Coupling of Transcription and Translation in *Dictyostelium discoideum* Nuclei†

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ABSTRACT: The nuclei of *Dictyostelium discoideum* cells have been found to contain polyribosomes active in protein synthesis. mRNA molecules enter nuclear polyribosomes while they are still being synthesized. "Non sense mediated mRNA decay" occurs in the nucleus, through the interaction of the mRNAs containing a nonsense codon with newly formed nuclear ribosomes, rather than with cytoplasmic ribosomes, as previously generally supposed.

Transcription and translation of mRNAs in prokaryotes are coupled (*1, 2*). It has been assumed for decades that in eukaryotes this coupling cannot exist, since transcription occurs in the nucleus and translation in the cytoplasm. We have recently found that newly assembled 40S and 60S ribosomal subunits still retained in the nuclei of *Dictyostelium* cells are fully active in protein synthesis in vitro (*3*). Furthermore, cytoplasmic ribosomal subunits still containing immature RNA enter polyribosomes with the same efficiency as mature ribosomal subunits (*4*). This has suggested the possibility that transcription and translation may be coupled even in the nuclei of eukaryotes.

The experiments reported here show that in *Dictyostelium* nascent molecules of specific mRNAs are included in nuclear polyribosomes active in protein synthesis. They also show that the destabilization of mRNAs containing internal nonsense codons ("non sense mediated mRNA decay"), which had been postulated to require the interaction of mRNAs with cytoplasmic ribosomes (*5*−*7*), occurs in *Dictyostelium* while the mRNAs are still bound to newly formed nuclear ribosomes.

EXPERIMENTAL PROCEDURES

Cell Growth and Development. Dictyostelium discoideum strain AX2 was grown and allowed to develop as previously described (*8*).

RNA and Protein Labeling. Cells were labeled during development at the stage of first finger (12 h after starvation) with [^{3}H]uracil to label newly formed nuclear ribosomes, with [^{32}P]orthophosphate to label preferentially nascent chains of RNA, and with [^{35}S]methionine to label newly synthesized or nascent polypeptide chains. To label cytoplasmic ribosomes, [^{14}C]uracil was added to developing cells 2 h after starvation; 8 h later, 10 mM cold uracil was added, and cells were harvested and lysed after 2 more h. Labeling conditions are further detailed in the text and in figure legends.

Nuclei Isolation. Cells were lysed by vortexing at 0 °C in 50 mM HEPES−KOH, pH 7.5, 5 mM MgOAc, 10% sucrose, and 2% Tergitol at 10^8 cells/mL. Nuclei were isolated by differential centrifugation in an Eppendorf minifuge as described in ref *3*. The purity and the integrity of the nuclei preparation were indicated by the fact that nuclei did not contain any detectable ^{14}C label when derived from cells labeled for 8 h with [^{14}C]uracil and chased for 2 h with 10 mM cold uracil, while cytoplasm did not contain any ^{3}H-labeled 36S RNA (the large precursor of ribosomal RNA) when cells had been labeled with [^{3}H]uracil for 30 min.

Polyribosome Isolation from Nuclei. Nuclei were lysed in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 50 mM KCl. The lysate was loaded on an 11 mL sucrose gradient (15−35%) made in the same buffer and centrifuged in an SW40 rotor in a Beckman ultracentrifuge at 33 000 rpm for 15 h at 4 °C. Fractions were collected with a polystaltic pump and analyzed as described in the figure legends. The 80S peak in Figure 1A was identified on the basis of several considerations: (a) the peak disappeared if cells were treated with puromycin 10 min before lysis or if the sucrose gradient was made in 0.2 mM $MgCl_2$ (see Results); (b) the major peak contained both pre-17S and pre-26S ribosomal RNAs, while the first peak of the gradient contained only pre-17S RNA and the second peak contained only pre-26S RNA.The 80S peak in Figures 3 and 5 was identified by analogy with Figure 1A.

Ribosomal RNA Analysis. RNA was extracted from ribosomal subunits and 80S monosomes isolated through the gradients shown in Figure 1A,B by Ultra-spec II RNA and was analyzed by gel electrophoresis by the procedure described in ref *3*.

