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Growth-regulated expression and G_0-specific turnover of the mRNA that encodes URH49, a mammalian DExH/D box protein that is highly related to the mRNA export protein UAP56

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ABSTRACT

URH49 is a mammalian protein that is 90% identical to the DExH/D box protein UAP56, an RNA helicase that is important for splicing and nuclear export of mRNA. Although *Saccharomyces cerevisiae* and *Drosophila* express only a single protein corresponding to UAP56, mRNAs encoding URH49 and UAP56 are both expressed in human and mouse cells. Both proteins interact with the mRNA export factor Aly and both are able to rescue the loss of Sub2p (the yeast homolog of UAP56), indicating that both proteins have similar functions. UAP56 mRNA is more abundant than URH49 mRNA in many tissues, although in testes URH49 mRNA is much more abundant. UAP56 and URH49 mRNAs are present at similar levels in proliferating cultured cells. However, when the cells enter quiescence, the URH49 mRNA level decreases 3–6-fold while the UAP56 mRNA level remains relatively constant. The amount of URH49 mRNA increases to the level found in proliferating cells within 5 h when quiescent cells are growth-stimulated or when protein synthesis is inhibited. URH49 mRNA is relatively unstable ($T_{\frac{1}{2}}$ = 4 h) in quiescent cells, but is stabilized immediately following growth stimulation or inhibition of protein synthesis. In contrast, there is much less change in the content or stability of UAP56 mRNA following growth stimulation. Our observations suggest that in mammalian cells, two UAP56-like RNA helicases are involved in splicing and nuclear export of mRNA. Differential expression of these helicases may lead to quantitative or qualitative changes in mRNA expression.

INTRODUCTION

The production of mature mRNA in eukaryotes involves a complex series of nuclear processing reactions, including addition of the 5′ cap, removal of introns and addition of the 3′ poly(A) tail, followed by the export of the fully processed mRNA to the cytoplasm. These reactions are highly interdependent and occur co-transcriptionally (1–3). In metazoans, the removal of introns results in the deposition of a protein complex (the exon junction complex) on the RNA molecule immediately upstream of the splice site (4). Proteins in this complex play important roles in nuclear export, cytoplasmic localization and translation, as well as nonsense-mediated decay of mRNA (5–8).

RNA processing and export involve multiple sequential interactions between the substrate RNA and a variety of proteins and ribonucleoprotein complexes (9,10). Remodeling of many of these RNA–RNA or RNA–protein complexes is facilitated by members of a conserved family of proteins that contain a DExH/D motif (11,12). Proteins in this family are believed to function as RNA helicases that disrupt RNA–RNA or RNA–protein complexes. Genetic and biochemical analyses have shown that at least eight different RNA helicases are required during the splicing reaction in *Saccharomyces cerevisiae* (11), and even more appear to be important in mammalian cells (9,10). The specific reactions that are carried out by many of the helicases have been defined (13–17).

UAP56 (also known as Sub2p, HEL, BAT1, HLA-B-associated transcript 1, RAF-2p48 and NPI-5) is a DExD/H protein that has been shown to play important roles in the splicing reaction as well as in nuclear export of mature mRNA. In the splicing reaction, UAP56 facilitates the association of U2 snRNP with the splice branch point, presumably by dissociating U2AF65 from the polypyrimidine track downstream of the splice branchpoint (18,19). This conclusion is supported by the observation that in *S.cerevisiae*, SUB2 can be made dispensable by deleting the gene encoding Mud2p, the yeast homolog of mammalian U2AF65 (20), indicating that Sub2p and Mud2p counteract each other's function.

UAP56 also has a second important role in coupling mRNA splicing with nuclear export (21–23). UAP56, one of the

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components of the exon junction complex, recruits the adaptor protein Aly/REF (Yra1p in *S.cerevisiae*) to the mRNA, and Aly/REF subsequently interacts with the heterodimeric export factor TAP/NXF1:p15, leading to the efficient export of the mRNA through the nuclear pore complex (24,25). Inactivation of UAP56 in *Drosophila*, *Caenorhabditis elegans* and *S.cerevisiae* leads to nuclear retention of poly(A)$^+$ mRNA, presumably because Aly is not attracted to the mRNA (24–27). Interestingly, Sub2p is required for nuclear export of mRNA derived from intron-containing as well as intronless genes (25), so the association of the helicase with the mRNA molecule is not necessarily coupled with the splicing reaction. Recent studies have shown that UAP56 associates with the mRNA molecule co-transcriptionally (28–30).

Although there is only a single gene encoding UAP56 in *S.cerevisiae* and *Drosophila*, a search for proteins similar to UAP56 revealed that there is a second protein, which we have designated URH49 (UAP56-related helicase, 49 kDa), which is highly related to UAP56 in mouse and human cells. Since the amino acid sequences of the two proteins are highly similar, it is likely that the proteins have similar or redundant functions. However, it is possible that the two helicases have different expression profiles or interact with non-overlapping populations of mRNAs or mRNA export proteins, which may lead to quantitative or qualitative differences in mRNA production.

