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Selenium-Regulated Translation Control of Heterologous Gene Expression: Normal Function of Selenocysteine-Substituted Gene Products

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Abstract In eukaryotes, the synthesis of selenoproteins depends on an exogenous supply of selenium, required for synthesis of the novel amino acid, selenocysteine, and on the presence of a "selenium translation element" in the 3′ untranslated region of mRNA. The selenium translation element is required to re-interpret the stop codon, UGA, as coding for selenocysteine incorporation and chain elongation. Messenger RNA lacking the selenium translation element and/or an inadequate selenium supply lead to chain termination at the UGA codon. We exploited these properties to provide direct translational control of protein(s) encoded by transfected cDNAs. Selenium-dependent translation of mRNA transcribed from target cDNA was conferred by mutation of an in-frame UGU, coding for cysteine, to UGA, coding for either selenocysteine or termination, then fusing the mutated coding region to a 3′ untranslated region containing the selenium translation element of the human cellular glutathione peroxidase gene. In this study, the biological consequences of placing this novel amino acid in the polypeptide chain was examined with two proteins of known function: the rat growth hormone receptor and human thyroid hormone receptor β1. UGA (*opal*) mutant-STE fusion constructs of the cDNAs encoding these two polypeptides showed selenium-dependent expression and their selenoprotein products maintained normal ligand binding and signal transduction. Thus, integration of selenocysteine had little or no consequence on the functional activity of the *opal* mutants; however, *opal* mutants were expressed at lower levels than their wild-type counterparts in transient expression assays. The ability to integrate this novel amino acid at predetermined positions in a polypeptide chain provides selenium-dependent translational control to the expression of a wide variety of target genes, allows facile ^{75}Se radioisotopic labeling of the heterologous proteins, and permits site-specific heavy atom substitution.

Selenoproteins form a unique group of polypeptides that contain the unusual amino acid, selenocysteine. This small group of proteins is found in both prokaryotes and eukaryotes and includes the prokaryotic enzymes in the formate dehydrogenase family [1,2], and several eukaryotic polypeptides, including the glutathione peroxidase (GPx) enzymes [3–6], type I iodothyronine deiodinase [7], and selenoprotein P [8]. In all these proteins, the universal termination codon, UGA, is used to direct selenocysteine incorporation into the growing polypeptide chain [9]. In addition, both specific secondary structural elements in the mRNA [10,11], and a unique selenocysteine-charged $tRNA^{[Ser]SeC}$ containing the UCA anticodon [12,13] are required to interpret the UGA correctly as coding for selenocysteine. Selenocysteine synthesis is also unique and this novel amino acid is formed by the enzyme-catalyzed substitution of selenium for the phosphate of a phosphoserine-charged $tRNA^{[Ser]SeC}$ [14,15]. Since selenium is essential to synthesize selenocysteine [16], removal of selenium from the diet or culture medium leads to a marked reduction in the cellular levels of all selenoproteins [17–20], presumably due to an inability to translate the UGA codon. Thus, the

Abbreviations: The abbreviations used are: T_3, 3′,3,5-triiodothyronine; hTR, human thyroid hormone receptor; GHRs, short-form of the growth hormone receptor; hGPx, human glutathione peroxidase; STE, selenium translation element; DMEM, Dulbecco's Modified Eagle's medium; G418, Geneticin, HEPES, 4-(2-hydroxethyl)-1-piperazineethanesulfonic acid.

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selenium supply determines whether the UGA triplet in a selenoprotein transcript serves as a selenocysteine codon or a translational stop signal.

Bacteria and mammals differ in the mechanisms that are used by the translational apparatus to distinguish between a UGA triplet coding for selenocysteine, or signaling chain termination. In the mRNA encoding *Escherichia coli* formate dehydrogenase, selenocysteine incorporation depends on a 40-nucleotide stem-loop located immediately downstream from the UGA codon and on several critical bases located in the middle of this stem-loop [21,22]. In mammals, chain elongation at a UGA codon also depends upon stem-loop structure(s) in the mRNA, but these are located in the 3′ untranslated region (3′UTR) of selenoprotein transcripts and have been identified as the "selenocysteine-insertion sequence" (SECIS) [10] or the "selenium translation element" (STE). All mammalian STEs share a similar potential stem-loop conformation of approximately 100 nucleotides; however, within the motif, only three short, 2–4 nucleotide segments are highly conserved [10,11,23–25]. For example, deletions or multiple substitution mutations in any of the three short sequence elements in the STE element of the mammalian cellular glutathione peroxidase gene, *GPX1,* severely diminishes expression of full-length glutathione peroxidase [23,25]. On the other hand, single nucleotide substitutions are readily tolerated when these mutations do not alter the overall the potential stem-loop configuration [25].

