and U2 nuclear RNA. Neither the nuclear U1 and U2 RNA nor the cytoplasmic ladders react significantly with nonimmune IgG (Fig. 1, lanes *2* and *3*).

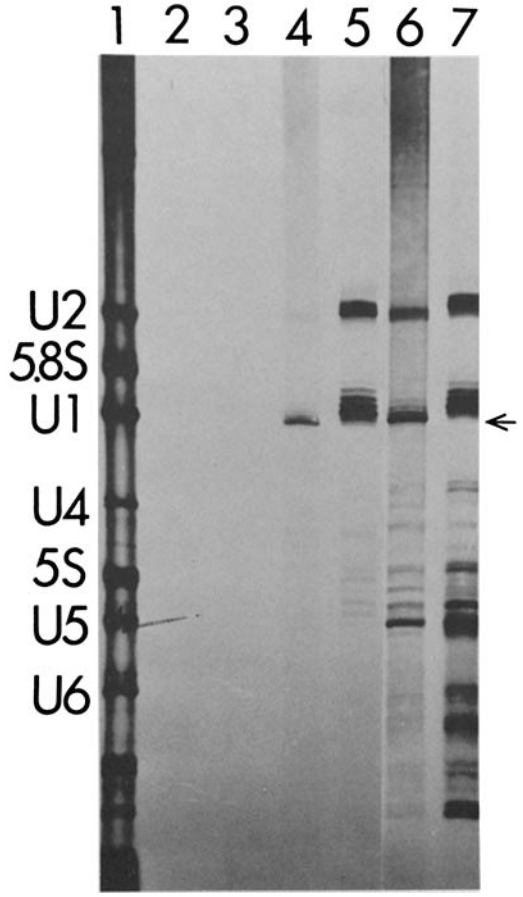

FIGURE 1 Pulse-labeled cytoplasmic U1 RNA reacts with autoantibodies. HeLa cells were resuspended in fresh medium at 3×10^6 cells/ml and incubated at 37°C for 45 min with [^{3}H]uridine (125 μCi/ml). The cells were fractionated in reticulocyte standard buffer (RSB, 0.01 M NaCl, 1.5 mM $MgCl_2$, 0.01 M Tris-HCl, pH 7.0) and nuclei were washed once in RSB before preparation of nuclear extracts by sonication (23). Cytoplasmic fractions were the post-nuclear supernatant in RSB. After reaction with antibodies or nonimmune IgG, antigenic material was recovered on protein A-Sepharose (23) and, after elution, RNA was phenol-extracted, ethanol-precipitated, and displayed by electrophoresis in 10% polyacrylamide gels containing 7 M urea followed by fluorography, as described previously (9, 23, 24). Lane *1*: markers of total HeLa small nuclear RNA from cells labeled with [^{3}H]uridine for 22 h. Lane *2*: nucleus, nonimmune IgG. Lane *3*: cytoplasm, nonimmune IgG. Lane *4*: nucleus, anti-U1/U2. Lane *5*: cytoplasm, anti-U1/U2. Lane *6*: nucleus, anti-Sm. Lane *7*: cytoplasm, anti-Sm. The arrow to the right of lane *7* indicates the position of mature U1 RNA. (The streak extending from U5 rightward across lane *2* is an autoradiographic artifact.) The material loaded in lanes *2–7* corresponds to the RNA from 1.5×10^7 cells.

For the remainder of this paper we will focus exclusively on U1 RNA, although bands related to U2 will also appear in some of the gels due to the specificities of the autoantibodies being used. Experiments similar to those shown in Fig. 1, using an antibody more specific for U1 RNP (22–24), gave results identical to the U1 regions of lanes *4* and *5* in Fig. 1 (data not shown).

Another autoantibody, Sm, reacts with RNP forms of U1, U2, U4, U5, and U6 small nuclear RNAs (22–24), due to the presence of an antigen or antigens shared by all five RNPs (26, 27). Lanes *6* and *7* of Fig. 1 show the results of an experiment using Sm antibody. In the nuclear fraction (Fig. 1, lane *6*), pulse-labeled material corresponding to mature-size U1 RNA is seen, as well as three to four faint bands running behind U1 (these are also visible in the anti-U1/U2 experriment [Fig. 1, lane *4*]). In the cytoplasm (Fig. 1, lane *7*), there is more antibody-reactive radioactivity and, like the case with anti-U1/U2 antibodies (Fig. 1, lane *5*), it consists of a ladder of bands running behind the position of mature U1. (The complex pattern of bands in the lower portions of lanes *6* and *7* in Fig. 1 represents RNAs unrelated to U1. These include U4 RNA precursors and will be the subject of a subsequent report.) Note that the spacing and relative intensities of bands in the Sm-reactive U1 RNA ladder (Fig. 1, lane *7*) are virtually identical to those seen with anti-U1/U2 (Fig. 1, lane *5*). At least eight bands can be resolved in both cases, and as many as 15 can be seen in these ladders with longer autoradiographic exposures (not shown).