To begin to explore these possibilities, we have compared the regulation of UAP56 and URH49 expression in mouse and human cells. We found that URH49 interacts with Aly in the same manner as UAP56. Both UAP56 and URH49 are able to complement a yeast deletion in Sub2. However, there are large differences in the expression of the mRNAs encoding the two proteins in different tissues and following growth stimulation of cultured cells. These differences are caused (at least in part) by destabilization of URH49 mRNA in quiescent cells.

MATERIALS AND METHODS

Plasmids

The human URH49 cDNA was isolated from a HeLa pMyr-cDNA library of the CytoTrap yeast two-hybrid system (Stratagene). To generate the pGST-URH49 expression vector, the full-length URH49 coding region was isolated from the pMyr vector following digestion with EcoRI and XhoI, and ligated into the corresponding sites in the polycloning region of the pGEX-5X-1 bacterial expression vector (Amersham Pharmacia Biotech). To generate the pGST-UAP56 expression vector, the UAP56 coding region was cloned into the pGEX-5X-3 bacterial expression vector (Amersham Pharmacia Biotech). The pHis-Aly expression vector was created by cloning the Aly coding region into the pRSET bacterial expression vector (Invitrogen) (24). The pGST-UAP56 and pHis-Aly plasmids were kindly provided by Robin Reed.

To examine the ability of UAP56 and URH49 to complement the *sub2* deletion in yeast, HA-tagged versions of UAP56 (UAP56-HA) and URH49 (URH49-HA) were PCR-amplified and placed under the yeast *GAL1* promoter control in p415GAL1 (31). The UAP56-HA open reading frame (ORF) was amplified using primers UAP56-1 and UAP56-2. Primer UAP56-1 (CCCGGTCTAG**AAAAA**ATGGCAGAG-AACGAT) contains an optimal yeast translation start sequence (in bold) upstream of the initiation codon (ATG) of UAP56; primer UAP56-2 [ACATGACTCGAG(CTAAG-CGTAGTCTGGGACGTCGTATGGGTA)CCGTGTCTGTT-CAATGTAGGAGGAGATGTC; HA-epitope in parentheses] is complementary to the last 30 nucleotides of the UAP56 ORF. The ~1.7-kb PCR product was digested with XbaI and XhoI (sites underlined in primer sequences) and cloned into p415GAL1 to yield plasmid UAP56-HA/p415GAL1. Plasmid URH49-HA/p415GAL1 was constructed in a similar manner, using primer URH49-1 (CCCGGTCTAG**AAAAA**ATGGCA-GAACAGGAT) and primer URH49-2 [AAGCCGCTCGAG-(CTAAGCGTAGTCTGGGACGTCGTATGGGTA)CCGGC-TCTGCTCGATGTATGTGGAGATGTC; HA-epitope in parentheses] as PCR primers. The XbaI- and XhoI-digested PCR product was then cloned into p415GAL1 to yield plasmid URH49-HA/p415GAL1. Plasmid *SUB2*/p415GAL1, a positive control, was constructed by isolating a 2-kb SalI (blunted)–XbaI fragment from pCG788 (*SUB2/CEN/URA3*), which was cloned into p415GAL1. pCG788 (*SUB2/CEN/URA3*) was a gift from Christine Guthrie (20).

Small-scale plasmid preparations were performed using a QIAprep Spin Miniprep kit (Qiagen). Large-scale plasmid preparations were performed using a Plasmid Maxi Kit (BioRad). The sequences of all plasmid inserts were confirmed by DNA sequence analysis.

Protein analysis

Bacterial expression plasmids glutathione *S*-transferase (GST)–UAP56 and GST–URH49 were transformed into DH5α. The His-Aly expression plasmid was transformed into BL21 (DE3) (Invitrogen). Cultures were grown to an absorbance (at 600 nm) of 0.6 and then induced for 4 h by adding isopropylthio-β-galactoside (IPTG) to a final concentration of 0.1 mM for the GST fusion proteins and 1 mM for His-Aly. The cells were collected by centrifugation and stored at –20°C. Cell pellets were resuspended in binding buffer [phosphate-buffered saline (PBS) containing 0.1% Triton X-100], disrupted by sonication on ice and clarified by centrifugation at 3000 *g* for 10 min. Approximately 200 µg of protein from bacteria expressing GST–UAP56, GST–URH49 or GST alone (negative control) was mixed with 200 µg of protein from the bacteria expressing His-Aly in a total volume of 1 ml of binding buffer. Twenty-five microliters of glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech) and 1 µl of 500 mM phenylmethylsulfonyl fluoride (PMSF) were then added and the samples were mixed by gentle rotation at room temperature for 2 h. The matrix was then mixed with 20 µl of 10 mM reduced glutathione (Sigma) dissolved in 50 mM Tris–HCl pH 8.0, 10 µl of NuPAGE LDS Sample Buffer (Invitrogen) and 4 µl of NuPAGE Sample Reducing Agent (Invitrogen), and heated at 70°C for 10 min. The eluted proteins were separated by electrophoresis on a NuPAGE 10% Bis-Tris gel (Invitrogen) and transferred to nitrocellulose. The membrane was blocked using Tris-buffered saline (pH 7.5) containing 0.05% Tween 20 (TBST) and 5% dried skimmed milk. Western blots were performed using anti-polyhistidine monoclonal antibodies (Sigma) or anti-UAP56 primary antibodies followed by anti-mouse IgG (Cell Signaling Technology) or anti-rabbit IgG (Santa Cruz

