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Effects of Nonsense Mutations on Nuclear and Cytoplasmic Adenine Phosphoribosyltransferase RNA

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We have analyzed Chinese hamster ovary (CHO) cell mutants bearing nonsense codons in four of the five exons of the adenine phosphoribosyltransferase (*aprt*) gene and have found a pattern of mRNA reduction similar to that seen in systems studied previously: a decrease in steady-state mRNA levels of 5- to 10-fold for mutations in exons 1, 2, and 4 but little effect for mutations in the 3′-most exon (exon 5). Nuclear *aprt* mRNA levels showed a similar decrease. Nonsense-containing *aprt* mRNA decayed at the same rate as wild-type mRNA in these cell lines after inhibition of transcription with actinomycin D. Nonsense-containing *aprt* mRNA is associated with polysomes, ruling out a model in which stable residual mRNA escapes degradation by avoiding translation initiation. A tetracycline-responsive form of the *aprt* gene was used to compare the stability of nonsense-containing and wild-type *aprt* mRNAs without globally inhibiting transcription. In contrast to measurements made in the presence of actinomycin D, after inhibition of *aprt* transcription with tetracycline, a nonsense-mediated destabilization of *aprt* mRNA was indeed demonstrable. The increased rate of decay of cytoplasmic *aprt* mRNA seen here could account for the nonsense-mediated reduction in steady-state levels of *aprt* mRNA. However, the low levels of nonsense-bearing *aprt* mRNA in the nucleus suggest a sensibility of mRNA to translation or translatability before it exits that compartment. Quantitation of the steady-state levels of transcripts containing introns revealed no accumulation of partially spliced *aprt* RNA and hence no indication of nonsense-mediated aberrancies in splicing. Our results are consistent with a model in which translation facilitates the export of mRNA through a nuclear pore. However, the mechanism of this intriguing nucleocytoplasmic communication remains to be determined.

Mutations that introduce premature translation termination codons into the protein-coding region of genes result more often than not in decreased steady-state levels of the corresponding mRNA. This nonsense mutation-mediated mRNA reduction has been found wherever sought among living organisms, in bacteria (47), yeasts (37), plants (60), and humans (17, 58). This phenomenon in yeasts has been termed nonsense-mediated mRNA decay, because it is clear that the stability of mature mRNA is affected (28, 35). Extensive site-directed mutagenesis studies of yeasts have identified sequences within the mRNA that are necessary to trigger accelerated mRNA decay (28, 48, 63, 64). The isolation of mutants resistant to general nonsense-mediated mRNA reduction has led to the identification of several genes required for this phenomenon: *upf* genes in *Saccharomyces cerevisiae* (16, 35) and *smg* genes in *Caenorhabditis elegans* (50). Genetic analysis of yeast cells has also suggested that the process of nonsense-mediated mRNA degradation differs in at least some steps from that of normal mRNA degradation (45). However, the molecular mechanism involved in this cytoplasmic decay has yet to be fully elucidated.

In mammalian cells, nonsense-mediated mRNA reduction appears to be a more complex process. In particular, nuclear rather than cytoplasmic RNA appears to be the target, raising the intriguing question of how the translatability of an mRNA can be sensed in the nucleus. The evidence for such nuclear involvement rests on three types of observations. First, decay rates of nonsense-containing mRNA measured in total or cytoplasmic RNA have shown no differences from those of wild-type mRNA; this result has been seen in several mammalian systems studied: human β-globin (3, 58), hamster dihydrofolate reductase (*dhfr*) (59), human triose-phosphate isomerase (*tpi*) (14), and murine T-cell receptor (*tcr*) (cited in reference 9b). Second, direct measurements of mRNA levels in the nuclear fraction of disrupted cells have shown reductions that mirrored those seen in total or cytoplasmic preparations (globin [3, 58], *tpi* [6, 14]). Third, in some systems, nonsense mutations are associated with aberrancies in a nuclear process: either pre-mRNA splicing is reduced (39, 46, 51) or the affected exon is skipped (20, 21, 26, 43). Moreover, in some cases, mRNA reduction has been shown to require the presence of intron sequences (*tpi* [13] or *tcr* [9a]).

Several years ago, in an effort to explain the pattern of reduced mRNA levels among more than 30 premature translation termination mutants and their revertants at the *dhfr* locus in Chinese hamster ovary (CHO) cells, we proposed two alternative models (59). The first model, called translational translocation, invoked translation as a process that could facilitate the transport of an RNA molecule from the nucleus through a nuclear pore into the cytoplasm. When a premature stop codon was encountered, the mRNA would stall in transit and the nuclear portion of the RNA molecule would be degraded by nuclear nucleases or be subject to aberrant splicing. In favor of this model was the sharp polarity seen for this effect within the mRNA: little or no reduction in mRNA levels was seen if the nonsense codons were located within about the distal 25% of the reading frame. Thus, as molecules in transit from the nucleus passed a point of no return, they would exit the nucleus with no further translation required. Also consistent with this idea was electron-microscopic evidence showing that mRNA is oriented as it exits the nucleus, with its 5′ end

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emerging first, and that mRNA can associate with polysomes immediately at the cytoplasmic side of the nuclear pore (42). In an effort to explain the severe polar effect of nonsense codons, a version of this model was elaborated to connect splicing to the translation process; the removal of the last intron would represent the point of no return mentioned above. However, evidence has accumulated against translation-coupled splicing. The splicing model predicts that the 3′-most intron should be the last to be spliced (for a nonsense mutation in the upstream exon to have an effect on splicing), and we have shown that this is not the case for two genes studied (32). The translational translocation model without the involvement of splicing remains viable for these cases. Furthermore, for the minute virus of mice and the immunoglobulin gene, in which a decreased splicing efficiency is found, splicing of an intron can be inhibited by a nonsense mutation located in a downstream exon, ruling out any direct connection to orthodox translation (39, 46).

In the second model, called nuclear scanning, internal exons would be screened for translatability before they are recruited for splicing. If an open reading frame was found to be compromised by a premature translational stop codon, splicing would be curtailed. This model has the appeal of helping to explain how true exons are distinguished from counterfeits that are bounded by good consensus splice site sequences; an open reading frame would be added to the constraints of the exon definition model (53). Against this model is the difficulty in seeing how a reading frame could be recognized in an exon that is yet to be spliced. Also, for *tpi*, in experiments explicitly designed to detect splicing aberrancies, none were found (14).

In view of the inadequacy of either model to explain all of the facts surrounding this phenomenon, we have extended our analysis to another gene to test several new notions about nonsense-mediated mRNA reduction in mammalian cells. The adenine phosphoribosyltransferase (*aprt*) gene is attractive for this purpose for two reasons. First, because of the facility in selecting deficient mutants, revertants, and transfectants by using drug-resistant phenotypes, this locus has been the subject of extensive mutational studies, especially in CHO cells (18, 19, 22, 49). As a result, numerous nonsense mutants have been described and a double-deletion mutant is available as a recipient for transfections (9). Second, although the *aprt* gene is typical in containing multiple introns (four introns), it is relatively small, with a primary transcript of only 2.3 kb. Thus, it is possible to detect partially spliced intermediates as well as the primary transcript by reverse transcription (RT) coupled to PCR (32).

