RNA (2001), *7*:445–456. Cambridge University Press. Printed in the USA.

Mammalian heat shock p70 and histone H4 transcripts, which derive from naturally intronless genes, are immune to nonsense-mediated decay

LYNNE E. MAQUAT and XIAOJIE LI

Department of Biochemistry and Biophysics, School of Medicine and Dentistry, University of Rochester, Rochester, New York 14642, USA

ABSTRACT

Nonsense-mediated decay (NMD), also called mRNA surveillance, is an evolutionarily conserved pathway that degrades mRNAs that prematurely terminate translation. To date, the pathway in mammalian cells has been shown to depend on the presence of a *cis*-acting destabilizing element that usually consists of an exon–exon junction generated by the process of pre-mRNA splicing. Whether or not mRNAs that derive from naturally intronless genes, that is, mRNAs not formed by the process of splicing, are also subject to NMD has yet to be investigated. The possibility of NMD is certainly reasonable considering that mRNAs of *Saccharomyces cerevisiae* are subject to NMD even though most derive from naturally intronless genes. In fact, mRNAs of *S. cerevisiae* generally harbor a loosely defined splicing-independent destabilizing element that has been proposed to function in NMD analogously to the spliced exon–exon junction of mammalian mRNAs. Here, we demonstrate that nonsense codons introduced into naturally intronless genes encoding mouse heat shock protein 70 or human histone H4 fail to elicit NMD. Failure is most likely because each mRNA lacks a *cis*-acting destabilizing element, because insertion of a spliceable intron a sufficient distance downstream of a nonsense codon within either gene is sufficient to elicit NMD.

Keywords: heat shock gene; histone gene; immunity to nonsense-mediated mRNA decay; naturally intronless genes

INTRODUCTION

Nonsense-mediated decay (NMD), also called mRNA surveillance, typifies all organisms that have been examined, including *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Homo sapiens* (for reviews, see Maquat, 1995, 2000; Li & Wilkinson, 1998; Culbertson, 1999; Hentze & Kulozik, 1999; Jacobson & Peltz, 2000). NMD eliminates mRNAs that harbor premature termination codons, presumably to preclude the synthesis of proteins that acquire new functions or are otherwise deleterious to cellular metabolism. Premature termination codons can arise as the consequence of genomic mutations or routine abnormalities in gene expression, such as inefficient or inaccurate splicing. Most mammalian genes harbor at least one intron, and studies of genes of this type indicate that NMD requires both splicing of product pre-mRNA and translation of product mRNA (Cheng et al., 1994; Carter et al., 1996; Moriarty et al., 1998; Zhang et al., 1998a, 1998b; Thermann et al., 1998; Sun & Maquat, 2000). Generally, a nonsense codon elicits NMD when followed by a splicing-generated exon–exon junction that resides more than 50–55 nt downstream (Nagy & Maquat, 1998, and references therein). This finding has led to the hypothesis that the process of pre-mRNA splicing deposits signature proteins at or near the exon–exon junctions of product mRNA, and these proteins remain bound to mRNA so as to influence translating ribosomes during or after translation termination. In support of this model, studies using HeLa-cell nuclear extract and in vitro-synthesized transcripts have found that a splicing-dependent complex of proteins binds near exon–exon junctions and remains bound after spliceosome dissociation, providing evidence that splicing does influence mRNP structure (Le Hir et al., 2000a, 2000b). Furthermore, mRNAs that derive from intron-containing pre-mRNAs have been shown in *Xenopus laevis* oocytes

Reprint requests to: Lynne E. Maquat, Department of Biochemistry and Biophysics, School of Medicine and Dentistry, University of Rochester, Rochester, New York 14642, USA; e-mail: lynne_maquat@urmc.rochester.edu.

to be either more efficiently translated (Matsumoto et al., 1998) or more efficiently transported from nuclei to the cytoplasm (Lou & Reed, 1999; Zhou et al., 2000) than their intronless counterparts, indicating that splicing-induced alterations to mRNP can influence downstream events. Consistent with the idea that splicing plays a critical role in NMD, the removal of all introns from the gene for either classical glutathione peroxidase 1 or triosephosphate isomerase results in mRNA that is immune to NMD (Moriarty et al., 1997; Zhang et al., 1998a).

A relatively small number of mammalian genes are notable because they naturally lack introns. Because the one or more introns of intron-containing genes are important for pre-mRNA stability, RNA 3′-end formation, and mRNA export to the cytoplasm (see, e.g., Ryu & Mertz, 1989; Morimoto, 1998; and references therein), naturally intronless genes are characterized by features that bypass the need for introns. For example, mouse histone H2a mRNA harbors a coding-region determinant that permits efficient cytoplasmic accumulation of intronless transcripts by promoting nuclear export (Huang et al., 1999). mRNAs from the naturally intronless thymidine kinase genes of herpes simplex virus and hepatitis B virus are characterized by similar determinants (Huang et al., 1999). In theory, mRNAs produced from naturally intronless genes may be subject to an intron-independent NMD pathway. If so, then a yet-to-be characterized *cis*-acting destabilizing element that functions analogously to an exon–exon junction must exist. Such an element may be related to the *cis*-acting destabilizing element, loosely defined by the consensus sequence 5′-YGCUGAUGYYYYY-3′, that typifies most *S. cerevisiae* mRNAs (Peltz et al., 1993; Hagan et al., 1995; Ruiz-Echevarría et al., 1998; González et al., 2000), which usually derive from naturally intronless genes.

Here, we present the results of experiments designed to determine if nonsense-harboring mRNAs from two naturally intronless genes—the mouse HSP70.1 (HSP70) gene and the human histone H4 (H4) gene—are subject to NMD. The HSP70 gene is interesting not only because it is intronless, but also because its transcription requires heat shock (Hunt & Calderwood, 1990). We demonstrate that heat shock for 1 h at 43 °C allows for sufficient pre-mRNA splicing and mRNA translation to support NMD by demonstrating that a nonsense codon-containing β-globin allele driven by the heat shock-dependent HSP70 promoter produces spliced mRNA that is subject to NMD. We next demonstrate that the HSP70 gene is immune to NMD with the findings that (1) the levels of mRNA from four nonsense-containing HSP70 alleles are comparable to the level of mRNA from the corresponding nonsense-free allele, and (2) the level of mRNA from the nonsense-free allele is unaffected when the NMD pathway is inhibited by overexpressing a dominant-negative human Upf1 protein. Our finding that nonsense-containing HSP70 mRNA is subject to NMD if it derives from a gene harboring an intron inserted downstream of any one of the four nonsense codons tested indicates that HSP70 mRNA lacks a *cis*-acting destabilizing element required for NMD rather than contains a stabilizing element that precludes NMD.

H4 mRNA is distinct from the vast majority of mammalian mRNAs not only because it derives from an intronless gene, but also because it has a 3′ end that is not generated by polyadenylation (reviewed in Dominski & Marzluff, 1999). The H4 gene is efficiently expressed in HeLa cells when stably but not transiently introduced. We have found by stably transfecting HeLa cells with either a nonsense-free or nonsense-containing H4 allele that none of three nonsense codons tested reduce mRNA abundance. This finding was corroborated by transiently transfecting HeLa cells with the same alleles harboring β-globin polyadenylation sequences in place of H4 sequences that direct H4 RNA 3′-end formation. Therefore, immunity is not attributable to the absence of polyadenylation. Because insertion of an intron a sufficient distance downstream of a nonsense codon results in NMD, we conclude that H4 mRNA, like HSP70 mRNA, lacks a *cis*-acting destabilizing element required for NMD.

RESULTS

Heat shock for 1 h allows for NMD

The mouse HSP70.1 (HSP70) gene is an intronless member of the heat shock family of genes. HSP70 gene expression is strictly dependent on elevated temperature (Hunt & Calderwood, 1990). To establish conditions optimal for expression, HeLa cells were transiently transfected with pmHSP70, a plasmid harboring the full-length HSP70 gene (Fig. 1), and phCMV-MUP, a plasmid harboring the mouse major urinary protein driven by the human cytomegalovirus (hCMV) promoter (Belgrader & Maquat, 1994). Measurements of MUP RNA were used to control for variations in the efficiencies of cell transfection and RNA recovery. The results of northern blot hybridization indicated that HSP70 RNA production in human HeLa cells is dependent on the HSP70 gene and incubation at elevated temperature, in this case 43 °C (Fig. 1, lanes 1–6). Identification of the 2.1-kb transcript as HSP70 RNA was corroborated by the observation of a similar-sized heat shock-dependent RNA in mouse NIH 3T3 cells (Fig. 1, lanes 7–8). The ratio of HSP70 mRNA to MUP mRNA in HeLa cells was 1.1, 1.6, or 2.3 after, respectively, 30, 60, or 120 min incubations at 43 °C (Fig. 1, lanes 4–6). Considering the deleterious effects of prolonged heat shock to cell viability, subsequent analyses were confined to incubations at elevated temperature for no more than 60 min.

