

# Evidence of Post-transcriptional Regulation of U6 Small Nuclear RNA\*

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**Mechanisms regulating the intracellular level of endogenous U6 small nuclear RNA were studied by transient transfection of ectopic U6 gene constructs into immortalized normal and malignant human cell lines. Transfection and expression of a modified U6 gene containing native promoter, capping, and termination sequences but lacking all highly conserved internal spliceosome sequences produced dose-dependent effects on endogenous U6 gene expression. At low transfection doses, no significant changes in endogenous U6 RNA levels or half-life were noted. However, as the dose of the transfected gene and its expression increased, native U6 RNA levels dramatically decreased in association with an apparent decrease in U6 RNA half-life. Down-regulation of native U6 RNA levels was transient, with recovery noted within 48–96 h in conjunction with declining expression of the ectopic gene. These modulatory effects appeared specific to endogenous U6 transcripts, because no changes were noted in 7sk, U1, U3, or 5S RNA levels or half-lives. Transfection with an unmodified U6 gene did not alter total U6 transcript levels but did produce a similar dose-dependent decrease in U6 RNA half-life. These studies suggest a hitherto unrecognized U6-specific intracellular regulatory mechanism, through which over-accumulation of U6 small nuclear RNA is prevented.**

U6 small nuclear RNA (snRNA)<sup>1</sup> is a highly conserved, abundantly transcribed, stable RNA species that plays a crucial role in the processing of premature mRNA within the spliceosome (1–4). Although the precise function of U6 within the spliceosome has not been clearly defined, evidence is mounting that it may be involved in the catalytic active site (5–7), cleaving at exon/intron junctions. U6 is unique from the other four major U-rich spliceosomal RNAs (U1, U2, U4, and U5) in that it is transcribed by RNA polymerase III rather than RNA polymerase II (8, 9), it obtains a  $\gamma$ -monomethylphosphate 5' cap rather than a trimethylguanosine 5' cap (10), and it has not been shown to require a cytoplasmic phase as part of its maturation. In addition, whereas other snRNA genes contain proximal sequence elements and distal sequence enhancers within their promoters, only U6 contains an essential TATA box that mediates polymerase specificity and defines the initiating nucleotide (11, 12).

The U6 snRNA gene not only has features distinct from other

snRNA genes but also has features distinct from other class III genes. Unlike the vast majority of class III genes (e.g. 5S, tRNA, EBER, adenovirus VA RNA), which have essential *cis*-acting promoter elements located intragenically (13), the U6 promoter lies entirely upstream from the initiating nucleotide (14, 15). Thus, the promoter of U6 defines an exact start site and will initiate and elongate in a sequence-independent fashion until it reaches the class III termination signal, a string of 4–6 thymine residues. These unique features of the U6 gene allow it to be exploited for intracellular RNA delivery as part of ribozyme-, antisense-, antigene-, or aptamer-encoding gene therapy strategies (16). Other U-rich snRNA genes are being investigated for similar purposes.

Given the potential use of U6 and other snRNA genes as gene-targeting RNA expression vectors, as well as the emergence of data implicating U6 as a key element within the spliceosome, more information is needed on regulatory mechanisms controlling the intracellular expression of various snRNA genes. Despite their diversity in polymerase specificity, promoter structure, and transcription factor requirements, snRNAs are maintained in relatively fixed proportions to one another intracellularly. The regulatory mechanisms that underlie snRNA homeostasis are still largely unknown. Although evidence for snRNA gene regulation has been reported during embryogenesis (17) and following partial hepatectomy (18), few studies have addressed more general mechanisms accounting for snRNA homeostasis (3, 19). To address this need, our studies focused on the following questions relating to U6 snRNA expression: (a) Do intracellular regulatory mechanisms exist that respond to altered U6 transcript levels?; (b) Are U6 regulatory mechanisms independent of or linked to expression of other snRNA genes? and (c) Are such mechanisms invoked by changes in transcript levels, rates of U6 snRNA synthesis, or U6 promoter activity?

