Precursors of U4 Small Nuclear RNA

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ABSTRACT The processing and ribonucleoprotein assembly of U4 small nuclear RNA has been investigated in HeLa cells. After a 45-min pulse label with [^{3}H]uridine, a set of apparently cytoplasmic RNAs was observed migrating just behind the gel electrophoretic position of mature U4 RNA. These molecules were estimated to be one to at least seven nucleotides longer than mature U4 RNA. They reacted with Sm autoimmune patient sera and a monoclonal Sm antibody, indicating their association with proteins characteristic of small nuclear ribonucleoprotein complexes. The same set of RNAs was identified by hybrid selection of pulse-labeled RNA with cloned U4 DNA, confirming that these are U4 RNA sequences. No larger nuclear precursors of these RNAs were detected. Pulse-chase experiments revealed a progressive decrease in the radioactivity of the U4 precursor RNAs coincident with an accumulation of labeled mature U4 RNA, confirming a precursor-product relationship.

The existence of a set of nuclear small RNAs, ranging in abundance from 10^5 to 10^6 molecules per cell, has been known for some time (1). However, only recently have clues to the function of these RNAs emerged. The 5′ end of U1 small nuclear RNA is complementary to donor splice sites in mRNA precursors (2, 3), and U1 RNA is base-paired to pre-mRNA in vivo (4, 5). U1 RNA has been implicated in mRNA splicing (6, 7) and site-specific poly(A) addition (8) in cell extracts. As other small nuclear RNAs, including U2 and U4, are also base-paired to pre-mRNA in vivo (9, 10), it is probable that they too function in mRNA processing.

The small nuclear RNAs may themselves be processed from precursor molecules. U1 RNA precursors 1–12 nucleotides longer than mature U1 RNA (165 nucleotides) (11–15) are found in the cytosol fraction of pulse-labeled HeLa cells (16), complexed with proteins that confer antigenicity for both Sm and RNP human autoantibodies (17–19). During a chase, radioactivity disappears from the U1 precursors and progressively appears as mature U1 RNA (16). We now present the first evidence that U4 small nuclear RNA is also processed from precursors, and that these precursor RNAs are associated with proteins characteristic of small nuclear RNPs.

MATERIALS AND METHODS

HeLa cells were maintained in suspension culture as previously described (20). [^{3}H]uridine labeling and pulse-chase conditions are specified in the figure legends. Cell fractionation was as previously described (20). As will be emphasized, this nuclear–cytoplasmic separation is strictly operational and we have no direct evidence on whether the U4 RNA precursor molecules have a nuclear or cytoplasmic localization in situ. For this reason, we deliberately use the cell fractionation term "cytosol" throughout this paper.

IgG was prepared from either Sm autoimmune patient sera (referred to in the text as autoantibody) or sera of healthy adults as described (21). Mouse Sm monoclonal antibody (22) was recovered from ascitic tumor fluid (23). The procedures used for preparation of nuclear extracts, antibody reactions, isolation of antigen-antibody complexes on *Staphylococcus aureus* protein A-Sepharose columns, extraction of antibody-selected RNA and electrophoresis have all been described in our previous papers (18, 19, 24). All RNA samples were heated at 90°C for 2 min in 80% formamide before electrophoresis.

The pU4/5 clone of human genomic DNA described by Hammarstrom et al. (25) was cut with PstI and an 85-base-pair fragment containing a sequence homologous to nucleotides 4–69 of U4 RNA was isolated and ligated into the PstI site of bacteriophage M13mp8 replicative form DNA. After transfection of *Escherichia coli* JM 103, plaques were picked and grown up in 10-ml cultures. A M13/U4 subclone containing the desired strand of U4 DNA was identified by the ability of purified phage DNA to hybrid-select U4 RNA from a preparation of HeLa cell nuclear RNA, which had been 3′-end-labeled with ^{32}P-pCp and T4 RNA ligase.