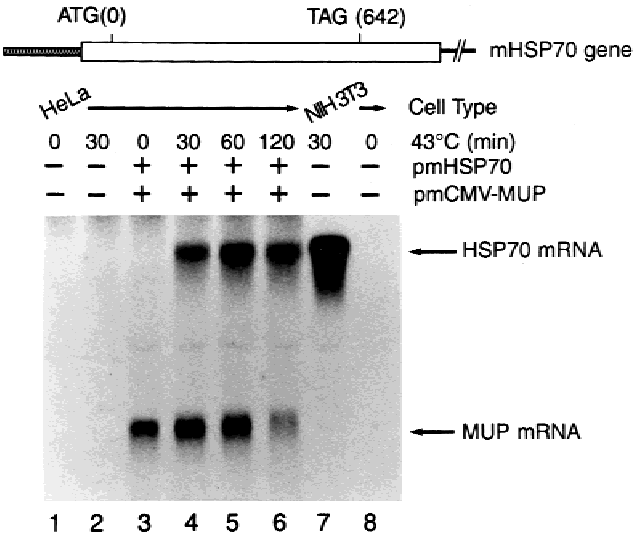

FIGURE 1. HSP70 gene expression in HeLa cells is dependent on heat shock. The upper diagram represents the mouse (m) HSP70 gene, where ATG(0) and TAG(642) specify translation initiation and termination codons, respectively. HeLa cells ($1–2 \times 10^6$) were transiently transfected with 18 μg of pHSP70 test plasmid and 6 μg of phCMV-MUP reference plasmid using calcium phosphate, cultured for 40 h, and incubated at 43 °C for the times specified. Total RNA was then isolated, and HSP70 and MUP transcripts were detected by RNA blot hybridization. RNA (15 μg) from transfected (+) HeLa cells or untransfected (−) HeLa or NIH 3T3 cells were denatured, electrophoresed in 1.5% agarose, and transferred to nylon. HSP70 and MUP transcripts were detected using uniformly ^{32}P-labeled DNAs. Notably, the HSP70 probe did not cross-react with HeLa-cell HSP70 RNA, and the slight alteration in the size of MUP mRNA with heat shock that is evident here was not characteristic of three other independently performed experiments.

HSP70 mRNA contains an AU-rich element (ARE) in the 3′ untranslated region yet fails to be rapidly degraded during heat shock despite its active translation (Moore et al., 1987). A recent study demonstrated that heat shock is associated with a block in the degradation of ARE-mRNAs due to the nuclear sequestration of AUF1, which promotes ARE-mediated decay, in a complex with HSPp70, translation initiation factor eIF-4G and poly(A) binding protein (Laroia et al., 1999). Because heat shock can alter mRNA decay, and HSP70 gene transcription is strictly dependent on heat shock, we first aimed to determine if NMD is active during heat shock. To this end, HeLa cells were transiently transfected with two plasmids: (1) a test pmHSP70-Gl plasmid harboring a hybrid human-mouse β-globin (Gl) gene (Zhang et al., 1998b), in which the promoter and 5′ untranslated region had been substituted for 579 bp of 5′ flanking DNA and 224 bp of 5′ untranslated region of the mouse HSP70 gene so that transcription was likely to be heat-shock dependent (for reviews, see Fernandez et al., 1994; Morimoto, 1998), and (2) the reference plasmid phCMV-MUP that generates MUP transcripts independent of heat shock (Belgrader & Maquat, 1994). The Gl gene was either nonsense-free (Norm) or harbored a nonsense codon at position 39 (39Ter). The Gl gene was chosen for study because it is known to encode mRNA that undergoes NMD (Thermann et al., 1998; Zhang et al., 1998b). However, it differs from the HSP70 gene in having two introns that must be removed from pre-mRNA by splicing for proper expression. Because heat shock at elevated temperatures has been reported to block pre-mRNA splicing (see, e.g., Kay et al., 1987) and translation initiation (see, e.g., Duncan & Hershey, 1989; Feigenblum & Schneider, 1996; Vries et al., 1997), we chose to assay the effect of heat shock for 1 h at the standard 43 °C as well as the less stringent 39 °C.

The results of control transfections, which assayed test pmCMV-Gl plasmids harboring the nonsense-free or 39Ter-containing Gl allele driven by the heat-insensitive mouse (m) CMV promoter, demonstrated that 39Ter reduced the level of Gl mRNA to ~20% of normal with or without heat shock at 43 °C (Fig. 2A). Considering the heat-shock-insensitive nature of the mCMV-Gl alleles, nothing could be learned about the heat sensitivity of NMD because most transcription (and, therefore, NMD) took place prior to heat shock. In contrast, the pHSP70-Gl alleles were heat-shock sensitive: HSP-Gl mRNA production was not evident in the absence of heat shock, barely detectable after heat shock at 39 °C, and required heat shock at 43 °C (Fig. 2B; data not shown). Because HSP-Gl mRNA production required splicing, we can conclude that heat shock at 43 °C did not preclude pre-mRNA splicing. Furthermore, the level of 39Ter-containing HSP-Gl mRNA was 30% the level of nonsense-free HSP-Gl mRNA after heat shock (Fig. 2B), indicating that heat shock at 43 °C did not preclude NMD for at least some of the time after the transcriptional induction.

Nonsense codons within HSP70 mRNA fail to elicit NMD

Normally, translation of HSP70 mRNA terminates at position 642, where the initiation codon is designated position 0. To determine if HSP70 mRNA is subject to NMD, HSP70 alleles that harbored a nonsense codon at positions 34, 209, 357, or 547 were generated (Fig. 3). Furthermore, an allele in which the normal termination codon was converted to a missense codon so that translation terminated at position 660 was also generated (Fig. 3). Each allele was transiently expressed in HeLa cells together with the reference MUP allele. Results indicated that none of the nonsense mutations or the missense mutation elicited NMD (Fig. 3).

Evidence that HSP70 mRNA is immune to NMD because it lacks a *cis*-acting destabilizing element required for NMD

Most simply, the apparent immunity of HSP70 mRNA to NMD may be attributable to one of three possibilities:

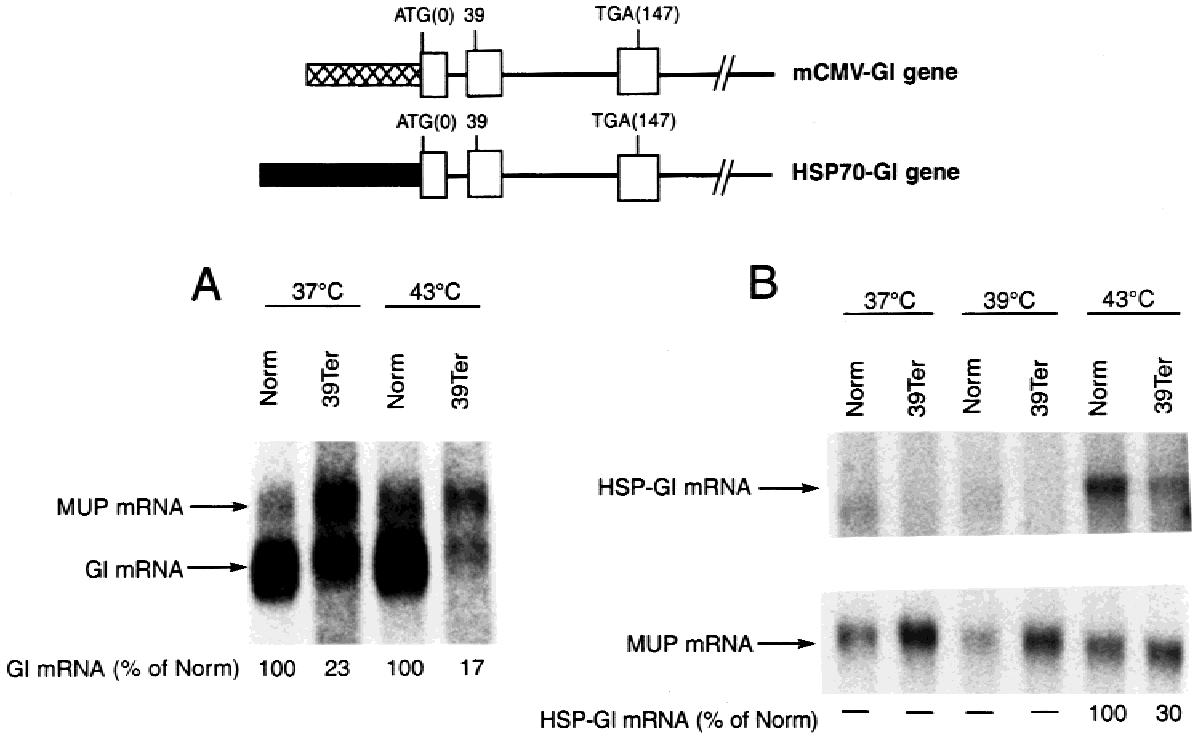

FIGURE 2. NMD is detectable after heat shock. Upper diagrams represent mCMV-GI and HSP-GI genes, where hatched and solid regions specify mCMV and HSP70 promoters, respectively; ATG(0) and TGA(147) specify translation initiation and termination codons, respectively; and 39 positions the normal (CAG) or nonsense (TAG, Ter) codon. HeLa cells were transfected as described in the legend to Figure 1, except the test plasmid consisted of either pmCMV-GI (**A**) or pHSP70-GI (**B**), each of which was either nonsense free (Norm) or 39Ter. Cells were incubated at the specified temperature for 1 h prior to RNA isolation. HSP-GI and MUP transcripts were detected by RNA blot hybridization using uniformly ^{32}P-labeled DNAs. The level of mRNA from each mCMV-GI and HSP70-GI allele was normalized to the level of MUP mRNA in order for the analysis to be quantitative. Normalized values were then calculated as a percentage of the normalized value for either mCMV-GI^{Norm} mRNA (i.e., nonsense-free mRNA) or HSP-GI^{Norm} mRNA from the 43 °C incubation, each of which was considered to be 100. Dashes for % of Norm indicate that the level of HSP-GI mRNA was undetectable. Percentages from two independently performed experiments did not differ by more than 7%. At 43 °C, the level of GI mRNA produced using the HSP70 promoter was generally 15% the level of GI mRNA produced using the mCMV promoter.

HSP70 mRNA may (1) be a natural substrate for NMD, (2) lack a destabilizing element required for NMD, or (3) contain a stabilizing element that precludes NMD. HSP70 mRNA could be a natural substrate for NMD if its normal termination codon resides a sufficient distance upstream of a destabilizing element so as to elicit NMD. Under such circumstances, the introduction of a nonsense codon would be of no consequence to mRNA abundance because two nonsense codons in *cis* reduce mRNA abundance to the same extent as one nonsense codon (reviewed in Maquat, 1995). Notably, if such a destabilizing element does exist, it must also function to elicit NMD brought about by termination at position 660 considering that conversion of the normal termination codon to missense (so that translation terminates at position 660) is also of no consequence to mRNA abundance.

To determine if HSP70 mRNA is a natural substrate for NMD, HeLa cells were transiently transfected with pmHSP70, phCMV-MUP, and either pCI-Neo-hUPF1 Wt or pCI-neo-hUPF1 R844C. pCI-neo-hUPF1 Wt produces wild-type (Wt) hUpf1 protein (p) and is of no consequence to NMD in HeLa cells, whereas pCI-neo-hUFP1 R844C produces hUpf1p that harbors an arginine-to-cysteine change at position 844 and has a dominant-negative effect on both nucleus-associated and cytoplasmic NMD (Sun et al., 1998). If HSP70 mRNA is a natural substrate for NMD, then its abundance should be increased by R844C hUpf1p and unaffected by Wt hUpf1p. Results indicated that Wt hUpf1p and R844C hUpf1p did not differentially affect the abundance of HSP70 mRNA (Fig. 4). Therefore, the apparent immunity of HSP70 mRNA to NMD is not because the mRNA is a natural substrate for NMD.

To determine if immunity is due to the lack of a *cis*-acting destabilizing element required for NMD or the presence of a *cis*-acting stabilizing element that precludes NMD, a spliceable intron from the gene for human triosephosphate isomerase (TPI) was inserted between positions 435 and 436 of the nonsense-free and various nonsense-containing HSP70 alleles (Fig. 5). Based on studies of naturally intron-containing transcripts in which this or another intron was inserted at one of a number of sites (Cheng et al., 1994; Thermann

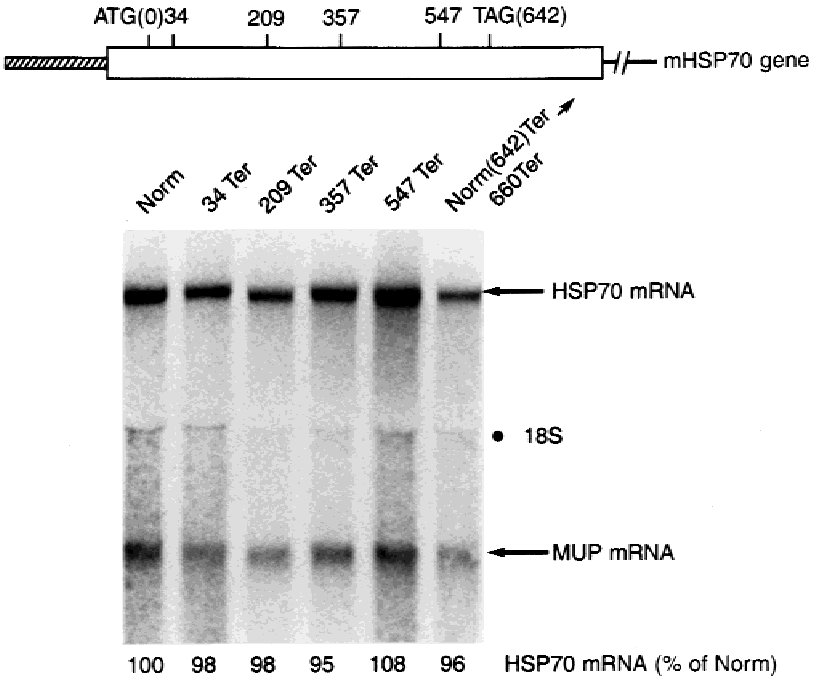

FIGURE 3. Nonsense codons within the HSP70 gene do not decrease the abundance of HSP70 mRNA. HeLa cells were transiently transfected with the specified HSP70 test allele and MUP reference allele, and subjected to 43 °C for 1 h prior to RNA purification. RNA was blot hybridized as described in the legend to Figure 1. The level of each HSP70 mRNA was normalized to the level of MUP mRNA, and normalized values were then calculated as a percentage of the normalized value for HSP70^{Norm} mRNA, which was considered to be 100. Percentages from two independently performed experiments did not differ by more than 8%.

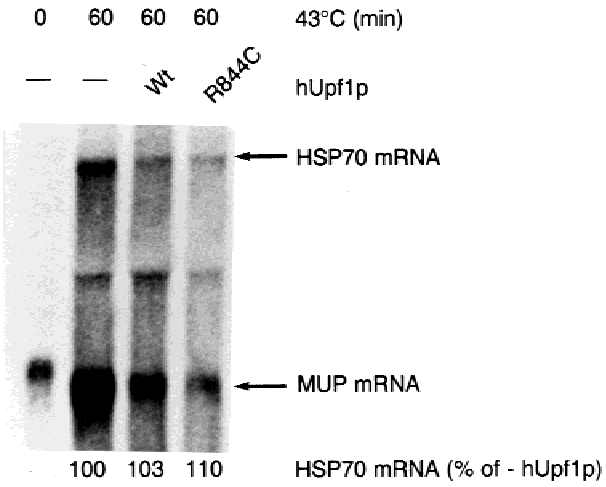

FIGURE 4. HSP70 mRNA is not a natural substrate for NMD. HeLa cells were transiently transfected using calcium phosphate with a total of 20 μg of plasmid DNA (8 μg of pmHSP70^{Norm} or pmCMV-Gl^{Norm} or pmCMV-Gl^{39Ter}; 2 μg of phCMV-MUP; and 10 μg of either pCI-neo-hUPF1 Wt, which harbors an unmutagenized hUpf1p reading frame, pCI-neo-hUPF1 R(844)C, which harbors an arginine-to-cysteine change at amino acid position 844, or pCI-neo, which consists only of vector sequences). Prior to RNA purification, cells were incubated at 43 °C for 1 h. RNA was blot hybridized as described in the legend to Figure 1. The level of HSP70 mRNA from each HSP70 allele was normalized to the level of MUP mRNA, and normalized values were then calculated as a percentage of the normalized value of HSP70^{Norm} mRNA in the absence of either hUPF1 allele. Percentages differed between two independently performed experiment by no more than 10%.

et al., 1998; Zhang et al., 1998b), and provided that HSP70 mRNA simply lacks a destabilizing element, nonsense codons at positions 34, 209, and 357, which reside more than 50–55 nt upstream of the inserted intron, would be expected to elicit NMD; in contrast, the nonsense codon at position 547, which resides downstream of the inserted intron, would not be expected to elicit NMD. If results counterindicated this simple scenario, then the possibility of a stabilizing element would have to be entertained. The only stabilizing element demonstrated to date for mammalian transcripts consists of a efficiently utilized translation reinitiation site that nullifies NMD when located downstream of and in frame with a nonsense codon so as to restore the site of translation termination to normal (Zhang & Maquat, 1997). In theory, however, a stabilizing element could be modeled after one of the two additional types described for mRNAs of *S. cerevisiae*. One type prevents upstream termination codons from eliciting NMD, presumably by modifying the termination complex and its interaction with downstream destabilizing element(s) (Ruiz-Echevarría et al., 1998; Ruiz-Echevarría & Peltz, 2000). The other type prevents downstream termination codons from eliciting NMD, presumably by modifying the translation elongation complex and its interaction with downstream destabilizing element(s) (Peltz et al., 1993).