The results in Fig. 1 indicate that after a 45-min pulse-label there is much more antibody-reactive U1 RNA in the cytoplasm than in the nucleus, which suggests that the cytoplasmic molelcules may be precursors of mature nuclear U1 RNA. This possibility is supported by the results of continuous labeling experiments, as shown in Fig. 2. After only a 15-min label, a ladder of antibody-reactive cytoplasmic U1 RNA bands can be detected (Fig. 2, lane *4*), whereas no labeled U1 RNA is seen in the nucleus (Fig. 2, lane *1*). The amount of radioactivity in the cytoplasmic U1 ladder increases when the label time is extended to 30 min (Fig. 2, lane *5*), but there is still little labeled nuclear U1 (Fig. 2, lane *2*). After 45 min, the amounts of label in the cytoplasmic ladder and nuclear U1 RNA come to reflect closely those seen in Fig. 1, where the label time was also 45 min. This labeling pattern strongly suggests that the bands of the cytoplasmic ladder are precursors of nuclear U1 RNA, and that assembly of these into autoantibody-reactive ribonucleoprotein occurs in the cytoplasm. Although there are small amounts of antibody-reactive bands larger than U1 in the nuclear fractions after 30 and 45 min of labeling (Fig. 2, lanes *2* and *3*), their pattern of appearance with increasing label time is not compatible with nuclear assembly.

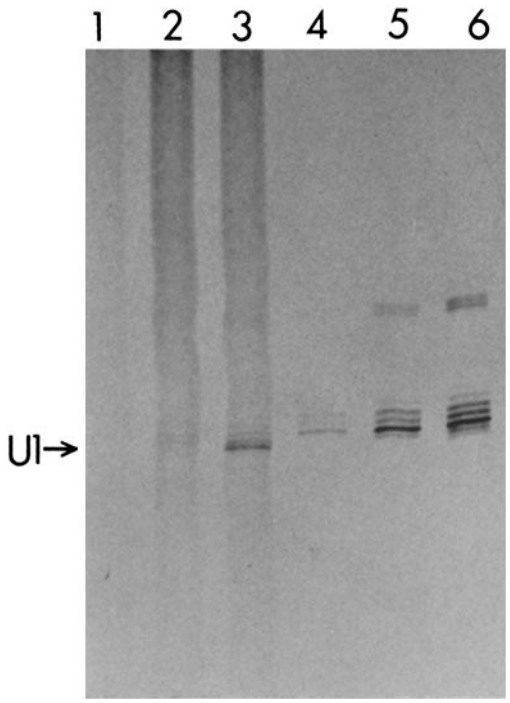

FIGURE 2 Continuous labeling kinetics. HeLa cells (3×10^6/ml) were labeled for 15, 30, or 45 min with [^{3}H]uridine, and nuclear and cytoplasmic extracts were reacted with anti-U1/U2 antibody and analyzed as in Fig. 1. Lanes *1–3*: nucleus: 15, 30, and 45 min, respectively. Lanes *4–6*: cytoplasm: 15, 30, and 45 min, respectively. The position of mature nuclear U1 RNA is indicated to the left of lane *1*.

