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An exon that prevents transport of a mature mRNA

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ABSTRACT **In *Caenorhabditis elegans*, pre-mRNA for the essential splicing factor U2AF^{65} sometimes is spliced to produce an RNA that includes an extra 216-bp internal exon, exon 3. Inclusion of exon 3 inserts an in-frame stop codon, yet this RNA is not subject to SMG-mediated RNA surveillance. To test whether exon 3 causes RNA to remain nuclear and thereby escape decay, we inserted it into the 3′ untranslated region of a *gfp* reporter gene. Although exon 3 did not affect accumulation or processing of the mRNA, it dramatically suppressed expression of green fluorescent protein (GFP). We showed by *in situ* hybridization that exon 3-containing *gfp* RNA is retained in the nucleus. Intriguingly, exon 3 contains 10 matches to the 8-bp 3′ splice-site consensus. We hypothesized that U2AF might recognize this octamer and thereby prevent export. This idea is supported by RNA interference experiments in which reduced levels of U2AF resulted in a small burst of *gfp* expression.**

An important element in intron recognition in vertebrates is the polypyrimidine tract, which functions by binding the essential splicing factor U2AF to facilitate the interaction between U2 small nuclear ribonucleoprotein (snRNP) and the branch point just upstream (1). In *Caenorhabditis elegans,* introns lack both branch-point and poly(Y) tract consensus sequences, but, instead, have a highly conserved U_4CAG/R consensus at the 3′ splice site (2). This sequence has been shown to be vital for recognition of the 3′ splice site (3, 4), and we have hypothesized that association of some splicing factor with that sequence is the key event in intron and outron (the intron-like sequence at the 5′ ends of pre-mRNAs that gets removed by trans-splicing) recognition in *C. elegans*. Because the polypyrimidine tract is important for 3′ splice-site recognition and the U_4CAG/R consensus contains a run of five pyrimidines, we suggested that this octamer is the site of U2AF binding (5).

C. elegans U2AF^{65} is encoded by *uaf-1* (5) and is a close homolog of mammalian and fly U2AF^{65}. *uaf-1* specifies two classes of RNAs, an mRNA of 1.7 kb that is translated to give U2AF^{65}, and a 1.9-kb RNA that includes an extra exon (exon 3). Remarkably, this exon contains 10 U_4CAG/R repeats, although there is no indication that they are ever used as splice sites. The exon 3-containing RNA has a premature termination codon (PTC) in the third exon of six, but no truncated protein was detected (5). We hypothesized that the 1.9-kb RNA instead serves a regulatory role, related to the presence of the 10 conserved U_4CAG/R octamers. Interestingly, the relative abundance of the two alternatively spliced RNAs is regulated by growth conditions (5), consistent with the idea that the alternatively spliced product has a regulatory function.

The possibility remained that under rapid growth conditions, the 1.9-kb RNA levels are low because of the PTC, which the *C. elegans* Smg pathway for mRNA surveillance normally recognizes (6). Here we show that *uaf-1* exon 3-containing RNA is not subject to Smg-mediated decay in spite of its containing a PTC. Although it is not yet understood how the components of the pathway act to recognize and degrade aberrant mRNAs, Smg is likely to operate at the site of protein synthesis. Because the yeast homolog of SMG-2, UPF1, has been localized to the cytoplasm at the site of polyribosomes (7), an mRNA that fails to leave the nucleus might well be unaffected by the Smg pathway. Hence, we reasoned that exon 3-containing RNA may never leave the nucleus, and exon 3 added to an mRNA could be sufficient to cause nuclear retention of that mRNA. In this report we demonstrate that when exon 3 is inserted into the 3′ untranslated region (UTR) of a *gfp* reporter, it prevents expression of mature mRNA by causing nuclear retention.

MATERIALS AND METHODS

Worms and Transgenics. General procedures for maintaining and handling *C. elegans* were used (8). Transgenic strains were obtained by gonadal injection of plasmid DNAs coinjected with marker plasmid pRF5 [*rol-6* (*su1006*)] (9, 10) into N2 worms [BL3310–3313 (gfpex3) and BL3320–23 (gfpex3R)] or into TR1332 [*smg-2 (r863)*], [BL3314–17 (gfpex3) and BL3324–26 (gfpex3R)]. BL3314–3316 and BL3324–3326 were outcrossed twice to wild type (N2) to remove the *smg-2*(*r863*) mutation from each strain. The *smg*+ derivatives, BL3334–3339, were tested by probing a Northern blot of RNAs derived from these strains as well as the *smg-2* parental strains with a probe to SRp20 (11) to confirm the difference in the *smg*+ and *smg*− strains.

For integration of strains, strains BL3316 (gfpex3) and BL3325 (gfpex3R) were γ-irradiated (3,800 rad, Cs source) and cloned to select strains segregating 100% rollers. BL3340 and BL3345 (both *smg-2*) acted like chromosomal integrants and were used for *in situ* hybridization analysis.