Biotechnology) secondary antibodies that were conjugated with alkaline phosphatase. The anti-UAP56 antibodies were polyclonal rabbit antibodies raised against GST–UAP56 (18) and were kindly provided by Michael Green. Blots were developed using nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Invitrogen).

Mammalian cell culture

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Colorado Serum). WI38 human diploid fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen). WI38 cells were growth-arrested by incubating confluent monolayers of the cells for 3 days in DMEM in the absence of serum (32). They were then growth-stimulated by replacing the medium with fresh DMEM supplemented with 20% FBS. Swiss mouse 3T3 cells (33) were maintained in DMEM + 10% Nu-serum (BD Biosciences). The 3T3 cells were growth arrested by incubating confluent monolayers of cells in DMEM supplemented with 5% Nu-serum for 7 days, with medium changes on days 2 and 4. Quiescent cells were stimulated to proliferate by replacing the medium with fresh DMEM supplemented with 20% Nu-serum. 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; Sigma) (34) was dissolved in dimethyl sulfoxide (DMSO) and added to the cell culture media to a final concentration of 80 μg/ml. Actinomycin D (Sigma) was dissolved in DMSO and added to the culture media to a final concentration of 5 μg/ml. Cycloheximide (Sigma) was dissolved in PBS and added to the culture media to a final concentration of 5 μg/ml.

Yeast methods

Plasmids URH49-HA/p415GAL1, UAP56-HA/p415GAL1 and *SUB2*/p415GAL1 were transformed into yeast strain yCG470 (20), which contains the *sub2Δ::HIS3* chromosomal deletion and pCG788. yCG470 was provided by Christine Guthrie. Growth of these strains was examined on synthetic medium lacking leucine and containing galactose and 5-fluoroorotic acid (5-FOA) for counter-selecting pCG788 (35).

RNA isolation

Mouse tissues were isolated from a 45-day-old normal male mouse. Total cellular RNA was isolated from tissues or cultured cells using the Absolutely RNA reverse transcriptase (RT)–PCR miniprep kit (Stratagene). Samples of RNA isolated from human tissues were from the Human Total RNA Master Panel II (Clontech) and were kindly provided by James Lang.

Real-time PCR

Real-time PCR was performed in 96-well plates using the BioRad iCycler iQ Real-Time Detection System. cDNA was made using the SuperScript first-strand synthesis system for RT–PCR (Invitrogen). An oligo dT primer and 0.1 μg of total RNA were used in each 20 μl cDNA reaction. Each 25-μl real-time PCR consisted of a 1 μl sample of the cDNA preparation, 12.5 μl of iQ SYBR Green Supermix (BioRad), 1 μl of each 10 μM primer and 10.25 μl of water. Each cDNA preparation was analyzed in triplicate. The cycling parameters were 15 min at 94°C (initial incubation), 15 s at 94°C, 30 s at 62°C (human) or 63°C (mouse), and 30 s at 72°C.

The human UAP56 forward primer (in exon 2) was 5′-GACAGCAGCTGGGGGAGATG-3′ and the human UAP56 reverse primer (spanning exons 2 and 3) was 5′-CTCATG-CTGGACTTCTGACG-3′. The human URH49 forward primer (in exon 2) was 5′-GCCCCAGGCTCCTCAAGAGA-3′ and the human URH49 reverse primer (spanning exons 2 and 3) was 5′-CTCATGCTGGACCTCAGAAG-3′. The PCR products for both helicases are 166 nt in length. Primer specificity was confirmed by the presence of a BamHI site in the URH49 PCR product and a BsmAI site in the UAP56 PCR product.

The mouse UAP56 forward primer (in exon 2) was 5′-CTGGGGCAGATGGGACCGAG-3′. The mouse UAP56 reverse primer (spanning exons 2 and 3) was 5′-CACTCA-TGCTGGACCTCTGA-3′. The mouse URH49 forward primer (in exon 2) was 5′-CACCCCAGGAGAGCACTCCA-3′ and the mouse URH49 reverse primer (spanning exons 2 and 3) was 5′-CACTCATGCTGGACCTCTGA-3′. The PCR products for both helicases were 160 nt in length. Primer specificity was confirmed by the presence of a BstX1 site in the mouse URH49 product and a TseI site in the mouse UAP56 product.

