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RNA processing and ribonucleoprotein assembly studied *in vivo* by RNA transfection

(calcium phosphate-mediated RNA uptake/U2 small nuclear RNA/3′ processing/5′ cap hypermethylation/nuclear targeting)

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ABSTRACT **We present a method for studying RNA processing and ribonucleoprotein assembly *in vivo*, by using RNA synthesized *in vitro*. SP6-transcribed ^{32}P-labeled U2 small nuclear RNA precursor molecules were introduced into cultured human 293 cells by calcium phosphate-mediated uptake, as in standard DNA transfection experiments. RNase protection mapping demonstrated that the introduced pre-U2 RNA underwent accurate 3′ end processing. The introduced U2 RNA was assembled into ribonucleoprotein particles that reacted with an antibody specific for proteins known to be associated with the U2 small nuclear ribonucleoprotein particle. The 3′ end-processed, ribonucleoprotein-assembled U2 RNA accumulated in the nuclear fraction. When pre-U2 RNA with a 7-methylguanosine group at the 5′ end was introduced into cells, it underwent conversion to a 2,2,7-trimethylguanosine cap structure, a characteristic feature of the U-small nuclear RNAs. Pre-U2 RNA introduced with an adenosine cap (ApppG) also underwent processing, small nuclear ribonucleoprotein assembly, and nuclear accumulation, establishing that a methylated guanosine cap structure is not required for these steps in U2 small nuclear ribonucleoprotein biosynthesis. Beyond its demonstrated usefulness in the study of small nuclear ribonucleoprotein biosynthesis, RNA transfection may be of general applicability to the investigation of eukaryotic RNA processing *in vivo* and may also offer opportunities for introducing therapeutically targeted RNAs (ribozymes or antisense RNA) into cells.**

The U1, U2, U5, and U4/U6 small nuclear ribonucleoprotein (snRNP) complexes are attractive model systems for investigating RNA processing, ribonucleoprotein (RNP) assembly, and trafficking of RNP from cytoplasm to nucleus (1–9). Except for U6 RNA (10, 11), the major small nuclear RNAs (snRNAs) are synthesized by RNA polymerase II and the primary transcripts contain a 7-methylguanosine (^{7}mG) cap (12). snRNAs, like most eukaryotic RNAs, are synthesized as precursors. The precursors of human U1, U2, and U4 RNAs contain 3′ end extensions ranging in length from 1 to 15 nucleotides (nt) (refs. 3–5, 13, and references cited therein). In contrast to most other eukaryotic RNAs, however, snRNAs are exported to the cytoplasm prior to processing (7). After nuclear export, snRNA precursors are assembled into snRNP complexes (3–9) and the ^{7}mG cap becomes hypermethylated to 2,2,7-trimethylguanosine (^{2,2,7}mG) (13, 14). After 3′ RNA processing, the snRNP complex is imported back into the nucleus (2–9, 13).

Our current understanding of snRNP biosynthesis has come from pulse-labeling studies in mammalian cells (1, 3–8, 13) or from injection of snRNA genes, or their transcripts, into *Xenopus* oocytes (2, 9). However, these approaches are not without limitations. Mammalian cell pulse-labeling studies illuminate endogenous snRNA processing and snRNP assembly but do not readily lend themselves to experimental manipulation—for example, to learn the specific snRNA sequence elements that direct 3′ processing, snRNP assembly, and nuclear targeting. In contrast, the *Xenopus* oocyte system (2, 9) offers important experimental opportunities but represents a highly specialized nongrowing cell. Moreover, oocyte microinjection requires special expertise and equipment. To exploit and combine the strongest features of both systems, we have developed an alternative approach. Our idea was to introduce a snRNA precursor into human cells by using calcium phosphate-mediated uptake, as in standard DNA transfection (15).

MATERIALS AND METHODS

The U2 RNA precursor used for these experiments (shown in Fig. 1*A*) was transcribed with T7 RNA polymerase from a *Bam*HI-linearized human U2 template, termed pG2U2^{pre}, that this laboratory has described (18). The presence of an 18-nt 5′ leader sequence (Fig. 1*A*) results in a "maxi-U2" precursor RNA, the distinctive size of which was essential for these experiments. Transcription was primed with 7-methylguanosine(5′)triphospho-(5′)guanosine (^{7}mGpppG) in all cases unless otherwise specified.