We exploited the selenium-dependent nature of selenocysteine synthesis and the requirement for a STE to direct selenocysteine incorporation at a UGA codon to provide translational control over heterologous protein expression. Selenium-dependent translation of the polypeptide is achieved by mutating a UGU (encoding cysteine) to a UGA (encoding selenocysteine or termination) in the coding region of the cDNA, then fusion of the mutated coding region to a 3′UTR containing the STE from human cellular glutathione peroxidase. We previously showed that such a construct programmed the incorporation of ^{75}Se into a UGA mutant of rab5b [23], a small G protein with a molecular mass similar to that of glutathione peroxidase. In this study, we examine the levels of protein expression and the functional consequences of the introduced selenocysteine residue for two selenocysteine mutants of proteins of known function, the circulating form of the rat growth hormone receptor (rGHRs) and the β1 isoform of the human thyroid hormone receptor (hTRβ1).

EXPERIMENTAL PROCEDURES

Materials

The 1.2-kb rat adipocyte GHRs cDNA [26] and the 1.7-kb human TRβ1 cDNA [27] were generously provided by Drs. H.M. Goodman and R.M. Evans, respectively. The human GPXI cDNA [28] served as the source of the STE. The T_3-dependent reporter plasmid, pF2H-luc [29], and anti-TRβ1 IgG were the gifts of Dr. W.W. Chin. Antisera directed against the C-terminus of GHRs (A1615) was provided by Dr. H.M. Goodman. COS7 and CV-1 kidney cells and C6 astrocytoma cells were obtained from the American Tissue Culture Collection. T_3 was obtained from Henning GmBH, Berlin, GBR. Dulbecco's modified Eagle's medium (DMEM), Ham's F12 medium (F12), antibiotics, Geneticin (G418), Hank's solution, and 0.25% trypsin were obtained from Gibco, Grand Island, NY and defined bovine calf serum (heat-inactivated) from HyClone, Boulder, CO. Culture flasks were obtained from Nunc; 6- and 24-well tissue culture plates were obtained from Costar, Fisher Sci, MA. All other reagents used were of the highest purity commercially available.

Production of *opal* (UGA) Mutants

The cDNAs encoding rGHRs and hTRβ1 were each subcloned into the *Eco*R1 site of pALTER-1 and oligonucleotide-directed mutagenesis performed according to manufacturer's instructions (Promega). The TGT (wt) to TGA (*opal*) mutations at the codons for (1) amino acid 56 in GHRs, and (2) amino acid 290 in TRβ1 were confirmed by sequencing with the fmol DNA sequencing kit (Promega, Madison, WI). Both *opal* and wild-type cDNAs were cut from pALTER-1 with *Eco*R1, the 5′ overhangs filled in with the T4 DNA polymerase, and the blunt-ended cDNAs purified by agarose gel electrophoresis. *Bst*XI adaptors were then ligated to the eluted *opal* and wild-type cDNAs, using T4 DNA ligase and unligated adaptors, were removed by chromatography on Sepharose Cl-6B spin columns (Boehringer Mannheim).

Eukaryotic Expression Constructs

All cDNAs were ligated into the *Bst*XI site of the eukaryotic expression plasmid, p*OPAL* de-

rived from pRC/CMV (Invitrogen, San Diego, CA). The p*OPAL* expression plasmid was prepared by ligating the 212 nucleotide *Avr*II–*Xba*I restriction fragment of the *GPXI* gene, containing the 3′UTR STE (nt920–1132) [29], into the *Xba*I site of pRC/CMV, generating p*OPAL,* and orientation was confirmed by DNA sequencing. Constructs carrying the wild-type (TGT) and *opal* (TGA) mutations of rGHRs and hTRβ1 were completed by ligation of the individual cDNAs into the BstXI site of p*OPAL*. All constructs were confirmed by DNA sequencing.

