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LETTER TO THE EDITOR

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

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INTRODUCTION

Selenoprotein mRNAs of mammalian cells normally (1) derive from a pre-mRNA harboring at least one intron (Knudsen & Brunak, 1997) and (2) contain one or more UGA triplets that direct incorporation of the nonstandard amino acid selenocysteine (Sec) into the encoded protein (reviewed in Low & Berry, 1996). Selenium (Se) deprivation leads to inefficient utilization of the UGA codon as a Sec codon and reduces the abundance of many selenoprotein mRNAs, including those for Se-dependent glutathione peroxidase 1 (Se-GPx1) (Saedi et al., 1988; Toyoda et al., 1990; Lei et al., 1995; Weiss et al., 1996; P.M. Moriarty, C.C. Reddy, & L.E. Maquat, in prep.), type I iodothyronine deiodinase (Hill et al., 1992), selenoprotein P (Hill et al., 1992), and selenoprotein W (Vendeland et al., 1995). Considering that all organisms appear to have developed mechanisms that reduce the abundance of mRNAs that prematurely terminate translation because of an in-frame nonsense codon (reviewed in Peltz et al., 1994; Maquat, 1995, 1996), it is reasonable to attribute the reduction in selenoprotein mRNA abundance during Se deficiency to recognition of the UGA codon as nonsense. Consistent with this idea, under Se-supplemented conditions of cell growth, converting the TGA codon of the rat type I iodothyronine deiodinase (5′ DI) gene to a TGC cysteine codon has been shown to increase the level of 5′ DI protein (Berry et al., 1994), and converting the TGA codon of the rat Se-GPx1 gene to a TGC cysteine codon has been shown to increase the level of Se-GPx1 mRNA (P.M. Moriarty, C.C. Reddy, & L.E. Maquat, in prep.).

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A recent Letter to the Editor by Knudsen and Brunak (1997) proposed that selenoprotein pre-mRNAs assume a "kissing loop" secondary structure that incorporates part of the exon containing the UGA codon as well as part of the downstream intron. The letter went on to speculate that the structure offers new insight into the mechanism by which nonsense codons can be recognized in the nucleoplasm. The mechanism, referred to as "nuclear scanning," has been proposed by some investigators to reduce the quantity of nuclear mRNA, in some cases by influencing pre-mRNA splicing (see, e.g., Dietz & Kendzior, 1994; Aoufouchi et al., 1996), and in other cases by mediating the decay of nuclear mRNA (see, e.g., Carter et al., 1996; Li et al., 1997).

We have experimentally addressed the provocative ideas put forth in the letter by analyzing rat Se-GPx1 gene expression. This gene harbors a single in-frame TGA codon and a single intron located 105-bp downstream (Ho & Howard, 1992). Although Knudsen and Brunak (1997) did not analyze the rat Se-GPx1 gene, they did analyze the Se-GPx1 genes of human, mouse, and *Schistosoma mansoni*, along with five other selenoprotein genes. Therefore, their data imply that kissing loops would be characteristic of the rat Se-GPx1 gene, if not all intron-containing selenoprotein genes. In fact, comparing the 204-bp kissing loop region of the mouse Se-GPx1 gene to the corresponding region of the rat Se-GPx1 gene reveals only 18 differences or 88% identity.

We have analyzed expression of the rat Se-GPx1 gene, with and without the single intron, in cells grown in Se-supplemented and Se-deficient media. Data described here and elsewhere (P.M. Moriarty, C.C. Reddy, & L.E. Maquat, in prep.) indicate that inefficient incorporation of Sec at the UGA codon of Se-GPx1 mRNA

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results in the nonsense-mediated decay of cytoplasmic mRNA without an effect on nuclear RNA metabolism. Therefore, at least for Se-GPx1 transcripts, Knudsen and Brunak were correct in hypothesizing that recognition of the UGA codon as nonsense does not alter nuclear RNA metabolism. However, as shown here, there is no effect of Se deficiency on the nuclear metabolism of Se-GPx1 transcripts that derive from an intronless gene. Therefore, the nonsense-mediated decay of Se-GPx1 mRNA appears to be restricted to the cytoplasm, and the intron is not required to preclude nuclear recognition of the UGA codon as nonsense.