Human 293 cells were grown in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum. Except where otherwise noted, 100-mm dishes of subconfluent cells plated 20–24 hr previously were used for RNA transfection. To prepare calcium phosphate-bound RNA, ^{32}P-labeled G2U2^{pre} RNA was combined with 10 μg of calf liver tRNA in a final volume of 250 μl of water. $CaCl_2$ (1 M, 83 μl) was added to the RNA, and this mixture was then added dropwise to 333 μl of 0.2 M NaCl/50 mM Hepes/1.5 mM Na_2HPO_4, pH 7.1. After formation of calcium phosphate precipitate (10 min, 20–22°C), 10 ml of prewarmed (37°C) culture medium was added and this mixture was then used to replace the medium in each dish of cells.

After the desired period of time (ranging from 4 to 24 hr in various experiments), the cells were harvested and washed two times in phosphate-buffered saline (150 mM NaCl/10 mM sodium phosphate, pH 7.2). The cells were resuspended in 10 mM NaCl/3 mM $MgCl_2$/10 mM Tris·HCl, pH 7.4, allowed to swell for 10 min (4°C), and broken in a Dounce homogenizer. For analysis of whole-cell homogenates, NaCl was added to 0.1 M, the nuclei were disrupted by sonication, the sample was centrifuged at 13,000 × *g* for 3 min, and the supernatant was recovered. For preparation of cytoplasmic and nuclear fractions, the nuclei were collected after cell

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Abbreviations: RNP, ribonucleoprotein; sn, small nuclear; nt, nucleotide(s); ^{7}mG, 7-methylguanosine; ^{2,2,7}mG, 2,2,7-trimethylguanosine.

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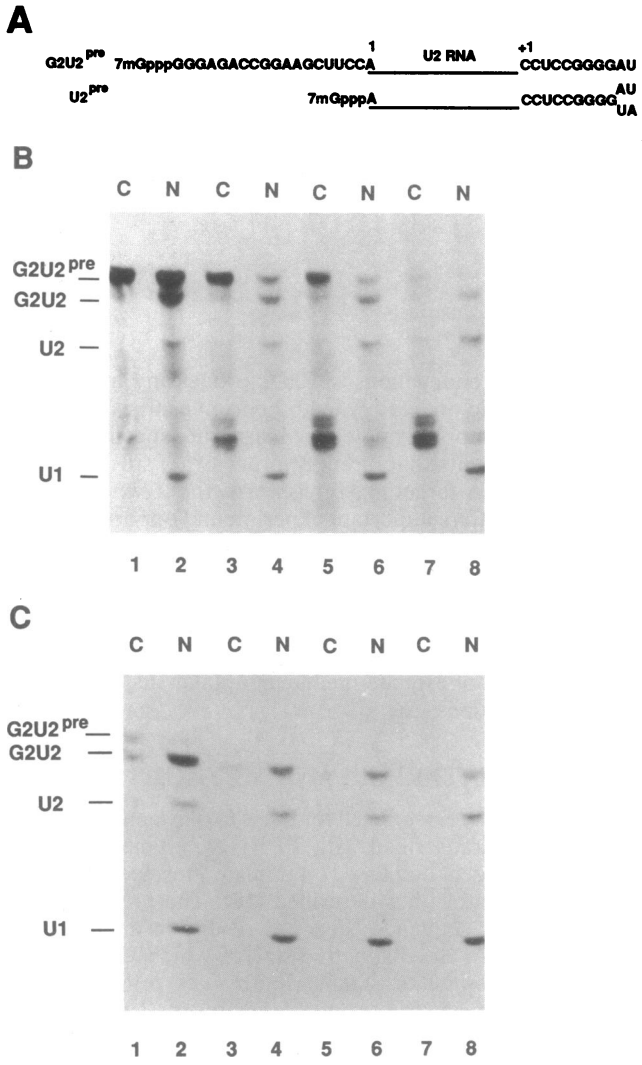