Cell Culture

COS7, CV-1, and C6 cells were maintained in 75-cm^2 culture flasks (Nunc) and grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C in DMEM supplemented with 15 mM sodium bicarbonate, 33 mM glucose, 1 mM sodium pyruvate, and 15 mM HEPES, pH 7.4, supplemented with 10% (v/v) calf serum, 50 U/ml penicillin and 90 μg/ml streptomycin. Culture media was changed 3 times weekly.

Transient Transfection

One day prior to transfection, COS7 or CV-1 cells were seeded as indicated at 75,000 cells/well into six-well cluster plates, or 50,000 cells/well in 24-well cluster plates, and transfected by the $CaPO_4$ co-precipitation (30) method with test plasmids (see individual experiments) and 0.2 μg of the transfection control plasmid, pSVβgal (Promega). After 4 h, the transfection medium was replaced with serum-free DMEM/F12 medium supplemented with insulin (20 μg/ml), transferrin (10 μg/ml), hydrocortisone (100 nM), bovine serum albumin (BSA) (1 mg/ml), penicillin (50 U/ml) and streptomycin (90 μg/ml). As indicated in individual experiments, increasing concentrations of T_3 and/or 40 nM ^{75}Se as Na^{75} selenite (150 Ci/g) were also added. Cells were then grown for 48 h at 37°C in a humidified atmosphere containing 5% CO_2.

Stable Transfection

C6 astrocytoma cells (100,000 cells/25-cm^2 flask) were transfected by $CaPO_4$ co-precipitation with 10 μg of either p*OPAL* (vector), p*OPAL*-TRwt (TRwt), or p*OPAL*-TRm290 (TR*opal*). After a 24-h recovery period, growth medium was changed to DMEM containing 10% bovine serum, 15 mM HEPES buffer (pH 7.1), penicillin (50 U/ml), and streptomycin (90 μg/ml) and supplemented with 200 μg/ml G418 (Life Technologies). After 14 d, individual colonies of G418-resistant cells were isolated by limiting dilution in the presence of G418 (200 μg/ml). Fifteen G418-resistant cell lines expressing the TR*opal* and 24 G418-resistant cell lines expressing TRwt were obtained. Vector cells were prepared from a pool of G418-resistant cells transfected with p*OPAL* without clonal isolation.

Analytical Methods

GHR expression was determined by measuring the quantity of protein bound ^{125}I-labeled rGH precipitated with an antisera directed against the C-terminal 17-amino acids (A1615) of GHRs as described by Frick and Goodman [26]. ^{75}Se incorporation into secreted GHRs was done by immunoprecipitation, followed by γ-counting.

Functional TR expression was determined using a T_3-dependent reporter system described previously [29]. COS7 cells (50,000 cells/well) were seeded into 24-well cluster plates (Costar) and each well transfected by $CaPO_4$ co-precipitation with 0.5 μg pF2H-luc (T_3-reporter), 0.2 μg pRSVβgal (control, Promega), and 1 μg of either p*OPAL* (vector), p*OPAL*–TRwt (TRwt), or p*OPAL*–TRm290 (TR*opal*). After a 24-h recovery period, medium was changed to serum-free DMEM/F12 medium, ±40 nM selenium and ±100 nM T_3 and cells were grown for an additional 72 h. Luciferase and β-galactosidase activities were determined using commercial kits according to manufacturer's instructions.

Immunoprecipitation of metabolically labeled hTRβ1 was done by pulse-labeling cells expressing the TRwt and/or TR*opal* with 10 μCi/ml ^{35}S-Met (1,000 Ci/mmol, DuPont) in serum-free DMEM/F12 for 6 h. Cell nuclei were isolated as previously described [31], the TRs extracted with 0.3 M KCl, and then immunoprecipitated with anti-TRβ1 IgG(32). Immunoprecipitates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), under reducing conditions, and radiolabeled TR determined by autoradiography.

Immunocytochemistry

Clone 9 and C6TRwt cells were grown on poly-*l*-lysine (10 μg/ml)-coated coverslips in serum-free DMEM/F12 medium, ±40 nM selenium and ±100 nM T_3 and cells were grown for 48 h. Cell monolayers were washed free of defined medium, fixed with 4% paraformaldehyde,

and permeabilized with iced methanol. Myelin basic protein (MBP) was visualized by indirect immunofluorescence, using antimyelin basic protein IgG (Boheringer-Mannheim) and antirabbit IgG conjugated with Texas Red (Amersham, Arlington Heights, IL). TRs were visualized using anti-TRβ1 antisera and antirabbit IgG conjugated with Texas Red (Amersham).