By transiently transfecting U6 gene constructs into human cell lines, we provide evidence of a regulatory mechanism that acts to prevent U6 RNA overexpression, similar to that previously described for U1 RNA (19). This mechanism appears to act primarily through a tightly regulated post-transcriptional enhancement of U6 degradation and be relatively specific for U6 RNA. These results suggest that the use of the U6 gene for gene-targeting strategies may not affect the levels of other snRNA but may lead to transient dose-dependent down-regulation of endogenous U6 snRNA.

## MATERIALS AND METHODS

**U6 Expression Vectors**—Plasmids containing the normal human U6 gene or a mutant sequence with bases +25 to +55 replaced by an *Xho*I restriction site were generously provided by G. Kunkel and T. Pederson (14, 20). The mutant U6 sequence was modified further by site-directed mutagenesis at bases +86 and +88 to create a unique *Nsi*I restriction site, as described previously (16). The internal *Xho*I/*Nsi*I sequence of this mutant U6 gene was then replaced by a synthetic 38-base pair duplex fragment having no homology to the native U6. The resulting gene, referred to as U6ON, encodes an 82-nucleotide hybrid transcript,

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<sup>1</sup> The abbreviation used is: snRNA, small nuclear RNA.

sharing only the first 25 and the last 19 nucleotides with native U6. Both U6 and U6ON expression constructs were cloned within pGEM1 plasmids (Promega, Madison, WI).

**Cell Culture and Gene Transfection**—The human embryonic kidney cell line, 293, and the human breast cancer cell line, MDA-453 (ATCC, Rockville, MD), were transfected by electroporation (Bio-Rad) with 5–40  $\mu$ g of U6ON, U6, or promoterless plasmid control DNA. Cell viability after transfection, ectopic gene expression, and native U6 levels were comparable between the two cell lines. 293 cells were cultured in minimal essential medium with Earle's basic salt solution and 10% fetal calf serum supplemented with 100 units/ml penicillin and streptomycin in 5% CO<sub>2</sub> incubators. MDA-453 cells were cultured in Leibovitz L-15 medium containing 10% fetal calf serum and supplemented with 100 units/ml penicillin and streptomycin in the absence of CO<sub>2</sub>.

**RNA Isolation, Northern Blotting, and snRNA Quantitation**—Total cellular RNA was isolated 48, 72, 96, or 120 h post-transfection by 4 M guanidinium isothiocyanate extraction at pH 5.5 followed by cesium chloride ultracentrifugation (21). Equal amounts of RNA/lane were electrophoresed in 8% polyacrylamide/7 M urea gels, electroblotted onto nylon filters, and UV cross-linked for 2 min as described previously (16). Probes to detect native U6 or U6ON RNA were made by random priming of an 800-base pair *Bam*HI/*Eco*RI fragment taken from the U6 or U6ON plasmid, respectively. Probes to detect 7sk and 5S RNA were also made by random priming from plasmids generously provided by R. Reddy. After hybridization and autoradiography, snRNA levels were quantitated by scanning densitometry.