DNA Clones. Gene GM27 was cloned by G. Mangiarotti and sequenced by S. Chung. Its expression was studied in ref *9*. Gene SC253 was cloned and sequenced by S. Chung, and its expression was studied in ref *10*. Gene Actin15 was cloned and sequenced in ref *11*. Gene AC914 was cloned and sequenced by A. Ceccarelli, and its expression was studied in ref *12*. For the experiment shown in Figure 4, the segment of each gene corresponding to the 5′UTR, the coding region, and the 3′UTR was subcloned (*13*). For the experiment of Figure 5, a c-myc oligonucleotide (*14*) was inserted

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in-frame in the coding region of each gene to allow detection only of the mRNA transcribed from the plasmid.

DNA Dot Hybridization. It was carried out as described in ref *8*.

RESULTS

Nuclei Contain Polyribosomes Active in Protein Synthesis. The distribution in the nucleus of four specific mRNAs, AC914 and GM27, which are pre-spore-specific, SC253, which is pre-stalk specific, and Actin15, which is common to both pre-spore and pre-stalk cells (*15*), was examined. Developing cells were labeled with [^{32}P]orthophosphate for 15 min, to label preferentially nascent chains of RNA, and with [^{3}H]uracil for 30 min, to label preferentially newly assembled ribosomal subunits, still retained in the nuclei (*3, 4, 16*). The nuclear lysate was fractionated on a sucrose gradient. The dot DNA hybridization analysis made on the fractions of the gradient and reported in Figure 1A shows that no ^{32}P-labeled mRNA was found free in the nucleoplasm; all sedimented with structures of 200−250 S, detected by the [^{3}H]uracil labeling. If 1 mg/mL puromycin was added to cells 10 min before lysis, the ^{3}H-labeled structures sedimented as expected for free ribosomal subunits, while the analyzed mRNAs (labeled with ^{32}P) sedimented at about 10−12 S (Figure 1B). The free ribosomal subunits contained immature pre-17S and pre-26S RNAs as expected for newly assembled particles (*3*) (Figure 2).

The same result shown in Figure 1B was observed if 200 μg of pactamycin was added to cells 10 min before lysis, or if the Mg^{2+} concentration in the sucrose gradient was lowered to 0.2 mM (data not shown).

In an experiment similar to the one shown in Figure 1A, cells were labeled with 100 μCi [^{35}S]methionine for 10 min before lysis, rather than with [^{32}P]orthophosphate. Most of the TCA-precipitable ^{35}S-label was found to sediment at the top of the sucrose gradient, but 25−30% of it cosedimented down the gradient with the ^{3}H-label (Figure 3A). The latter fraction of ^{35}S-label disappeared from the gradient if the [^{35}S]methionine pulse was followed by a 10 min chase with 10 mM unlabeled methionine before cell lysis (Figure 3B) or if cells were treated with 1 mg/mL puromycin for 5 min after the 10 min pulse (data not shown). The ^{35}S-labeled molecules cosedimenting with polyribosomes were very likely nascent polypeptide chains.

The reported data indicate that the test mRNAs were incorporated in nuclear polyribosomes active in protein synthesis.

Nascent mRNAs Are Included in Nuclear Polyribosomes. To verify whether the mRNAs labeled with a 15 min pulse of [^{32}P]orthophosphate and found in polyribosomes were completed molecules or nascent chains, the mRNAs extracted from nuclear polyribosomes were hybridized to spotted DNA molecules containing segments of the genes corresponding to the 5′ untranslated region, to the coding region, and to the 3′ untranslated region of the mRNAs. While the mRNAs hybridized strongly with the first two types of DNAs, they hybridized very poorly with the third type of DNA (Figure 4A). In a control experiment, cells were labeled with [^{32}P]orthophosphate for 90 min, and the labeled RNA was extracted from the cytoplasm. Each of the four test mRNAs reported in Figure 4A hybridized strongly also with the DNA