All experiments were performed at least three times. Statistical analysis was done by Student's *t*-test.

RESULTS

GHR Expression

Figure 1 shows the schematic representation of the cDNA-STE constructs used. The 212-nucleotide *Avr*II–*Xba*I restriction fragment containing the GPx STE provided the downstream stem-loop and sequence elements necessary to interpret UGA as a selenocysteine codon in all constructs. A coding region TGA (*opal*) mutant was generated by mutating the TGT (cysteine) codon to TGA at amino acid position 56 of rGHR (upper pair of constructs in Fig. 1) and at amino acid position 290 of hTRβ1 (lower pair of constructs). The *opal* mutant and wild-type cDNAs were inserted 5′ to the GPx STE and transcription was terminated by the bovine growth hormone polyadenylation signal in the eukaryotic expression vector p*OPAL*, derived from pRC/CMV (Invitrogen).

As shown in Figure 2A, transient expression of the *opal* mutant GHRs fusion construct, rGHRm56-STE, in COS7 cells programmed the synthesis and secretion of a full-length, immunoprecipitable growth hormone receptor. Since the antireceptor antibody is directed against the C-terminus of GHRs, translation products truncated at amino acid 56, due to chain termination at the UGA in the absence of selenium, are not detected in this functional assay [26]. Co-precipitation of ^{125}I-labeled growth hormone (open bars) indicates that the *opal* mutant rGHRm56-STE construct retains ligand binding activity, and the quantity of GHR*opal* secreted was proportional to the quantity of plasmid transfected. Immunoprecipitation of ^{75}Se-labeled rGHRs-*opal* (hatched bars) indicates that selenocysteine was incorporated into the full-length polypep-

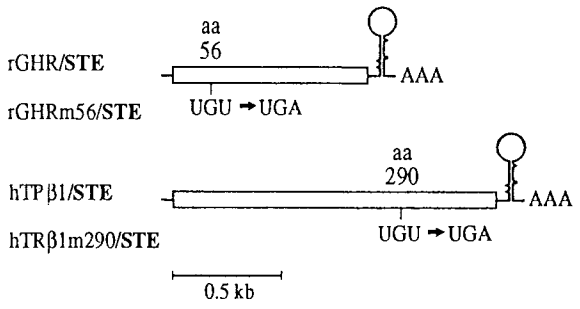


Fig. 1. Schematic representation of the cDNA-STE constructs.

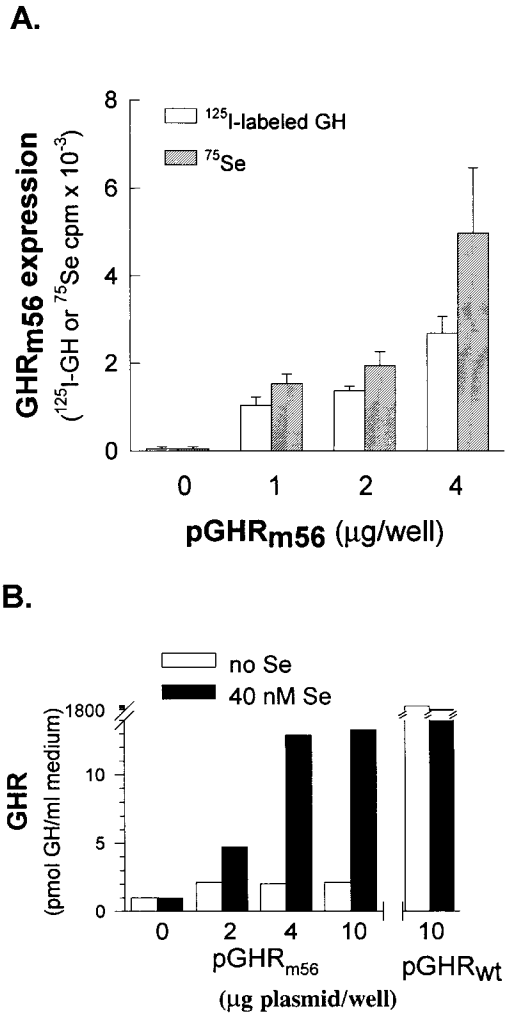


Fig. 2. **A:** Expression of *opal* mutant GHRs. COS7 cells (75,000 cells/well) were seeded into six-well clusters plates (Costar) and transfected in triplicate with increasing amounts of p*OPAL*-GHRm56 as described in Experimental Procedures. GHRs expression was determined in triplicate in 300-μl aliquots of medium by the method of Frick and Goodman [25] and ^{75}Se incorporation into the GHRs immunoprecipitate determined by γ-counting. Data are reported as mean ± SE (n = 4). **B:** Selenium-dependent expression of *opal* mutant GHRs. Triplicate wells of COS7 cells were transfected with increasing amounts of p*OPAL*-GHRm56 or p*OPAL*–GHRwt, as described in Experimental Procedures, grown for 48 h in DMEM/F12 with or without 40 nM selenium, and GHRs determined. Data are reported as the mean of quadruplicate wells.