For normalization purposes, the ribosomal protein L4 (rpL4) mRNA was also measured. The human rpL4 forward primer (spanning exons 2 and 3) was 5′-CTTTTGGAAA-CATGTGTCGTGG-3′ and the reverse primer (in exon 3) was 5′-CTTTAGACATGACCAGTGCTGG-3′. The mouse rpL4 forward primer (spanning exons 2 and 3) was 5′-CCTTTG-GAAATATGTGTCGTGG-3′ and the reverse primer (in exon 3) was 5′-CTTTAGACATCACCAAAGCTGG-3′. Both PCR products were 150 nt in length. Primer specificity was confirmed by the presence of a TseI site in the rpL4 PCR products.

The efficiencies of each primer pair were calculated by generating a standard curve and were between 90 and 100% for each set of primers. Real-time PCR data were analyzed using the comparative C_T method (36,37).

RESULTS

The UAP56/URH49 helicases are evolutionarily conserved

Comparison of the predicted amino acid sequences of UAP56 (human UAP56: NCBI LocusLink ID No. 7919; mouse UAP56: NCBI LocusLink ID No. 53817) and URH49 (human URH49: NCBI LocusLink ID No. 10212; mouse URH49: NCBI LocusLink ID No. 68278) revealed that the proteins are 90% identical to each other. Both are 49 kDa proteins that are ~82% identical to HEL and ~60% identical to Sub2p, the *Drosophila* and *S.cerevisiae* homologs of UAP56, respectively. The greatest variation between UAP56 and URH49 occurs within the first 30 amino acids. Otherwise, the two proteins have only minor variations in their sequences across the remainder of their ORFs. All of the conserved helicase motifs (38) that are found in UAP56 are also present in URH49. The gene that encodes human UAP56 is located on chromosome 6, while the gene that encodes human URH49 is on chromosome 19. The coding regions of both genes are highly conserved and both genes are interrupted by 10 introns at the same positions in the ORF. The sequences of the introns,

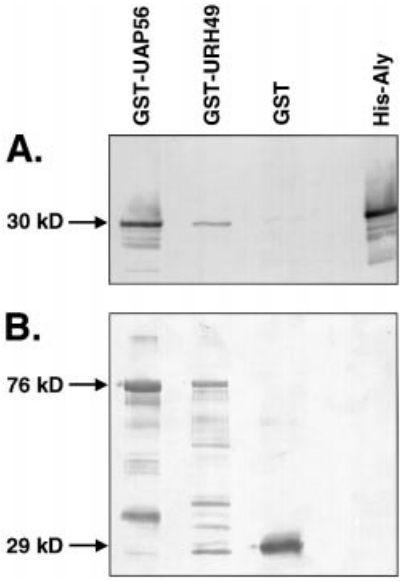

Figure 1. His-Aly interacts with UAP56 and URH49. Extracts from bacteria expressing His-Aly were mixed with extracts from bacteria expressing GST–UAP56, GST–URH49 or GST alone, and incubated with glutathione beads. Duplicate samples of bound proteins were separated by polyacrylamide gel electrophoresis and analyzed using western blots that were probed with anti-His (**A**) or polyclonal anti-GST–UAP56 (**B**) antibodies. His-Aly is a sample of extract isolated from bacteria expressing the His-Aly protein that was run on the SDS polyacrylamide gel as a marker. The electrophoretic mobilities of His-Aly (30 kDa), GST–UAP56 or GST–URH49 (76 kDa) and GST (29 kDa) are indicated.

as well as the 5′ and 3′ untranslated regions of the mRNAs, are not conserved.

UAP56 and URH49 interact with Aly/REF

UAP56 has been shown previously to interact physically with the mRNA export factor Aly (24). To determine if URH49 also has this property, extracts from bacteria expressing GST–URH49, GST–UAP56 or GST alone (control) were mixed with extracts from bacteria expressing His-Aly, and the resulting protein complexes were isolated by affinity chromatography on glutathione–Sepharose. Proteins eluted from the matrix were analyzed by western blot analysis using anti-His antibodies. Figure 1A shows that His-Aly interacted with GST–URH49 as well as GST–UAP56, but not with GST alone. Western blots of duplicate samples probed with polyclonal antibodies to GST–UAP56 (Fig. 1B) show that the amount of GST–UAP56 was greater than the amount of GST–URH49, which is in line with the relative amounts of His-Aly detected (Fig. 1A). When equal amounts of GST–UAP56 and GST–URH49 were used, equal amounts of His-Aly were observed (data not shown).