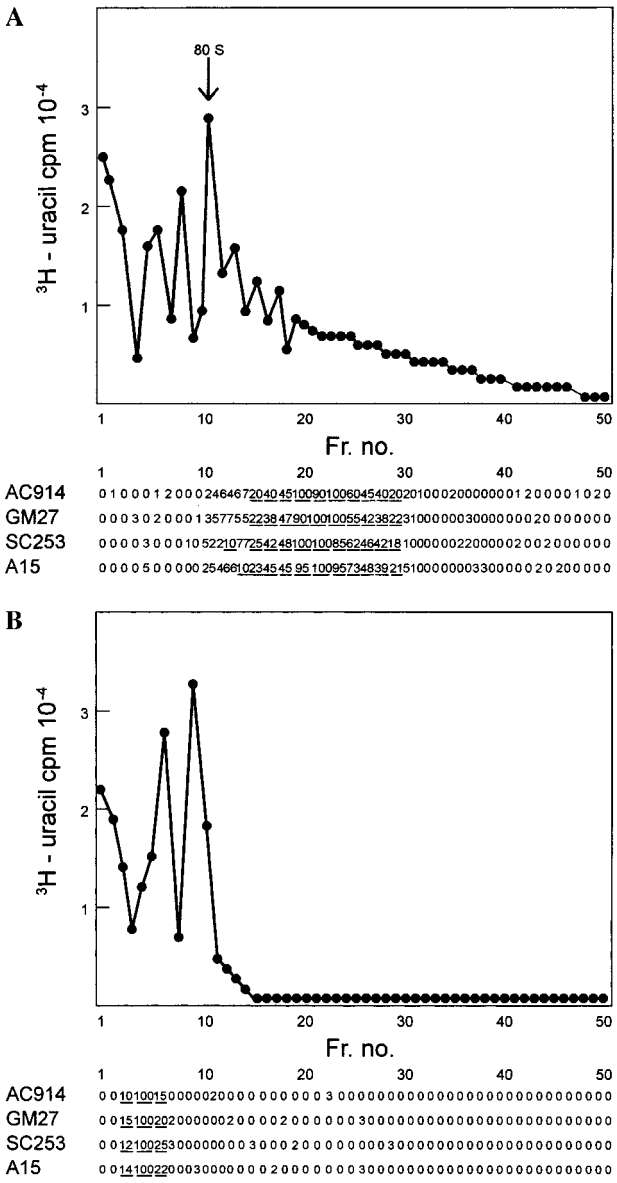

FIGURE 1: Distribution on a sucrose gradient of mRNAs contained in nuclei. AX2 cells at the first finger stage were labeled with 200 μCi of [^{3}H]uracil for 30 min to allow the detection of newly formed ribosomal subunits and with 1 mCi of [^{32}P]orthophosphate for 15 min to allow the detection by hybridization of newly synthesized mRNA molecules. In the experiment described in panel B, cells were treated with 1 mg/mL puromycin before being lysed. The nuclear lysate was centrifuged on a sucrose gradient as described under Experimental Procedures. The ^{3}H-label present in each fraction was determined in a Kontron scintillation counter. 2 μg of DNA for each indicated clone was spotted onto Amersham Hybond-N paper and hybridized to each fraction of nuclear RNA after extraction with Ultraspec II RNA. The amount of ^{32}P present in each hybridization spot was determined with a Bio-Rad Phosphoimager and is indicated in arbitrary units by a number under each fraction.

segments corresponding to the 3′ untranslated region of the mRNAs (Figure 4B).

The results shown in Figure 4 indicate that most of the ^{32}P-labeled mRNAs found in nuclear polyribsomes were uncompleted RNA molecules. This finding may be surprising because the length of the ^{32}P-pulse (15 min) is certainly greater than the time required to synthesize an entire mRNA chain. However, the incorporation of [^{32}P]orthophosphate into RNA is very slow in the first 10−12 min of labeling, due to

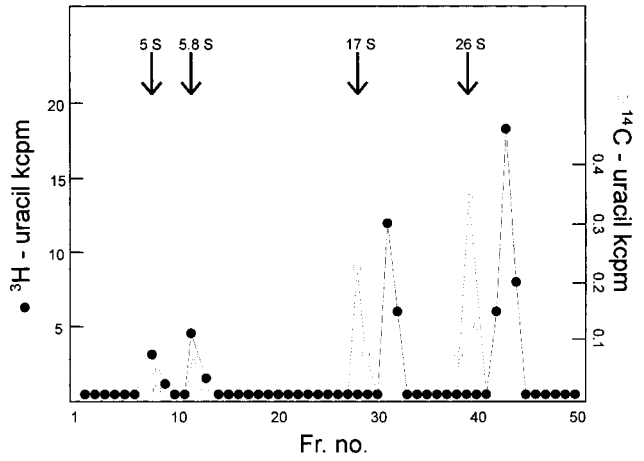

FIGURE 2: Gel electrophoretic analysis of the RNA contained in the 40S and 60S subunits isolated through the sucrose gradient shown in Figure 1B. The subunits were pooled, and the RNA was extracted and analyzed as described under Experimental Procedures.