tide. Scatchard analysis (data not shown) revealed that the *opal* mutation of rGHRs did not affect ligand affinity; however, the number of *opal* rGHR molecules secreted into the culture medium was only 1–3% of that secreted by cells expressing the wild-type, rGHRwt-STE construct. As shown in Figure 2B, expression of the *opal* mutant rGHRm56-STE construct was entirely dependent on the presence of selenium in the culture medium. By contrast, alterations in the selenium content of the growth medium had no effect on expression of the wild-type rGHR-STE construct. While the 3'UTR located STE is essential for expression of the *opal* mutant of the rGHRs, the presence of this element in the 3'UTR of the wild-type rGHR construct had little or no effect on expression of the wild-type receptor and the absolute levels of wild-type receptor were 30- to 100-fold greater than that of the *opal* mutant of GHRs in transiently transfected COS7 cells (Fig. 2B).

TR Expression

The *opal* mutant of the thyroid hormone receptor was examined using a functional reporter assay that requires both ligand binding and signal transduction. This heterologous reporter system uses a T_3-responsive reporter plasmid (pF2H-luc) [29] consisting of a thyroid hormone response element (TRE) from the chick lysozyme gene (TRE_{F2H}), the inducible thymidine kinase (TK) promoter, and the cDNA encoding luciferase. Shown in Figure 3 are the results obtained when pF2H-luc was co-transfected into COS7 cells along with the different thyroid hormone receptor constructs. Transient expression of the *opal* mutant of the thyroid hormone receptor, hTRm290-STE, in selenium-replete cells produces a thyroid hormone receptor that yielded a >2-fold, T_3-dependent increase in luciferase expression when compared to cells grown in the absence of T_3. Like the *opal* mutants of rGHR, the level TR*opal* expression was less than that of the wild type transfectants, presumably due to lower receptor number. In cells grown in selenium-free medium, both the *opal* mutant hTRm290-STE and wild-type TR-STE controls repressed luciferase expression by 30–60% in the absence of T_3, when compared to the p*OPAL* vector controls ($P > 0.05$ TRwt $P > 0.02$ TRm290), presumably due to the well-described dominant negative effect of an unliganded thyroid hormone receptor [27,33]. Only the wild-type TR mediated T_3-dependent luciferase expression in the absence of selenium; the TR*opal* mutant failed to show hormonal control of reporter expression in the absence of selenium.

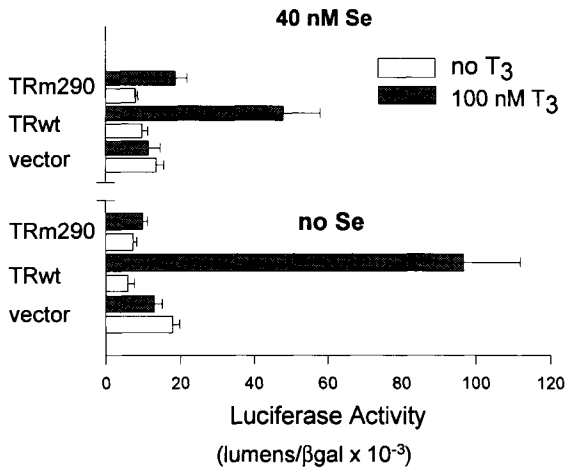


Fig. 3. Selenium-dependent expression of functional *opal* mutant TRβ1. COS7 cells (50,000 cells/well) were seeded into 24-well cluster plates (Costar) and transfected by $CaPO_4$ coprecipitation with 0.5 μg pF2H-luc, 0.2 μg pRSVβgal (Promega), and 1 μg of either p*OPAL* (vector), p*OPAL*–TRwt, or p*OPAL*–TRm290. After a 24-h recovery period, medium was changed to DMEM/F12 medium ±40 nM selenium and ±100 nM T_3 and cells were grown for an additional 72 h. Luciferase and β-galactosidase activities were determined using commercial kits according to manufacturer's instructions. Data are reported as the mean ±SE of quadruplicate wells.