Plasmid Construction. Exon 3 from *uaf-1* was inserted in both orientations into the 3′ UTR of a *vit*/*gfp* plasmid. The *vit*/*gfp* plasmid contains 247 bp of 5′ upstream sequence of *vit-2*, followed by 11 bp of 5′ UTR and 12 bp of *vit-2* coding sequence [derived from pJ247 (12)] fused to TU61 [a promoterless *gfp* construct containing multiple cloning sites, a synthetic intron of 42 bp and a nuclear localization signal (NLS)] (13). The *unc-54*-derived 3′ UTR of TU61 was replaced by a fragment from *vit-6* containing the *vit-6* polyadenylation signal, 3′ UTR, and adjoining 3′ sequence.

uaf-1 exon 3 fragment was generated by PCR with oligonucleotides U2ex3us (5′-CGCGCGGTCGACACACTCCTGCTGTC-3′) and U2ex3ds (5′-CCCGGGGTCGACCTGAATTTTGCAAGATTATTTG-3′), digested with *Sal*I and cloned into the *Sal*I site in the 3′ UTR of *vit*/*gfp* in both orientations.

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Abbreviations: UTR, untranslated region; GFP, green fluorescent protein; PTC, premature termination codon; dsRNA, double-stranded RNA; RNAi, RNA interference.

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Characterization of RNA. Mixed-stage populations of transgenic worms, enriched in adults, were grown and harvested and total RNA was prepared (3). Synchronous cultures of wild type (N2) and TR1332 [*smg-2* (*r863*)] (6) obtained by alkaline hypochlorite digestion of adults and hatching the resulting embryos without food (14) were grown to particular larval stages and harvested. RNA samples (5–15 μg each) were separated electrophoretically on a formaldehyde-agarose gel (15) and blotted onto Hybond N (Amersham) 16 hr in 10× SSC. Blots were hybridized overnight with ^{32}P random-prime-labeled PCR-amplified fragments; hybridization was quantified by using IMAGEQUANT software on a Molecular Dynamics PhosphorImager. The Northern blot in Fig. 3 was hybridized with a *vit-2* probe for normalization of the number of adults and with *rol-6* to estimate the number of Roller transgenics in the mixed populations used for RNA preparation. The ratios expressed in the figure legend express the level of *gfp/(vit-2) (rol-6)* because only the Roller adults should be expressing green fluorescent protein (GFP).

RNase protection assay (16) was done with 20 μg of total RNA for each strain, treated with RNase-free DNase I (1 unit, 30 min at 37°C). The probe was transcribed from the 449-bp *Nde*I fragment of gfpex3 cloned into the *Nde*I site of pGEM (Ampliscribe kit; Epicentre Technologies, Madison, WI). The probe is complementary to the 5′ region of *vit-2*, the 5′ coding region of *gfp*, and associated vector sequence including the 42-bp intron (Fig. 1, RPA probe).

Primer extension with ddG (16) was done using purified oligo GFint2ds (5′-CGCTACCCTCCAAGGGTCCTC-3′) and 10 μg of each DNase-treated RNA sample.

The site of polyadenylation was detected in an RNase H experiment by using 20 μg of total RNA for each lane, oligo(dT)$_{40}$ (T ×40) and vit6UTRds (5′-TGGTAGCGACC-GGCGCTC-3′), and RNase H treatment at 30°C for 1 hr. After digestion, the samples were phenol/chloroform-extracted and precipitated with ethanol, and the pellets were dried, resuspended, and separated electrophoretically on an 8% polyacrylamide, 7 M urea gel. The gel was electroblotted overnight at 200 mA in 25 mM $NaPO_4$, and the blot was hybridized with a random-primed, ^{32}P-labeled, PCR-amplified fragment containing *uaf-1* exon 3.

***In Situ* Hybridization.** Synchronous cultures of integrant strains BL3340 (gfpex3) and BL3345 (gfpex3R) were grown to obtain gravid adults for hybridization. Worms were cut with a 25-gauge needle in PBS (+2.5 mM levamisole) to extrude intestines and gonads on polylysine-subbed slides. The cut worms were immediately fixed by adding an equal volume of 5% paraformaldehyde, coverslipping, and freezing on dry ice. Slides were processed (17), treated with Proteinase K at 5 μg/ml or 20 μg/ml (resulting in better signals), and hybridized for 16 hr to a digoxigenin-labeled RNA probe complementary to the entire coding region of gfp, but without complementarity to either *uaf-1* exon 3, the *vit-2* 5′ UTR, or the *vit-6* 3′ UTR. After hybridization and washing, slides were incubated for another 16 hr with antidigoxigenin antibody (Boehringer Mannheim) at 4°C, developed with NBT/BCIP, and stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were photographed with Ektachrome 400, and the photographic slides were scanned to create digital images processed with Adobe PHOTOSHOP to make the figures shown.