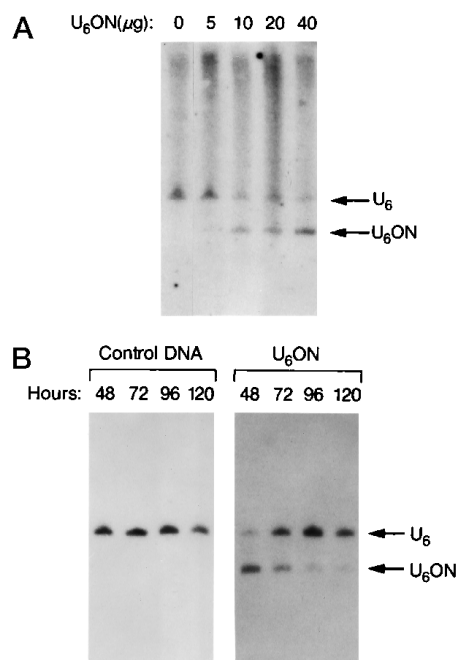
**Transcription Arrest and snRNA Half-life Assessment**—Intracellular half-lives of snRNAs were measured after halting total cellular transcription with 10  $\mu$ g/ml actinomycin D (Sigma) administered to culture medium 48 h after transfection with 5–40  $\mu$ g of the U6 or U6ON gene. At 0, 0.5, 1, 2, 4, and 8 h after actinomycin D treatment, cells were washed twice with phosphate-buffered saline, and total cell RNA was extracted with guanidinium isothiocyanate. Northern blotting was performed as described above to quantitate U6, U6ON, and other snRNA transcript levels. Half-life values were estimated assuming a first-order model of RNA degradation.

## RESULTS

The feasibility of using specific regulatory elements from the U6 gene to create a chimeric gene capable of abundant nuclear expression of sequence-specific RNA has previously been reported (16). This study uses one such chimeric gene, U6ON, along with an unmodified U6 gene to explore regulation of endogenous U6 gene expression.

Fig. 1 illustrates the dose-dependent (Fig. 1A) and post-transfection time-dependent (Fig. 1B) down-regulation of endogenous U6 snRNA with increasing expression of U6ON RNA. At low U6ON transfection doses (5  $\mu$ g/10<sup>7</sup> cells), U6 snRNA levels are near their normal steady-state levels, and U6ON RNA is only modestly expressed. However, as U6ON transfection doses increase to 20–40  $\mu$ g/10<sup>7</sup> cells, U6 snRNA levels drop markedly in association with a sharp rise in U6ON expression. Because both the U6ON and the U6 probes were radiolabeled to equal specific activity, it is notable that the U6ON RNA band density at 40- $\mu$ g transfection dose is similar to the U6 band density at 0- $\mu$ g transfection dose and that summing the U6 and U6ON bands at intermediate doses gives rise to nearly constant total levels of U6-promoted transcripts. Transfection doses greater than 40  $\mu$ g/10<sup>7</sup> cells were not analyzed because they resulted in unacceptable levels of cell death.

We have previously demonstrated the kinetic profile of U6ON expression to be a rapid rise to steady-state within the first 12 h post-transfection, maintenance of steady-state levels 12–48 h post-transfection, and a slow decline in U6ON RNA levels 48–168 h post-transfection (16). In clear correspondence with this profile, U6 snRNA down-regulation is observed both at 24 and 48 h post-transfection, with recovery occurring as the level of U6ON expression decreases 48–120 h post-transfection. Fig. 1B illustrates these time-dependent complimentary changes in U6 and U6ON RNA levels following transient transfection of 293 cells with 20  $\mu$ g of U6ON. No such changes in U6



**FIG. 1. Evidence of dose-dependent and time-dependent U6 down-regulation in the presence of U6ON.** A, 293 cells were transfected with increasing doses of the U6ON gene, and RNA was isolated 48 h post-transfection. B, 293 cells were transfected with a 20- $\mu$ g dose of the U6ON gene or control DNA, and RNA was isolated after increasing time periods. In both Northern blots, U6 RNA and U6ON RNA were probed independently.

snRNA levels are seen when cells were transfected with promoterless plasmid control DNA. As in Fig. 1A, the sum of the U6 and U6ON band intensities at each time point results in nearly constant levels of total U6-promoted transcripts.