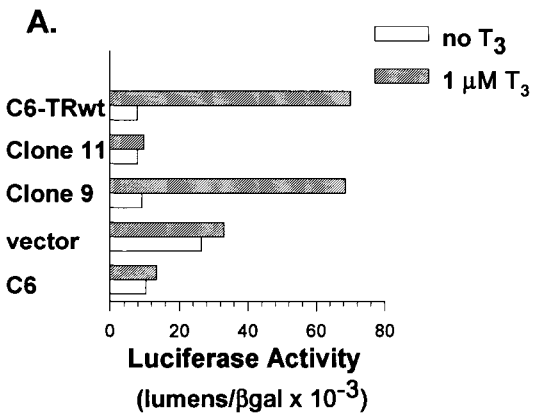
While both the rGHR*sopal* and TR*opal* mutant-STE fusion constructs directed the selenium-dependent incorporation of selenocysteine at UGA codons and produced selenium-containing, functional receptor molecules, the levels of heterologous protein expression fell well below the wild-type controls in the transient transfection experiments. This presumably results from the demands of multiple plasmid copy number, the strong promoters used to overexpress the introduced gene products, and the limiting supply of selenocysteine-charged $tRNA^{[Ser]SeC}$. We therefore turned to expression of the TR*opal* mutant in cells stably expressing this protein.

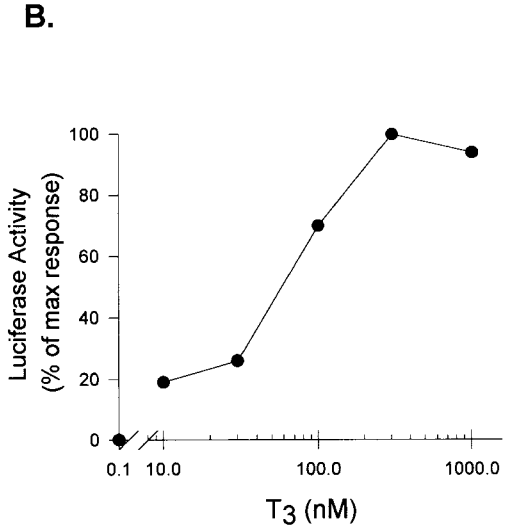
Figure 4A shows the levels of functional TR obtained in selected clonal lines of rat C6 astrocytoma cells that were stably transfected with either the *opal* mutant, TRm290-STE construct, wild-type TRwt-STE construct, or p*OPAL* expression vector controls and selected using G418 resistance. Transient transfection of selected, TR expressing cell lines with the pF2H-luc reporter plasmid, and analysis of T_3-dependent reporter activity revealed that cells harboring the *opal* mutant TRm290-STE con-

struct (clone 9) yielded ~7-fold, T_3-dependent increase in reporter expression, and suppressed reporter expression in the absence of the T_3 by ~60% when compared to vector controls. Importantly, expression of the TR*opal* equaled that obtained with TRwt. Several other G418-resistant C6 clones failed to express the *opal* TR mutant (e.g., Fig. 4A, clone 11), presumably due to incomplete integration of a full-length TR cDNA into the genome. The T_3-dependent C6 cell line, clone 9, was used in all subsequent experiments.

As shown in Figure 4B, clone 9 cells showed the expected dose-response relationship for T_3-dependent transactivation of reporter gene expression suggesting adequate receptor affinity and number for TR*opal*. Scatchard analysis (Fig. 4C) of T_3 binding to isolated nuclei showed no significant difference between the ligand affinity or receptor number between the TRs derived from TRwt-STE or the *opal* mutant TRm290-STE constructs (Table I).