Double-Stranded (ds) RNA Interference (RNAi). dsRNAs were prepared by transcribing both strands of genes inserted in pGEM vector with T7 and SP6 (Ampliscribe kit). Approximately 1 μg/μl of each strand was mixed and injected into L4 or young adults in either the intestine or gonad (18). Injected animals were monitored for GFP expression and for the hatching of their embryonic progeny.

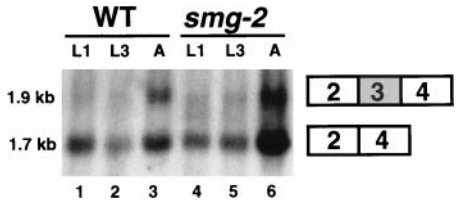

FIG. 1. Exon 3-containing RNA is not subject to mRNA surveillance. RNA (15 μg/lane) from wild-type and *smg-2* (*r863*) strains was analyzed by Northern blot for alternatively spliced forms of *uaf-1* mRNA. Lanes 1–3 contain RNA from L1 and L3 larvae and adult-stage worms of a wild-type strain. Lanes 4–6 contain RNA from the same stages of *smg-2* (*r863*) worms. *uaf-1* RNAs were detected with a random-primed *uaf-1* cDNA probe. The 1.9-kb band represents the RNA that retains exon 3; the 1.7-kb RNA is *uaf-1* mRNA. The faint 1.8-kb band in lane 4 is unidentified.

RESULTS

Smg Degradation System: Effect on *uaf-1* RNA Levels. The exon 3-containing *uaf-1* RNA with the PTC was tested for degradation by the Smg mRNA surveillance system. Surprisingly, relative levels of the two alternatively spliced forms of *uaf-1* pre-mRNA do not change in *smg-2* mutant worms (Fig. 1). As a control, the same RNAs were hybridized with a SRp20 probe, which has been shown previously to detect an increased level of the larger RNA species containing a PTC in *smg-2*

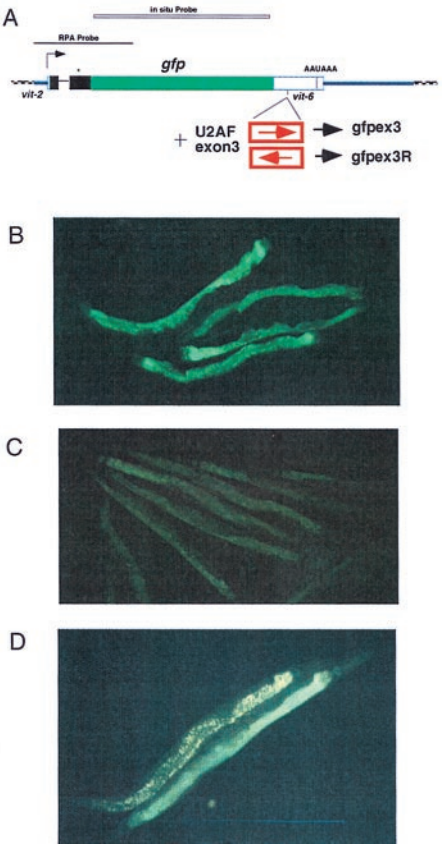

FIG. 2. Exon 3 prevents expression of a *vit/gfp* reporter. Exon 3 was inserted in both orientations into the 3′ UTR of the *vit/gfp* construct shown in *A* to create gfpex3 and gfpex3R. Probes used for RNase protection and *in situ* hybridization are shown. GFP expression in adult hermaphrodite (*smg-2*) worms: BL3345, gfpex3R: high-level GFP (*B*); BL3340, gfpex3 (*C*). (*D*) One worm each of BL3340 and BL3345 for comparison: gfpex3 (*Left*) has yellow color because of autofluorescent gut granules; gfpex3R (*Right*) has abundant GFP expression, identical to *vit/gfp* (not shown). *A–D* are at approximately the same magnification.

Table 1. GFP expression in transgenic strains

	smg-2			*smg+*			
Strain	++	+	−	++	+	−	PCR*
gfpex3							
BL3314	0	7	13	0	0	20	
	0	15	11				
BL3315	0	17	3	0	0	20	
	0	6	21				
BL3316	0	11	9	0	0	20	
	0	15	10				
BL3307				0	0	50	Y
BL3310				0	0	40	Y
BL3312				0	0	32	Y
BL3308				0	0	50	Y
BL3313				0	0	167	Y
BL3309				0	0	77	Y
gfpex3R							
BL3324	16	4	0	0	10	10	
BL3325	15	2	2	0	5	15	
BL3326	19	1	0	12	8		
BL3306				0	0	25†	
BL3320				0	0	9	
BL3321				0	40	34	
BL3322				0	25	27	

Zeiss Axioskop was used to score worms for fluorescence as (++) strong, (+) weak, or (−) no intestinal GFP expression. The number of worms in each category is listed for each strain. *Smg+* transgenic strains arose from germ-line injection into N2 (BL3306–3310, BL3312–3313, BL3320–3322) or by outcrossing *smg-2* strains BL3314–3316 and BL3324–3326 with wild type (N2).