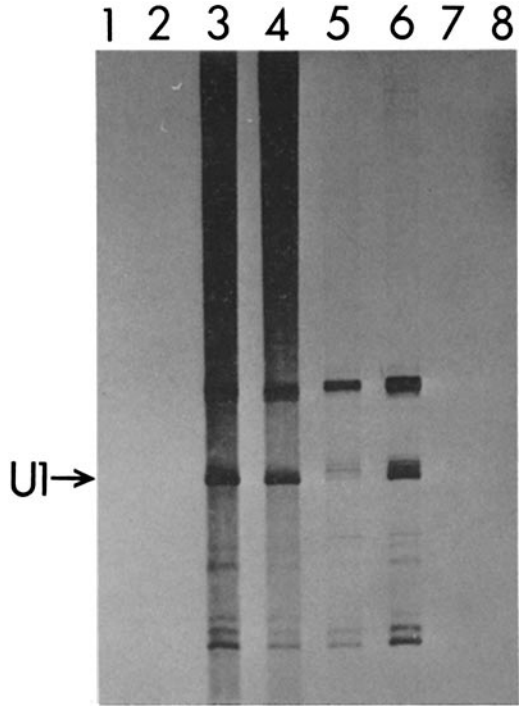

FIGURE 3 Isotonic vs. hypotonic cell fractionation. HeLa cells were labeled for 45 min with [^{3}H]uridine and homogenized in hypotonic buffer (RSB) as described in Materials and Methods or in an isotonic buffer consisting of 0.15 M NaCl, 1.5 mM $MgCl_2$, 0.01 M Tris-HCl, pH 7.5, and 0.5% Nonidet P-40. Lanes *1* and *2*: hypotonic nucleus and cytoplasm, respectively; nonimmune IgG. Lane *3*: hypotonic nucleus; anti-U1/U2. Lane *4*: isotonic nucleus; anti-U1/U2. Lane *5*: hypotonic cytoplasm; anti-U1/U2. Lane *6*: isotonic cytoplasm; anti-U1/U2. Lanes *7* and *8*: isotonic nucleus and cytoplasm, respectively; nonimmune IgG.

To investigate the possibility that the cytoplasmic U1 RNA ladder represents nuclear material that is released during cell fractionation, we homogenized pulse-labeled (45 min) cells in either hypotonic or isotonic buffers (see legend, Fig. 3) and compared the amounts of antibody-reactive U1 RNA in the nuclear and cytoplasmic fractions. A monoclonal Sm antibody (26) was used for this experiment. A U1 RNA ladder is seen in both cytoplasmic fractions (Fig. 3, lanes *5* and *6*) and there is actually more with isotonic fractionation (Fig. 3, lane *6*). Although it remains possible that nuclear leakage of pulse-labeled U1 RNA occurs extensively with both isotonic and hypotonic buffers, we note that anhydrous fractionation of HeLa cells also leads to the recovery of pulse-labeled U1 RNA precursor molelcules in the cytoplasmic fraction (21).

To more conclusively identify the cytoplasmic bands as U1 RNA sequences, we hybridized gel blots of antibody-reactive nuclear and cytoplasmic RNA with cloned U1 DNA. Fig. 4 shows three different autoradiographic exposures of the same blot. Cytoplasmic and nuclear RNA reacting with nonimmune human IgG is shown in Fig. 4, lanes *2* and *3*, respectively, where it can be seen that minor amounts of U1 RNA are recovered, as is common experience. In the material selected by reaction with anti-U1/U2 antibody (Fig. 4, lanes *4* and *5*), the major hybridization signal in the nuclear material corresponds to mature-size U1 RNA (Fig. 4, lane *5*) (the band below U1 is a routinely observed U1 RNA fragment, e.g., see Fig. 7 in reference 6 and Fig. 3 in reference 21). However, the antibody-reactive cytoplasmic RNA (Fig. 4, lane *4*) can be seen to contain a series of bands above the position of mature U1 that hybridize with the U1 DNA probe and that correspond to the ladders in experiments with pulse-

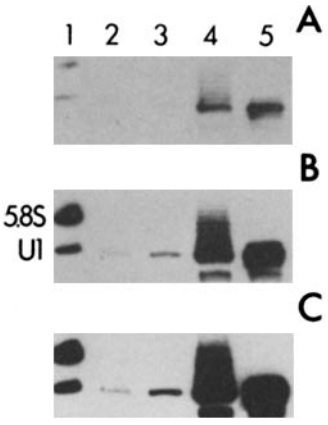

FIGURE 4 RNA gel blot hybridization with U1 RNA. Nuclear or cytoplasmic extracts (in RSB) were reacted with either nonimmune IgG or anti-U1/U2. RNA was electrophoresed as in Fig. 1, transferred to diazobenzyloxymethyl paper and hybridized with an avian U1 DNA probe (14) labeled with ^{32}P by nick-translation. Three different autoradiographic exposures are shown: *A*, 16 h; *B*, 4 d; and *C*, 10 d. Lane *1*: Markers of HeLa 5.8S and U1 RNA, obtained by labeling total nuclear RNA with ^{32}P-cytidine-3′,5′-bisphosphate (38). Lanes *2* and *3*: Cytoplasmic and nuclear RNA, respectively; nonimmune IgG. Lanes *4* and *5*: Cytoplasmic and nuclear RNA, respectively; anti-U1/U2.

labeled RNA (Fig. 1). In the medium exposure (Fig. 4*B*), eight bands can easily be counted above the position of mature U1. Although the autoradiographic resolution is superior in the ^{3}H-labeling experiments (Figs. 1 and 2) to that in the blot hybridizations (where ^{32}P is used), the concordance of the cytoplasmic ladders is striking.