FIG. 1. Transfection, processing, and nuclear localization of U2 RNA. (*A*) Sequence of G2U2^{pre} RNA compared to human U2 RNA precursor. As indicated, the 3′ terminal dinucleotide of human pre-U2 RNA is divergent (16, 17). The constructed human U2 clone, pG2U2^{pre} (18), contains a 5′ leader of an extra 18 nt. T7 RNA polymerase produces from this template a U2 "maxi-transcript," the distinctive length of which was essential for these experiments. (*B*) Total RNA isolated from cytoplasmic (lanes C) and nuclear (lanes N) fractions of transfected cells. The 35-mm dishes of cells were grown to subconfluency and each was incubated in medium containing a calcium phosphate precipitate to which 1.5×10^6 dpm of ^{32}P-labeled G2U2^{pre} RNA had been initially bound (specific activity = 2.1×10^8 dpm/μg). Cells were incubated for the following number of hours. Lanes: 1 and 2, 12 hr; 3 and 4, 16 hr; 5 and 6, 20 hr; 7 and 8, 24 hr. Cells were then harvested, washed, and fractionated. RNA was purified from nuclear and cytoplasmic fractions by phenol extraction, electrophoresed on 8% polyacrylamide/8.3 M urea gels, and visualized by autoradiography. The electrophoretic mobilities of G2U2^{pre}, G2U2 (processed G2U2^{pre}), and endogenous U2 and U1 RNAs are designated. (*C*) Sm monoclonal antibody-selected RNA from cytoplasmic (lanes C) and nuclear (lanes N) fractions. No detectable RNA was recovered when murine nonimmune IgG was used in place of Sm antibody (data not shown). Lanes 1–8 were from cells incubated as in *B*.

homogenization by centrifugation at 800 × *g* for 5 min. The supernatant was removed as the cytoplasmic fraction and the nuclei were resuspended in 10 mM NaCl/3 mM $MgCl_2$/10 mM Tris·HCl, pH 7.4 and processed as detailed above for whole-cell homogenates.

The efficiency of RNA transfection, as determined by measurement of cell-associated ^{32}P radioactivity after the second phosphate-buffered saline wash, varied from 0.5 to 2.0% of the initial RNA added as a calcium phosphate precipitate. (This reflects the amount of radioactivity that had entered and remained in the cells during the 12- to 24-hr transfection period and does not specifically address the amount of intact G2U2 RNA present at a particular time.)

Immunoselection with Sm monoclonal antibody (19) or trimethylguanosine-specific antibody (20) was performed as described (21). RNA was analyzed by electrophoresis as described (22).

RESULTS

To produce a distinctively sized U2 RNA precursor, we used a U2 clone (22) to construct a human U2 maxi-gene (18), containing a 18-nt extension at the 5′ end and one of the two alternate 11-nt 3′ extensions that are present on human U2 RNA precursors (Fig. 1*A*). A representative transfection experiment with maxi-pre-U2 RNA is shown in Fig. 1*B*. At 12 hr, the introduced precursor RNA (G2U2^{pre}) was evident in both cytoplasmic and nuclear fractions, whereas the processed product (G2U2) was observed predominantly in the nuclear fraction (Fig. 1*B*, lanes 1 and 2). At subsequent times, the precursor was more prevalent in the cytoplasmic fraction (lanes 3, 5, and 7) and the processed RNA continued, as at 12 hr, to be present predominantly in the nuclear fraction (lanes 4, 6, and 8). Additional experiments revealed that there is no detectable intracellular G2U2^{pre} RNA at 0 hr and a nearly constant amount of G2U2 RNA (precursor plus processed) at 4, 8, and 12 hr (data not shown).

We noted that in addition to the introduced U2 RNA precursor and its processed product, other species of RNA also became labeled. Two of these have the sizes and subcellular localizations expected for endogenous U2 and U1 RNAs, and, as will be shown below, are selected both by Sm and ^{2,2,7}mG antibodies. We interpret their labeling to reflect reutilization of ^{32}P-labeled nucleotides arising from turnover of the introduced RNA.

We next determined whether the transfected G2U2^{pre} RNA underwent assembly into a snRNP complex. A portion of each of the fractions analyzed in Fig. 1*B* was immunoselected with Sm monoclonal antibody (Fig. 1*C*). This antibody is specific for three snRNP proteins, termed B, B′, and D (23). At all time points analyzed, processed G2U2 RNA was Sm antibody-selectable. Densitometry (data not shown) indicated that the relative amounts of processed G2U2 RNA selected by the antibody from the nuclear fractions were the same as for the endogenous U2 RNA, demonstrating a comparable extent of Sm antibody reactivity with snRNP complexes assembled on the introduced and endogenous U2 RNAs. Also apparent in Fig. 1 *B* and *C* is the fact that the introduced and assembled G2U2 RNA is less stable than endogenous U2 RNA. We determined that only ≈20% of the processed G2U2 RNA present in the nuclear fraction after 12 hr remained at 24 hr.