UAP56 and URH49 both complement a yeast *sub2* deletion

An earlier study showed that UAP56 can rescue the lethal deletion of *sub2* in yeast (39). To determine if URH49 could also complement the *sub2* deletion, we placed HA-epitope-tagged versions of UAP56 (UAP56-HA) and URH49 (URH49-HA) under the control of the yeast *GAL1* promoter

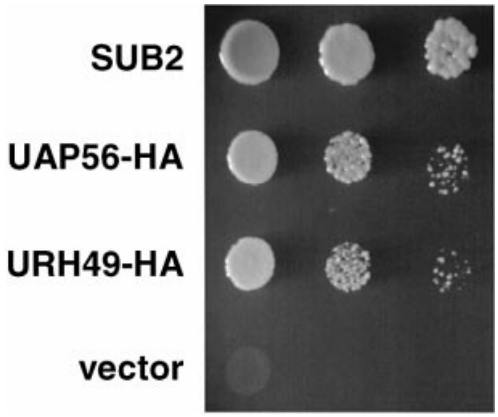

Figure 2. URH49 rescues the lethal *sub2* deletion in yeast. Shown are the growth phenotypes of the *sub2*-deletion strains containing plasmids expressing *SUB2*, UAP56-HA and URH49-HA, which were placed under the control of *GAL1* promoter. Transformation with vector alone was used as a negative control. Cells were grown to mid-log phase, 10-fold serially diluted, and spotted on a leucine drop-out plate containing 5-FOA and galactose. The plate was incubated at 30°C for 5 days.

and transformed the plasmids into a yeast tester strain containing a chromosomal *sub2* deletion complemented by a *URA3*-marked plasmid carrying *SUB2*. A *LEU2*-marked plasmid that expresses *SUB2* from the *GAL1* promoter was used as a control. As expected, after 5-FOA counter-selection of the *URA3*-marked *SUB2* plasmid, galactose-induced *SUB2* expression fully rescued the chromosomal *sub2* deletion (Fig. 2). Consistent with the previous report (39), under the same galactose-induced conditions, UAP56-HA complemented the *sub2* deletion, although to a much lesser extent than *SUB2*. Likewise, URH49-HA allowed cells to grow in the absence of *SUB2* in a manner nearly identical to that of the UAP56-HA. We conclude that both URH49 and UAP56 are capable of replacing *SUB2*'s function in yeast.

Expression of UAP56 and URH49 mRNAs in tissues

Since mRNA splicing and export occur in all living tissues, UAP56 (or URH49 if it has an equivalent function) must be present in all cells. To determine if the relative expression of the UAP56 and URH49 genes varies under different physiological conditions, we measured the amount of mRNA corresponding to both proteins in human and mouse tissues using real-time PCR. To correct for differences in mRNA recovery from the different tissues, the amounts of UAP56 and URH49 mRNA were normalized to the amount of rpL4 mRNA, which was likely to be expressed at comparable levels under a wide variety of conditions. Figure 3 shows that UAP56 and URH49 mRNAs were present at similar levels in many of the tissues examined in this study. UAP56 mRNA was usually present at a higher concentration than URH49 mRNA. However, in testes, the amount of URH49 mRNA was much greater than the amount of UAP56 mRNA. Similar observations were made with RNA isolated from human and mouse tissues, although the amount of UAP56 mRNA was generally greater in the latter. We also found that UAP56 and URH49 mRNAs are expressed at equivalent levels in exponentially growing human WI38 and mouse 3T3 fibroblasts.

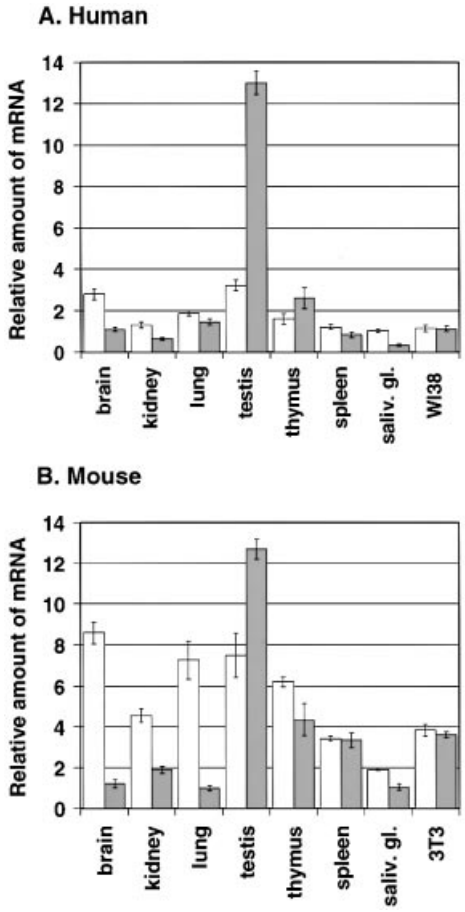

Figure 3. Helicase mRNA levels in tissues. The amount of UAP56 (open bars) and URH49 (filled bars) mRNA was determined for the indicated human and mouse tissues or exponentially growing cultured cells. All values were normalized to the levels of rpL4 mRNA to correct for differences in mRNA recovery. The corrected values were then normalized to the level of URH49 mRNA in the brain, which was set at 1.0. Each RNA sample was analyzed at least six times. Standard deviations are indicated.