*Six nonexpressing gfpex3 strains were checked by PCR to verify that they contained the inejcted plasmid DNA (Y).

†Some GFP expression was seen in this strain although the frequency was not recorded.

(11). Our results were identical to those of Morrison *et al.* (11), verifying the normal operation of the Smg pathway (not shown). We infer that exon 3-containing RNA may be sequestered from SMG-2, which presumably acts in the cytoplasm similar to its homolog UPF1 in yeast (7). In the following experiments, we tested the hypothesis that exon 3 inclusion causes nuclear retention and thereby prevents degradation by the Smg system.

Exon 3 Insertion into a Reporter Gene UTR Reduces Expression. The 216-bp exon 3 was inserted in both orientations into the 3′ UTR of a *vit/gfp* reporter to generate gfpex3 and gfpex3R (antisense insert) plasmids (Fig. 2*A*). Transgenic GFP expression (Fig. 2 *B–D*) controlled by the vitellogenin promoter was confined to adult hermaphrodite intestines, as expected (11). Fig. 2 *B* and *C* shows transgenic worms from gfpex3R (*B*) and gfpex3 (*C*) strains; Fig. 2*D* compares the two: the intestine of a very low expressing strain (gfpex3) is to the left of one expressing GFP at a high level (gfpex3R). The gfpex3 worm is indistinguishable from a nontransgenic strain (only yellow, punctate gut granules fluoresce). In contrast, the gfpex3R intestine is filled with diffuse GFP, obscuring the gut granules (identical to *vit/gfp*). Strikingly, levels were much lower in all gfpex3 strains than in gfpex3R controls (Table 1). The lowered GFP levels were apparent in the intensity of fluorescence, the percentage of transgenic animals with GFP, and the extent of the GFP-expressing regions.

GFP Levels Are Higher in *smg-2* Strains. Initially, the two constructs were injected into wild-type (*smg+*) worms and the levels of expression were low even in gfpex3R and undetectable in gfpex3 (Table 1). Subsequently, a second set of strains was made by injections into *smg-2* mutants. Levels of GFP were higher, yet the dramatic difference between the GFP levels with the two constructs remained. Because the gfpex3 RNA is primarily nuclear (see below) and the Smg pathway most likely

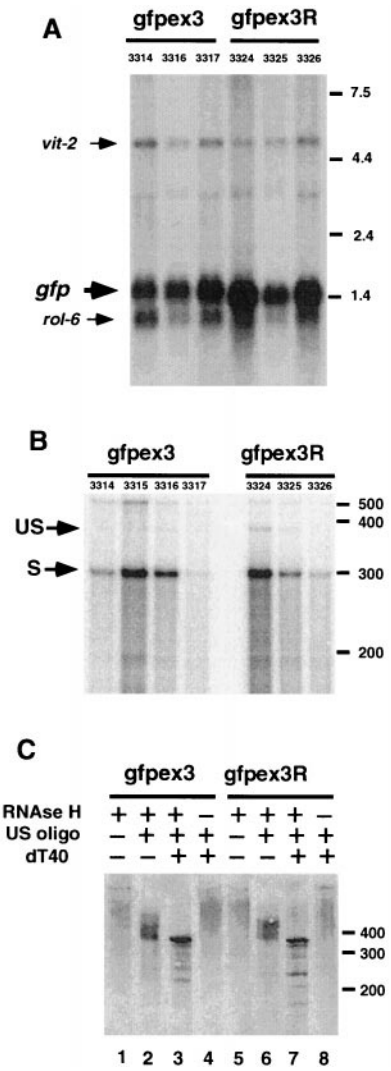

FIG. 3. Gfpex3 RNA is spliced and polyadenylated. (*A*) Northern blot analysis of total RNA from three strains each of gfpex3 and gfpex3R. The blot was probed sequentially with *gfp*, *vit-2*, and *rol-6*. Ratios of signals from the three probes: *gfp/(vit-2)(rol-6)* (gfpex3 strains) BL3314, 2.2; BL3316, 5.7; BL3317, 3.9; (gfpex3R strains) BL3324, 19; BL3325, 6.5; BL3326, 4.8. (*B*). Splicing of gfpex3 RNA analyzed by RNase protection of DNased total RNA from four strains of gfpex3 and three strains of gfpex3R, hybridized to a ^{32}P-labeled RNA probe diagrammed in Fig. 2*A*. Unspliced RNA protects a product of 380 nt (US), and spliced RNA protects products of 297 nt (S) and 43 nt (not visible). (*C*) 3′ End formation of gfpex3 RNA. DNased total RNA from strain BL3315 (gfpex3) and BL3324 (gfpex3R) was analyzed by cleavage with RNase H in the presence of US oligo and oligo(dT)$_{40}$. The blot of separated products was hybridized with a random-primed probe to *uaf-1* exon 3. Molecular mass markers for *A–C* are RNA, with lengths expressed in kb (*A*) or nt (*B* and *C*).