The results presented so far demonstrate that the bands in the cytoplasmic RNA ladder are U1 RNA sequences (Fig. 4)

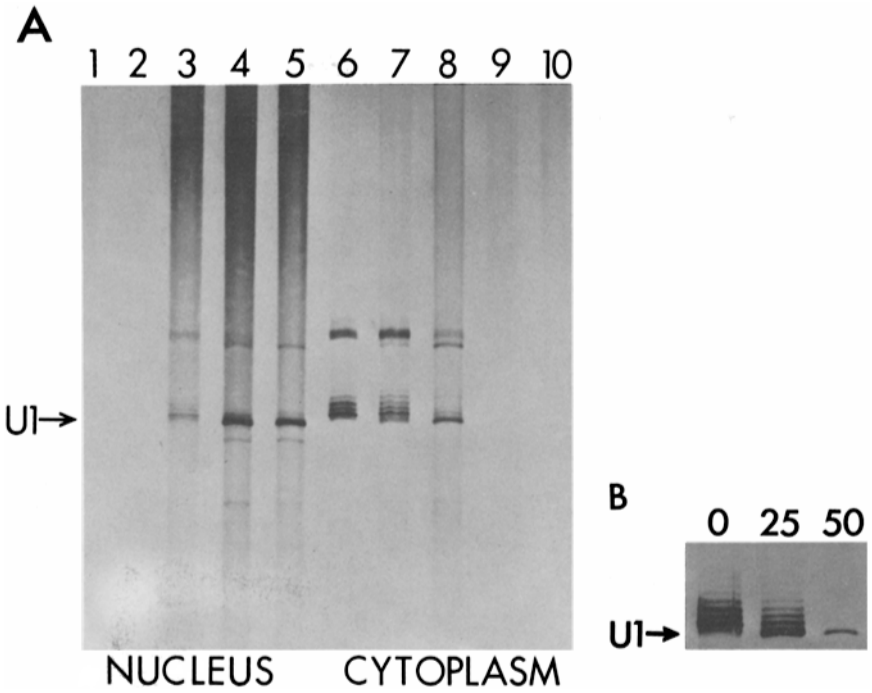

FIGURE 5 Pulse-chase experiments. HeLa cells (3×10^6/ml) were labeled for 25 min with [^{3}H]uridine (200 μCi/ml). One-third of the cells was then harvested ("0 min"), and to the remainder actinomycin was added to 0.1 μg/ml. This is a very low concentration of actinomycin which nevertheless completely inhibits U1 RNA transcription in HeLa cells (39). One-half of the remaining cells were harvested after 25 min and the other half after 50 min. Nuclear and cytoplasmic extracts were prepared and analyzed with anti-U1/U2 as in Fig. 1. (*A*) Experiment 1. Lanes *1* and *2*: nucleus: 0 and 50 min, respectively; nonimmune IgG. Lanes *3–5*: nucleus: 0, 25, and 50 min, respectively; anti-U1/U2. Lanes *6–8*: cytoplasm: 0, 25, and 50 min, respectively; anti-U1/U2. Lanes *9–10*: cytoplasm: 0 and 50 min, respectively; nonimmune IgG. Each lane corresponds to 1.25×10^7 cells. (*B*) Experiment 2. Antibody-reactive cytoplasmic RNA after the chase times indicated (in minutes).

and also hint that they are precursors of mature nuclear U1 RNA (Fig. 2). To examine this latter possibility further, we conducted pulse-chase experiments (see legend of Fig. 5 for details). After 25 min of [^{3}H]uridine labeling, the familiar ladder of antibody-reactive U1 RNA is seen in the cytoplasm (Fig. 5*A*, lane *6*), whereas only a very small amount of U1 radioactivity is seen in the nucleus (Fig. 5*A*, lane *3*), confirming the 30-min labeling data presented earlier (Fig. 2, lanes *2* and *5*). However, during a subsequent chase, radioactivity disappears from the cytoplasmsic ladder (Fig. 5*A*, lanes *7* and *8*), while radioactivity in mature-size nuclear U1 RNA increases. In addition, there appears to be a movement of radioactivity "down the ladder" in the cytoplasmic U1 RNA during the chase (Fig. 5*A*, lanes *6–8*), suggesting that each band may be a precursor of the one below it. This chase behavior of the cytoplasmic ladder is reproducible, as shown by Fig. 5*B*, lanes *1–3*, which are comparable data from another experiment.