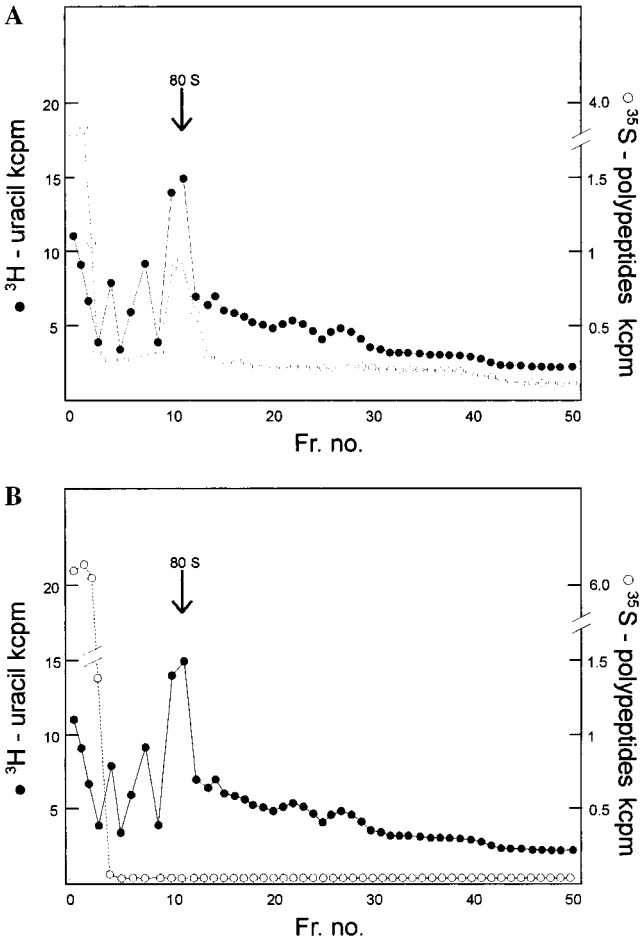

FIGURE 3: Distribution along a sucrose gradient of ^{35}S-polypeptides contained in a nuclear lysate derived from cells labeled for 30 min with [^{3}H]uracil and for 5 min with [^{35}S]methionine (A). In (B), the 5 min pulse with labeled methionine was followed by a 10 min chase with unlabeled methionine before cell lysis. (●) [^{3}H]Uracil; (○) TCA-precipitable ^{35}S-polypeptides.

the presence of a large pool of cold ribonucleotides in the cell [developing cells degrade ribosomes synthesized during growth, while synthesising new ribosomes (*17*−*19*)]. The rate of incorporation then increases exponentially until it reaches a plateu at about 30−40 min. Most of the ^{32}P-label found in the mRNAs chains analyzed in Figure 4A is

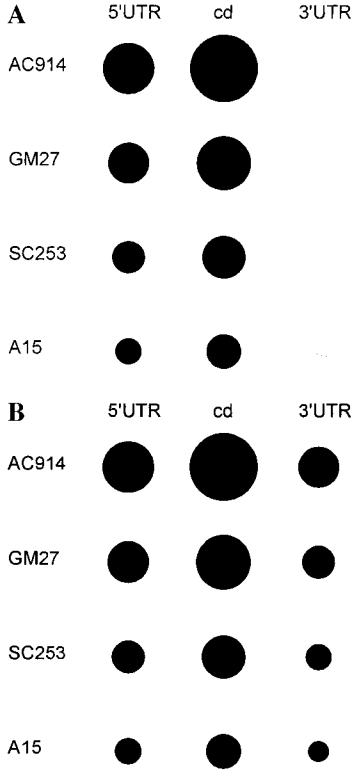

FIGURE 4: Determination of the size of several species of mRNA contained in nuclear polyribosomes. DNA segments corresponding to the 5′UTR, the coding region (cd), and the 3′UTR of the indicated genes were subcloned. 2 μg of each DNA was hybridized to the ^{32}P-labeled RNA localized on the sucrose gradient described in Figure 1A, after extraction with Ultraspec II RNA. The amount of ^{32}P present in each hybridization spot was determined with a Bio-Rad Phosphoimager (A). In (B), the ^{32}P-labeled RNA was extracted from the cytoplasm of cells labeled for 90 min.

therefore present in molecules whose synthesis probably started in the last 2−3 min before cell lysis. Since all four test mRNAs have a 5′UTR of 4−600 nucleotides and a coding region longer than 3000 nucleotides, several minutes are required before an RNA polymerase can synthesize an entire chain of mRNA.