To test whether these complimentary changes in U6 snRNA levels were specific to U6 or indicative of a more general effect on RNA metabolism induced by U6ON expression, levels of other class II and class III snRNA were monitored in the presence of U6ON. Fig. 2 demonstrates the lack of any significant change in U1, U3, 7sk, or 5S RNA levels resulting from increasing expression of U6ON. In addition, as U6ON RNA levels decline 48–120 h after transfection, no compensatory changes are noted in 7sk or U1, snRNAs arising from genes with promoter structures very similar to U6. To search for interruption of spliceosomal function or general alterations in RNA metabolism, levels of glyceraldehyde-3-phosphate dehydrogenase and *c-myc* mRNA were assessed by Northern blotting of agarose gels. No significant changes in glyceraldehyde-3-phosphate dehydrogenase or *c-myc* mRNA levels were noted following U6ON transfection, nor was there any detectable evidence of dysregulated mRNA splicing in the form of premature mRNA species of lower mobility. As well, co-transfection of a  $\beta$ -HCG expression vector showed no decline in  $\beta$ -HCG protein expression in the presence of U6ON (22). Thus, based on these transient transfection assays, the regulatory effects of high level U6ON expression appear specific to the U6 gene.

Despite the lack of dose-dependent or time-dependent effects of U6ON expression on 7sk, a gene that shares the same polymerase specificity, 5' cap, general promoter structure, and subcellular localization with U6, we explored further the possibility that U6 down-regulation results from transcription factor quenching by U6ON. Fig. 3 illustrates the results of an experiment in which MDA-453 cells were transfected with 40  $\mu$ g of linearized U6ON plasmid, closed circular U6ON plasmid, truncated plasmid (wherein the promoter and first half of the gene is retained and the termination sequence removed), or

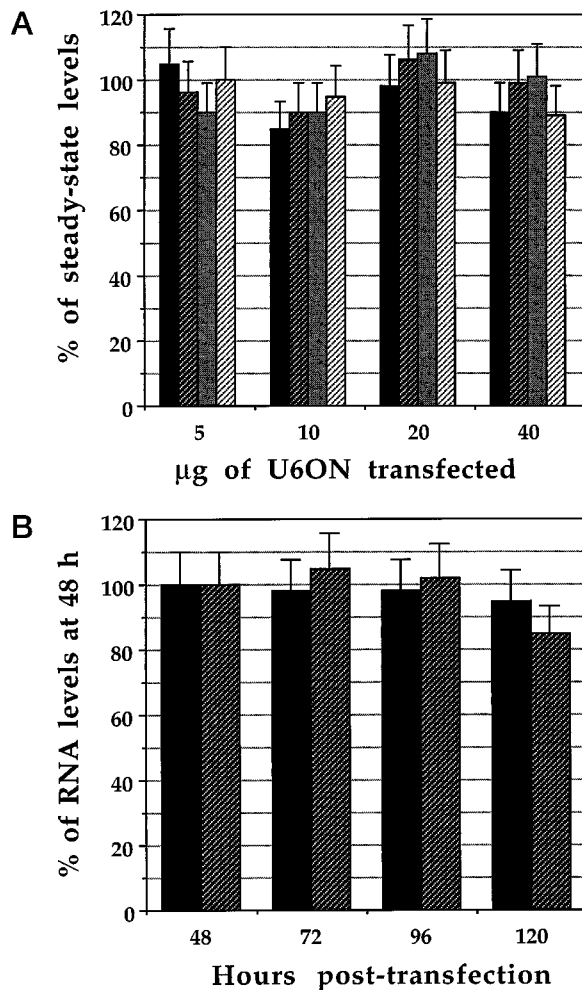


FIG. 2. **RNA down-regulation is specific to U6.** 293 cells were treated as described in the legend to Fig. 1 but probed for levels of other snRNA. Scanning densitometry was performed on the resulting Northern blots, and values were plotted as a percentage of either the corresponding RNA level at 0 µg of U6ON transfection (A) or the corresponding RNA level at 48 h post-transfection (B). A, black bars, 7sk RNA; black bars with white hatching, U3 RNA; shaded bars, U1 RNA; white bars with black hatching, 5S RNA. B, black bars, 7sk RNA; hatched bars, U1 RNA.