acts in the cytoplasm, it must act on the small amounts of gfpex3 RNA that leave the nucleus to result in the low-level expression of GFP seen in the *smg-2* background (Table 1). When we crossed the *smg-2* strains with wild type (N2) to remove the *smg-2* mutant allele, we saw the same relative levels as with the original strains. In all cases, levels of GFP decreased in *smg+*; yet, the difference between the gfpex3 and the gfpex3R strains remained. The *smg-2* transgenic strains were used exclusively for the remainder of the work reported here. We do not know why the 216-bp of exon 3, in either orientation, inserted into *vit/gfp* 3′ UTR results in SMG-mediated decay of this mRNA. There is nothing about the sequence of either exon 3 or exon 3R that would *a priori* lead one to expect

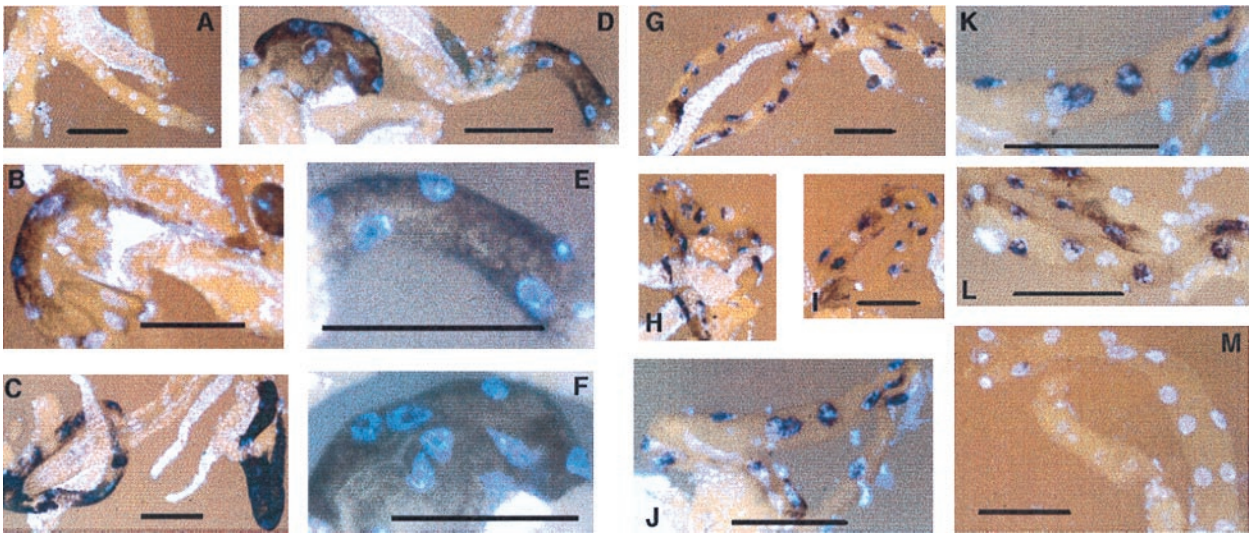

FIG. 4. Exon 3 causes nuclear retention of *vit/gfp* mRNA. Micrographs of dissected worms hybridized *in situ* with digoxigenin-labeled *gfp* probes. Light blue/whitish color is 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei; dark color is the product of alkaline-phosphatase coupled to the antidigoxigenin antibody used for detection of *in situ* hybridization. *A–M* show hybridization to the antisense probe unless otherwise indicated. Controls: N2 (wild type nontransgenic) (*A*); BL3340 (gfpex3), sense probe (*M*). Experimentals (all *smg-2*): gfpex3R–BL3345 (*B–F*); gfpex3–BL3340 (*G–L*). Bars = 100 μ. *H* and *I* are at the same magnification and share a scale bar.

that insertion between the natural *gfp* stop codon and the poly(A) site would make it subject to Smg-mediated RNA degradation. Perhaps it interrupts downstream-sequence elements required for recognition of the true chain-termination signal (19).

***gfp* mRNA Levels Are High in All Transgenic Strains.** To determine the cause of the reduced gfpex3 expression, we examined levels of *gfp* mRNA. Despite the lack of GFP in the gfpex3 strains, we found high levels of *gfp* mRNA by Northern blot analysis (Fig. 3*A*). The *gfp* mRNA levels were normalized to *vit-2* and *rol-6* controls, and the ratios (Fig. 3*A*, see legend) indicate similar levels of *gfp* RNA in gfpex3 and gfpex3R strains with very different levels of GFP. The predicted mRNA size for both constructs is 1.3 kb, [not including poly(A)], which is the approximate size calculated from the Northern blot; however, the gfpex3 mRNA appears slightly larger. The same population of molecules also was seen by a *uaf-1* exon 3 probe (data not shown), demonstrating that exon 3 was not eliminated from the *gfp* transcripts by RNA processing.