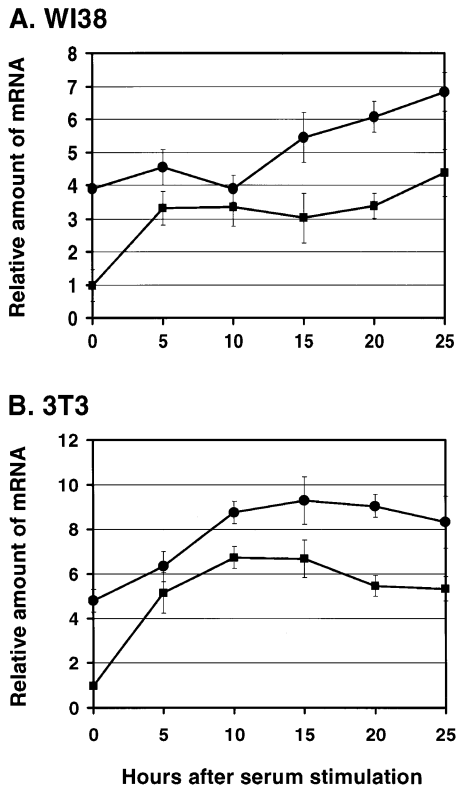

Figure 4. Regulation of helicase mRNA levels in growth-stimulated cells. The amount of UAP56 (circles) and URH49 (squares) mRNA was determined by real-time PCR in human WI38 cells and mouse 3T3 cells that were growth-stimulated for the indicated times. The data were normalized to rpL4 mRNA levels to correct for differences in recovery. The corrected values were then normalized to the level of URH49 mRNA at 0 h, which was set at 1.0. Data from at least three independent experiments are included in the graph. Standard deviations are indicated.

Regulation of UAP56 and URH49 mRNA in growth-stimulated cells

To determine the effect of proliferation rate on UAP56 and URH49 gene expression, we measured the amount of mRNA corresponding to both helicases in quiescent and growth-stimulated cells using real-time PCR. The values were again normalized to rpL4 mRNA levels to correct for differences in RNA recovery. In WI38 cells, the amount of UAP56 mRNA gradually increased following growth stimulation, approximately doubling by 25 h (Fig. 4A). In 3T3 cells the UAP56 mRNA level doubled by 15 h and then remained relatively constant. In contrast, the amount of URH49 mRNA increased rapidly during the first few hours following growth stimulation and then remained relatively constant (Fig. 4B). The initial increase was particularly striking in 3T3 cells, where URH49 mRNA content increased 5-fold within 5 h, and 7-fold within 10 h following growth stimulation.

The immediate increase in URH49 gene expression following growth stimulation is reminiscent of the expression of the 'immediate early' genes that are rapidly induced under these conditions. The increase in mRNA corresponding to many immediate early genes (e.g. c-*fos* and c-*myc*) is controlled at the transcriptional level and is not blocked by the presence of inhibitors of protein synthesis (40–42). To determine if the increase in URH49 mRNA is regulated in a similar manner, we measured the amount of URH49 mRNA in 3T3 and WI38 cells that were growth-stimulated for 5 h in the presence of cycloheximide. Figure 5 (top) shows that URH49 mRNA content increased 3.5-fold in WI38 and 6-fold in 3T3 cells that were serum stimulated in the presence or absence of cycloheximide. Unexpectedly, the same increases in URH49 mRNA content were also observed when quiescent cells were treated with cycloheximide alone. (In this experiment, the inhibitor was added directly to the medium on the quiescent cells to avoid any growth stimulation that would result from adding fresh medium to the cells.)

These analyses were also performed for UAP56 mRNA (Fig. 5, bottom). When quiescent cells were serum-stimulated in the presence of cycloheximide or treated with cycloheximide alone, the amount of UAP56 mRNA increased to a greater extent than when cells were treated with serum alone. In WI38 cells, the increases were comparable to those observed with URH49 mRNA. However, in 3T3 cells, the increases were much less than those observed with URH49 mRNA.

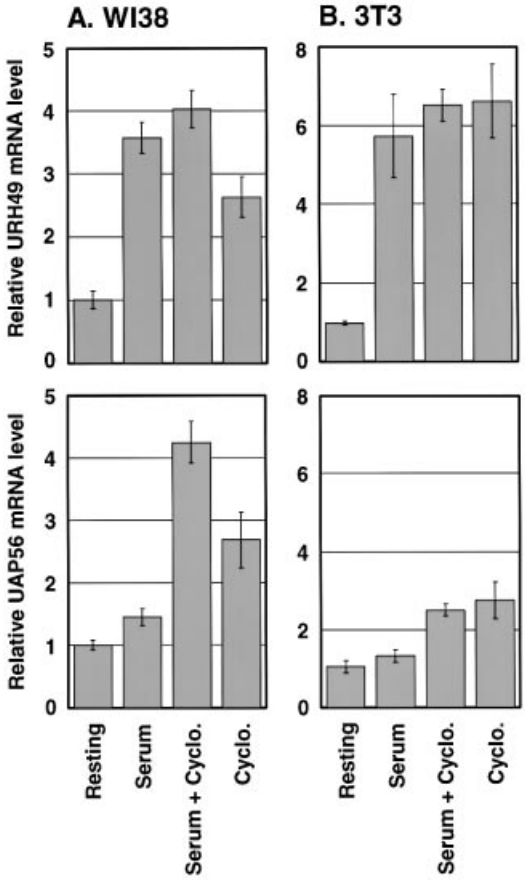

Figure 5. An increase in URH49 mRNA occurs in the absence of protein synthesis. The amount of URH49 and UAP56 mRNA was determined by real-time PCR 5 h after the addition of serum and/or cycloheximide to resting WI38 or 3T3 cells, and normalized to the amount of rpL4 mRNA. The corrected values were then normalized to the amount of URH49 mRNA in resting cells at 0 h, which was set at 1.0. The data are from at least two independent experiments. Standard deviations are indicated.