promoterless plasmid control DNA. The first three ectopic U6 genes have identical promoter constructs but differ in their inherent capacity for U6ON expression (closed circular > linear >> truncated). Therefore, if the large number of exogenously introduced U6ON promoters were merely binding and saturating requisite transcription factors and preventing them from binding to native chromosomal U6 promoters, then each of these ectopic U6 genes should equally down-regulate endogenous U6 snRNA levels relative to control DNA. As shown in Fig. 3, this phenomenon did not occur. U6 snRNA down-regulation was most apparent in the lane marked *U<sub>6</sub>ON*, where U6ON expression was highest. (Note that the truncated transcript was too small to be pelleted by GITC extraction/cesium chloride centrifugation (23)). Thus, U6 down-regulation appears to be more a function of the intracellular levels of U6-promoted transcripts than a function of the intracellular levels of U6 promoters.

Given that U6 snRNA has been shown to be a highly stable transcript with a half-life value estimated at ~24 h (24, 25), it is consistent that the near total down-regulation of U6 (seen in Fig. 1A, lane 5, and Fig. 3, lane 2) could not be explained solely by a decrease in the initiation rate of endogenous U6. Even in the extreme case of complete cessation of U6 initiation follow-

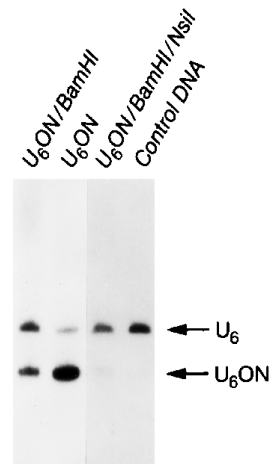
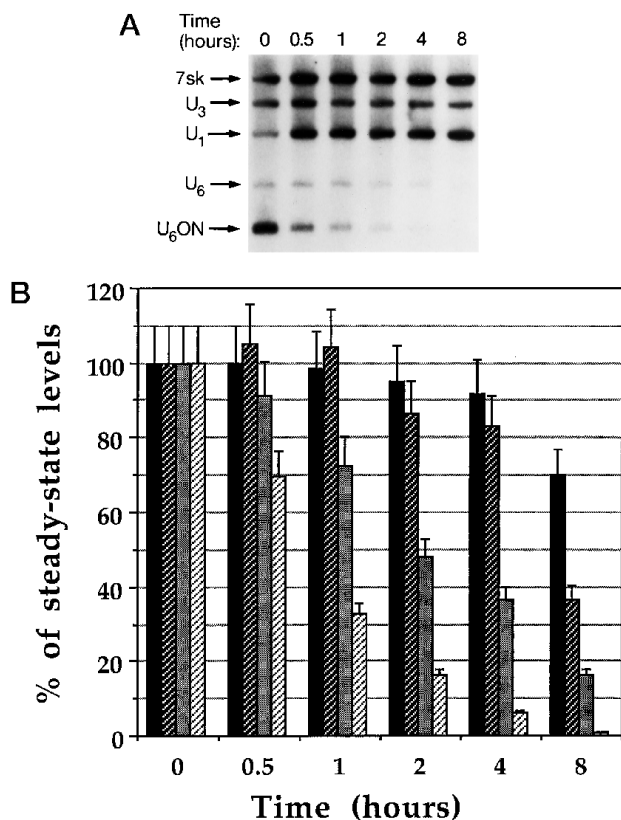


FIG. 3. **U6 RNA down-regulation is dependent upon U6 promoter activity.** MDA-453 cells were transfected with 40 µg of U6ON or control DNA. In the *U<sub>6</sub>ON/BamHI* lane, the DNA was linearized with a *Bam*HI digestion; in the *U<sub>6</sub>ON* lane, the DNA was closed circular; in the *U<sub>6</sub>ON/BamHI/NsiI* lane, the U6ON gene was truncated with a *Bam*HI/*Nsi*I digestion. In the *Control DNA* lane, the DNA was closed circular promoterless plasmid control DNA. For each cell sample, RNA was isolated at 48 h post-transfection.

ing U6ON transfection, 25% of U6 snRNA would be expected 48 h post-transfection. To test an alternate hypothesis that U6 down-regulation is being mediated primarily by a decrease in transcript stability rather than a decrease in transcript production, U6 snRNA half-life was measured in the presence of increasing levels of U6ON expression.