EVIDENCE THAT THE UGA Sec CODON REDUCES THE ABUNDANCE OF RAT Se-GPx1 mRNA BY ELICITING NONSENSE-MEDIATED DECAY IN THE CYTOPLASM

The Se dependence of Se-GPx1 gene expression that characterizes the endogenous gene of rat liver has been recapitulated by transiently transfecting NIH-3T3 cells with a pmCMV-GPx1 test plasmid and a pmCMV-Gl reference plasmid. The test plasmid harbors a rat Se-GPx1 allele driven by the mouse cytomegalovirus (mCMV) promoter (Fig. 1), and the reference plasmid harbors a β-globin allele similarly driven by the mCMV promoter. The amount of RNA produced from the reference allele was used to normalize the amount of RNA produced from the test allele, so as to control for variations in the efficiencies of cell transfection and RNA recovery.

Using RT-PCR to quantitate the level of test and reference transcripts from transfected cells that had been propagated for 48 h in either Se-deficient or Se-supplemented medium, Se deficiency was found to reduce the level of rat Se-GPx1 mRNA in total-cell RNA to 50% the level observed in Se-supplemented cells (P.M. Moriarty, C.C. Reddy, & L.E. Maquat, in prep.). The extent of the reduction was comparable to the extent observed for Se-GPx1 mRNA from the endogenous gene of NIH-3T3 cells and other cultured cells, even after 20 days in Se-deficient medium (Chada et al., 1989; P.M. Moriarty, C.C. Reddy, & L.E. Maquat, in prep.). Therefore, changing to another type of cultured cell or incubating longer in Se-deficient medium (i.e., performing stable transfections) would not lead to a larger decrease in Se-GPx1 mRNA abundance.

The analysis of nuclear and cytoplasmic RNA revealed that Se did not alter the metabolism of either nuclear pre-mRNA or nuclear mRNA; only cytoplasmic mRNA was reduced in abundance by Se deficiency (Fig. 2, GPx1 gene; P.M. Moriarty, C.C. Reddy, & L.E. Maquat, in prep.). Changing the TGA Sec codon to either a TAA nonsense codon or a TGC cysteine codon eliminated the sensitivity of cytoplasmic Se-GPx1 mRNA to Se deficiency, indicating that the TGA codon was critical to the regulation of Se-GPx1 gene expression by Se (P.M. Moriarty, C.C. Reddy, & L.E. Maquat, in prep.). Furthermore, relative to the TGA-containing allele expressed under Se-deficient conditions, the ratio of cytoplasmic-to-nuclear Se-GPx1 mRNA was 1.7-fold lower for the TAA-containing al-