gfpex3 mRNA Is Spliced Normally. To determine whether reduced expression of gfpex3 might be a result of failure to splice the 42-bp intron, we performed RNase protection analysis of RNA isolated from transgenic strains. The probe crosses the intron and extends into the adjacent DNA 5′ of the site of transcription initiation, thus allowing discrimination between unspliced RNA and spliced RNAs, as well as any contaminating DNA. As seen in Fig. 3*B*, the predominant species of RNA is about 300 bp, as predicted for spliced RNA. Unspliced RNA was visible as a minor band in some strains, but it was most prevalent in one of the gfpex3R strains. We conclude that lack of GFP in the gfpex3 strains is not a result of failure of the mRNA to be spliced correctly.

We confirmed the above result with another experiment that analyzed the prevalence of spliced vs. unspliced *gfp* RNA. Using an oligonucleotide complementary to the 3′ splice junction, we showed that primer extension with ddG results in different-length products with spliced and unspliced RNA. The first cytosine encountered in the intron is 2 nt after the end of the primer and 5 nt for the spliced product. The results showed that the majority of the product in the gfpex3 strains was extended 5 nt as expected for spliced RNA (not shown).

gfpex3 mRNA Is Polyadenylated Normally. To ask whether the mRNA for gfpex3 is polyadenylated and whether the site of cleavage and polyadenylation is at the site predicted 10–15 bases 3′ of the AAUAAA signal, we performed an RNase H experiment [RNase H cleaves RNA/DNA duplexes (20)]. We used an oligonucleotide complementary to sequence in the 3′ UTR upstream of the insertion of exon 3, with and without oligo(dT)$_{40}$, and detected the digested RNAs with an exon 3 probe (Fig. 3*C*). In both of the strains examined, one strain each for gfpex3 and gfpex3R, digestion with the upstream oligo alone produced a collection of RNA molecules that ranged in size from about 400 to 500 nt. The addition of oligo(dT)$_{40}$, complementary to poly(A), resulted in a product of about 380 nt, as predicted for a message that is cleaved and polyadenylated just downstream of the AAUAAA site. The size of the RNA products appeared identical for the two strains, indicating that aberrant 3′ end processing of the gfpex3 message does not account for lack of GFP. However, the poly(A) tail may be longer in gfpex3, which may account for the slightly larger size of gfpex3 RNA (Fig. 3*A*). We conclude from these experiments that the transgenes from gfpex3 and gfpex3R strains both make fully mature mRNA, present at similar levels; both are spliced and polyadenylated.

gfpex3 mRNA Is Primarily Nuclear. To test whether failure to express the gfpex3 mRNA is a result of failure to transport it to the cytoplasm, we performed *in situ* hybridization to detect *gfp* mRNA in tissues of two strains bearing integrated copies of gfpex3 and gfpex3R. *In situ* hybridization of cut-worm samples of these strains with an RNA probe specific for *gfp* mRNA (Fig. 2*A*) detected RNAs confined to the intestines of the transgenic adult hermaphrodites, where it is abundant, whereas nontransgenic worms exhibited no signal (Fig. 4*A*). In the gfpex3R strain, the hybridized probe is present throughout the cytoplasm of many intestinal cells (Fig. 4 *B–F*). In sharp contrast, in gfpex3 strains, signal is clearly localized over intestinal nuclei (Fig. 4 *G–L*). There is little hybridization in cytoplasmic regions in the gfpex3 strain, although some small patches can be found (Fig. 4 *H*, *I*, and *L*), consistent with occasional small patches of GFP in the gfpex3 worms (Table 1). It was difficult to see whether hybridization in gfpex3R is confined to the cytoplasm or is in some nuclei as well because of heavy staining in many samples. In the less heavily stained gfpex3R worms (Fig. 4 *B*, *D*, *E*, and *F*), most of the nuclei are visible and are free of localized RNA. Controls hybridized with sense strand *gfp* probes, which should detect only DNA or

aberrant RNA transcribed from the opposite strand, had insignificant levels of signal, all of which is confined to nuclei. The intensity of stain with the sense-strand probe (Fig. 4*M*) is insignificant compared with that of the antisense probe in gfpex3 (Fig. 4*G*). In those cases in which nuclear staining of gfpex3 with the antisense probe is the clearest, the nuclei often are stained uniformly, but in other cases the stain occupies only one region of the nucleus (Fig. 4 *J–L*). In any event, the data of Fig. 4 make it clear that gfpex3 RNA is almost entirely nuclear, which explains why these strains do not show GFP fluorescence.