In Fig. 4A, 293 cells were transfected with 20 µg of U6ON and then treated with actinomycin D at 48 h to halt total cellular transcription. The steady-state level of various snRNAs is shown at 0 h, and at subsequent time points the intensity of each snRNA band declines in accordance with its individual intracellular rate of degradation. At the onset of actinomycin D exposure (0 h), consistent with results shown in Figs. 1, 2, and 3, U6 snRNA levels are reduced (relative to other snRNAs) in the presence of high U6ON RNA expression. For 7sk, U1, and U3 snRNAs, stable and highly abundant transcripts with half-lives comparable with that of U6 snRNA (3, 24), there is no apparent reduction in stability resulting from U6ON expression. In contrast, U6 stability is markedly reduced, with scanning densitometry suggesting an 8-fold reduction in intracellular half-life (from ~24 to ~3 h). As expected, U6ON, missing all known protein binding sites (aside from the La protein) and capacity for 3' hybridization with U4, demonstrates a half-life of only ~1 h. Fig. 4B demonstrates that this decrease in U6 snRNA half-life can be titrated back toward higher values by decreasing the dose of transfected U6ON. The near normal levels of U6 snRNA after a 5-µg U6ON transfection dose (Fig. 1A) and after 96 h post-transfection (Fig. 1B) provide internal consistency to these results. It is interesting to note that although the half-life of U6 appears highly dependent upon levels of U6ON, the half-life of U6ON appears independent of its own level expression as well as of total U6 snRNA levels. U6ON half-life after a 5-µg transfection is nearly identical to that following a 20-µg transfection.

Because these observations suggest that U6 transcript levels are at least in part regulated by changes in U6 snRNA degradation, a similar set of transfection experiments was performed using an unmodified ectopic U6 gene to explore further the relationship between U6 snRNA levels and U6 snRNA half-life. Analogous to experiments transfecting the U6ON gene (Fig. 1A), cells were transfected with increasing doses of the unmodified U6 gene, and Northern blotting was performed to measure



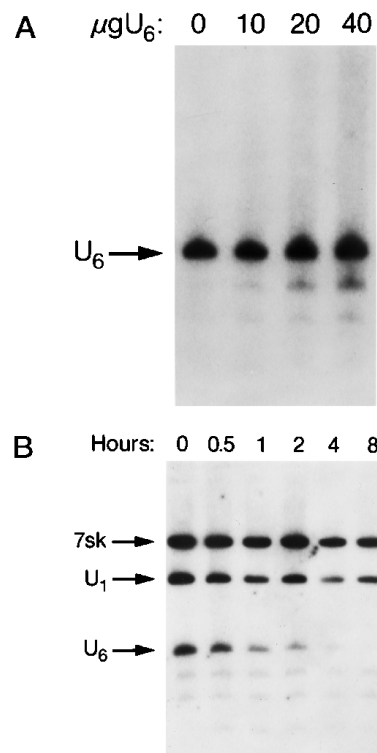
**FIG. 4. U6 RNA is destabilized by U6ON.** 293 cells were treated with 20  $\mu$ g (A) or increasing levels (B) of the U6ON gene, and after 48 h post-transfection, cells were treated with 10  $\mu$ g/ml of actinomycin D to halt total cellular transcription. At given time points after actinomycin D administration, RNA was isolated. In B, data from Northern blots were quantitated by scanning densitometry, and the values were plotted as a function of RNA levels at 0 h. Black bars, U6 (5  $\mu$ g of U6ON); black bars with white hatching, U6 (10  $\mu$ g of U6ON); shaded bars, U6 (20  $\mu$ g of U6ON); white bars with black hatching, U6ON (20  $\mu$ g of U6ON).