Pulse-labeled HeLa nuclear or cytosol RNA was hybridized to M13/U4 DNA immobilized on nitrocellulose (26). 50-μg aliquots of M13/U4 DNA were loaded as dots on Schleicher & Schuell BA85 nitrocellulose filters (0.45-μm pore size; Schleicher & Schuell, Inc., Keene, NH), which were then washed with 4X standard saline-citrate buffer (SSC) (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate), and baked at 80°C for 2 h under vacuum. Prehybridization was with 50% formamide, 0.6 M NaCl, 0.1 M PIPES, pH 6.6, and 100 μg/ml polyadenylic acid, for 1 h at 50°C. Nuclear and cytosol RNA was dissolved in water, adjusted to the same concentrations of formamide, NaCl, and PIPES as the prehybridization buffer (total volume = 1.0 ml), and hybridized with filter-bound M13/U4 DNA for 16 h at 50°C. Filters were washed with (*a*) 0.2 M NaCl, 0.1 M PIPES, pH 6.6, 0.5% SDS at 60°C; (*b*) 0.2 M NaCl, 0.1 M PIPES,

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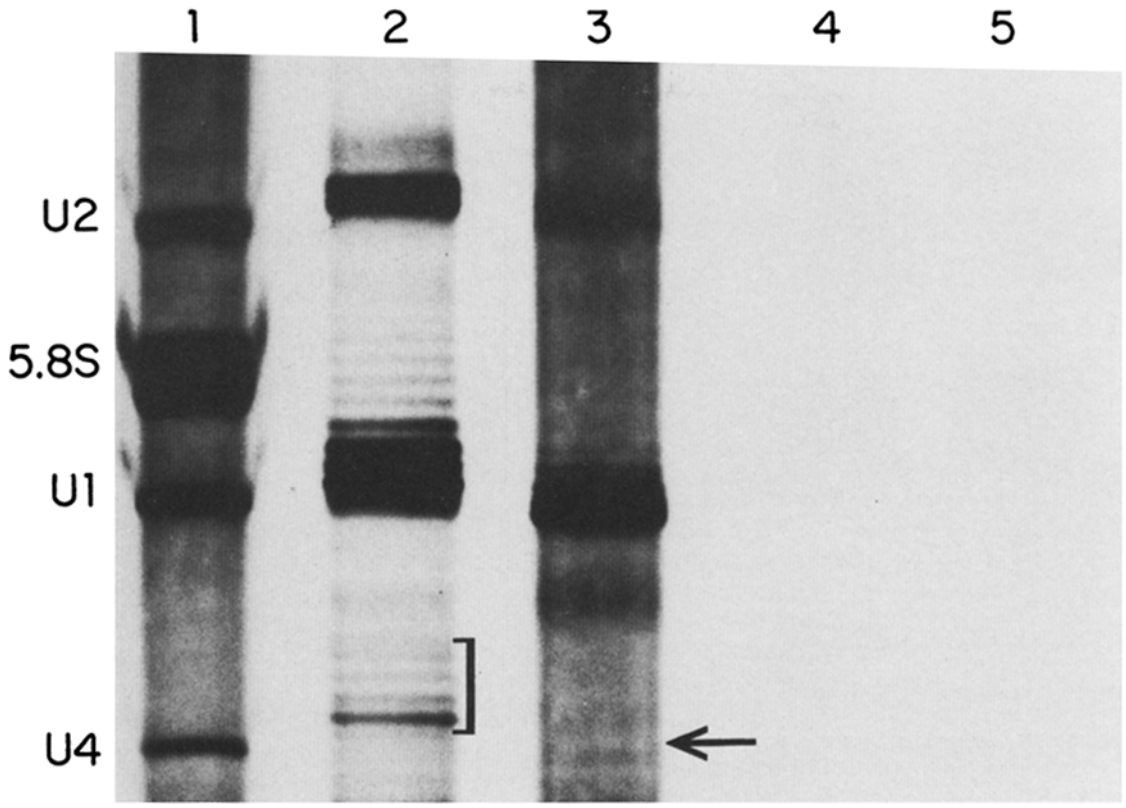