Since the introduced G2U2^{pre} RNA was shortened (Fig. 1), we determined whether it had undergone accurate 3′ processing. The 3′ ends of both the introduced and endogenous U2 RNAs were mapped using a U2 RNA-specific ribonuclease protection assay (22). Fig. 2*A* shows electrophoresis of aliquots of gel-purified G2U2^{pre}, processed G2U2, and endogenous U2 RNAs, which were then separately used for RNase mapping. As shown in Fig. 2*B*, lanes 2 and 3, the majority of the G2U2 RNA was processed accurately, as indicated by the nucleotide-for-nucleotide coalignment of the 3′ end of G2U2 RNA with that of endogenous U2 RNA.

Another step in the maturation of U2 RNA is hypermethylation of the ^{7}mG cap to ^{2,2,7}mG. We, therefore, asked whether this modification occurs on the introduced G2U2^{pre} RNA. The transfected RNA contained a ^{7}mG cap structure

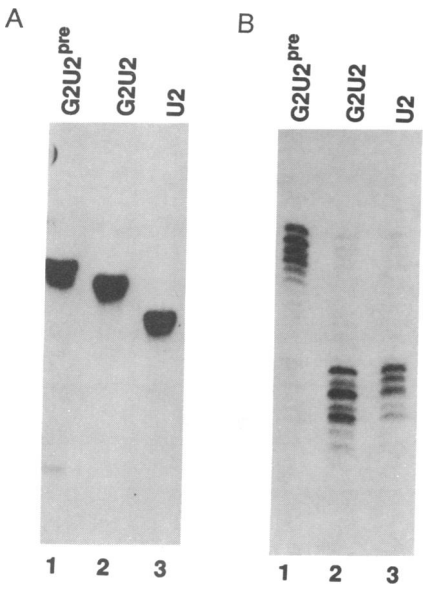

FIG. 2. RNase protection mapping of the 3′ ends of introduced and endogenous U2 RNAs. Four 100-mm dishes of cells were each transfected with 1.2×10^7 dpm of ^{32}P-labeled G2U2^{pre} RNA (specific activity = 4×10^8 dpm/μg). After 16 hr, the cells were harvested and nuclear RNA was prepared and separated by electrophoresis. After autoradiography, the desired gel bands were excised and the RNA was eluted (22). (*A*) Purity of an aliquot of each recovered RNA. For RNase mapping, an M13 clone containing sequences complementary to the last 39 nt of mature U2 RNA and 90 nt of 3′ flanking sequence was used (22). For mapping of endogenous U2 RNA, 30 μg of DNA was used and, for mapping of transfected U2 RNAs, 7.5 μg of DNA was used. Hybridization and RNase digestion were performed as described (22) except that the final concentration of RNase A was 1 μg/ml. The heterogeneity observed for the protected fragments is typical of RNase (22) and S1 nuclease (16, 17) protection assays performed on the 3′ ends of mammalian U2 RNAs.

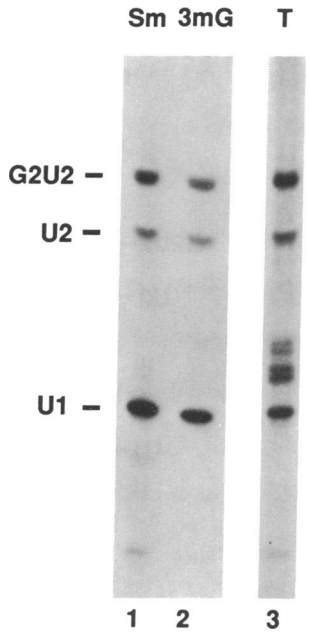

FIG. 3. The 5′ cap of transfected U2 RNA becomes hypermethylated. A 100-mm dish of cells was transfected with 8×10^6 dpm of ^{32}P-labeled G2U2^{pre} RNA (specific activity = 8×10^7 dpm/μg) for 24 hr. Total cell extracts were prepared and immunoselected with Sm monoclonal antibody (lane 1) or with a rabbit polyclonal ^{2,2,7}mG antibody (20) (lane 2). Lane T contains total cell RNA isolated from the same number of cells as for antibody selection. The autoradiographic exposure for the antibody-selected samples was 9 days and for total RNA was 1 day. The electrophoretic mobilities of G2U2 RNA and of endogenous U2 and U1 RNAs are indicated.

due to the use of ^{7}mGpppG as a primer during its *in vitro* transcription. Immunoselection of whole-cell extracts with an antibody specific for ^{2,2,7}mG (20) was compared to selection with Sm monoclonal antibody (Fig. 3). It can be seen that the same relative amounts of G2U2 RNA and endogenous U2 and U1 RNAs were selected with each of the two antibodies, indicating that the ^{7}mG cap of G2U2 RNA had been converted to a ^{2,2,7}mG cap.