We have analyzed CHO cell mutants bearing nonsense codons in four of the five exons of the *aprt* gene and have found that the pattern of mRNA reduction follows that seen in systems studied previously: an mRNA reduction of 5- to 10-fold for mutations in exons 1, 2, and 4 but little effect for mutations in the 3′-most exon (exon 5). Nuclear *aprt* mRNA levels showed a similar decrease. Nonsense-containing *aprt* mRNA was found to be associated with polysomes, ruling out a model in which stable residual mRNA escapes degradation by avoiding translation. A tetracycline-repressible form of the *aprt* gene was used to compare the stability of nonsense-containing and wild-type *aprt* mRNAs without globally inhibiting transcription. In this system and in contrast to measurements made in the presence of actinomycin D, a nonsense-mediated destabilization of *aprt* mRNA was indeed demonstrable. Finally, an analysis of the steady-state levels of intron-containing transcripts revealed no accumulation of partially spliced *aprt* RNA and thus no indication of nonsense-mediated splicing aberrancies.

MATERIALS AND METHODS

Cell culture. To study the effect of nonsense mutation in the *aprt* gene, we obtained from Mark Meuth, University of Utah, a set of CHO cell lines (S1, S20, S23, S62, S69, XA50, and XA52) carrying nonsense mutations in either exon 1, 2, 4, or 5 of the *aprt* gene (44, 49). As a wild-type standard, we used the parental hemizygous cell line CHO D422, which bears a single copy of the *aprt* gene (9). As a recipient for transfection experiments, we used the *aprt* deletion mutant U1S (9). CHO cells were grown as described previously (59).

Plasmid constructions. Plasmid pH2 contains the CHO genomic *aprt* gene (38). pSP72D was made by deleting an *Xho*I-*Acc*I fragment from the multiple-cloning site of the cloning vector pSP72 (Promega) to eliminate some restriction sites for further cloning. For the purpose of further manipulation, the genomic *aprt* gene was transferred to the vector pSP72D. pWTaprt was constructed by cloning a 2,737-bp *Bam*HI-*Xba*I fragment from pH2 into the *Xba*I-*Bgl*II sites of pSP72D. pS20aprt is a similar construct but contains the nonsense mutation of cell line S20 in exon 4 (49). Total RNA from S20 cells was amplified by reverse transcription followed by PCR (RT-PCR) to produce a fragment representing a region from intron 3 to exon 5 (32). A 478-bp *Pst*I-*Bst*XI fragment from the PCR product was cloned into pWTaprt between the *Pst*I and *Bst*XI sites. The S20 nonsense mutation destroys a *Bal*I restriction site; therefore, we confirmed the existence of the mutation by using that restriction enzyme. To construct *aprt* genes under the control of a modulatable promoter, we used the tetracycline-responsive system described by Gossen and Bujard (27). pTETaprtWT and pTETaprtS20 contain the natural or the mutant *aprt* gene, respectively, driven by a chimeric promoter consisting of basal elements of the cytomegalovirus early promoter preceded by operator sequences of the tetracycline resistance (Tet^R) gene (27). The wild-type and the mutant *aprt* genes were PCR amplified from pWTaprt and pS20aprt, respectively, using a 5′ primer consisting of a sequence from the 5′ untranslated region modified to produce a *Sac*II restriction site (5′ *Sac*II [see below]) and a 3′ primer from a region downstream of the *aprt* polyadenylation site that contains a natural *Xba*I site. The amplified product starts 12 bp before the ATG codon. The *Sac*II-*Xba*I fragment from the PCR product was cloned into the same sites in the tetracycline-responsive cloning vector pUHD-10 (27).

Transfection. The Tet^R transactivator plasmid pUHD15-1 (27) (10 μg) was cotransfected with 1 μg of pHEBo (57) into the CHO *aprt* deletion mutant U1S by the calcium phosphate method (61). After 8 days of selection for resistance to hygromycin (300 μg per ml), colonies were isolated and screened for Tet^R transactivator activity as follows. The hygromycin-resistant clones and U1S cells (as a control) were grown for 48 h in F12 medium containing tetracycline (1 μg/ml) and transfected with 10 μg of pTETaprtWT for 5 h. The cells were washed twice with the same medium, trypsinized, and split into two dishes, with or without tetracycline in the medium. After 48 h, cell extracts were prepared and assayed for Aprt enzyme activity (10). Different clones exhibited a 2- to 15-fold induction of enzyme activity in response to the removal of tetracycline. TAT is the clone that showed the greatest enzyme induction (15-fold) and was used for all further experiments.

To isolate permanent transfectants bearing tetracycline-responsive *aprt* genes, TAT cells were transfected with 10 μg of pTETaprtWT or pTETaprtS20 DNA together with 1 μg of pNEO-BPV100 DNA (40). After 8 days of selection for resistance to G418 (400 μg of the active compound per ml), several clones were isolated and checked by RT-PCR for *aprt* mRNA levels in the presence or absence of tetracycline. Clone TAT-S20A gave 10-fold induction of the mutant *aprt* mRNA, and TAT-WTA showed a 30-fold induction of the wild-type *aprt* mRNA.

Cell fractionation. Cell fractionations to isolate nuclei were performed by three methods. The simple nonionic detergent (Triton X-100) method has been described previously (24). The citric acid method was carried out with 5×10^7 cells exactly as described by Birnie and Graham (8). For these two methods, equal amounts of RNA (1 μg of nuclear, cytoplasmic, or total RNA) were analyzed for *aprt* mRNA by RT-PCR. Nonidet P-40-plus-deoxycholate fractionation was performed by the method of Kugler et al. (34) and is briefly described in the legend to Fig. 7.

RNA analysis. Total RNA was extracted from exponentially growing cells by a guanidinium thiocyanate method (29). For detection of intron-containing molecules, the total RNA was treated with DNase as described previously (32). cDNA was synthesized by RT with random hexamers as primers or, in some cases, with a specific *aprt* primer (primer 13 in reference 32). The cDNA was amplified by PCR with radioactive dATP as a substrate to facilitate quantitation by phosphorimaging exactly as described previously (32). Under these conditions, the RT-PCR responds linearly to the amount of input RNA (Fig. 1) (12). PCR cycles were from 18 to 21 for mRNA and 23 to 25 for intron-containing molecules. The primers used to amplify mRNA and pre-mRNA regions were as follows (a number following the description refers to Table 1 of reference 32): for *aprt*, 5′ ex1, no. 1; 5′ ex2, no. 5; 5′ ex3, no. 9; 5′ ex4, GGCCAGAAAGTGGT TGTTGT; 5′ *Sac*II, GTGCCCGCGGCTATGGCGGAATCTG; 3′ int2, no. 2; 3′ int3, no. 6; 3′ int4, no. 10; 3′ ex5, no. 12; 3′ ex5B, no. 13; 3′ *Xba*I, GTCACTC TAGAGACTACTGATGAAA; for *dhfr*, 5′ ex1, no. 19; 3′ ex2, no. 26; for *hprt*, 5′ ex2, ATGAACCAGGCTATGACCT; 3′ ex3, TCTACAGTCATGGGAATG GA; for β-globin, 5′ ex1, AGAAGTCTGCCGTTACTGCC; 3′ ex3, GCACAC AGACCAGCACGTTG.