Uaf-1 RNA Interference Suggests U2AF Mediates Exon 3 Nuclear Retention. To test the hypothesis that U2AF itself binds the U_4CAG/R octamers in exon 3 to mediate nuclear retention, we performed RNAi experiments with *uaf-1* dsRNA (18). Injection of dsRNA results in a drastic reduction/elimination of the targeted mRNA and consequent loss of function. Elimination of U2AF in our system eventually should result in release of retained RNA from the nucleus (after endogenous U2AF protein levels drop) if U2AF plays a major role. In three RNAi experiments (Fig. 5), the levels of GFP increased in the injected worms in which *uaf-1* mRNA was eliminated. The amount of GFP in each case was very small; yet, in these blind-scored experiments, injection of ds U2AF RNA resulted in large increases in the percentage of animals scored as expressing GFP. In all three experiments, after injection, the percentage of GFP-positive worms was increased dramatically in individuals treated with ds *uaf-1*. Full-length intestinal GFP was also more frequent after *uaf-1* RNAi. Controls were injected with buffer (TE) or with an unrelated dsRNA [specific to a component of 3′ end processing, CstF64, (21)]. In all of these experiments, the dsRNA for these essential genes was 100% effective in producing embryonic lethality in progeny of the injected worms. RNA specific for *uaf-2*, the gene encoding U2AF[35] (33), also stimulated GFP but was less effective than *uaf-1*. This result is consistent with both subunits participating in RNA binding (22). Injections with mixtures of RNA from *uaf-1* and *uaf-2* were not dramatically different from *uaf-1* alone.

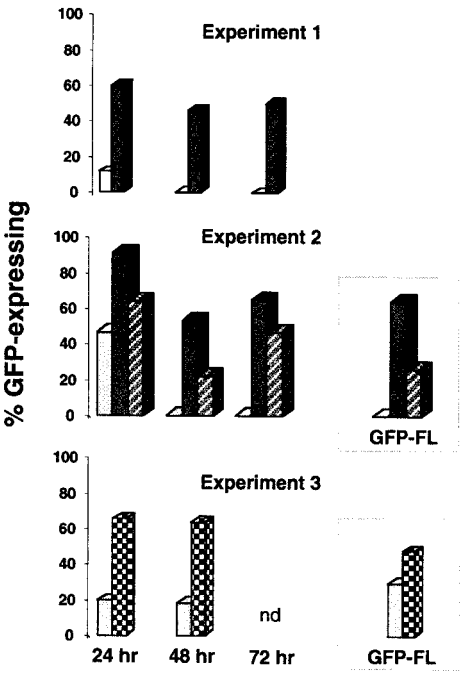

FIG. 5. U2AF reduction by RNA interference increases GFP expression in gfpex3 strains. dsRNA (1 μg/μl) was injected in BL3340 (gfpex3), and surviving worms were scored blindly for fluorescence at 24, 48, and 72 hr after injection. Experiment 1: Young adults were injected in the distal gonad, and numbers of worms represented by each bar were 16, 15, 12, 13, 7, and 10, respectively. Control: TE buffer. Experiments 2 and 3: Most injected worms were L4 larvae, followed by young adults. Most frequent site of injection was intestine, followed by gonad and pseudocoelom. Controls: CstF64 RNAi. Experiment 2: Numbers of worms for each bar were 9, 20, 15, 8, 16, 14, 9, 18, 16, 9, 20, and 16, respectively. Experiment 3: Numbers of worms for each bar were 34, 29, 29, 28, 34, and 29, respectively. Boxed graphs represent percentages of the same worms that fluoresced in a long intestinal region from anterior to posterior (full-length: GFP-FL). Open bar, TE; lightly shaded bars, CstF64; darkly shaded bars, *uaf-1*; striped bars, *uaf-2*; checkered bars, *uaf-1* + *uaf-2* dsRNAi.

DISCUSSION

***uaf-1* Exon 3-Containing RNA Is Not Degraded by the Smg RNA Surveillance System.** There are two alternatively spliced *uaf-1* RNAs: the smaller (1.7 kb) encodes U2AF[65], whereas the larger, exon 3-containing RNA (1.9 kb), contains a PTC (5). Nevertheless, the Smg pathway for mRNA surveillance (6) does not affect the levels of the latter RNA. It is likely that this pathway acts in the cytoplasm at the level of translation where the mechanisms exist to detect premature termination signals. *Smg-2* is homologous to the *S. cerevisiae* UPF1 gene whose product is required for nonsense-mediated decay in yeast and has been localized to the cytoplasm on polyribosomes (7). In vertebrates, decay is also dependent on ribosomes, leading to the hypothesis that events in the nucleus and cytoplasm are somehow coupled (23). The simplest interpretation of our result that *uaf-1* exon 3-containing RNA is not subject to Smg-mediated decay is that it is inaccessible because it is retained in nuclei.