The biological significance of the trimethylguanosine cap structure on U1–U5 snRNAs is unknown. It has been shown that ^{7}mG cap hypermethylation precedes the 3′ RNA processing and nuclear entry of U2 snRNP (13) and requires snRNP assembly (14). It has been suggested that the trimethylguanosine cap prevents the cytoplasmic precursors of U1–U5 RNAs from becoming engaged with the translational machinery (24). Another possibility is that the hypermethylated cap is a signal for nuclear targeting.

To explore this question, we transcribed three G2U2^{pre} RNAs, carrying ^{7}mGpppG, ApppG, or pppG at their 5′ ends (Fig. 4). These RNAs were transfected into cells, and nuclear and cytoplasmic fractions were prepared and immunoselected with Sm monoclonal antibody. The relative amounts of ^{7}mGpppG- and ApppG-capped RNAs selected from the nuclear fraction were the same (Fig. 4*A*, lanes 2 and 4), showing that the ^{2,2,7}mG cap is not required for either snRNP assembly or nuclear import of U2 snRNP (or for U2 RNA 3′ processing). It is likely that the 5′ end of the ApppG-U2 RNA remained capped during the transfection, since the uncapped RNA was not stable (Fig. 4*A*, lane 6). To control for the unlikely removal and replacement of the ApppG cap with ^{7}mG, which could then have undergone hypermethylation, we asked whether the ApppG-capped RNA could be selected with the ^{2,2,7}mG antibody after transfection. None of the nuclear ApppG-capped RNA was selected with ^{2,2,7}mG antibody (Fig. 4*B*, lane 4) although the endogenous U2 and U1 molecules were, as expected. Thus, the ApppG cap is not converted to ^{2,2,7}mGpppG during the experiment. Note again that the ApppG-capped U2 RNA is Sm antibody-reactive (Fig. 4*B*, lane 3), showing that snRNP assembly does not require a ^{7}mG or a ^{2,2,7}mG cap structure.

DISCUSSION

We have presented a method for introducing a small RNA (219 nt) into cultured human cells. Other reports of RNA uptake by cells have appeared (refs. 25–27 and references cited therein), but these studies were not aimed at RNA processing or RNP assembly, as we have presented here. At its current stage of development our method is a "tracer" approach, in which the transfected RNA enters the on-going pathway of endogenous snRNA processing, RNP assembly, and intracellular traffic. We calculate from the data shown in Fig. 1 that 3×10^3 molecules of G2U2 RNA are introduced per cell (assuming that ≈20% of the cells take up RNA; see ref. 28). The cytoplasmic concentration of endogenous U2 RNA precursor has been estimated to be 2×10^4 molecules per cell (in mouse fibroblasts growing with a 30-hr doubling time; ref. 29).

Since our objective was to employ "tracer" levels of transfected RNA to probe RNA processing, RNP assembly, and nuclear import we have not attempted to maximize the efficiency of RNA uptake. From the data presented by Malone *et al.* (27), we calculate that they introduced 1.8×10^5 molecules of RNA per cell (assuming that 100% of the cells took up RNA in these liposome-mediated experiments). Whether our calcium phosphate-based method can be opti-