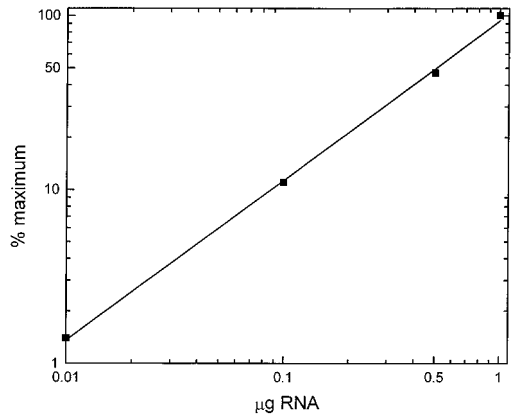

FIG. 1. Quantitation of *aprt* mRNA by RT-PCR. The indicated amounts of total RNA from CHO D422 (carrying one copy of the *aprt* gene) were mixed with total RNA from the *aprt* deletion mutant U1S to make a total of 1 μg. The RNA was subjected to RT, and the cDNA was amplified with a pair of primers from the first and last exons of the *aprt* gene. *dhfr* cDNA was amplified in a parallel reaction. After gel electrophoresis and phosphorimaging, the *aprt* band values were divided by the *dhfr* values. These ratios are plotted relative to the value for a 1-μg input, which was set to 100%.

RNA decay rates in the presence of actinomycin D (5 μg/ml) were determined as described previously; under these conditions, transcription is inhibited more than 98% within 5 min (32).

Polysome analysis. Cells (2×10^7) were exposed to 50 μg of cycloheximide per ml for 5 min, harvested by trypsinization, washed twice with phosphate-buffered saline (PBS) at 4°C, and resuspended in 0.5 ml of cold reticulocyte standard buffer (54). The trypsin and PBS also contained 50 μg of cycloheximide per ml. After addition of an equal volume of reticulocyte standard buffer containing 1% Triton X-100, 1% deoxycholate, and 2% Tween 40, the suspension was Dounce homogenized and centrifuged for 10 min at 10,000 × *g*. The supernatant was loaded onto a gradient of 10 to 50% (wt/vol) sucrose in 10 mM Tris (pH 7.6)–75 mM KCl–3 mM $MgCl_2$. After centrifugation in an SW40 rotor for 2 h at 36,000 rpm and 4°C, the gradient was collected from the top into 18 fractions and the A_{260} was monitored with an ISCO fractionator. Sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5%, and each fraction was extracted with phenol and then twice with phenol-chloroform. The nucleic acids in the aqueous phase (the bottom phase at high sucrose concentrations) were precipitated with ethanol, resolubilized in water, and assayed for *aprt* and *hprt* mRNA by RT-PCR.

RESULTS

Nonsense mutations decrease *aprt* mRNA levels. The small size of the *aprt* gene and the wealth of mutations that have been isolated at this locus in mammalian cell lines make it an attractive system for investigating the effects of premature translation termination on RNA metabolism. We first asked whether, as in other systems, nonsense mutations in this gene decrease *aprt* mRNA levels. We obtained from Mark Meuth a set of mutant CHO cell lines carrying nonsense mutations in several different exons of the *aprt* gene (44, 49). The names of these mutants and the locations of the mutations are presented in Fig. 2. Quantitative RT-PCR was used to determine the steady-state level of *aprt* mRNA in the different mutants: we subjected total RNA to RT, PCR amplified the resulting cDNA with primers from the first and last exons, and measured the radioactivity in the amplified DNA with a PhosphorImager (see Materials and Methods for validation of the quantitation). We also amplified the same cDNA preparation with primers for hypoxanthine phosphoribosyltransferase (*hprt*) mRNA, which served as an internal control for the RT and a parallel control for the PCR. A representative analysis of the electrophoretically separated products derived from several different mutants is presented in Fig. 3. Mutant *aprt* mRNA levels are summarized in Fig. 2, in which the height of the bars indicates

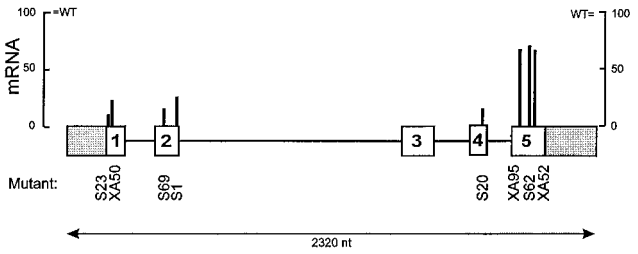

FIG. 2. Map of the *aprt* gene showing the intron-exon organization and the location of the nonsense mutations studied. The height of the bar at each mutation location represents the steady-state level of *aprt* mRNA exhibited by that mutant relative to that of the single-copy parental D422 cells. nt, nucleotides; WT, wild type.

the amount of *aprt* mRNA relative to the parental hemizygous cell line (CHO D422), which carries a single copy of the *aprt* gene (9). Nonsense mutations in exons 1, 2, and 4 resulted in a 5- to 10-fold reduction in *aprt* mRNA levels. However, mutations in exon 5 (the last one) caused only a 40% reduction. These results, including the polar effect, are similar to those obtained previously for the hamster *dhfr* gene (59), the human triose-phosphate isomerase gene (17), and the mouse immunoglobulin gene (4, 15, 39), among others. It should be noted that no shorter mRNA species were detected in these experiments, even with more amplification cycles and longer exposures (data not shown). Thus, no evidence for skipping of exons containing nonsense mutations was found.

***aprt* mRNAs with nonsense mutations are associated with polysomes.** In several previous studies of nonsense-mediated mRNA reduction in mammalian cells, it has been found that the half-life of total or cytoplasmic mRNA has not been affected (see, e.g., references 14, 58, and 59). Our measurements of *aprt* mRNA decay following inhibition of transcription with actinomycin D produced the same result: *aprt* mRNA decayed with a half-life of 16 h in both wild-type and nonsense mutant cells (see below). In several cases, it has been shown that

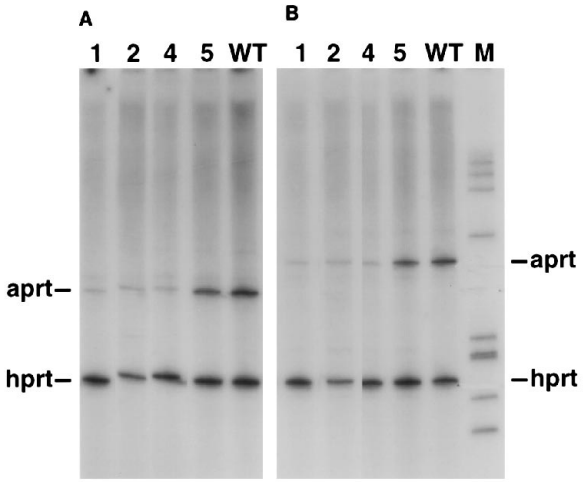

FIG. 3. Autoradiogram of a representative RT-PCR assay of steady-state mRNA levels in the nonsense mutants studied. (A) Lanes: 1, mutant S23; 2, S1; 4, S20; 5, S62; WT, D422. The lane numbers refer to the exons in which the mutations are located. The upper bands represent RT-PCR products from *aprt* mRNA amplified with primers in exons 1 and 5; the lower bands are derived from *hprt* mRNA primers, included as an internal control. (B) Same as panel A but with a more distal 3′ primer in *aprt* exon 5 (primer 3′ ex5B [see Materials and Methods]). Lane M, markers, end-labeled ϕX174 *Hae*III fragments.