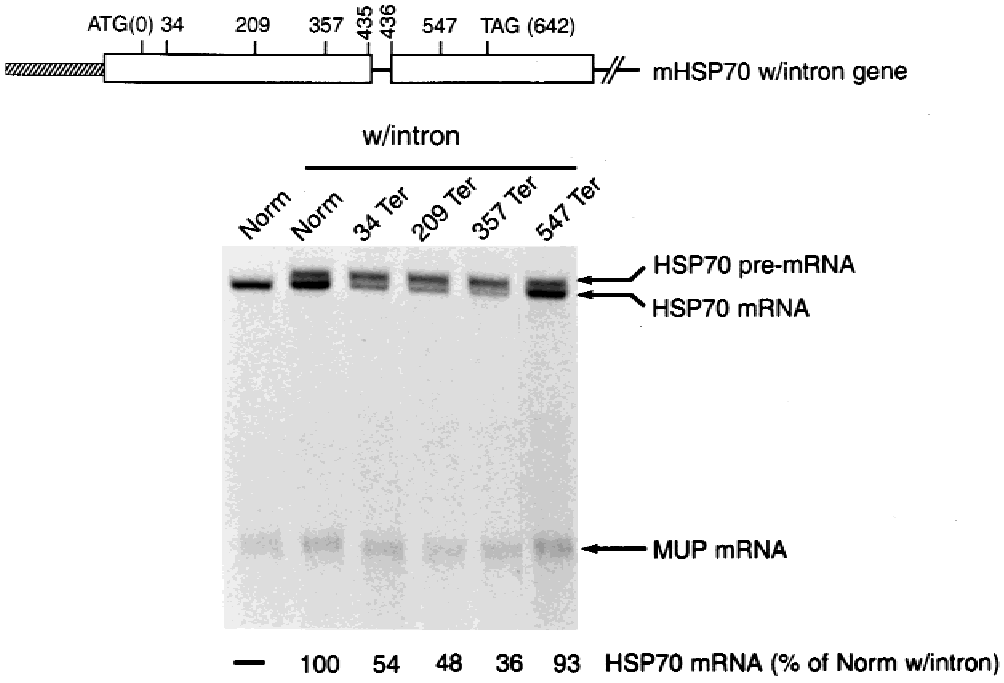

FIGURE 5. Inserting TPI intron 6 between codons 435 and 436 of the HSP70 alleles harboring 34Ter, 209Ter, or 357Ter but not 547Ter elicits NMD. Transfections and RNA analyses were as described in the legend to Figure 3, except that HSP70 mRNA levels are presented as a percentage of Norm w/intron, which was considered to be 100. Percentages differed between two independently performed experiment by no more than 5%. The amount of HSP70 transcript (unspliced and spliced) from the nonsense-free intron-containing allele was 160% the amount of HSP70 transcript from the nonsense-free intronless allele.

HeLa cells were transiently transfected with phCMV-MUP and each of the intron-containing HSP70 alleles. Two HSP70 transcripts were evident for each allele (Fig. 5). The faster migrating transcript was the product of pre-mRNA splicing, consistent with its migrating identically to the transcript from the intronless HSP70 gene. Sequence analysis of the faster migrating product of PCR-amplified HSP70 cDNA, obtained using primers that spanned the site of intron insertion, indicated that the intron was accurately removed by splicing (data not shown). The slower migrating transcript corresponded to intron-containing RNA as evidenced by its hybridization to the TPI intron (data not shown). Its prevalence, which is likely attributable to the intron residing within an exonic sequence that is suboptimal for splicing, did not interfere with the analysis of spliced mRNA. Relative to the level of spliced mRNA from the nonsense-free intron-containing HSP70 allele, which was defined as 100, the level of spliced mRNA harboring 34Ter, 209Ter, 357Ter, or 547Ter was 54%, 48%, 36%, or 93%, respectively (Fig. 5). As would be predicted from a number of studies (see, e.g., Cheng & Maquat, 1993), the level of intron-containing RNA was unaffected by any Ter (Fig. 5). Therefore, the inserted intron together with those nonsense codons located 234 nt or more upstream are capable of eliciting the NMD of spliced mRNA, whereas the intron and the nonsense codon located 333 nt or, in the case of the normal termination codon, 618 nt downstream are incapable of eliciting NMD. We conclude that 34Ter, 209Ter, 357Ter, and 547Ter do not elicit NMD of HSP70 mRNA that derives from the intronless gene because the gene lacks an appropriately positioned destabilizing element.

Human H4 mRNA harboring one of several nonsense codons is not a substrate for NMD

To determine if immunity to NMD characterizes the transcript of a different intronless gene, nonsense codons were generated at positions 22, 44, or 77 of the human histone 4 (H4) gene driven by the mCMV promoter and harboring 44 nt of mCMV 5′ untranslated region (Fig. 6). Normally, translation terminates at position 105, where the initiation codon is designated position 0. Because H4 gene expression is barely detectable in transient transfections unless sequences that direct H4 RNA 3′-end formation are replaced with sequences that direct polyadenylation (W.S. Marzluff, pers. comm.; our unpubl. data), each allele was inserted into a plasmid that confers resistance to G418 and stably introduced into HeLa cells. Several individual G418-resistant clones expressing each construct were isolated and expanded for analysis, and pools of transfectants that expressed each construct were established with the remaining G418-resistant cells. Both H4 and G418 transcripts were

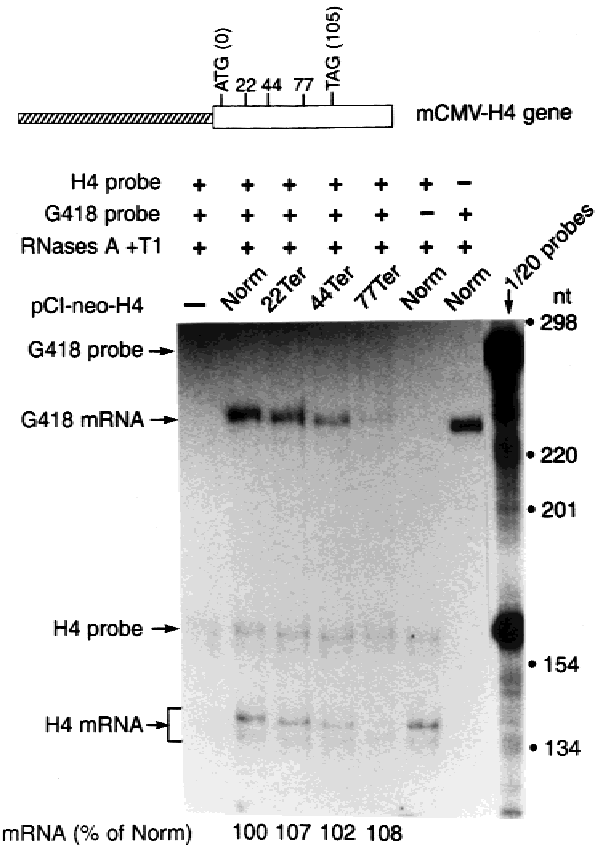

FIGURE 6. Nonsense codons within the H4 gene do not affect the abundance of H4 mRNA. HeLa cells were stably transfected using lipofection with pCI-neo-H4 alleles (either Norm, 22Ter, 44Ter, or 77Ter). Encoded H4 and G418-resistance (G418) transcripts were quantitated by RNase mapping using uniformly ^{32}P-labeled antisense RNAs (probes; 1/20 of which are shown in the right-most lane) and RNases A+T1. Notably, H4 mRNA generated multiple RNase-resistant bands due to either incomplete or excessive RNase digestion, and there was always some RNase-resistant H4 probe regardless of the amount of RNase A+T1 or probe used. The level of each H4 mRNA was normalized to the level of G418 mRNA, and normalized values were then calculated as a percentage of the normalized value for H4^{Norm} mRNA, which was considered to be 100. For each mCMV-H4 construct analyzed, percentages from at least five independently performed experiments using at least four independently derived G418-resistant clonal HeLa-cell lines as well as a pool of minimally 50 HeLa-cell transfectants did not differ by more than 8%.

quantitated from each of the clonal transfectants by RNase mapping. The presence of mCMV sequences provided a means to distinguish transcripts from the introduced H4 allele and transcripts from the endogenous H4 gene (Fig. 6). Results indicated that none of the three nonsense codons elicited NMD (Fig. 6; data not shown for additional clonal transfectants and pools of transfectants).