FIGURE 1 Pulse-labeled HeLa cell RNAs detected by Sm autoantibody. 200 ml of cells (3×10^5/ml) were resuspended in fresh medium at 3×10^6 cells/ml and incubated at 37°C for 45 min with [^{3}H]uridine (125 μCi/ml). The cells were harvested, washed, and fractionated as described (17–20), and nuclear and cytosol extracts were incubated with Sm autoantibody or nonimmune IgG. After selection of antigen-antibody complexes on protein A-Sepharose and elution, the RNA was deproteinized by phenol extraction, denatured (see Materials and Methods), and analyzed by electrophoresis on a 10% polyacrylamide gel containing 7 M urea, followed by fluorography. Lane *1*: Markers from HeLa cells labeled for 22 h with [^{3}H]uridine; lane *2*: cytosol pulse-labeled RNA reacting with Sm autoantibody; lane *3*: nuclear pulse-labeled RNA reacting with Sm autoantibody; lanes *4* and *5*: same as lanes *2* and *3*, respectively, but with human nonimmune IgG. The bracket in lane *2* indicates the pre-U4 RNAs; the arrow in lane *3* marks the position at which mature nuclear U4 RNA would migrate.

pH 6.6 at 60°C; (*c*) 20 mM NaCl, 10 mM PIPES, pH 6.6, at 60°C; and (*d*) 1 mM EDTA, 10 mM Tris-HCl, pH 7.4 at 20°C. Hybridized RNA was then eluted by adding 0.5 ml of water to the filters and incubating at 80°C for 5 min. Calf thymus transfer RNA was added as carrier, the RNA was precipitated with ethanol and electrophoresed in a 10% polyacrylamide gel containing 7 M urea (18), and the [^{3}H]uridine radioactivity was detected by fluorography.

RESULTS

After HeLa cells were pulse-labeled for 45 min with [^{3}H]-uridine, labeled RNA molecules the sizes of mature U1 and U2 RNA, which are 165 and 189 nucleotides, respectively, became detectable in the nucleus in ribonucleoprotein particles reactive with Sm autoantibody (Fig. 1, lane *3*). In contrast, the cytosol fraction (lane *2*) contained antibody-reactive pulse-labeled molecules larger than mature U1 and U2 RNA. We have previously established that the molecules running behind the position of U1 are precursors of mature, nuclear U1 RNA (16), and we tentatively regard the species migrating behind U2 RNA as precursors also (see Fig. 4 of this paper).[1] We now focus on U4, which is 146 nucleotides in length (27). As can be seen in Fig. 1, lane *3* (arrow), no labeled U4 RNA was present in antibody-reactive form in the nucleus after a 45-min pulse. In contrast, the cytosol (lane *2*) contained a series of at least six bands (bracketed) that migrated just behind the position of mature U4 RNA (see markers, lane *1*), and which were complexed with proteins reactive with the Sm antibody. The results of another experiment of this kind are shown in Fig. 2, in which cells were again pulse-labeled for 45 min and cytosol was reacted with an Sm autoantibody (lane *1*), a monoclonal Sm antibody (lane *2*), or nonimmune human IgG (lane *3*). In addition to the U1 RNA precursors, and probable U2 precursors,[1] both antibodies reacted with a series of at least seven bands migrating just behind the position of mature U4 RNA. As can be seen in lane *3*, none of these RNA precursors reacted with nonimmune IgG. The concentrations of mature U1 and U2 RNA are about 10^6 molecules per nucleus whereas the concentration of mature U4 RNA is about 10^5 molecules per nucleus. It appears that the respective precursors may have roughly comparable relative concentrations. Hence the U1 and U2 ladders are overexposed (film silver grain saturation) when the U4 precursors are cleanly resolved (Fig. 2, lanes *1* and *2*).

To verify that the bands under analysis are indeed U4 RNA sequences, we hybridized nuclear or cytosol RNA from [^{3}H]-uridine pulse-labeled cells to cloned human U4 DNA (see Materials and Methods). The hybrid-selected RNA was eluted, electrophoresed under denaturing conditions, and

[1] The pulse-labeled bands migrating behind the position of mature U2 RNA (lane *2*, Fig. 1) have now been identified as U2 RNA sequences by hybrid-selection on human U2 RNA cloned in bacteriophage M13. (Nenninger, J., G. Kunkel, and T. Pederson, unpublished results.)