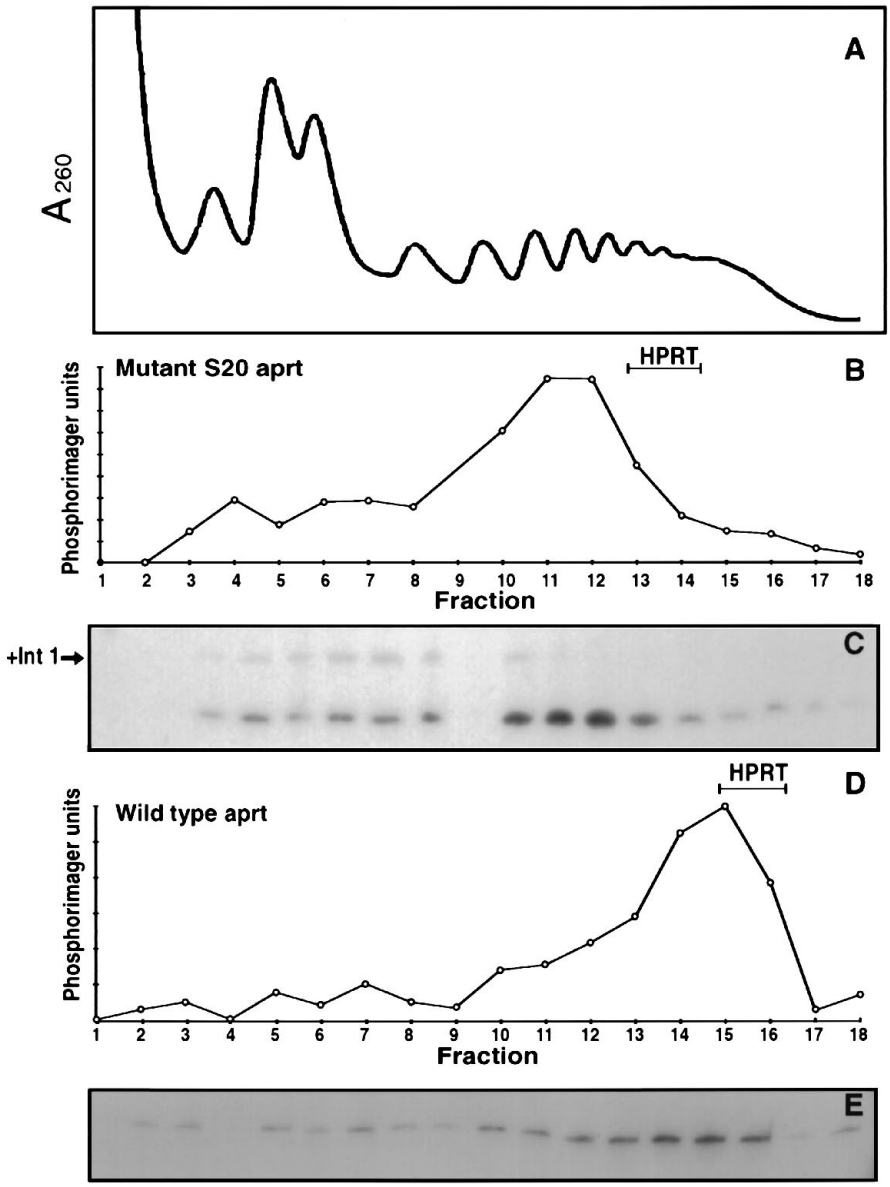

FIG. 4. Polysome distribution of *aprt* mRNA in wild-type and nonsense mutant cells. Cytoplasmic extracts were prepared with nonionic detergents and subjected to sucrose gradient centrifugation. RNA was prepared from fractions by phenol extraction and assayed for *aprt* and *hprt* mRNA by RT-PCR and phosphorimaging. (A) Absorbance profile of a polysome preparation from the S20 nonsense mutant. (B) Distribution of S20 *aprt* mRNA in the polysome preparation depicted in panel A. The bar shows the position of the *hprt* mRNA peak as assayed separately in the same fractions. (C) Autoradiogram of the S20 *aprt* RT-PCR product distribution shown in panel B. The minor upper band (Int 1) corresponds to the size of molecules that retain intron 1. (D) Polysome distribution of *aprt* mRNA from wild-type cells. The wild-type *aprt* mRNA is associated with slightly larger polysomes than the S20 mRNA, which carries a nonsense mutation in exon 4. (E) Autoradiogram of the wild-type *aprt* RT-PCR product distribution shown in panel D.

nonsense-mediated mRNA degradation requires translation (5, 28, 63). We therefore considered the idea that the majority of nonsense-containing mRNA molecules are indeed very unstable but that there exists a minority class of mRNA that is stable because it fails to be translated, avoiding association with polysomes. According to this notion, it is the decay of this relatively stable residual *aprt* mRNA that is being measured after the experimental inhibition of transcription.

To test this idea, polysomes from the wild-type (D422) line and two nonsense mutants (S23 and S20, with mutations in exons 1 and 4, respectively) were displayed by sucrose gradient centrifugation. RNA was extracted from the collected fractions, and the distribution of *aprt* mRNA was measured by RT-PCR. As can be seen in Fig. 4D and E, the great majority of wild-type *aprt* mRNA is associated with polysomes slightly smaller than those associated with *hprt* mRNA, measured as a control. The relative sizes of the *aprt* and *hprt* polysomes are in agreement with the relative subunit molecular weights of these two proteins, 19,500 and 24,500 (23, 62). Mutant S20 mRNA is associated with polysomes of four to five ribosomes (compare Fig. 4A and B). This size is somewhat smaller than the wild-type case, as expected if ribosomes terminate translation prematurely at the predicted position corresponding to 85% of the wild-type open reading frame (Fig. 4B and C). As in the wild-type case, the great majority of the S20 mutant mRNA was associated with polysomes, indicating no anomalous initiation

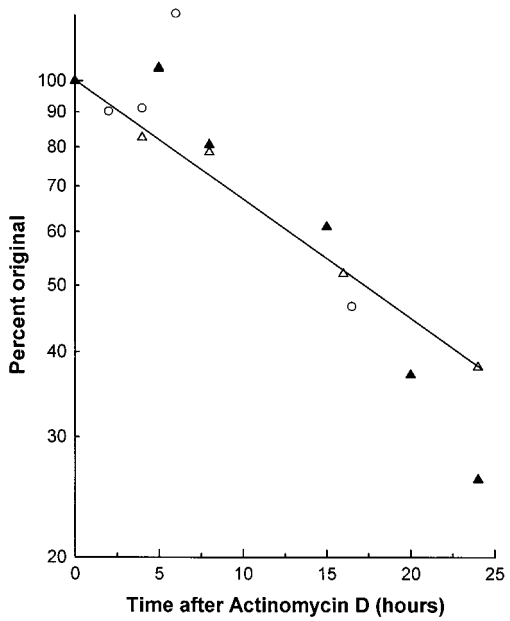

FIG. 5. Stability of *aprt* mRNA in the presence of actinomycin D. To stop transcription, actinomycin D (5 μg/ml) was added to a series of dishes containing either wild-type cells (D422 [solid triangles]), nonsense mutant cells (S23 [open triangles]), or a transfectant clone (STN) carrying the tetracycline-responsive *aprt* gene with the S20 nonsense mutation (circles). At the times indicated, total RNA was extracted and *aprt* mRNA in 1 μg of total RNA was amplified by RT-PCR and quantified by phosphorimaging.

of protein synthesis on this template. A similar result was obtained with mutant S23, which contains a nonsense codon in exon 1; this mRNA was associated with mono- and disomes (data not shown).

We have previously shown that *aprt* RNA from which all introns but intron 1 have been spliced accumulates as a minority species in CHO cells (32). The data presented in Fig. 4 show that these intron 1-containing molecules can be considered a form of *aprt* mRNA, because they are also associated with polysomes. These polysomes are small, consistent with the occurrence of a predicted translation termination codon 1 base after the start intron 1 (19).