H4 mRNA is unique in lacking poly(A) at the 3′ end. To determine if immunity is attributable to the absence of polyadenylation, sequences within each allele that direct H4 3′-end formation were replaced with sequences from the mouse β-globin gene that direct polyadenylation (Fig. 7). The resulting constructs were transiently introduced into HeLa cells together with the reference phCMV-MUP plasmid. Test and reference transcripts were then analyzed by blot hybridization, which was possible because β-globin sequences within H4-GI RNA provided a means to distinguish H4-GI mRNA from the endogenous H4 mRNA. Again, results indicated that none of the three nonsense codons elicited NMD (Fig. 7). Notably, the H4-GI transcripts were polyadenylated (and, as a consequence, detectably heterogenous in length) as evidenced by their binding to oligo(dT) along with MUP transcripts, in contrast to endogenous H4 transcripts that did not bind to oligo(dT) (data not shown). Therefore, the immunity of H4 mRNA to NMD is not due to the absence of polyadenylation at the 3′ end.

To gain additional insight into the immunity of H4 mRNA to NMD, the TPI intron was inserted between codons 87 and 88 of the nonsense-free and each nonsense-containing H4-GI allele. By so doing, the intron was located within the resulting H4-GI pre-mRNA 195 nt, 129 nt, or 30 nt downstream of 22Ter, 44Ter, or 77Ter, respectively. Notably, H4-GI alleles rather than H4 alleles were used for the intron insertion experiments, not only because the consequence could be assayed by transient transfection, but also because introns are known to target transcripts to the cleavage and polyadenylation pathway, and an intron insertion

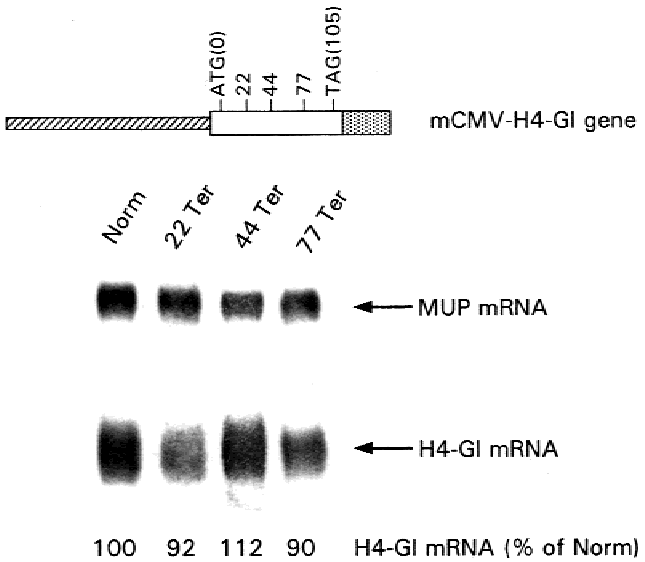

FIGURE 7. Nonsense codons within the H4-GI gene do not decrease the abundance of H4-GI mRNA. HeLa cells were transiently transfected with the specified test pmCMV-H4-GI constructs and the MUP reference construct. Total RNA was quantitated by blot hybridization. The level of mRNA from each pmCMV-H4-GI allele was normalized to the level of MUP mRNA, and normalized values were then calculated as a percentage of the normalized value for H4-GI^{Norm} mRNA, which was considered to be 100. Percentages from two independently performed experiments did not differ by more than 19%.

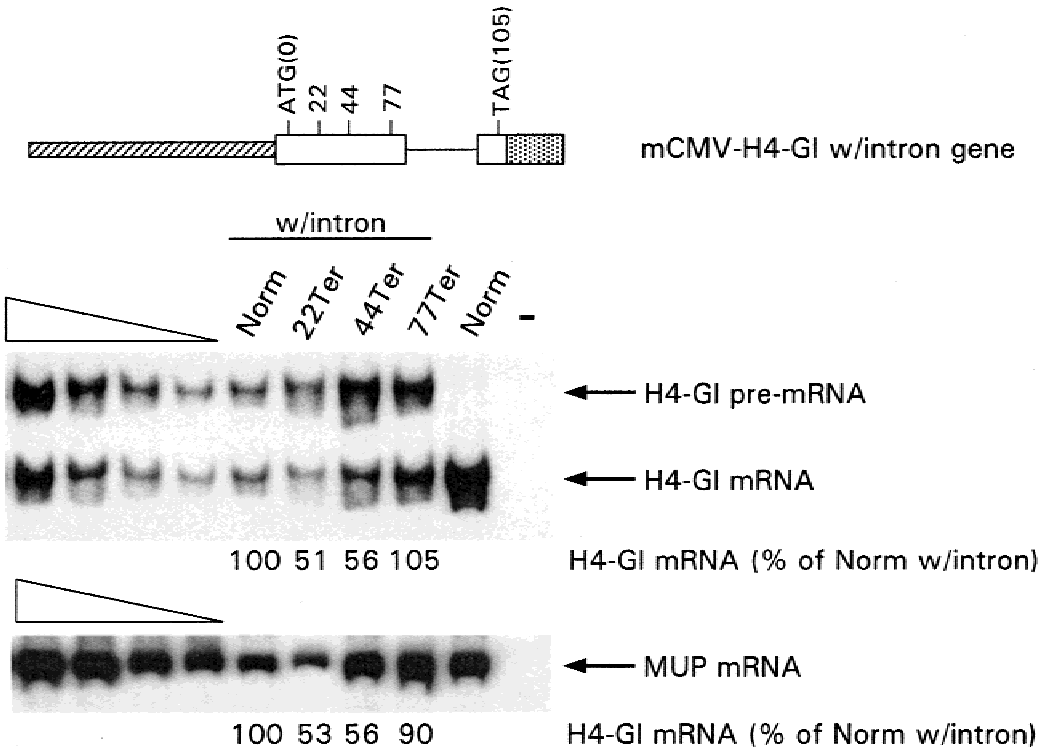

FIGURE 8. Inserting TPI intron 6 between codons 87 and 88 of H4-GI alleles harboring 22Ter or 44Ter elicits NMD, but the same intron insertion into the H4-GI allele harboring 77Ter does not elicit NMD. Transfections were as described in the legend to Figure 7. H4-GI and MUP transcripts were quantitated using RT-PCR, and H4-GI mRNA levels are presented as a percentage of Norm w/intron using either H4-GI pre-mRNA (upper panel) or MUP mRNA (lower panel) as a standard. The first four lanes demonstrate a linear relationship between the amount of RNA analyzed and the intensity of the RT-PCR products. The lane labeled with a dash analyzes RNA from untransfected cells. Percentages from four independently performed experiments did not differ by more than 8% except for data using 77Ter shown here. The amount of H4 transcript (unspliced and spliced) from the nonsense-free intron-containing allele was 135% the amount of H4 transcript from the nonsense-free intronless allele.

in the absence of a bone fide *cis*-residing cleavage/polyadenylation sequence would likely facilitate use of one or more cryptic cleavage/polyadenylation sequences (Pandey et al., 1990). RT-PCR rather than northern blot hybridization was used to assess the effect of the intron insertions because the H4-GI mRNA RT-PCR product was a discrete size, unlike the product of northern blot hybridization (compare Figs. 7 and 8), which facilitates quantitation. Using the level of either MUP mRNA or H4-GI pre-mRNA as a reference, 22Ter and 44Ter reduced H4 mRNA abundance to ~55% of normal and, thus, elicited NMD whereas 77Ter did not significantly reduce mRNA abundance (Fig. 8), as would be predicted from the rule for termination codon position (Nagy & Maquat, 1998). Sequence analysis of PCR-amplified H4-GI cDNA, obtained using primers that spanned the site of intron insertion, indicated that the intron was accurately removed by splicing (data not shown). Therefore, 22Ter and 44Ter fail to elicit NMD of H4 mRNA that derives from the naturally intronless gene because there is no appropriately positioned destabilizing element. The same situation most likely explains why 77Ter fails to elicit NMD.

DISCUSSION

Here, we demonstrate that naturally intronless genes for HSP70 and H4 generate mRNAs that are immune to NMD by demonstrating that none of the nonsense codons introduced into the gene for either mRNA elicited NMD (Figs. 3 and 6). Immunity is most likely attributable to the lack of a destabilizing element rather than the presence of a stabilizing element, because insertion of an intron resulted in NMD, provided the intron was located a sufficient distance downstream of a nonsense codon. In the case of HSP70 mRNA, an intron located either 234 nt, 678 nt, or 1,203 nt downstream of a nonsense codon elicited NMD (Fig. 5). In the case of H4 mRNA, an intron located only 30 nt downstream of a nonsense codon failed to elicit NMD, but an intron located either 129 nt or 195 nt downstream of a nonsense codon did elicit NMD (Fig. 8). Notably, the NMD of either HSP70 or H4 mRNA resulted in only a twofold reduction in mRNA abundance. Although this level of reduction is less than the ≫50-fold reduction characteristic of T-cell receptor mRNA (Carter et al., 1996) or the four- to fivefold reduction

characteristic of TPI or β-globin mRNA (Thermann et al., 1998; Zhang et al., 1998a, 1998b), it is comparable to the reduction that typifies glutathione peroxidase 1 mRNA (Moriarty et al., 1998; Sun et al., 2000).