total U6 RNA levels. As shown in Fig. 5A, transfections of up to 40  $\mu$ g of the U6 gene failed to significantly alter the total cellular level of U6 RNA. Furthermore, with increasing U6 transfection dosage a progressive increase in U6 RNA degradative products (bands appearing below the full-length U6 RNA band) was clearly detectable. Using 5'- and 3'-specific labeled antisense oligonucleotide probes, we attempted to determine whether this degradation was occurring predominantly from the 5' or 3' end of the U6 RNA; however, these results were inconclusive, suggesting that U6 RNA degradation was not occurring by unidirectional exonuclease activity (data not shown).

Additional actinomycin D transcription arrest experiments were performed to detect alterations in U6 RNA stability associated with transfection of the unmodified U6 gene. As shown in Fig. 5B, at the highest gene transfection dose (40  $\mu$ g/ $10^7$  cells) U6 RNA half-life was found to be only 1–2 h, corresponding to a >10-fold decrease in U6 RNA stability from normal values. As in previous experiments, U1 and 7sk RNA retain their stability. Interestingly, in Fig. 4A U6 is down-regulated at time 0 and degrades rapidly from this level, whereas in Fig. 5B, it degrades rapidly from steady-state levels at time 0. If a first-order model of RNA degradation is assumed, then these differences in initial U6 RNA levels do not affect half-life determinations.

#### DISCUSSION

This study attempted to identify mechanisms regulating intracellular U6 snRNA. Our results indicate that U6, much like



**FIG. 5. U6 RNA stability but not total RNA levels are down-regulated by ectopic U6.** In A MDA-453 cells were treated with increasing doses of the unmodified ectopic U6 gene. RNA was isolated after 48 h post-transfection. In B MDA-453 cells were treated with 40  $\mu$ g of the ectopic U6 gene and subjected to actinomycin D treatment after 48 h, as described for Fig. 4.

U1 (19), is capable of compensatory regulation of its snRNA product using a servo-mechanism triggered by supranormal U6 promoter activity and accumulation of U6-promoted RNA. Contrary to initial expectations, the most profound cellular response to introducing increasing doses of an active U6 promoter is not a decrease in endogenous U6 transcript production but rather an increase in endogenous U6 transcript degradation. This response does not reflect general cellular injury, metabolic interferences, or ribonuclease saturation but instead represents a specific mechanism regulating total U6 RNA that does not substantially affect other spliceosomal or nonspliceosomal snRNAs.

Cells with considerably reduced levels of U6 RNA (and increased levels of U6ON RNA) 48 h post-transfection were phenotypically indistinguishable from cells with normal levels of U6 RNA. Over this brief time span, none of the transfected cell populations showed any morphological or biochemical evidence of toxicity or abnormal splicing of either glyceraldehyde-3-phosphate dehydrogenase or *c-myc* mRNA. In preliminary experiments to examine the long term effects of U6ON expression and U6 RNA down-regulation, we stably transfected the U6ON gene into MDA-453 cells under the selective pressure of G418 (data not shown). The majority of G418-resistant clones did not sustain cell division sufficient for RNA isolation. However, of the cell colonies that survived and amplified, U6 RNA levels were normal with only very low levels of detectable U6ON expression. No colonies with U6ON overexpression or U6 down-regulation could be isolated. These preliminary findings suggest that although transient U6 down-regulation may be compatible with survival, the cell may not be able to continue to function normally with U6 RNA at subnormal levels and/or subnormal stability.