A nonsense mutation destabilizes cytoplasmic *aprt* mRNA. In several cases of nonsense-mediated mRNA reduction in yeasts, a dramatic decrease in the stability of the mRNA accompanies the introduction of a nonsense codon (e.g., *PGK1* and *HIS4* [28] and cytochrome oxidase [63]), as measured after inhibition of transcription. In mammalian cells, the results are less clear; in some cases, overall mRNA stability is decreased (e.g., retroviral mRNA [1], androgen receptor [25], and β-globin in erythroid cells [36, 41]), whereas in others, no change in overall mRNA stability is evident (globin [31, 58], *dhfr* [59], and *tpi* [14]). To determine whether nonsense codons promoted the degradation of *aprt* mRNA, we measured the rate of disappearance of *aprt* mRNA after inhibition of new RNA synthesis with actinomycin D, the method used in the mammalian studies cited above. As can be seen in Fig. 5, an mRNA half-life of approximately 16 h was found both in the wild type (D422) and in mutant S23, the nonsense mutant exhibiting the lowest steady-state level of *aprt* mRNA. Thus, the *aprt* gene apparently fell into the second category of mammalian systems, in which nonsense codons reduce mRNA levels without affecting mRNA decay rates. However, since actinomycin D treatment (and perhaps any global inhibition of transcription) could be interfering with a nonsense-mediated destabilization process, we decided to pursue this question further by measuring *aprt* mRNA decay without inhibiting overall RNA synthesis.

Toward this end, we substituted a modulatable promoter to drive wild-type and nonsense mutant versions of a cloned *aprt* gene, so as to compare mRNA decay rates after the exclusive deactivation of these genes. We used the tetracycline-inhibitable promoter system developed by Gossen and Bujard (27) for this purpose. The natural *aprt* promoter in a cloned genomic *aprt* gene (pH2 [38]) was replaced with a chimeric promoter consisting of the basal elements of the cytomegalovirus early promoter preceded by operator sequences of a Tet^R gene (27). At the same time, we isolated a stable transfectant of the *aprt* deletion mutant U1S (9) that carried a chimeric transcriptional activator gene in which the activator domain of the mammalian transcription factor VP16 was fused to the operator-binding domain of the tetracycline repressor protein (pUHD15-1 [27]). This transfectant cell clone, termed TAT, served as the recipient for the chimeric *aprt* genes in transient- and stable-transfection experiments (described below). In these cells, in the absence of tetracycline, the chimeric transcriptional activator binds to the Tet^R operator and activates transcription. In the presence of tetracycline, transcription is deactivated, since when the activator protein binds tetracycline, it loses affinity for the operator (27).

We first measured the decay rate of *aprt* mRNA in a transient-transfection experiment: wild-type or nonsense mutant (S20) versions of the Tet^R-driven *aprt* gene were introduced into TAT cells along with a plasmid carrying a β-globin gene as a control. At 48 h after transfection, tetracycline was added to stop *aprt* gene transcription. Cultures were harvested at various times thereafter for RT-PCR measurement of *aprt* and β-globin and/or *hprt* mRNA levels. The results of two experiments are shown in Fig. 6A. After addition of tetracycline, mRNA levels decayed with exponential kinetics over the next 6 h. *hprt* mRNA levels were not reduced by the tetracycline treatment (data not shown). The average half-life of wild-type *aprt* mRNA degradation was 7.5 h, representing about twice the decay rate measured in the presence of actinomycin D. Thus, actinomycin D treatment stabilized *aprt* mRNA against normal degradation. Such stabilization has been noted frequently in the past, although it seems to depend on the specific mRNA in question (31, 52). More significantly for this study, the nonsense-bearing mRNA of the S20 mutant exhibited an average half-life of 2 h following deactivation (Fig. 6A). This fourfold-greater lability can account for most if not all of the lower steady-state level of S20 *aprt* mRNA (16% of wild type [Fig. 2 and 3]).

The increased lability of S20 mRNA was also evident in a stable transfectant. Stable transfectants of TAT cells were isolated by cotransfection with a plasmid carrying the *neo* gene and either the wild-type or S20 version of the tetracycline-responsive *aprt* gene. G418-resistant transfectant clones were then screened for tetracycline responsiveness by measuring *aprt* mRNA levels. One transfectant clone of each type (STW and STN for the wild-type and S20 nonsense mutant genes, respectively) that exhibited a maximum tetracycline response was chosen for further study. As can be seen in Fig. 6B, wild-type *aprt* mRNA decayed with a half-life of 5.5 h in this stable transfectant, somewhat faster than in the transient-transfection experiments, and substantially faster than the 16-h half-life exhibited by the endogenous *aprt* mRNA in actinomycin D-treated wild-type cells. Also shown in Fig. 6B is a half-life of 1.5 h for S20 nonsense mutant *aprt* mRNA. As in the transient-expression experiments, the mutant *aprt* mRNA was degraded at about four times the rate of the wild type. The increased

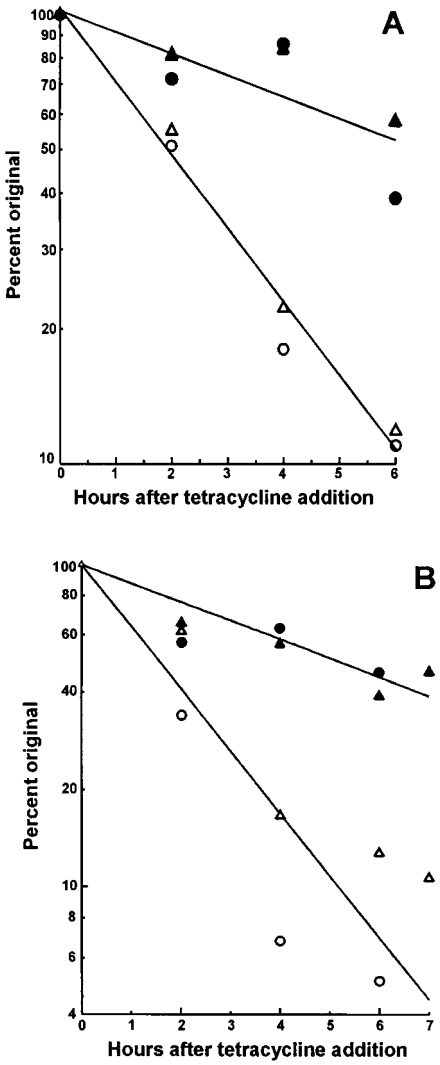

FIG. 6. *aprt* mRNA stability after deactivation of transcription from a tetracycline-responsive promoter. (A) Transient-expression experiments. Cells of the *aprt* deletion mutant U1S, carrying the Tet^R activator gene, were cotransfected with a plasmid carrying either a wild-type (solid symbols) or a nonsense mutant (S20 [open symbols]) *aprt* gene driven by a tetracycline-responsive promoter, as well as a control β-globin gene. At 48 h after transfection, tetracycline (1 μg/ml) was added to inhibit *aprt* transcription. At various times thereafter, total RNA was extracted and *aprt* and β-globin and/or *hprt* mRNA levels were quantified by phosphorimaging. The *aprt* values were normalized to either the cotransfecting β-globin (triangles) or the endogenous *hprt* (circles) mRNA. (B) In permanent transfectants. Cloned cell lines carrying the Tet^R activator gene and either the wild-type (STW [solid symbols]) or nonsense mutant (STN [open symbols]) tetracycline-responsive *aprt* genes were treated with tetracycline and at various times, total (circles) or cytoplasmic (triangles) RNA was extracted and *aprt* and *hprt* mRNA was quantified as above. The *aprt* values were normalized to *hprt* mRNA.