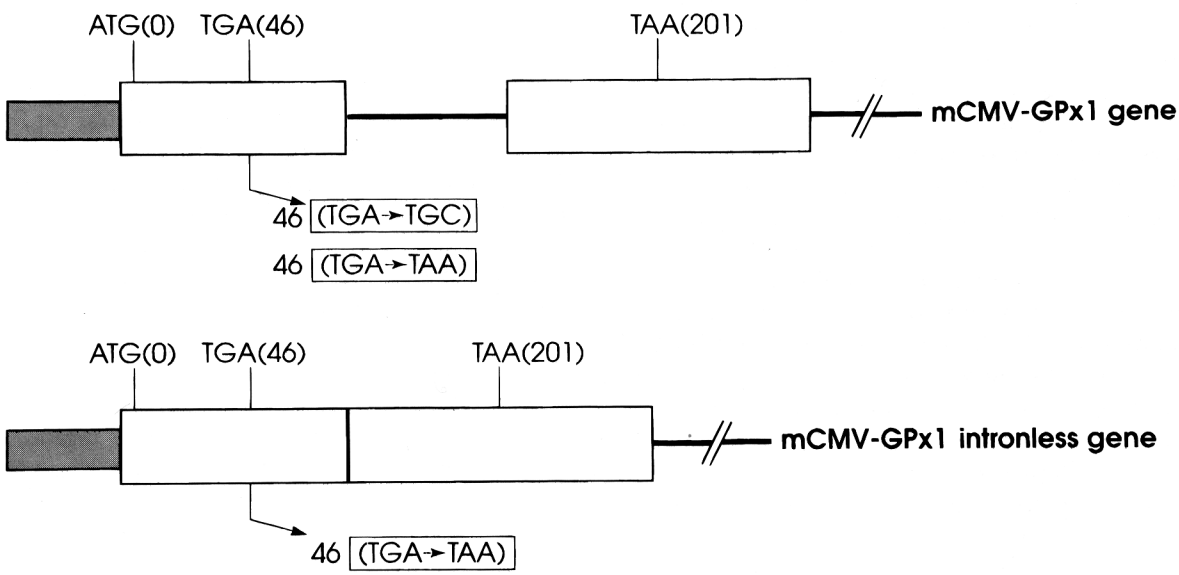

FIGURE 1. Structures of the mCMV-GPx1 gene, the mCMV-GPx1 intronless gene, and derivative alleles. Gray boxes specify sequences from mouse cytomegalovirus (mCMV) that contain the promoter (P.M. Moriarty, C.C. Reddy, & L.E. Maquat, in prep.). Open boxes specify exons, the intervening line of the upper structure specifies the single 216-bp intron, and the right-most line in both structures specifies 3′-flanking DNA. ATG(0), TGA(46), and TAA(201) indicate, respectively, the initiation codon, Sec codon, and termination codon. Mutations that convert the Sec codon to either a cysteine codon (TGC) or a premature termination codon (TAA) are designated below the gene structures.

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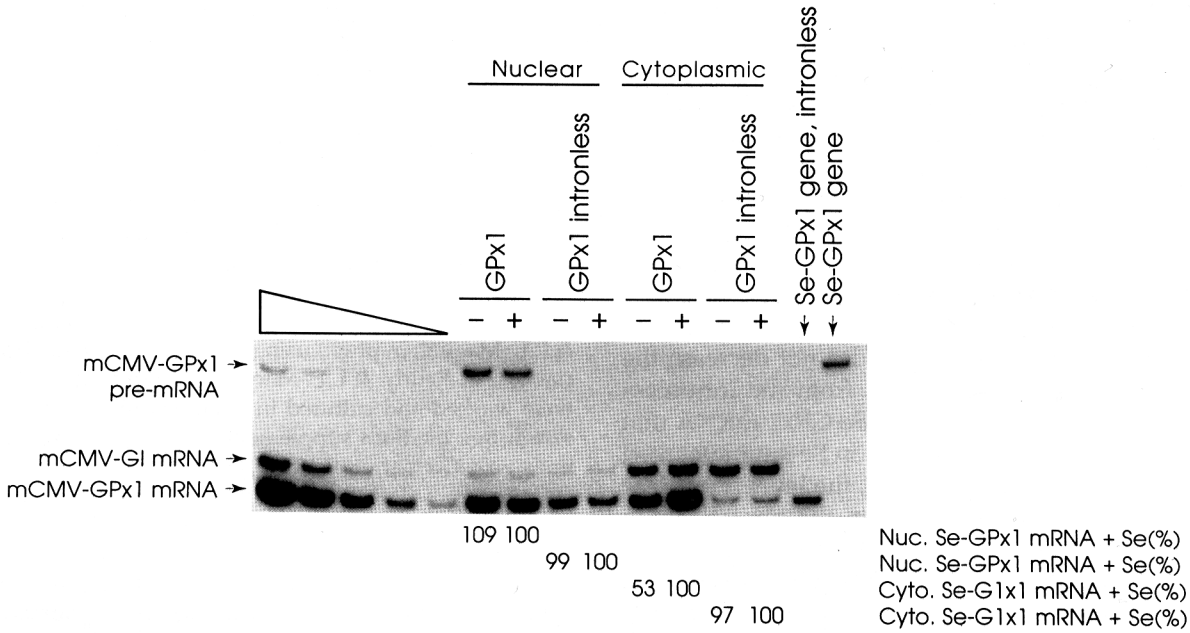

FIGURE 2. Deleting the intron from pmCMV-GPx1 does not elicit the decay of nuclear Se-GPx1 RNA when recognition of the Sec UGA as nonsense in increased by Se deficiency. NIH-3T3 cells were transiently transfected with a test plasmid, either pmCMV-GPx1 or pmCMV-GPx1 intronless, and the reference pmCMV-Gl plasmid. After 12 h, the cells were transferred to either Se-deficient (−) medium or Se-supplemented (+) medium (P.M. Moriarty, C.C. Reddy, & L.E. Maquat, in prep.). After an additional 48 h, cells were harvested, nuclear (Nuc.) and cytoplasmic (Cyto.) RNA was isolated, and mCMV-GPx1 and mCMV-Gl transcripts were quantitated by RT-PCR as described in Materials and Methods. The five left-most lanes represent twofold serial dilutions of RNA from Se-supplemented cells transfected with pmCMV-GPx1 and pmCMV-G1. These lanes demonstrate a linear relationship between the amounts of input RNA and RT-PCR products. The level of mCMV-GPx1 mRNA in the each of the next eight lanes was normalized to the level of mCMV-Gl mRNA in that lane. The normalized value for mCMV-GPx1 mRNA in each Se-deficient sample was then calculated as a percentage of the normalized value for mCMV-GPx1 mRNA in the corresponding Se-supplemented sample, which was defined as 100. Values represent an average of two independently performed experiments. The two right-most lanes, which constitute the PCR analyses of intronless pmCMV-GPx1 DNA and pmCMV-GPx1 DNA, provided size standards for pre-mRNA and mRNA, respectively.