Given the important function of U6 RNA within the spliceo-

some, it is reasonable that cells have evolved a servo-mechanism based on U6 RNA degradation to prevent an excess accumulation of this critical snRNA species relative to others in the spliceosome complex. Based on the normal half-life of U6 RNA (~24 h), it would be difficult to down-regulate cellular levels simply by decreasing the rate of U6 RNA. For example, even with complete halting of endogenous U6 transcript initiation, at least 25% of normal steady-state U6 RNA would still be present after 48 h. In contrast, by reducing U6 RNA stability, intracellular U6 RNA levels can be modulated with a more rapid feedback response. Additional study, however, is necessary to explore the possibility that unobserved regulatory pathways exist that modulate the rate of U6 RNA initiation.

The molecular mechanisms mediating the rates of U6 RNA degradation are still unclear. U6 RNA stability is thought to be dependent upon its 5' cap, 3' U-rich tail, and capacity for self and/or U4 hybridization (25, 26). One hypothesis that could only partially explain our observations is saturation of the U6 capping enzyme and/or substrates, resulting in a population of uncapped and unstable U6 RNA. If such an enzyme or substrate were in limiting supply then the presence of increasing levels of U6ON RNA, which is at least partially capped (16), would likely compete with endogenous U6 RNA capping in a dose-dependent fashion. Three facts support the hypothesis that U6ON enhances U6 RNA degradation by saturating the U6 capping mechanism. Firstly, the observed 8-fold reduction in U6 half-life in the presence of U6ON corresponds perfectly to the half-life value obtained when uncapped U6 RNA are micro-injected into oocytes (25, 26). Secondly, unlike capping of mRNA and other U-rich snRNA, the capping of U6 RNA is not coupled to U6 transcription (27). Thirdly, endogenous U6 capping activity has been found to be a saturable enzymatic mechanism (28). Inconsistencies with this hypothesis include the lack of destabilization of 7sk RNA (which acquires the same  $\gamma$ -monomethylphosphate cap (29) and shares at least one essential component with the U6 capping mechanism (28)) and the first-order (as opposed to biphasic) destabilization of U6 RNA.

A second mechanistic hypothesis invokes another saturable, stabilizing protein factor that associates with U6 RNA within its first 25 or its last 19 nucleotides (those common to both U6 and U6ON). Whereas the La protein (30), which transiently binds the U-rich 3' end of U6 (and presumably U6ON), fits this description, the lack of any U6ON effect on 5S or 7sk transcript levels or stability make it an unlikely candidate. The decreased stability of U6 RNA in the presence of a large transfection dose of the unmodified ectopic U6 gene could have arisen from a saturation of available U4 RNA for hybridization; however, the U6ON gene has the internal portion of U6 replaced with a sequence that is incompatible with U4 hybridization, and, therefore, this explanation cannot fully account for the reduction in U6 RNA stability. Additional studies are needed to elucidate the mechanism of destabilization.

This report demonstrates the utility of modified snRNA gene constructs in exploring cellular mechanisms regulating endogenous snRNA homeostasis. It also provides additional information on potential limitations to the use of snRNA promoters to

deliver high levels of short sequence-specific RNA for gene regulation purposes. In our initial report (16), we overestimated the number of copies/cell of RNA that could be delivered by such a system because we failed to recognize the extent to which normal U6 was down-regulated in the presence of U6ON. We now recognize that as U6ON RNA levels increase, U6 RNA levels fall to keep the total number of U6-promoted transcripts relatively constant, as has also been seen with U1 (19). The existence of such cellular servo-mechanisms may present serious limitations to the use of snRNA promoters for long term gene therapy strategies by resulting in cellular toxicity that is unintended or not due to the sequence-specific snRNA insert. However, the lack of toxicity under short term experimental conditions indicates that these gene expression systems may be effective for transient intracellular production of ribozymes or antisense or triplex-forming RNA, which could serve as useful research tools for investigating gene function and regulation.

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