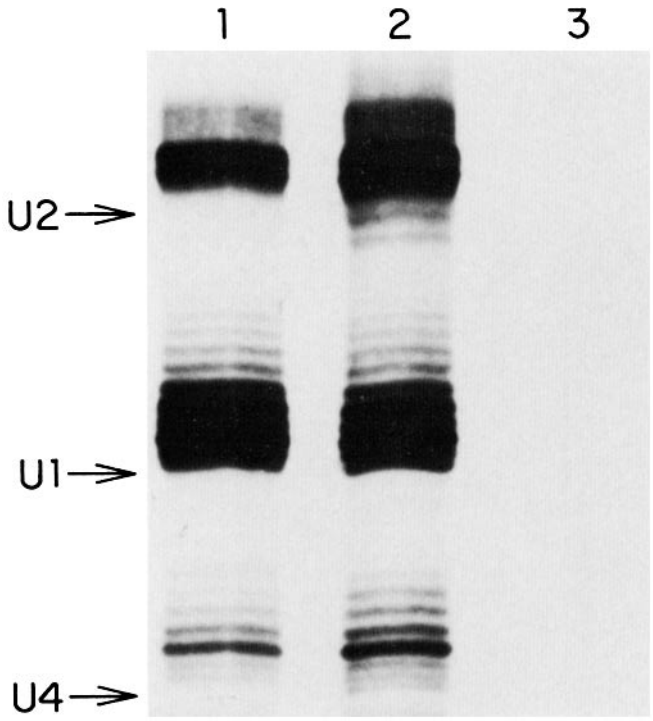

FIGURE 2 Monoclonal Sm antibody reacts with pre-U4 RNAs. This experiment was performed as in Fig. 1, except that a monoclonal Sm antibody was used in comparison with Sm autoantibody from patient serum. Lane *1*: cytosol pulse-labeled RNA reacting with Sm autoantibody; lane *2*: Sm monoclonal autoantibody; lane *3*: human nonimmune IgG. The positions of mature nuclear RNAs are indicated at the left of lane *1* (determined by migration of marker RNAs in adjacent lanes). Each lane represents 4×10^7 cell equivalents.

fluorographed. Fig. 3 shows that the hybrid-selected nuclear RNA (lane *2*) consisted of a single band the size of mature U4 RNA (compare with markers in lane *1*). In contrast, the hybrid-selected cytosol RNA (lane *3*) contained several bands running behind mature U4 and coincident with the pulse-labeled bands in Figs. 1 and 2. Thus, these molecules contain U4 RNA sequences.

Pulse-chase experiments confirm a precursor-product relationship between the cytosol U4 molecules and nuclear U4 RNA. Fig. 4 shows the results of an experiment in which HeLa cells were pulse-labeled for 25 min and then chased for 25 or 50 min. Nuclear and cytosol extracts were reacted with Sm antibody and the RNAs were displayed by electrophoresis. After the pulse, the cytosol contained the usual series of bands migrating behind the position of mature U4 (lane *4*). After 25 min of chase (lane *5*), radioactivity appeared in a smaller band (arrow), and after 50 min of chase (lane *6*), radioactivity in this band intensified coincident with a decrease of radioactivity in the larger bands. Meanwhile in the nuclear fraction, labeled RNA the size of mature U4 progressively appeared (lane *1–3*). Note also the chase behavior of the U1 RNA precursors, confirming the precursor-product relationship previously established (16), and the similar pattern for the U2 RNA molecules, suggesting that they too are precursors.

DISCUSSION

The experiments reported here provide the first evidence that U4 small nuclear RNA is derived from larger precursor molecules. These RNAs appear promptly in the cytosol fraction as Sm antibody-reactive RNP complexes, apparently before the accumulation of processed U4 RNA in the nucleus. From the known antigenic properties of small nuclear RNP proteins, the reaction of the cytosol U4 RNA molecules with Sm monoclonal antibody indicates that these precursors are complexed with the B and/or D small nuclear RNP proteins (28). We emphasize that it is entirely possible that these precursor particles are actually nuclear but leak into the cytosol fraction with our cell homogenization procedure. Clearly this would be a selective process as little, if any, labeled mature-size U4 RNA is recovered in this fraction. Precursors of U1 and U2 RNAs (11–14, 16) have been found in HeLa cell cytosol using a nonaqueous fractionation method (15). We are continuing to examine the subcellular localization of small nuclear RNA precursors by other techniques.