The incompleted mRNA chains present in nuclear polyribosomes had probably been released from the template DNA by the detergent used to lyse cells. To support this interpretation, in a control experiment cells were lysed in 0.5% Tergitol, rather than in 2% Tergitol. The lysis was poor, but nuclei were obtained in a sufficient amount to be analyzed on a sucrose gradient; 30% of the ^{3}H-label (ribosomes) and 90% of the ^{32}P-labeled test mRNAs sedimented at the bottom of the tube. If the nuclear lysate was treated with 1 mg/mL DNase I for 10 min before the sucrose gradient centrifugation, a pattern very similar to the one reported in Figure 1A was obtained (data not shown).

Destabilization of mRNAs Containing Internal Nonsense Codons Occurs in Nuclei. The finding that nuclear mRNAs are already associated with polyribosomes suggested the possibility that the destabilization of mRNAs containing internal nonsense codons may not necessarily occur after the mRNAs have entered partially or completely the cytoplasm (*7*), but while they are still in the nucleus. To explore this possibility, a nonsense mutation was introduced about 250 nucleotides 3′ to the first AUG codon of each of the 4 test mRNAs (*13*). This position was chosen not only for technical

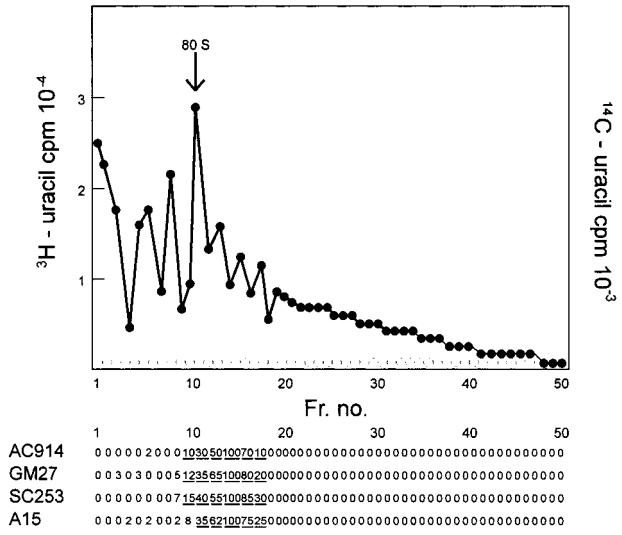

FIGURE 5: Interaction of nuclear mRNAs containing internal non sense codons with nuclear ribosomes. Nuclear RNA was labeled with 200 μCi of [^{3}H]uracil for 30 min to allow detection of newly formed ribosomal subunits and with 5 mCi of [^{32}P]orthophosphate for 15 min to allow detection by hybridization of newly synthesized mRNAs. To allow detection of cytoplasmic ribosomes eventually attached to nuclei, cells were prelabeled with 100 μCi of [^{14}C]uracil for 8 h and chased for 2 h with 10 mM cold uracil before being lysed. The nuclear lysate was centrifuged on a sucrose gradient as in Figure 1. The ^{3}H- and ^{14}C-labels present in each fraction were counted in a Kontron scintillation counter. After extraction with Ultraspec II RNA, each fraction of the gradient was hybridized to 2 μg of the c-myc oligonucleotide utilized to make the mRNA coded by the plasmid distinguishable from the endogenous mRNA. The amount of ^{32}P-label present in each hybridization spot was determined with a Bio-Rad PhosphoImager and is indicated in arbitrary units by a number under each fraction. The graph represents the data obtained with cells transfected with the AC914 clone containing the c-myc oligonucleotide. The patterns obtained with the other three clones were superimposable. (●) [^{3}H]uracil; (○) [^{14}C]uracil.