DISCUSSION

In this investigation we have confirmed and extended previously reported evidence for cytoplasmic U1 RNA precursors in HeLa cells, and have demonstrated their association, in the cytoplasm, with proteins reactive with Sm and RNP autoantibodies. We cannot exclude the possibility that these proteins first combine with U1 RNA precursors in the nucleus but in a conformation not recognized by the autoantibodies, or that these proteins associate with the U1 RNA precursors at the moment they move through the nuclear envelope. Nor can we rule out the existence of large nuclear precursors of U1 RNA that are associated with proteins nonreactive with Sm or RNP antibodies, for example hnRNP proteins (28–32).

The ladder of cytoplasmic U1 RNA sequences we have characterized contains a multiple array of closely spaced bands under electrophoresis conditions similar to those of RNA sequencing gels. It is therefore likely that each band in the ladder is one nucleotide longer than the one below it. Accordingly, the uppermost bands detected in the ladder would correspond to molecules at least eight nucleotides longer than mature U1 RNA (Figs. 1, 4, and 5). As the pulse-chase experiments (Fig. 5) suggest that each band may be a precursor of the next below it, it is possible that the processing reaction involves removal of one nucleotide at a time, although a more complex set of cleavages cannot yet be ruled out. Recent RNase T1 digestion experiments of ^{32}P-end-labeled U1 precursors suggest that processing takes place at the 3′ ends of these molecules (33). It is possible that the last few nucleotides are not removed until the U1 RNA returns to the nucleus, as evidenced by the one to four bands of antibody-reactive RNA that are consistently observed just above mature-size U1 in nuclear fractions (Figs. 1, 2, 4, and 5*A*).

A cytoplasmic assembly of U1 RNA precursors into ribonucleoprotein structures is compatible with recent experiments showing that U1 RNA microinjected into enucleated *Xenopus* oocytes forms RNP complexes that are autoantibody-reactive (34), although this may be an exceptional case due to the cytoplasmic storage of many nuclear proteins in the amphibian oocyte. We have shown that U1 RNA forms antibody-reactive U1 RNP when added (without exogenous mRNA translation) to a rabbit reticulocyte lysate (Fig. 3 in reference 24), which is a cytoplasmic extract of a somatic mammalian cell. However, both of these cases (24, 34) involved mature, nuclear U1 RNA, so the relevance of its observed RNP assembly to the association of U1 RNA precursors themselves with proteins is unclear.

We do not know whether the cytoplasmic pre-U1 RNA associates with newly synthesized or previously made Sm and RNP antigens. The existence of a small cytoplasmic pool of these proteins would not be incompatible with their predominantly (but not exclusively) nuclear location as revealed by immunofluorescence (e.g., reference 26). The small nuclear RNAs are metabolically stable and thus their rates of synthesis are low. The assembly of newly made U1 into RNP would

therefore not require a particularly large pool of cytoplasmic snRNP proteins, relative to the amount of such protein associated with the much greater number of stable nuclear U1 RNA molecules, about 10^6 per HeLa cell (1).

The biological rationale for a cytoplasmic maturation of an RNA that is destined to function in the nucleus remains enigmatic. It appears that, in eucaryotes, all nuclear-transcribed RNA species that undergo processing do so in a cell compartment other than the one in which they ultimately function. Thus, ribosomal RNA, messenger RNA, 5S RNA, and transfer RNA are all processed in the nucleus (35–37) but function in the cytoplasm. Conversely, as we have been discussing, the stable nuclear RNA U1 apparently undergoes a significant part of its maturation in the cytoplasm. The unusual maturation of U1 RNA raises a number of intriguing questions about nuclear-cytoplasmic interactions and RNA traffic. The movement of U1 RNA precursors to the cytoplasm and back to the nucleus must reflect specific signals in either the precursor RNA sequence, secondary structure or the proteins that associate with these RNAs during their maturation. Because U1 RNP can now be assembled in vitro (9, 24), it may be possible to identify RNA sequence elements or proteins that signal movement in one direction or the other, by using microinjection into oocytes, for example. This approach might also lead to the identification of catalytic proteins involved in U1 RNA processing. We have recently found that U1 RNA precursors are complexed with the La antigen (40), a protein previously implicated in processing of transfer RNA and 5 S ribosomal RNA (36), and this may be another clue to the processing of U1 snRNA.

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Note Added in Proof: The paper cited as "B. Setyono and T. Pederson, manuscript submitted for publication," is now in press (Evidence that U1 small nuclear RNA is a ribonucleoprotein when base-paired with pre-messenger RNA in vivo, *J. Mol. Biol.*, 1984). We have now identified cytoplasmic precursors of U4 snRNA in pulse-chase experiments and RNA gel blot hybridizations with a cloned human U4 DNA probe (Madore, S. J., E. D. Wieben, G. R. Kunkel, and T. Pederson, manuscript in preparation).

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