Our findings are consistent with the general rule that a nonsense codon elicits NMD provided an intron is located at least 50–55 nt downstream (Cheng et al., 1994; Nagy & Maquat, 1998; Thermann et al., 1998; Zhang et al., 1998a, 1998b). Notable exceptions to the rule include (1) T-cell receptor-β mRNA, which can be subject to NMD when an intron is located less than 10 nt downstream of a nonsense codon (Carter et al., 1996) and (2) instances where ill-defined exonic sequences can apparently function in the absence of an intron provided at least one upstream intron exists (Cheng et al., 1994; Zhang et al., 1998b). Our findings also correct a limitation previously attributed to the rule: namely, that a nonsense codon located more than 416 nt upstream of an intron fails to elicit NMD (Zhang et al., 1998a; Sun & Maquat, 2000). As a nonsense codon located well beyond this distance can elicit NMD for both HSP70 and H4 transcripts, we believe that the failure of a nonsense codon within TPI exon 1 to elicit NMD in the absence of all but the first of six TPI gene introns does not indicate that NMD requires a minimum distance between a nonsense codon and a downstream intron, but reflects a mechanism that has yet to be understood but appears to be particular to the specific TPI construct.

A number of other interesting observations were made during the course of this study. First, studies of the β-globin gene driven by the HSP70 promoter so that transcription is dependent on heat shock indicate that heat shock at 43 °C for 1 h fails to block NMD (Fig. 2). Because NMD of HSP-Gl mRNA requires pre-mRNA processing and mRNA translation, neither processing of HSP-Gl pre-mRNA nor translation of HSP-Gl mRNA must be inhibited to an extent that precludes NMD for at least some of the time after transcriptional induction. Consistent with this finding, other studies found an inhibition of mRNA translation does not occur until after 1 h of heat shock at 44 °C (Laroia et al., 1999). Second, although it is debatable if NMD in *S. cerevisiae* is triggered by a loosely defined downstream destabilizing element (see, e.g., González et al., 2000) or the absence of proper spatial arrangement of the translation termination codon with respect to the polyadenylation site (see, e.g., Hilleren & Parker, 1999; Muhlrad & Parker, 1999; Jacobson & Peltz, 2000), our finding that NMD can be elicited in mammalian cells by insertion of an intron, which does not change the spatial arrangement of the translation termination codon with respect to the polyadenylation site, provides evidence that a splicing-generated exon–exon junction can function as the *cis*-acting destabilizing element in mammalian cells. This result is consistent with the finding that deletion of the last four introns (introns 3–6) of a TPI allele harboring a termination codon located 24 bp upstream of intron 2 (the new 3′-most intron) failed to elicit NMD even though the same termination codon in the context of the full-length allele did elicit NMD (Zhang et al., 1998a). Because there is no difference between the two alleles in the distance between the termination codon and polyadenylation site, the failure to elicit NMD in the case of the first allele must be due to the change in distance between the termination codon and downstream intron(s). Also consistent are studies that convert a normal termination codon to one that elicits NMD with insertion of a downstream intron (Thermann et al., 1998).

It will be of interest to determine if splicing-generated exon–exon junctions can function to elicit NMD in *S. cerevisiae.* Also of interest is the mechanism by which the transcripts of several intron-containing mammalian genes appear to be immune to NMD despite harboring a termination codon followed by an intron located more than 50–55 nt downstream (X. Sun, X. Li, P.M. Moriarty, T. Henics, J.P. LaDuca, and L.E. Maquat, in press).

MATERIALS AND METHODS

Mouse HSP70 gene constructions

pmHSP70^{Norm}, which harbors the mouse heat shock 70.1 gene, was generated by inserting the 3.5-kbp *Eco*RI-*Hin*dIII fragment from PM70.1 (a gift from Clayton Hunt), into the *Eco*RI and *Hin*dIII sites of pBluescript II KS(+). Nonsense mutations at positions 34 (AAC → TAG), 209 (CTG → TAG), 357 (ACC → TAG), and 547 (ACC → TAG), and a missense mutation at position 642 (TAG → CAG) were introduced using the sense mutagenic primers 5′-GCCAACGACCAGG*GCT AGC*GCACGACCC-3′ (where italicized nucleotides constitute a *Nhe*I site), 5′-CGTGTCCA*TCTAGA*CGATCGACGAC GGC-3′ (where italicized nucleotides constitute a *Xba*I site), 5′-GGACTTCTTCAACGGGC*GCTAGC*TGAACAAGAGC-3′ (where italicized nucleotides constitute a *Nhe*I site), 5′-CCTATGCCT*TCTAGA*TGAAGAGCGCC-3′ (where italicized nucleotides constitute a *Xba*I site), and 5′-GGAGGTGGA TCAGAGGCCTCTGCTGGC-3′, respectively, where underlined nucleotides are mutagenic. After the mutagenesis was confirmed by DNA sequencing, the 529-bp *Msc*I-*Not*I fragment harboring 34Ter, the 238-bp *Not*I-*Bss*HII fragment harboring 209Ter, the 626-bp *Bss*HII-*Nae*I fragment harboring 357Ter, the 270-bp *Nae*I-*Psh*AI fragment harboring 547Ter, or the 429-bp *Psh*AI-*Nsi*I fragment harboring the missense mutation were used to replace the corresponding fragment of pmHSP70^{Norm}.

pmHSP70-Gl^{Norm} was generated by ligating the 830-bp *Eco*RI-*Nco*I fragment, which includes vector DNA, 580 bp of 5′-flanking DNA, and the entire 224 bp of 5′-untranslated region of the HSP70 gene, to the 427-bp *Nco*I-*Bam*HI and 1,521-bp *Bam*HI-*Xba*I fragments of pmCMV-Gl^{Norm} (Zhang & Maquat, 1997), which together include the entire Gl reading frame, 3′-untranslated region, 3′-flanking DNA, and most vector sequences of pmCMV-Gl^{Norm}. Prior to ligation, the *Bam*HI site and *Eco*RI sites were Klenow filled. pmHSP70-Gl^{39Ter} was generated by substituting the 1.95-kbp *Nco*I-*Xba*I frag-

ment from pmHSP70-GI^{Norm} with the corresponding fragment of pmCMV-GI^{39Ter}.

To generate pmHSP70^{Norm} w/intron, intron 6 of the human gene for TPI was generated by PCR, cleaved with *Pml*I and *Kpn*I, Klenow filled (Cheng et al., 1994), and inserted into the *Sma*I site of pHSP70 that resides between codons 435 and 436. Prior to insertion, the *Sma*I site in the pUC10 portion of pHSP70 was destroyed by Klenow chewing the adjacent *Kpn*I site. Derivatives harboring either 34Ter, 209Ter, or 547Ter were generated by substituting the 626-bp *Bss*HII-*Nae*I fragment of pmHSP70 w/intron with the corresponding fragment from pmHSP70^{34Ter}, pmHSP70^{209Ter}, or pmHSP70^{547Ter}, respectively. A derivative harboring 357Ter was generated by ligating the 581-bp *Not*I-*Bgl*I fragment from pmHSP70^{357Ter} to the 638-bp *Bgl*I-*Xho*I and *Xho*I-*Not*I fragments from pmHSP70^{Norm} w/intron.

Human H4 gene constructions

pMT-H4^{Norm} was generated by inserting the 504-bp *Bam*HI-*Xba*I fragment of the human H4 gene of pJUC50 (Sierra et al., 1993) into the *Bam*HI and *Mbo*II sites of pUC13. Prior to insertion, the *Xba*I and *Mbo*II sites were Klenow filled. pmCMV-H4, a plasmid harboring the H4 gene driven by the mouse cytomegalovirus (mCMV) promoter was generated in two steps. First, the 510-bp *Bam*HI-*Hin*dIII fragment from pMT-H4 was inserted into the *Bam*HI and *Hin*dIII sites of pBluescript II KS(+) (Stratagene). Second, the 585-bp *Xba*I-*Eco*RI fragment from pM-H4 (a gift from Jack Gauldie) that harbors the mCMV promoter was Klenow filled and inserted into the *Hin*cII site. Nonsense mutations at positions 22 (TTG → TAG), 44 (AAG → TAG) and 77 (AAG → TAG) were introduced using antisense mutagenic primers 5′-GTCTC TCTAGACCTTGCG-3′, 5′-GATCCGCTAAACGCCGCC-3′ and 5′-CTTGCGCTAGGCGTGCTC-3′, respectively, where underlined nucleotides are mutagenic. The translational reading frame was then sequenced in entirety.

pmCMV-H4-GI^{Norm}, a derivative of pmCMV-H4 harboring the mouse β^{major}-globin polyadenylation sequence in place of sequences that determine H4 mRNA 3′-end formation, was generated by inserting a 138-bp *Pvu*II-*Hin*cII fragment that spans the mouse β^{major}-globin polyadenylation sequence and includes 68 bp of exon 3 and 70 bp of 3′-flanking DNA (Nesic et al., 1995) 8 bp downstream of the translation termination codon at an artificially created *Bam*HI site in pmCMV-H4^{Norm}. The 138-bp fragment was generated by PCR (Nesic et al., 1995), and the *Bam*HI site was created using the mutagenic antisense primer 5′-GCAAAGCT*GGATCC*GCGGC CTAGC-3′ (where italicized nucleotides constitute a *Bam*HI site). pmCMV-H4-GI containing either 22Ter, 44Ter, or 77Ter was similarly made using the 225-bp fragment from the corresponding nonsense-containing pmCMV-H4 construct.