reasons, but also because the nonsense codon-mediated decay is known to require that the nonsense codon is located in a region proximal to the 5′ end of the mRNA (*6*, *7*). All four genes analyzed in this paper contain only two small introns (100−200 nucleotides) in the middle of the coding region. Therefore, in all four mutated mRNAs, the nonsense codon precedes the first intron. To recognize the mutated mRNAs, a c-myc oligonucleotide (*14*) was inserted in-frame in their coding region. The four mutated mRNAs were found only in the nucleus, and not in the cytoplasm, even if the labeling time with [^{32}P]orthophosphate was extended to 1 h (data not shown). A pulse−chase experiment with ^{32}P (*20*) showed that they decayed with a half-life of less than 12 min (data not shown), about 40-fold faster than the wild-type mRNAs (*8*). Most importantly, the mutated mRNAs were associated only with nuclear ^{3}H-labeled ribosomes. No cytoplasmic ^{14}C-labeled ribosomes were associated with intact nuclei or with the polyribosomes containing the mutated mRNAs (Figure 5).

DISCUSSION

The data reported in Figure 1A,B and 3 clearly show the presence of polyribosomes active in protein synthesis in the nuclei of *Dictyostelium* cells. The ribosome aggregates that sediment along the sucrose gradient are not aspecific: they contain mRNA molecules and nascent polypeptide chains and are dissociated by puromycin, an inhibitor of protein synthesis which competes with the binding of aminoacyl-tRNA to ribosome, and their formation is prevented by pactamycin, which inhibits the movement of 40S subunits from the Cap to the first AUG codon. The presence of polyribosomes in the nuclei could not be confirmed by electron microscopy because the degree of resolution obtained was insufficient.

Obviously, the finding reported here cannot be extended "a priori" to other eukaryotic organisms. In yeast and in vertebrates, no microscopic evidence for the presence of translationally active nuclear ribosomes has been reported, though biochemical indications have been obtained that protein synthesis may occur within the nucleus (*21*). Two circumstances may favor the accumulation of polyribosomes in *Dictyostelium* nuclei: the newly assembled ribosomal subunits still containing immature RNA are functional in protein synthesis (*3*, *4*), and newly synthesized mRNA molecules usually spend several hours in the nucleus before entering the cytoplasm (*15*), despite the fact that introns are very rare.

The presence of active nuclear polyribosomes is a prerequisite for the coupling of transcription and translation. The existence of this phenomenon is supported by the data reported in Figure 4A, which show that the four test mRNAs enter polyribosomes while they are still incomplete. Since this is true for most of the detected mRNA molecules, it is very unlikely that these represent molecules which enter polyribomes after having detached incidentally from the template DNA. It seems reasonable to admit that mRNA molecules enter polyribosomes while they are still being synthesized.

The findings reported above also suggest a new mechanism for the non-sense-mediated mRNA decay. This process is generally considered to require the recognition of the internal nonsense codon by a translating ribosome. While in yeast there is evidence that the mRNA decay occurs in the cytoplasm (*5*), in vertebrates most nonsense mRNAs are degraded while maintaining an association with nuclei (*6*). To explain the latter finding, it has been postulated that the nonsense codon is recognized by a cytoplasmic ribosome which joins the mRNA molecule while it is exiting from the nucleus through a nuclear pore. The data reported in Figure 5 show that in *Dictyostelium* mRNAs containing a nonsense codon are degraded inside the nuclei while they are connected with nuclear ribosomes, in the absence of any interaction with cytoplasmic ribosomes. The nonsense codon must therefore be recognized by a nuclear ribosome.

Many authors have reported that the presence of a nonsense codon in a pre-mRNA molecule affects its splicing (*22*−*32*). The recognition of the nonsense codon by a nuclear ribosome could be the basis of this phenomenon. Other authors, however, have found that the splicing of other pre-mRNAs containing a nonsense codon occurs normally (*6* and references cited therein). The introns contained in the pre-mRNAs I have studied are too short to accumulate enough label to allow their detection by hybridization. Other techniques shall be used to determine whether the rapidly decaying mRNAs have been correctly spliced or not. Furthermore, more mutated genes shall be tested to obtain a statistically relevant answer.

Evidence for nonsense codon recognition occurring in the nucleus and affecting pre-mRNA splicing has recently been obtained for the T-cell receptor-β pre-mRNA (*33*).

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