lele and 3-fold higher for the TGC-containing allele. In contrast, the ratio of nuclear Se-GPx1 mRNA to nuclear Se-GPx1 pre-mRNA for either the TAA-containing allele or the TGC-containing allele was unaffected by the concentration of Se. These findings suggest that the premature termination of translation at position 46 mediates the decay of cytoplasmic Se-GPx1 mRNA, whether the codon be a TGA codon in Se-deficient cells or a TAA codon in either Se-deficient or Se-supplemented cells. Additionally, these findings suggest that some decay is also characteristic of cytoplasmic mRNA that derives from TGA-containing allele under Se-supplemented conditions.

DELETING THE INTRON FROM THE RAT Se-GPx1 GENE DOES NOT ELICIT THE DECAY OF NUCLEAR Se-GPx1 RNA

Having demonstrated that the efficiency with which the UGA codon of Se-GPx1 transcripts is utilized as a Sec codon affects only the metabolism of cytoplasic Se-GPx1 mRNA, we set out to test the hypothesis of Knudsen and Brunak (1997). These authors proposed that the metabolism of nuclear Se-GPx1 RNA would not be affected by the efficiency with which the UGA codon is utilized as a Sec codon because of a secondary structure, involving the UGA codon and the downstream intron, that hides the UGA codon from recognition in the nucleus (i.e., from nuclear scanning). If their proposal is correct, then deleting the intron from the Se-GPx1 gene would result in the decay of nuclear transcripts.

NIH-3T3 cells were transiently transfected with the pmCMV-Gl reference plasmid and the pmCMV-GPx1 intronless test plasmid. The test plasmid harbors an intronless rat Se-GPx1 allele containing the usual TGA codon at position 46 (Fig. 1). In control experiments, cells were transfected with the reference plasmid and pmCMV-GPx1, which harbors the usual intron-containing rat Se-GPx1 allele. Twelve hours after transfection, cells were placed in Se-deficient or Se-supplemented medium, and nuclear and cytoplasmic RNA was isolated 48 h later. RT-PCR was used to quantitate the levels of plasmid-derived Se-GPx1 and β-globin RNAs without interference from NIH-3T3-cell transcripts.

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Interestingly, deleting the Se-GPx1 gene intron eliminated the twofold decrease in the level of cytoplasmic Se-GPx1 mRNA caused by Se deficiency (Fig. 2). In other words, when the level of cytoplasmic Se-GPx1 mRNA that derived from the intronless allele in Se-supplemented cells was assigned a value of 100, the level of cytoplasmic Se-GPx1 mRNA that derived from the same allele in Se-deficient cells was 97 (Fig. 2). We conclude that the intron is required for Se responsiveness, which we have shown to be strictly dependent on the TGA codon (P.M. Moriarty, C.C. Reddy, & L.E. Maquat, in prep.). Se deficiency did not decrease the level of nuclear Se-GPx1 mRNA from the intronless allele: when the level of nuclear Se-GPx1 mRNA that derived from the intronless allele in Se-supplemented cells was assigned a value of 100, the level of nuclear Se-GPx1 mRNA that derived from the same allele in Se-deficient cells was 99 (Fig. 2). Because Se deficiency increases recognition of the UGA codon as nonsense (P.M. Moriarty, C.C. Reddy, & L.E. Maquat, in prep.), our finding that deleting the intron does not reduce the abundance of nuclear Se-GPx1 mRNA under Se-deficient conditions indicates that the intron does not sequester the UGA codon from nuclear recognition.

Consistent with the finding that the intron is required for Se-responsiveness of the TGA-containing allele, changing the TGA codon to a TAA nonsense codon in the absence of the intron did not change the level of Se-GPx1 mRNA (data not shown). Therefore, as has been found for the human triosephosphate isomerase gene (J. Zhang, Y. Qian, & L.E. Maquat, in prep.), the presence of at least one intron is required for nonsense-mediated mRNA decay.