Since the RNA preparations used in this study were denatured at 90°C in 80% formamide prior to electrophoresis and

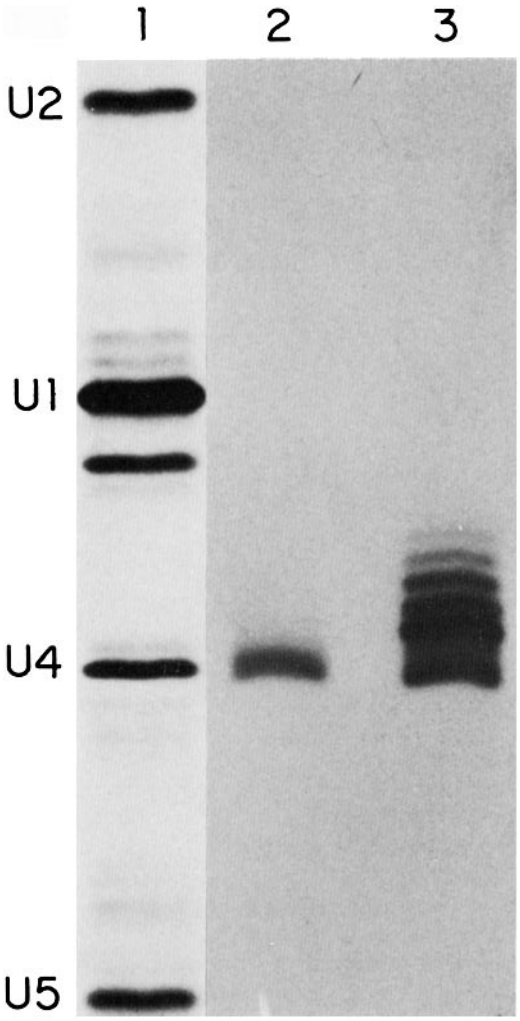

FIGURE 3 Sequence identity of U4 RNA precursors. HeLa cells were pulse-labeled as in Fig. 1 and nuclear and cytosol extracts were reacted with Sm monoclonal antibody. The antibody-selected RNPs were deproteinized and the RNA was hybridized to cloned U4 DNA as detailed in Materials and Methods. The hybrid-selected RNA was released, denatured, and displayed by electrophoresis and fluorography. Lane *1*: Sm antibody-selected RNA markers (from cells labeled for 22 h with [^{3}H]uridine); lane *2*: U4 DNA-selected, pulse-labeled nuclear RNA; lane *3*: U4 DNA-selected, pulse-labeled cytosol RNA.