To generate pmCMV-H4-GI^{Norm} w/intron, a *Hpa*I site was generated between codons 87 and 88 using overlap-extension PCR (Ho et al., 1989). The two overlapping fragments were a 272-bp fragment made using oligonucleotides 5′-CCTG CGGTCATGTCCGGCTGTGG-3′ (sense) and 5′-GCGCTT GAGCGC*GTTAAC*CACATCCATGGCTGTGACGG-3′ (antisense, where italicized nucleotides correspond to the *Hpa*I site), and a 250-bp fragment made using oligonucleotides 5′-GCCATGGATGTG*GTTAAC*GCGCTCAAGCGCCAGGG G-3′ (sense, where italicized nucleotides correspond to the *Hpa*I site) and 5′-AATACGACTCACTATAG-3′ (same as the antisense T7 primer from Stratagene). The resulting 482-bp fragment was digested with *Nco*I and *Not*I and inserted into the *Nco*I and *Not*I sites of pmCMV-H4-GI^{Norm}. TPI intron 6 was subsequently inserted into the *Hpa*I site. Derivatives of pmCMV-H4-GI^{Norm} w/intron harboring 22Ter, 44Ter, or 77Ter were then constructed using the 351-bp *Nco*I-*Not*I fragment from the corresponding nonsense-containing pmCMV-H4-GI construct.

pCI-neo-H4^{Norm} was generated by inserting the 1,080-bp *Apa*I-*Not*I fragment from pmCMV-H4^{Norm} into the *Bgl*II and *Dra*II sites of pCI-neo (Promega). Prior to insertion, all sites were Klenow treated. pCI-neo-H4 containing either 22Ter, 44Ter, or 77Ter were similarly made from the corresponding nonsense-containing pmCMV-H4 construct.

Notably, all PCR-generated fragments were sequenced in entirety in the context of the final construction.

Cell transfections and RNA purification

Human HeLa (CCL-2; ATCC) cells were grown to 60% confluency in 100-mm diameter dishes in minimal essential medium alpha medium (GIBCO/BRL/Life Technologies, Inc.) containing 10% fetal calf serum and antibiotics, and transiently transfected with 18 μg of a test pmHSP70 DNA and 6 μg of reference phCMV-MUP DNA (Belgrader & Maquat, 1994) using calcium phosphate (Wigler et al., 1979). For heat shock, dishes were sealed 40 h after transfection with parafilm, floated in a 43 °C water bath for 60 min, and harvested immediately. Alternatively, HeLa cells were grown to 60% confluency in 60-mm dishes as described above, transferred to antibiotic-free medium, and stably transfected with 2 μg of pCI-neo-H4^{Norm} or a nonsense-containing derivative using LipofectAMINE PLUS Reagent (GIBCO/BRL/Life Technologies, Inc.) according to the manufacturer's instructions. G418 (GIBCO/BRL/Life Technologies, Inc.) was added to 800 μg/mL 20 h following the start of transfection and raised after 3 days to 1 mg/mL. After an additional 15 days, individual G418-resistant colonies were isolated and expanded in 200 μg/mL of G418.

Northern blot analysis

To detect HSP70, H4, or MUP transcripts, total HeLa-cell RNA (5–15 μg) was denatured, electrophoresed in a 1.5% agarose gel, transferred to a nylon membrane, and hybridized to, respectively, a 518-bp *Xba*I-*Nsp*I fragment from the 3′-untranslated region of the HSP70^{Norm} gene, a 336-bp *Kpn*I-*Not*I fragment of the mCMV-H4^{Norm} gene, or an 800-bp *Pst*I-*Pst*I fragment from MUP cDNA (Belgrader & Maquat, 1994). Prior to hybridization, each fragment was ^{32}P-labeled by random priming. Hybridization was quantitated by phosphorimaging (Molecular Dynamics) and autoradiography.

RNase mapping

In the case of stable transfections, uniformly ^{32}P-labeled antisense RNAs for mapping H4 or G418-resistance transcripts were synthesized using T7 RNA polymerase, the MAXIscript T7/SP6 Kit (Ambion), and, respectively, pGEM-antiH4 that had been linearized with *Xba*I or pGEM-antiG418 that had

been linearized with *Hin*dIII. pGEM-antiH4 harbored a 140-bp PCR product of the H4 gene construct that consisted of 84 bp of the H4 gene, 44 bp of the mCMV promoter, and 12 bp of vector. The PCR product was synthesized using 5′-CGACC GCGTC*GGTACC*GTCG-3′ (sense) and 5′-CTTGCGGTGG *GAATTC*GCGCC-3′ (antisense). pGEM-antiG418 harbored a 232-bp *Hin*dIII-*Pst*I fragment from pCI-neo that consisted of 2,321 bp of the G418-resistance gene and 48 bp of vector DNA. The fragment was inserted into the *Hin*dIII and *Pst*I sites of pGEM-7Z. RNase mapping was performed using 5 μg of total HeLa-cell RNA, 35 ng (8×10^5 cpm) of each probe, and the RPA III Kit (Ambion) following the manufacturer's instructions. Samples were electrophoresed in 6% polyacrylamide-7 M urea gels, and radioactivity was quantitated using phosphorimaging and autoradiography.

RT-PCR

RNA (5 μg) was treated with 1 U of RNase-free RQ DNase I (Promega), and cDNA was synthesized from 2.5 μg using Moloney murine leukemia virus reverse transcriptase (Superscript II; GIBCO/BRL/Life Technologies, Inc). H4-GI and MUP transcripts were then PCR-amplified in separate reactions using Taq DNA polymerase (Promega) and primer pairs that amplify either H4-GI RNA from the mCMV 5′-untranslated region (Sun et al., 1998) through the first 5 nt of the 3′-untranslated region (antisense; 5′-GCCTAGCCTCCGAAGC CGTAGAGG-3′) or MUP RNA from exon 1 through exon 7 (Belgrader & Maquat, 1994). Separate reactions were required because H4-GI pre-mRNA and MUP mRNA generate similar-sized RT-PCR products.

ACKNOWLEDGMENTS

We thank Clayton Hunt for PM70.1 DNA. We are grateful to Bill Marzluff, Anand Gersappe, Xiaolei Sun, and Yasuhito Ishigaki for helpful discussions; Yasuhito Ishigaki for assistance generating figures; and Guillaume Serin for comments on the manuscript. This work was supported by Public Health Research Grants DK 33933 and GM 59614 from the National Institutes of Health to L.E.M.

Received November 1, 2000; returned for revision November 21, 2000; revised manuscript received December 1, 2000

REFERENCES

Belgrader P, Maquat LE. 1994. Nonsense but not missense mutations can decrease the abundance of nuclear mRNA for the mouse major urinary protein, while both types of mutations can facilitate exon skipping. *Mol Cell Biol 14*:6326–6336.

Carter MS, Li S, Wilkinson MF. 1996. A splicing-dependent regulatory mechanism that detects translation signals. *EMBO J 15*: 5965–5975.

Cheng J, Belgrader P, Zhou X, Maquat LE. 1994. Introns are *cis*-effectors of the nonsense codon-mediated reduction in nuclear mRNA abundance. *Mol Cell Biol 14*:6317–6325.

Cheng J, Maquat LE. 1993. Nonsense codons can reduce the abundance of nuclear mRNA without affecting the abundance of pre-mRNA or the half-life of cytoplasmic mRNA. *Mol Cell Biol 13*: 1892–1902.

Culbertson MR. 1999. RNA surveillance: Unforeseen consequences for gene expression, inherited genetic disorders and cancer. *Trends Genet 15*:74–80.

Dominski Z, Marzluff WF. 1999. Formation of the 3′ end of histone mRNA. *Gene* 239:1–14.

Duncan RF, Hershey JW. 1989. Protein synthesis and protein phosphorylation during heat stress, recovery, and adaptation. *J Cell Biol 109*:1467–1481.

Feigenblum D, Schneider RJ. 1996. Cap-binding protein (eukaryotic initiation factor 4E) and 4E-inactivating protein BP-1 independently regulate cap-dependent translation. *Mol Cell Biol 16*: 5450–5457.