CONCLUSIONS

We conclude that the UGA codon of rat Se-GPx1 mRNA is recognized as nonsense less frequently when cells are grown in Se-supplemented medium than when cells are grown in Se-deficient medium. Recognition as nonsense affects the metabolism of cytoplasmic Se-GPx1 mRNA exclusively. However, the lack of an effect on nuclear Se-GPx1 RNA does not require the presence of the intron within pre-mRNA. Therefore, the proposals by Knudsen and Brunak (1997) of (1) a nuclear scanning mechanism that recognizes nonsense codons within the nucleoplasm and (2) a "kissing loop" structure that precludes nonsense codon recognition during scanning are unnecessary.

MATERIALS AND METHODS

Plasmid DNAs

pmCMV-GPx1 intronless was generated from pmCMV-GPx1 (P.M. Moriarty, C.C. Reddy, & L.E. Maquat, in prep.) by replacing the 457-bp *Xma* I–*Dra* III fragment that extends from exon 1 into exon 2 with the corresponding 251-bp *Xma* I–*Dra* III fragment from pGPx1211 (Reddy et al., 1988), which harbors Se-GPx1 cDNA.

Cell transfections and RNA isolations

NIH-3T3 cells (1×10^7 cells/15 cm dish; 40–50% confluency) were transiently transfected with a test plasmid, either pmCMV-GPx1 (25 μg; P.M. Moriarty, C.C. Reddy, & L.E. Maquat, in prep.) or pmCMV-GPx1 intronless (25 μg), and the reference pmCMV-Gl plasmid (25 μg; J. Zhang, Y. Qian, & L.E. Maquat, in prep.) using calcium phosphate (P.M. Moriarty, C.C. Reddy, & L.E. Maquat, in prep.). After 12 h, cells were washed and cultured in either serum-free medium or serum-free medium supplemented with 50 ng/mL of selenous acid (Kelner et al., 1995) as described (P.M. Moriarty, C.C. Reddy, & L.E. Maquat, in prep.). Serum-free medium consisted of a 1:1 (vol:vol) mixture of Ham's F-12 (Life Technologies) and DMEM plus 25 μg/mL of bovine holotransferrin, 10 μg/mL of bovine insulin, 10 ng/mL of mouse epidermal growth factor, and 25 μg/mL of human high-density lipoprotein (Chiang et al., 1985). Cells were harvested after another 48 h. RNA was isolated from nuclear and cytoplasmic fractions following Method 1 of Belgrader et al. (1994), except that Trizol reagent (Life Technologies) rather than CsCl gradient centrifugation was used to purify nuclear RNA. RNA (25 μg) was treated with RQ1 DNase (1U; Promega Corp.) for 1 h at 37 °C.

RT-PCR analyses

For all RT-PCR (Cheng & Maquat, 1993; P.M. Moriarty, C.C. Reddy, & L.E. Maquat, in prep.), cDNA was synthesized using 0.16–5.0 μg of nuclear or cytoplasmic RNA using RT (Superscript, GIBCO) and random hexamers (Promega). Each PCR reaction contained 1/20 of the RT reaction mixture, 0.12 mM of each of the four deoxynucleotides, 4 μCi of [α-^{32}P]dATP (3,000 Ci/mmol; Amersham), 0.5 μM of each of two primers, and 2.5 units of Taq DNA polymerase (Promega). mCMV-GPx1 and mCMV-Gl cDNAs were amplified using a sense primer 5′-ACCACCGTAGAACGCAGATCG-3′ that corresponds to the common mCMV promoter region. The antisense primer used to amplify mCMV-GPx1 cDNA, 5′-CTTCTCACCATTCACCTCGCACTT-3′, corresponds to Se-GPx1 exon 2. The antisense primer used to amplify mCMV-Gl cDNA, 5′-CGGGGTGAAGCTCCTTGCCAAG-3′, corresponds to exon 3 of the mouse β-globin gene (Cheng & Maquat, 1993). Notably, Se-GPx1 cDNA that derived from the endogenous NIH-3T3-cell gene was not amplified. One-tenth of each PCR reaction was electrophoresed in a 4% denaturing polyacrylamide gel, and RT-PCR products were quantitated using a Phosphorimager and ImageQuant software (Molecular Dynamics).

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