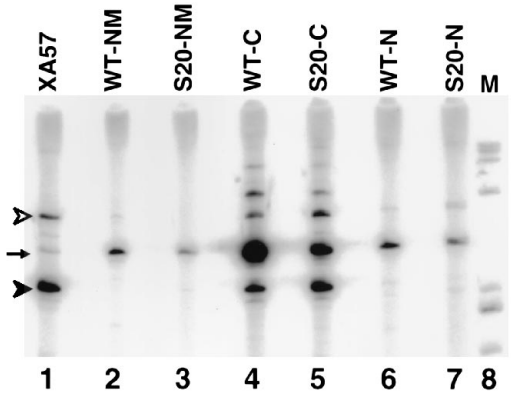

FIG. 7. Nuclear mRNA levels in a nonsense mutant are reduced. Near-confluent monolayers of D422 (WT) and mutant S23 (nonsense mutation in exon 1) cells were harvested by trypsinization and lysed with Nonidet P-40. Immediately after lysis, an equal volume of cytoplasm isolated in the same way from CHO XA57 cells was added to monitor adventitious association of cytoplasmic RNA with the nuclei. Because of a splicing mutation, XA57 cells produce *aprt* mRNA lacking exon 4, which can be distinguished from *aprt* mRNA of normal size (arrow) by the smaller size of its RT-PCR product (solid arrowhead, lane XA57). Some unspliced RNA retaining intron 4 was also produced (open arrowhead, lane XA57). After collection of the nuclei by centrifugation, the supernatant was taken as the cytoplasmic fraction (C). The nuclear pellet was treated with deoxycholate to strip off the nuclear membrane. The supernatant was collected as the nuclear membrane fraction (NM), and the final pellet was considered the nuclear fraction (N). RNA was extracted from the three fractions, and equal cell equivalents were analyzed for *aprt* mRNA by RT-PCR. An autoradiograph of the electrophoretic separation of radioactive RT-PCR products is shown. Lane M: markers, end-labeled ϕX174 *Hae*III fragments.

lability is evident in cytoplasmic mRNA (Fig. 6B) as well as in total RNA.

Placing the *aprt* genes under the control of the tetracycline-responsive promoter altered the 5′ untranslated region of the tetracycline-responsive *aprt* mRNA molecules. To test the idea that it was this alteration, rather than actinomycin D treatment, that accounted for the difference between the earlier actinomycin D decay rate and the tetracycline deinduction decay rate, we measured the decay rate of the tetracycline-responsive S20 *aprt* mRNA in cell line STN in the presence of actinomycin D. Once again, actinomycin D stabilized the mRNA, yielding a half-life of about 16 h (Fig. 5), compared with the 1.5-h value without the drug (Fig. 6B). We conclude that measurement of mRNA decay in the presence of actinomycin D is not valid in the case of *aprt* and that tetracycline deinduction provides a more reliable indicator of mRNA stability.

Nuclear *aprt* mRNA levels are reduced by nonsense mutations. The greater lability of the nonsense-containing S20 mRNA in the cytoplasm could explain the low overall steady-state levels of this *aprt* mRNA. However, in several other systems, there is also evidence for a nonsense-mediated decrease in nuclear RNA processing or stability (14, 20, 39, 59). To investigate the effects of nonsense mutations on nuclear mRNA levels, we fractionated mutant and wild-type cells by either simple nonionic detergent lysis (mutant S23) or treatment with citric acid (low pH; mutant S20). Nuclear and cytoplasmic *aprt* mRNA levels were quantified by RT-PCR with *hprt* as the standard for RNA extraction and the RT reaction. Both methods yielded the same result: nonsense-containing mRNA levels were depressed to 8 to 12% of wild-type levels in nucleus-associated mRNA (data not shown). In a third experiment, we used a more rigorous fractionation, in which the nuclear membrane was stripped off with deoxycholate after cell lysis by Nonidet P-40 (34); the nucleus-associated fraction here consists of both the nuclear membrane fraction and the remaining nuclear pellet. The results of this fractionation comparing wild-type D422 cells with the exon 1 nonsense mutant S23 are shown in Fig. 7. About 10% of *aprt* mRNA was associated with the nucleus, about equally divided between the nuclear membrane fraction and the nuclear pellet. The *aprt* mRNA level was reduced in these nuclear fractions to the same extent as it was in the cytoplasm (Fig. 7).

The conclusion that a nonsense mutation can reduce the level of nuclear *aprt* mRNA rests heavily on the purity of the

TABLE 1. Relative pre-mRNA levels[a] in nonsense mutants

Primer pair	Primers[b]	Relative pre-mRNA level in:					
		S23 (exon 1)	S1 (exon 2)	S69 (exon 2)	S20 (exon 4)	S62 (exon 5)	Wild type
1	E1-E5 (mRNA)	11 ± 3	26 ± 4	14 ± 1	15 ± 2	67 ± 7	100
2	I3-I4	121 ± 30	153 ± 14	—[c]	125 ± 25	116 ± 16	100
3	E2-I2	115	200 ± 88	143	77 ± 4	84 ± 6	100
4	E3-I3	108 ± 6	202 ± 3	—	109 ± 8	87 ± 2	100
5	E4-I4	99 ± 16	146 ± 10	—	76 ± 11	124 ± 17	100
6	E1-I4	46 ± 5	67	70 ± 3	100 ± 15	71 ± 6	100
7	E3-I4	56	—	66	86	67	100

[a] Total RNA was extracted and quantified by RT-PCR. *aprt* RNA values were normalized to *hprt* mRNA values from parallel cDNA amplifications. Most values were derived from two RNA preparations and two or more RT-PCR analyses; the mean and standard error of the mean are shown.
[b] I, intron; E, exon.
[c] —, not done.

nuclear fraction. Others have shown that nuclei prepared by nonionic detergent lysis are free of mitochondrial RNA (1) and lack cytoplasmic tags as judged by electron microscopy (6). Here we performed a reconstruction experiment to measure the amount of cytoplasmic mRNA that becomes adventitiously associated with the nuclear fraction. Just after the addition of nonionic detergent to lyse the cell membrane, a cytoplasmic fraction from an equal number of cells of the CHO mutant XA57 (44) was added. XA57 cells carry a mutation at the 5′ splice site of *aprt* intron 4. As a result, these cells do not produce any correctly spliced *aprt* mRNA (Fig. 7) but, rather, a mixture of two aberrant products. The predominant product is a shorter RNA of a size expected for the skipping of exon 4; a minor RNA of a size expected for the retention of intron 4 is also accumulated (Fig. 7, lane XA57). The short *aprt* RNA serves as a marker for the cytoplasmic contamination of the nuclei from the experimental D422 and S23 cells. As can be seen in Fig. 7, the short XA57 RNA is seen in the cytoplasmic fraction but not in the nuclear or nuclear membrane fraction. PhosphorImager quantitation put an upper limit of 15% on the amount of nuclear signal ascribable to cytoplasmic contamination. Similar results were obtained with mutant S20. This assessment of contamination does not rule out the possibility that mRNA in the nuclear fraction contains molecules that are partially or fully in the cytoplasm but retain their association with their nucleus of origin. Molecules such as these may be considered to be still nucleus associated, perhaps still in the last stages of nuclear export.

We conclude that the level of *aprt* mRNA is reduced in the nuclei of nonsense mutants as well as the cytoplasm. Thus, increased cytoplasmic lability may not be sufficient to explain all the effects of nonsense mutations in reducing mRNA levels.