Fernandez M, O'Brien T, Lis JT. 1994. Structure and regulation of heat shock gene promoters. In: Morimoto RI, Tissieres A, Georgopolis C, eds. *The biology of heat shock proteins and molecular chaperones*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 375–393.

González CI, Ruiz-Echevarría MJ, Vasudevan S, Henry MF, Peltz SW. 2000. The yeast hnRNP-like protein Hrp1p/Nab4 marks a transcript for nonsense-mediated mRNA decay. *Mol Cell 4*:489–499.

Hagan KW, Ruiz-Echevarría MJ, Quan Y, Peltz SW. 1995. Characterization of *cis*-acting sequences and decay intermediates involved in nonsense-mediated mRNA turnover. *Mol Cell Biol 15*:809–823.

Hentze MW, Kulozik AE. 1999. A perfect mRNA: RNA surveillance and nonsense-mediated decay. *Cell 96*:307–310.

Hilleren P, Parker R. 1999. mRNA surveillance in eukaryotes: Kinetic proofreading of proper translation termination as assessed by mRNP domain organization? *RNA 5*:711–719.

Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. 1989. Site-directed mutagenesis by overlap extension using polymerase chain reaction. *Gene 77*:51–59.

Huang Y, Wimler KM, Carmichael GG. 1999. Intronless mRNA transport elements may affect multiple steps of pre-mRNA processing. *EMBO J 18*:1642–1652.

Hunt C, Calderwood S. 1990. Characterization and sequence of a mouse *hsp70* gene and its expression in mouse cell lines. *Gene 87*:199–204.

Jacobson A, Peltz SW. 2000. Destabilization of nonsense-containing transcripts in *Saccharomyces cerevisiae*. In: Sonenberg N, Hershey JBW, Mathews MB, eds. *Translational control of gene expression.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 827–847.

Kay RJ, Russnak RH, Jones D, Mathias C, Candido EP. 1987. Expression of intron-containing *C. elegans* heat shock genes in mouse cells demonstrates divergence of 3′ splice site recognition sequences between nematodes and vertebrates, and an inhibitory effect of heat shock on the mammalian splicing apparatus. *Nucleic Acids Res 15*:3723–3741.

Laroia G, Cuesta R, Brewer G, Schneider RJ. 1999. Control of mRNA decay by heat shock-ubiquitin-proteasome pathway. *Science 284*:499–502.

Le Hir H, Izaurralde E, Maquat LE, Moore MJ. 2000a. The spliceosome deposits multiple proteins 20–24 nucleotides upstream of mRNA exon–exon junctions. *EMBO J 19*:6860–6869.

Le Hir H, Moore MJ, Maquat LE. 2000b. Pre-mRNA splicing alters mRNP composition: Evidence for a stable association of proteins at exon–exon junctions. *Genes & Dev 14*:1098–1108.

Li S, Wilkinson MF. 1998. Nonsense surveillance in lymphocytes? *Immunity 8*:135–141.

Lou MJ, Reed R. 1999. Splicing is required for rapid and efficient mRNA export in metazoans. *Proc Natl Acad Sci USA 96*:14937–14942.

Maquat LE. 1995. When cells stop making sense: Effects of nonsense codons on RNA metabolism in vertebrate cells. *RNA 1*:453–465.

Maquat LE. 2000. Nonsense-mediated RNA decay in mammalian cells: A splicing-dependent means to down-regulate the levels of mRNAs that prematurely terminate translation. In: Sonenberg N, Hershey JBW, Mathews MB, eds. *Translational control of gene expression.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 849–868.

Matsumoto K, Wasserman KM, Wolffe AP. 1998. Nuclear history of a pre-mRNA determines the translational activity of cytoplasmic mRNA. *EMBO J 17*:2107–2121.

Moore M, Schaack J, Baim SB, Morimoto RI, Shenk T. 1987. Induced heat shock mRNAs escape the nucleocytoplasmic transport block in adenovirus-infected HeLa cells. *Mol Cell Biol 7*:4505–4512.

Moriarty PM, Reddy CC, Maquat LE. 1997. The presence of an intron within the rat gene for selenium-dependent glutathione peroxidase 1 is not required to protect nuclear RNA from UGA-mediated decay. *RNA 3*:1369–1373.

Moriarty PM, Reddy CC, Maquat LE. 1998. Selenium deficiency reduces the abundance of mRNA for Se-dependent glutathione peroxidase 1 by a UGA-dependent mechanism likely to be nonsense codon-mediated decay of cytoplasmic mRNA. *Mol Cell Biol 18*:2932–2939.

Morimoto RI. 1998. Regulation of the heat shock transcriptional response: Cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes & Dev 12*: 3788–3796.

Muhlrad D, Parker R. 1999. Aberrant mRNAs with extended 3′ UTRs are substrates for rapid degradation by mRNA surveillance. *RNA 5*:1299–1307.

Nagy E, Maquat LE. 1998. A rule for termination codon position within intron-containing genes: When nonsense affects RNA abundance. *Trends Biol Sci 23*:198–199.

Nesic D, Zhang J, Maquat LE. 1995. Lack of an effect of the efficiency of RNA 3′ end formation on the efficiency of removal of either the final or penultimate intron in intact cells. *Mol Cell Biol 15*:488–496.

Pandey NB, Chodchoy N, Liu TJ, Marzluff WF. 1990. Introns in histone genes alter the distribution of 3′ ends. *Nucleic Acids Res 18*:3161–3171.

Peltz SW, Brown AH, Jacobson A. 1993. mRNA destabilization triggered by premature translational termination depends on at least three *cis*-acting sequence elements and on transacting factor. *Genes & Dev 7*:1737–1754.

Ruiz-Echevarría MJ, Gonzaléz CI, Peltz SW. 1998. Identifying the right stop: Determining how the surveillance complex recognizes and degrades an aberrant mRNA. *EMBO J 17*:575–589.

Ruiz-Echevarría MJ, Peltz SW. 2000. The RNA binding protein Pub1 modulates the stability of transcripts containing upstream open reading frames. *Cell 101*:741–751.

Ryu WS, Mertz JE. 1989. Simian virus 40 late transcripts lacking excisable intervening sequences are defective in both stability in the nucleus and transport to the cytoplasm. *Virol 63*:4386–4394.

Sierra F, Stein G, Stein J. 1983. Structure and in vitro transcription of a human H4 histone gene. *Nucleic Acids Res 11*:7069–7086.

Sun X, Li X, Moriarty PM, Henics T, LaDuca JP, Maquat LE. 2001. Nonsense-mediated decay of mRNA for the selenoprotein phospholipid hydroperoxide glutathione peroxidase is detectable in cultured cells but masked or inhibited in rat tissues. *Mol Biol Cell*: in press.

Sun X, Maquat LE. 2000. mRNA surveillance in mammalian cells: The relationship between introns and translation termination. *RNA 6*:1–8.

Sun X, Moriarty PM, Maquat LE. 2000. Nonsense-mediated decay of glutathione peroxidase 1 mRNA in the cytoplasm depends on intron position. *EMBO J 19*:4734–4744.

Sun X, Perlick HA, Dietz HC, Maquat LE. 1998. A mutated human homologue to yeast Upf1 protein has a dominant-negative effect on the decay of nonsense-containing mRNAs in mammalian cells. *Proc Natl Acad Sci USA 95*:10009–10014.

Thermann R, Neu-Yilik G, Deters A, Frede U, Wehr K, Hagenmeier C, Hentze MW, Kulozik AE. 1998. Binary specification of nonsense codons by splicing and cytoplasmic translation. *EMBO J 12*:3484–3494.

Vries RGJ, Flynn A, Patel JC, Wang XM, Denton RM, Proud CG. 1997. Heat shock increases the association of binding protein-1 with initiation factor 4E. *J Biol Chem 272*:32779–32784.

Wigler M, Sweet R, Sim GK, Wold B, Pellicer A, Lacy E, Maniatis T, Silverstein S, Axel R. 1979. Transformation of mammalian cells with genes from prokaryotes and eucaryotes. *Cell 16*:777–785.

Zhang J, Maquat LE. 1997. Evidence that translation reinitiation abrogates nonsense-mediated mRNA decay in mammalian cells. *EMBO J 16*:826–833.

Zhang J, Sun X, Qian Y, LaDuca JP, Maquat LE. 1998a. At least one intron is required for the nonsense-mediated decay of triosephosphate isomerase mRNA: A possible link between nuclear splicing and cytoplasmic translation. *Mol Cell Biol 18*:5272–5283.

Zhang J, Sun X, Qian Y, Maquat LE. 1998b. Intron function in the nonsense-mediated decay of β-globin mRNA: Indications that pre-mRNA splicing in the nucleus can influence mRNA translation in the cytoplasm. *RNA 4*:801–815.

Zhou Z, Lou MJ, Straesser K, Katahira J, Hurt E, Reed R. 2000. The protein Aly links pre-messenger-RNA splicing to nuclear export in metazoans. *Nature 407*:401–405.