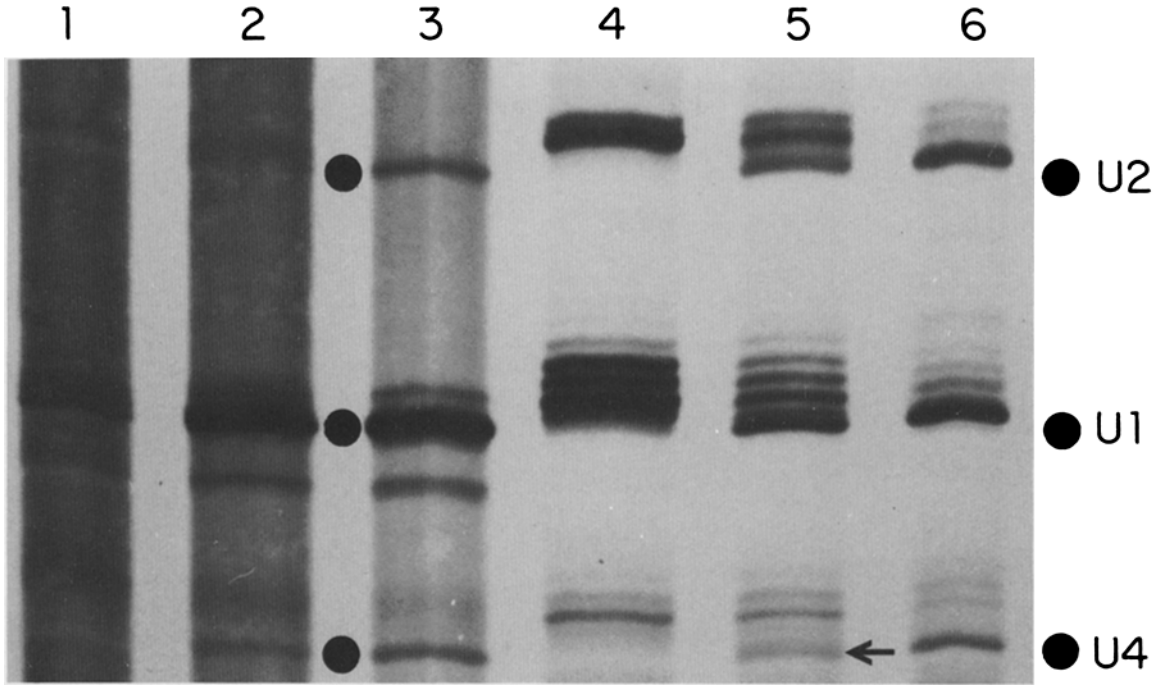

FIGURE 4 Pulse-chase behavior of U4 RNA precursors. 250 ml of cells (3×10^5/ml) were resuspended in 25 ml of fresh medium and labeled for 25 min with [^{3}H]uridine (125 μCi/ml). One-third of the cells was harvested and nuclear and cytosol extracts were prepared. To the remaining cells actinomycin was added to 0.1 μg/ml, and equal portions were harvested after an additional 25 or 50 min of incubation. All nuclear and cytosol fractions were reacted with Sm autoantibody and the RNAs were analyzed by electrophoresis and fluorography. The electrophoretic positions of mature U2, U1, and U4 RNA markers are indicated by the filled symbols between lanes *2* and *3* and to the right of lane 6. (The band beneath U1 in lanes *2* and *3* may correspond to "U1*," see reference 2.) Lanes *1–3*: Sm-reactive nuclear RNAs after pulse-label (lane *1*), 25-min chase (lane *2*), and 50-min chase (lane *3*). Lanes 4–6: Sm-reactive cytosol RNAs after pulse-label (lane *4*), 25-min chase (lane *5*), and 50-min chase (lane 6).

the gels contained 7 M urea, we interpret the cytosol U4 molecules to be longer than mature U4 RNA. It is possible, however, that altered methylation could be a factor in the electrophoretic mobilities of the U4 RNA molecules from the nuclear versus cytosol fractions.

It is of interest that U4 RNA-related molecules considerably longer than the cytosol species were never detected in nuclear or cytosol fractions, either with [^{3}H]uridine pulse-labeled RNA hybrid selected on U4 DNA (Fig. 3) or in Northern blot experiments (unpublished results). This argues against, but does not eliminate, the existence of high molecular weight U4 RNA primary transcripts. The transcription units for small nuclear RNAs are currently being investigated by several groups. We have evidence that transcription starts at or near the 5′ cap of mature U1 RNA (G. R. Kunkel and T. Pederson, unpublished results). We do not yet know the sequences of the 3′ ends of the HeLa cell small nuclear RNA precursors, but a U2 precursor has recently been identified in rat liver with at least 11 transcribed nucleotides at its 3′ end (29). We have recently obtained accurate processing of some of the small nuclear RNA precursors in an in vitro system, and this should facilitate progress on this new class of eucaryotic RNA processing and RNP assembly reactions.

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Note Added in Proof: We have now determined that the U4 RNA precursors react with an antibody against 2,2,7-trimethylguanosine, indicating that they contain 5′-cap structures.

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