***aprt* pre-mRNA levels are not affected by nonsense mutations.** There are several mechanisms that could account for a reduction in mRNA levels in the nucleus of nonsense mutants. These include a decreased transcription rate, a block in RNA splicing or polyadenylation, a defect in nuclear transport coupled with a rapid degradation of the untransported mRNA, and a direct effect increasing the rate of nuclear mRNA decay. To test the idea of a nonsense-mediated effect on transcription or splicing, we quantified *aprt* pre-mRNA levels in several nonsense mutants. The assumption behind these experiments was that a decreased transcription rate would decrease steady-state levels of *aprt* primary transcripts while a block to splicing would result in a higher steady-state level of pre-mRNA molecules upstream of the block, i.e., molecules containing one or more introns. RT-PCR was used to quantify intron-containing RNA molecules. Six primer pairs that amplified several regions of *aprt* RNA were used. The results of measuring the pre-mRNA levels in five nonsense mutants are summarized in Table 1, where they are expressed relative to the wild type. Autoradiograms from representative experiments are shown in Fig. 8. Amplifications involving a primer in intron 3 (Table 1, primer pairs 2 and 4) provide a measure of the *aprt* primary transcript, since this intron is the first to be spliced (32). The primer pairs exon 2-intron 2, exon 4-intron 4, exon 1-intron 4, and exon 3-intron 4 (Table 1, primer pairs 3, 5, 6, and 7, respectively) amplify splicing intermediates that retain the cor-

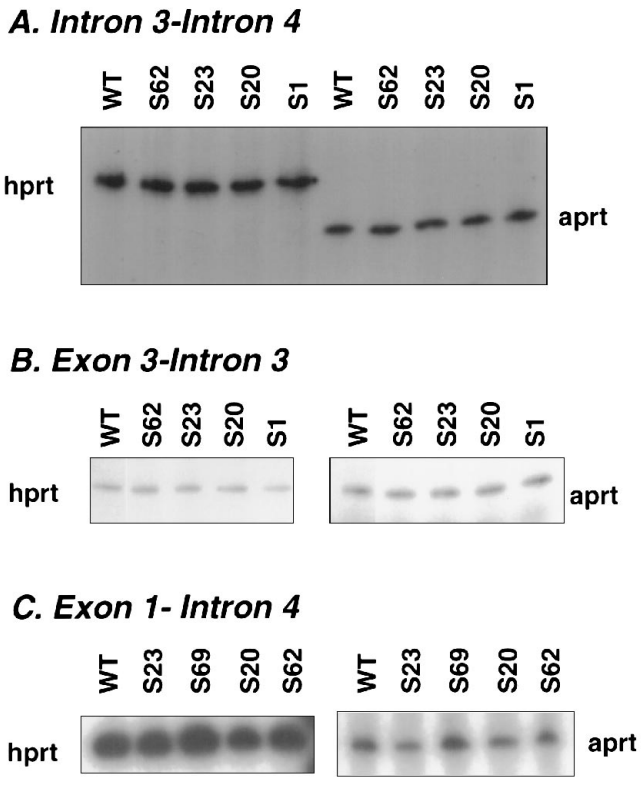

FIG. 8. *aprt* pre-mRNA levels in nonsense mutants. Total RNA was extracted from the indicated mutants as well as wild-type D422 cells, and various intron-plus-exon-containing regions were amplified by RT-PCR from 1 μg of RNA. The radioactive PCR products were then separated by gel electrophoresis and visualized by autoradiography. The *aprt* primer pairs used defined the region analyzed; the results with three primer pairs are shown here (right side). *hprt* pre-mRNA was amplified in parallel as a control (left side). (A) Primers in intron 3 and intron 4. (B) Primers in exon 3 and intron 3. (C) Primers in exon 1 and intron 4. The overall results were quantified by PhosphorImager analysis and are summarized in Table 1.

responding intron, as well as the primary transcript. The primer pairs exon 1-intron 4 and exon 3-intron 4 amplify one of the two last intermediates (retaining solely intron 4) of the *aprt* pre-mRNA splicing pathway (32). In each amplification, the *aprt* RNA level in total RNA was normalized to an *hprt* mRNA control, which was amplified from the same cDNA. Several features of these data are discussed below.

As can be seen in Table 1 (primer pairs 2 and 4) and Fig. 8A, nonsense mutants contained the same level of primary transcript as wild-type cells did. These molecules disappeared with a half-life of 5 to 10 min after inhibition of transcription with actinomycin D, consistent with a role as an mRNA precursor rather than the product of some dead-end pathway (data not shown; experiments similar to those described in reference 32). This result suggests that the transcription rate was not affected by the nonsense mutations, as has also been found by measurement of nuclear run-on rates in the cases of *dhfr*, β-globin, and *tpi* (3, 14, 59). Thus, the nonsense-mediated decrease in nuclear mRNA levels appears to be taking place at a posttranscriptional step in nuclear RNA processing.

Also comparable in the nonsense mutants and wild-type cells were the levels of other intron-containing molecules, representing partially spliced RNA as well as the primary transcript. Apparent twofold increases for intron 2- and intron 3-containing molecules in the S1 mutant can be seen in Table 1 (primer pairs lines 3 and 4). The exon 1-intron 4 primer pair amplifies one of two alternative last intermediates in the *aprt* splicing pathway, a triply spliced molecule that retains only intron 4 (Table 1, primer pair 6; Fig. 8C). The major last intermediate retains only intron 1 (32), but this molecule also appears in the cytoplasm and so is not a simple indicator of nuclear metabolism. In four of the five mutants studied, the level of this RNA appeared slightly (25 to 50%) reduced. Although this difference may be real, it is at the limit of the sensitivity of these measurements, which may include slight clonal variations unrelated to the *aprt* mutations.

Thus, within the approximately twofold precision of these experiments and in contrast to the decrease in nuclear mRNA levels, no consistent or dramatic difference in intron-containing RNA levels was found between wild-type cells and the set of five nonsense mutants tested.

DISCUSSION

Reduced cytoplasmic and nuclear mRNA in *aprt* nonsense mutants. Our examination of CHO mutants bearing nonsense mutations at the *aprt* locus adds this gene to the growing list of mammalian genes subject to nonsense-mediated mRNA reduction. As in the case of *dhfr*, *tpi*, β-globin, and immunoglobulin, mutations close to the natural translation termination site had almost no effect on mRNA levels: mutations in the 3′-terminal exon reduced mRNA levels by about 40% rather than 5- to 10-fold. This marked polarity has also been seen in yeasts, for which it has been shown that translation past certain mRNA sequences allows the mRNA to escape nonsense-mediated degradation (28, 48). However, there are a few exceptions to this polarity: nonsense mutations close to the 5′ end of the protein-coding regions of the *dhfr* (11) and immunoglobulin (15) genes can also display the low-mRNA phenotype.

The effects of nonsense mutations on the levels of nuclear RNA have been widely studied by using RNA viruses (1, 2, 55), in which the export of unspliced RNA represents an essential step in the viral life cycle. However, such measurements for cellular genes are less common. For *tpi* (6) and β-globin (34, 58), it was noted that nuclear mRNA levels decreased to about the same extent as did cytoplasmic mRNA levels in response to nonsense mutations. We have found a similar reduction in nuclear mRNA levels for *aprt*. This result takes on added importance in the case of *aprt*, because it represents the only evidence in this system for the nuclear recognition of nonsense codons (see the discussion below).