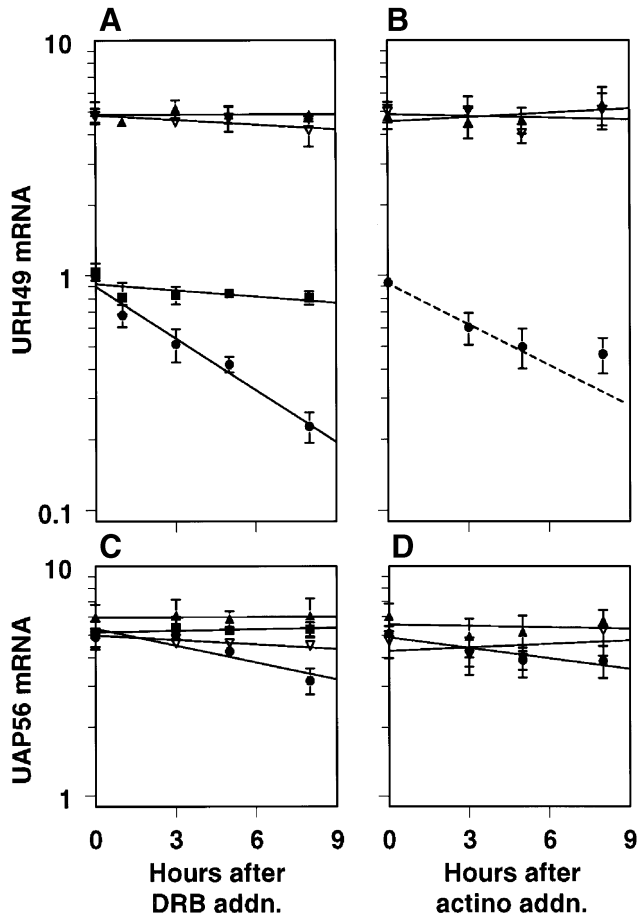

Figure 6. Stability of URH49 and UAP56 mRNA in 3T3 cells. The amount of URH49 and UAP56 mRNA was determined by real-time PCR at the indicated times following the addition of DRB or actinomycin D (actino), and normalized to the amount of rpL4 mRNA. The corrected values were then normalized to the amount of URH49 mRNA in resting cells at 0 h, which was set at 1.0. The following conditions were analyzed: resting cells (filled circles), resting cells treated with cycloheximide and DRB (filled squares), cells growth-stimulated for 5 h before the inhibition of transcription (filled triangles) and exponentially growing cells (open triangles). The dotted line represents a half-life of 5 h. The data are from at least two independent experiments. Standard deviations are indicated.

Stability of URH49 and UAP56 mRNA

It is well documented that inhibition of protein synthesis can decrease the rate of turnover of unstable mRNAs, thereby leading to an increase in mRNA content (43,44). The fact that the same increase in URH49 content was observed when cells were serum-stimulated as when they were treated with cycloheximide raised the possibility that URH49 mRNA turns over with a short half-life in quiescent cells, but is stabilized following growth induction.

To explore this possibility, we measured the half-life of the URH49 mRNA in quiescent, exponentially growing and growth-stimulated cells as well as in quiescent cells treated with cycloheximide. Transcriptional elongation was blocked using 80 μg/ml DRB (34) and the amount of URH49 mRNA was determined at various times thereafter by real-time PCR. To correct for differences in recovery, the values were normalized to the amount of rpL4 mRNA, which changed very little over the duration of the experiment. Figure 6A shows that URH49 mRNA turns over with a half-life of ~4 h in quiescent cells. However, the mRNA level was stable in exponentially growing cells or in cells that were growth-stimulated for 5 h before the addition of the transcriptional inhibitor. Simultaneous treatment of quiescent cells with DRB and cycloheximide also led to mRNA stabilization. Very similar observations were made when 5 μg/ml Actinomycin D was used as the transcriptional inhibitor (Fig. 6B). The content of UAP56 mRNA was also determined under the same conditions (Fig. 6C and D). We found that UAP56 mRNA had a half-life of ~15 h in quiescent 3T3 cells, but was stabilized following the addition of serum or cycloheximide. In addition, when these analyses of URH49 and UAP56 mRNA were performed on WI38 cells, similar results were obtained (data not shown).