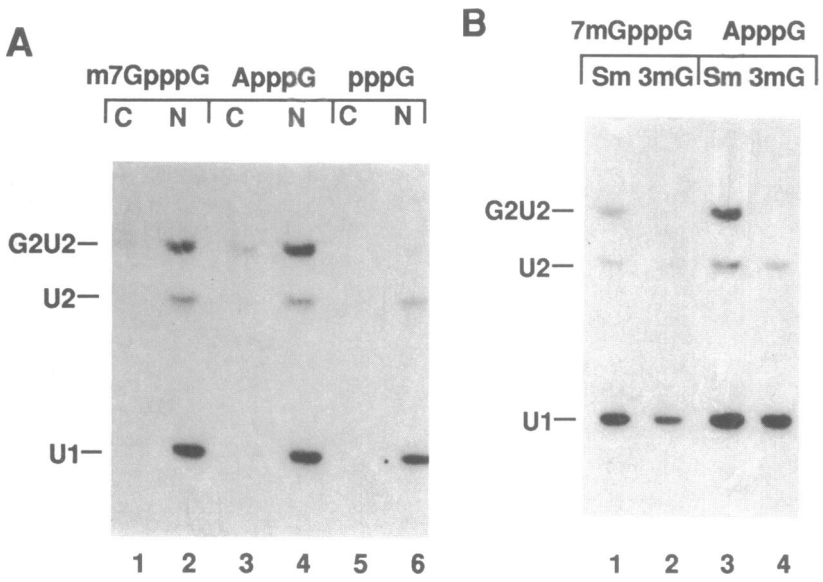

FIG. 4. A trimethylguanosine cap is not necessary for nuclear accumulation of U2 snRNP. Approximately 4×10^6 dpm of ^{32}P-labeled G2U2^{pre} RNA (specific activity = 2.7×10^7 dpm/μg) transcribed in the presence of ^{7}mGpppG, ApppG, or no cap analog was transfected. After 20 hr the cells were harvested and nuclear and cytoplasmic fractions were isolated. (*A*) Sm antibody-selected RNAs from cytoplasmic (lanes C) or nuclear (lanes N) fractions. ^{7}mGpppG, ApppG, and pppG refer to the 5′ end structures on the input RNAs. The mobilities of G2U2 and endogenous U2 and U1 RNAs are designated. (*B*) RNAs selected by Sm monoclonal antibody (Sm) or ^{2,2,7}mG antibody (3mG).

mized to a comparable (or superior) efficiency remains to be determined.

We point out that the cytoplasmic site of U2 RNA processing and RNP assembly may have been a serendipitous element in our experiments, as the introduced RNA almost certainly encounters this cell compartment first. The same point applies to the translation of introduced RNA (25–27).

We noted that the introduced G2U2 RNA is considerably less stable than endogenous U2 RNA (Fig. 1 *B* and *C*). This could be due to the presence of the 18-nt 5′ leader on G2U2 RNA as well as deficiencies in its post-translational modifications (ribose 2-*O*-methylation or pseudouridine formation) in the cell. It does not appear that incomplete or inaccurate snRNP assembly of G2U2 RNA can explain its lower metabolic stability, since the amount of Sm antibody-selected processed G2U2 RNA declined between 12 and 24 hr to the same extent as total G2U2 RNA (Fig. 1 *B* and *C*).

Although almost all of the processed G2U2 RNA was recovered in the nucleus, this fraction also contained some unprocessed G2U2^{pre} (Fig. 1*B*). This could reflect entry of some transfected RNA into the nucleus by a direct vesicular traffic pathway, in which case this subset of molecules would never encounter the cytoplasm. The fact that the unprocessed G2U2^{pre} RNA recovered with the nuclear fraction does not react with Sm antibody is also compatible with the possibility that it has not passed through the cytoplasm. It is also possible that some of the unprocessed G2U2^{pre} RNA that is in the cytoplasm binds avidly to the surface of nuclei during cell fractionation.

The mechanism by which calcium phosphate-bound DNA or RNA is internalized by cells is not well understood. Although the efficiency of pre-U2 RNA uptake we have described here is low (on the order of 0.5–2.0%), it is adequate for the types of experiments we have presented. Our purpose is neither phenotypic conversion (as in DNA transfection) nor heterologous protein expression (as in experiments that introduce mRNA). Instead, we present this approach in the context of investigating eukaryotic RNA processing and RNP assembly *in vivo*. We have also used this method to introduce mutant U2 RNA precursors into human cells. We find that numerous mutations in the 11-nt 3′ trailer of the U2 RNA precursor do not disrupt processing, whereas we have identified nucleotides within the mature U2 RNA 3′-vicinal sequence that are required for accurate processing (unpublished results). These results provide confidence that the RNA transfection approach will indeed generate insights into the principles that govern eukaryotic RNA processing *in vivo*.

The discovery of RNA molecules (ribozymes) that can cleave other RNAs (30, 31) has opened the possibility of using RNA as a therapeutic agent against viral or other diseases. Antisense (noncatalytic) RNAs have also been reported to inhibit the mRNA processing step of gene expression, at least *in vitro* (32). If ribozymes or antisense RNAs are ever to become a clinically significant modality by extracellular delivery, then the ways in which human cells take up RNA will need to be investigated. Although our own primary interest is how eukaryotic RNAs are processed and assembled into cellular structures, RNA transfection by this calcium phosphate method or other delivery systems may also be of use to investigators who wish to introduce RNA into cells for other purposes.

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