Mature gfpex3 mRNA Is Present yet Not Expressed as GFP. We tested the ability of exon 3 to cause nuclear retention of an RNA by inserting it into the 3′ UTR of a *gfp* reporter gene. In contrast to the control strains containing exon 3 in reversed orientation, where GFP expression was high, GFP levels were severely reduced with exon 3. Because the transcript contains 10 repeats of the U_4CAG/R 3′ splice-site consensus, we postulated that it is those repeats that impart exon 3's activity. These potential sites for spliceosome binding raise the possibility that the pre-mRNAs might be aberrantly spliced or polyadenylated; therefore, we characterized the RNA from gfpex3 and gfpex3R strains. Gfpex3 worms make abundant, exon 3-containing *gfp* mRNA from which the small intron has been spliced and that has poly(A) added at the normal site. Although the poly(A) tails may be somewhat longer, we believe this could result from their presence in the nucleus. The normal abundance and processing of gfpex3 mRNA suggest that another mechanism is required to account for its lack of expression. Because we see a correlation between the low-level GFP and small amounts of mRNA in the cytoplasm of gfpex3 strains, translation is apparently normal.

gfpex3 mRNA Is Nuclear-Localized. We investigated the localization of *gfp* RNA in strains of gfpex3 and gfpex3R by *in situ* hybridization and found dramatic differences. In the gfpex3 strains, nuclei contain high levels of the *gfp* RNA, whereas it is distributed throughout the cytoplasm in gfpex3R strains. All intestinal nuclei in gfpex3 worms are not stained uniformly, but the resolution of our analysis did not allow us to address the question of subnuclear localization. We have interpreted the nuclear localization as a result of nuclear retention but actually have no evidence that the RNA is not participating in export and import processes. If the RNA is shuttling, however, the net result is that the majority of the gfpex3 RNA is in nuclei.

How Is Nuclear Retention Accomplished? Normally, pre-mRNAs do not reach the cytoplasm. They are bound by various components of the splicing machinery resulting in spliceosome formation and subsequent intron removal; the presence of bound splicing factors may well prevent the export

of pre-mRNAs from the nucleus (24–28). Nuclear export does not result simply from the removal of splicing factors; other factors bound to mRNAs are also required to mediate their cytoplasmic transport [including some of the shuttling SR proteins, heterogeneous nuclear RNP (hnRNP) A1 and TAP] as well as the nucleoporins and the RAN/GTP system (28–31). SR proteins are also involved in splicing and splice-site selection (32), yet there must be a more complicated scenario than simple competition, such as cooperative binding of both types of factors, altered by splicing events in a way that allows bound nuclear export signals to be recognized. Such cooperative binding could involve exon-bound SR proteins and other splicing factors known to influence splice-site choice and intron removal and prevent the SR protein from participating in shuttling and nuclear export until the spliceosome is released. According to this model, it is not the presence of an intron that keeps a pre-mRNA in the nucleus, but rather the presence of bound splicing factors that prevent the full productive association of mRNA with proteins required for nuclear export.

The 10 3′ splice-site repeats (U_4CAG/R) of *uaf-1* exon 3 led us to propose that a splicing factor can bind those sequences (5). Indeed, we have suggested that U2AF itself may recognize and bind to the repeats, and our RNAi experiments indicate that reducing U2AF levels allows the release of some gfpex3 mRNA from the nucleus. Whether it is U2AF alone or in conjunction with other splicing components, the result is an abortive spliceosome because exon 3 is not removed from these RNAs. We suggest that this is the mechanism that keeps exon 3-containing RNAs in the nucleus. The proteins bound to exon 3, including U2AF, remain bound, and prevent the action of components (hnRNPs, SR proteins) required for nuclear export.

What Is the Role of the U2AF RNA Containing Exon 3? Our results suggest that the alternatively spliced *uaf-1* RNA is retained in the nucleus, where it could perform a regulatory role. The level of the 1.9-kb RNA increases when worms are starved, suggesting that when transcription decreases as a response to starvation, the need for splicing factors decreases and that this is a way of down-regulating functional mRNA levels for *uaf-1* (5). If this were the only function of the alternative splice, we might expect that that RNA would be degraded. However, the persistence of exon 3-containing RNA suggests that it could have a function; it might serve as a sink for the molecules that recognize the 3′ splice site. The presence of exon 3 with its multiple repeats of U_4CAG/R may provide a binding site for free splicing factors. Indeed, as we suggested above, nuclear retention was accomplished by binding of such splicing factors. During conditions such as starvation, in which transcription levels and, concomitantly, the need for splicing are reduced, free splicing factors could associate with exon 3 and be available as a resource that allows the cell to respond quickly when conditions improve. If, indeed, exon 3 functions in this way to maintain a reserve of U2AF, then it makes sense for the RNA to be retained in the nucleus, where splicing takes place.

Potential Uses of the Nuclear Retention Sequence. We have added a 216-bp sequence to a message, outside its coding sequence, and achieved a dramatic result, namely, nuclear retention of an mRNA. We imagine that this phenomenon could prove useful in other molecular and cellular contexts. Cells are engineered frequently to contain exogenous genes and other molecules; having the ability to target and retain a nucleic acid in the nucleus could prove useful in some situations.

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