Residual *aprt* mRNA in nonsense mutants is on polysomes. The apparent stability of the residual mRNA found in mammalian nonsense mutants points to the nucleus as the target for the nonsense effect. An alternative explanation is that the nonsense-bearing mRNA that gets translated is degraded very quickly, too quickly to have been detected. In this scenario, the residual mRNA, whose stability is measured, has escaped this rapid degradation because it has escaped translation initiation. In support of this idea is the demonstration by Belgrader et al. (5) that translation is necessary for the nonsense-mediated reduction of *tpi* mRNA. However, our analysis of polysomal mRNA showed that the majority of residual nonsense-containing *aprt* mRNA molecules are in fact associated with polysomes. Likewise, in wild-type mouse and CHO cells, a minority of cytoplasmic *aprt* transcripts retain intron 1; here, we show that these molecules are also associated with ribosomes or small polysomes. The size of these small polysomes is consistent with the predicted in-frame nonsense codon within intron 1. The level of this naturally occurring (in mouse as well as hamster cells [32]) nonsense-containing isoform of *aprt* mRNA is probably not further reduced by a nonsense mutation downstream in the reading frame, since the ratio of intron-containing to conventional mRNA was much higher in the S20 mutant than in wild-type cells (the intron-containing version is visible in Fig. 4C but not in Fig. 4D). It is reasonable to think that the relative level of this minor species would be higher were it not for the in-frame nonsense codon in the intron. In fact, the low level of this intron-containing RNA may be an example of how nonsense-mediated mRNA reduction prevents poorly spliced species from accumulating in the cytoplasm (50). mRNAs engineered to include unspliced introns were similarly found associated with polysomes in a yeast *upf1* mutant (30).

Nonsense mutations can destabilize *aprt* mRNA. Using actinomycin D to inhibit RNA synthesis, we measured identical half-lives of 16 h for both wild-type and nonsense-containing *aprt* mRNA molecules. However, the use of this drug has been known to stabilize some mRNA species (31, 33, 52, 56). There is no reason to think that such effects are due to actinomycin D per se: that is, any global inhibition of RNA synthesis carries with it a risk of perturbing RNA degradation. For this reason, we designed an experiment to measure the *aprt* mRNA decay rate after specifically inhibiting the transcription of the *aprt* gene. The tetracycline-responsive promoter-activator system developed and provided to us by Gossen and Bujard (27) enabled us to make these measurements. Although we did not achieve very high ratios of activation to deactivation when using transfected tetracycline-responsive *aprt* gene constructs in CHO cells, the 10-fold effect we did realize was sufficient for our purpose.

A comparison of wild-type and nonsense mutant *aprt* mRNA half-lives by measurement of the decay kinetics after deactivation (Fig. 6) showed that the nonsense mutation studied (S20) destabilized *aprt* mRNA, reducing the half-life about fourfold, from 8 h to about 2 h. Thus, the identical stabilities of wild-type and mutant mRNAs found in the actinomycin D experiment were not confirmed. Not only had actinomycin D treatment stabilized the mutant *aprt* mRNA, but also it had led to an overestimate of the stability of wild-type *aprt* mRNA (from an 8-h half-life to an apparent 16-h half-life). The decreased stability of nonsense mutant S20 mRNA is close to the decrease found in steady-state levels of this mRNA (sixfold). Thus, for *aprt*, the stability of nonsense-containing cytoplasmic mRNA

cannot be used as a compelling argument for a nuclear process being involved.

Our results with *aprt* stand in contrast to those of Cheng and Maquat (14), who conducted the same type of actinomycin D-free specific deinduction experiment with *tpi*. In that study, wild-type and nonsense mutant versions of *tpi* genes driven by the serum-responsive *fos* promoter were used in transient-transfection experiments. After serum starvation followed by serum readdition, a transient, documented burst of transcription ensued. No difference in *tpi* mRNA decay rate between wild-type and nonsense mutant mRNA was found, confirming these authors' own previous results with actinomycin D. Indeed, in this system, there was no mRNA decay during the 33-h experiment. In subsequent work, Belgrader and Maquat (7) did find a nonsense-mediated increased rate of degradation of serum-induced *fos-tpi* mRNA in the nucleus. The discrepancy between the *tpi* and *aprt* results could be due to different sensitivities of these two mRNA molecules to nonsense-mediated decay in the cytoplasm. Alternatively, multiple signal transduction pathways could have been affected by the serum starvation used in the *fos-tpi* experiment, one of which may have indirectly altered an RNA degradation pathway.

***aprt* pre-mRNA splicing intermediates are unaffected by nonsense mutations.** Perhaps the most puzzling effect of nonsense mutations on RNA metabolism is that pre-mRNA splicing aberrancies can accompany the reduction in mRNA levels. As mentioned in Introduction, increased levels of unspliced pre-mRNA or the skipping of exons that harbor nonsense mutations has been reported for the fibrillin, ornithine aminotransferase, α-iduronidase, fibroblast growth factor receptor 2, immunoglobulin, and minute virus of mice genes. In contrast, for one of the most intensively studied systems, the human *tpi* gene, wild-type and nonsense mutant genes accumulated similar levels of intron-containing *tpi* RNA molecules in transfected cells. Our results with CHO *aprt* nonsense mutants are in agreement with the *tpi* data: no differences in the steady-state levels of several different pre-mRNA molecules were detected in five mutants studied. Our data extend the *tpi* results in two ways. (i) We measured steady-state pre-mRNA levels produced by nonsense mutations in the chromosomal *aprt* gene, rather than studying transfected genes. (ii) We included the quantitation of splicing intermediates, i.e., molecules that had undergone at least one splicing event and therefore did not simply represent the primary transcript. Without this latter measurement, variations in splicing could be overlooked if the level of primary transcript were high compared with the levels of splicing intermediates. In previous work (32), we found that these intron-containing *aprt* RNA molecules disappeared with half-lives of 5 to 10 min, consistent with their role as mRNA precursors (as opposed to dead-end side products). Using sensitive RT-PCR methods, we did not detect the accumulation of either longer unspliced RNA or shorter mRNA molecules that had suffered exon skipping, although we could readily detect such molecules among frank splicing mutants of the small *aprt* gene (e.g., Fig. 7, lane XA57).

It is unclear why nonsense-mediated splicing aberrancies are found in some systems and not others. In some cases (e.g., fibrillin), skipping the nonsense-containing exon restores an open reading frame, leading to the possibility that a minor splicing pathway is being revealed by the decrease in the level of normally spliced mRNA (21; however, see reference 20). However, in other cases, there is no such reading frame restoration (e.g., ornithine aminotransferase [21]). It is possible, as Belgrader and Maquat (7) have argued, that the two effects are not directly related, i.e., that the reduction in mRNA level is coupled to a translation or a translation-like process while the splicing deficiencies are being caused by changes in RNA structure.

While the data presented here do not support an effect of nonsense mutations on splicing, the reduced level of nucleus-associated mRNA implies that some nuclear process may be affected by the presence of nonsense codons. The data could be consistent with the translational translocation model, in which premature termination blocks the export of mRNA from the nucleus, if it is postulated that mRNA in such a stalled state is rapidly degraded and thus fails to accumulate in the nucleus. Nuclear scanning of spliced mRNA by a translation-like process represents an alternative model. Explanations that circumvent the involvement of intranuclear events per se may also be considered. For example, if the majority of nucleus-associated mRNA represents molecules that have made their way back into the nucleus from the cytoplasm (e.g., via leakage or during mitosis), the reduced nuclear levels would simply reflect the enhanced cytoplasmic degradation shown here. Finally, despite its resistance to extraction with deoxycholate, the nucleus-associated mRNA may actually be localized on the exterior of the nucleus, perhaps still associated with nuclear pore complexes. At this location within the cell, it may be translatable and subject to cytoplasmic decay. Certainly, additional work is needed to clarify the nature of possible nuclear events tied to mRNA translatability.

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