DISCUSSION

URH49 is a DExD/H box protein that is 90% identical to UAP56, a presumed RNA helicase that is considered to be essential for the splicing and export of all mRNAs in all

eukaryotes. We show that URH49 and UAP56 are both able to interact with the mRNA export factor Aly. Furthermore, URH49 and UAP56 are both able to complement a *Sub2* deletion in yeast. Therefore, the two proteins are likely to have highly similar functions. The mRNAs for both helicases are expressed in a wide variety of mouse and human tissues and cell lines, although the relative expression of the two mRNAs varies considerably in different tissues. In cultured cells, the UAP56 mRNA is expressed at a relatively constant level while the URH49 mRNA level is closely correlated with cell proliferation. URH49 mRNA is present at low levels in quiescent cells in culture, but increases ~5-fold within a few hours following growth stimulation. The increased content of URH49 mRNA is due at least in part to the fact that URH49 mRNA has a relatively short (4 h) half-life in quiescent cells, but is stabilized immediately following growth stimulation.

There are several reasons why URH49 may not have been identified as an RNA splicing and export factor in earlier analyses. For example, Fleckner *et al.* (18) showed that UAP56 is essential for mRNA splicing in mammalian cells. However, in these analyses UAP56 function was inactivated using polyclonal antibodies that also would have inactivated URH49. In addition, a proteomic analysis did not identify URH49 as one of the constituents of human spliceosomes (10). However, URH49 may have been missed in this analysis due to the high degree of similarity between the two proteins (Robin Reed, personal communication). Inactivation of UAP56 function has been shown to result in nuclear retention of mRNA in *S.cerevisiae*, *C.elegans* and *Drosophila melanogaster* (25–27), which contain only a single protein corresponding to UAP56. However, this has not yet been demonstrated in mammalian cells. If UAP56 and URH49 carry out similar functions, it will be necessary to inactivate both helicases to observe this effect in mammalian cells.

The existence of two related helicases corresponding to UAP56 in mammalian cells raises the possibility that differential expression of the two helicases may be responsible for changes in mRNA production. Several possible scenarios can be imagined. For example, if UAP56 and URH49 have identical biological functions, increased expression of URH49 following growth stimulation may be important for the increased production of mRNA that occurs during the G_0 to G_1 transition. Alternatively, if the helicases have biological functions that do not completely overlap, changes in relative expression of the two helicases may lead to qualitative as well as quantitative changes in mRNA production. For example, URH49 may preferentially process or export mRNAs that are important in proliferating cells, but not in quiescent cells. We are currently exploring the biological functions of these two helicases by inactivating one or both helicases using RNA interference and determining the effects on mRNA production.

The high expression of URH49 mRNA in human and mouse testes suggests that URH49 may be particularly important in this tissue. This finding is not without precedent. The DEAD box RNA helicases p68, PL10, p68 and GRTH are also expressed at much higher levels in testes than in other tissues (45–47). GRTH expression has been shown to be upregulated by gonadotropin. It would be interesting to determine if URH49 is regulated in a similar manner. In addition to these DExD/H box helicases, two export factors, NXF2 and NXF3, are mainly expressed in the testes (48,49). These data indicate that a variety of mRNA export factors may have functions that are specific to the testes.

The rapid increase in URH49 mRNA that occurs following growth stimulation is due, at least in part, to an increase in the stability of the mRNA. URH49 mRNA is highly stable in cycling cells but has a half-life of ~4 h in quiescent cells. The mRNA is stabilized immediately after the quiescent cells are stimulated to proliferate and remains stable in proliferating cells. The turnover of URH49 mRNA in quiescent cells requires ongoing protein synthesis since the addition of cycloheximide to quiescent cells also leads to immediate stabilization of the mRNA. The mechanism responsible for the stabilization of URH49 and other mRNA species following the inhibition of protein synthesis is not known.

The rapid increase in URH49 mRNA is somewhat reminiscent of the behavior of a variety of immediate early mRNAs (e.g. c-*fos* mRNA) that increase rapidly following growth stimulation (44,50). In contrast to the situation observed with URH49 mRNA, the increase in the immediate early mRNAs is transient and regulated primarily at the transcriptional level. The half-lives of these early response mRNAs are generally much less than 1 h and do not change following growth stimulation. However, these mRNAs are stabilized when protein synthesis is blocked, similar to the situation observed with URH49 mRNA.

We are not aware of any other mRNAs that demonstrate the G_0-specific turnover that we have identified for URH49 mRNA. Earlier studies have shown that the decay rates for specific mRNAs can increase or decrease in response to a variety of stimuli. In several cases, the regulatory sequences and *trans*-acting factors that are responsible for regulating the turnover of these mRNAs have been identified (51–53). The mRNAs that have short half-lives frequently contain AU-rich elements in their 3′ untranslated region that serve as binding sites for proteins that either stabilize or destabilize the mRNA (54–57). However, there are no obvious AU-rich elements in the 3′ untranslated region of URH49 mRNA, so novel sequences and proteins may be involved. For this reason, we are currently attempting to identify the *cis*-acting sequences and *trans*-acting factors that are important for the unusual turnover characteristics of URH49 mRNA.

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