Shown in Figure 5A is the cellular distribution of the TR*opal* protein in clone 9 cells. Immunocytochemical analysis showed that cells grown in selenium containing medium contained abundant nuclear TR, while cells grown in the absence of selenium had little, if any, TR or truncated TR products either in the cytoplasm or in the nucleus. A comparison of the quantity of nuclear TR content for clone 9 cells and C6TRwt cells was done by direct immunoprecipitation of metabolically labeled of nuclear proteins. Figure 5B is a representative fluorogram of anti-TRβ1 IgG, immunoprecipitated, [^{35}S]-Met-labeled TRs after separation by SDS–PAGE. The *opal* mutant TRm290 and TRwt fusion constructs produce approximately equal amounts of receptor protein in selenium-replete medium; however,





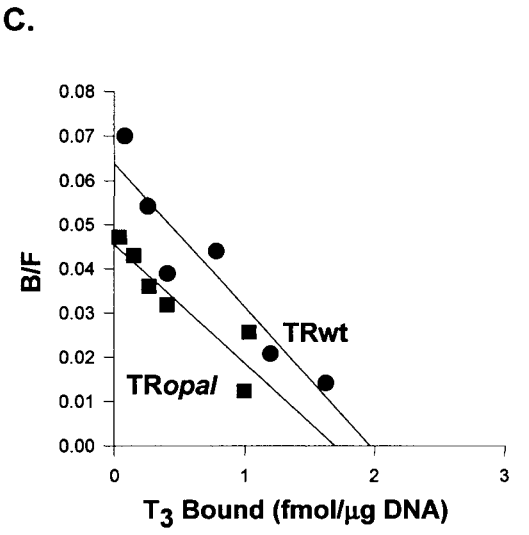


Fig. 4. **A:** Identification of C6 cell lines constitutively expressing the *opal* mutant of TRβ1. C6 astrocytoma cells (100,000 cells) were transfected by $CaPO_4$ coprecipitation with 10 μg of either p*OPAL* (vector), p*OPAL*–TRwt (TRwt), or p*OPAL*–TRm290 (TR*opal*), and G418-resistant cells lines cloned by limiting dilution. Selected cell lines were seeded (50,000 cells) in quadruplicate into 24-well cluster plates and transfected with 0.2 μg of pF2H-luc and 0.2 μg pRSVβgal by $CaPO_4$ coprecipitation and grown for 72 h in serum-free DMEM/F12 ±100 nM T_3. Luciferase and β-galactosidase activities were determined as described in the legend to Figure 2. **B:** T_3-dependent reporter expression in clone 9 cells. Clone 9 cells (75,000 cells/well) were transfected by $CaPO_4$ co-precipitation with 0.2 μg pF2H-luc and 0.2 μg of pRSVβgal and cells grown in serum-free DMEM/F12 supplemented with increasing concentration of T_3 in triplicate. Reporter activities were determined as described above. **C:** Scatchard analysis of nuclear T_3-binding in clone 9 and C6TRwt cells. Clone 9 cells and C6TRwt were grown in four 80-cm^2 flasks to confluence, cell nuclei prepared as described before [32], and TR determined by T_3-binding analysis [28,32]. Each point of the Scatchard plot was determined in triplicate.

TABLE I. Analysis of the Quantity of Nuclear TR in C6-TRwt and clone 9 Cells[a]

Cell line	Integrated cDNA	T3 binding (fmol/10^6 cells)	^{75}Se-labeled TR (fmol/10^6 cells)
C6-TRwt	hTRβ1-STE	9.5 ± 3.4 (4)	ND
Clone 9	hTRm290-STE	8.0 ± 2.0 (3)	7.5 ± 1.2 (3)

[a]T_3 binding capacity was determined by Scatchard analysis. ^{75}Se-labeled TR in clone 9 cells was determined in 0.3 M KCl extracts of isolated nuclei [32]. Data are presented as the mean ±SE; numbers in parentheses are the number of independent determinations.

no immunoreactive TR was found for clone 9 cells grown in the absence of selenium. Similarly, the quantity of the chromatin-associated, [^{75}Se]labeled, immunoprecipitated TR present in the clone 9 cell nucleus was in close agreement with the estimates made by ligand binding Scatchard analysis (Table I).

Finally, we examined the ability of the TR*opal* mutant to transactivate native gene(s) using the expression of the T_3-dependent gene, myelin basic protein [34]. Figure 6 presents photomicrographs of myelin basic protein (MBP) expression in clone 9 cells and in C6TRwt cells grown in the absence and presence of 40 nM selenium. As expected, clone 9 cells expressing TR*opal* showed normal T_3-dependence of myelin basic protein expression when selenium was present in the medium (top). Removal of selenium from the culture conditions, led to the complete loss of T_3-dependent expression of MBP in clone 9 cells (middle), while control cells harboring the wild-type TR retained full T_3-dependent expression of MBP in the absence of selenium (bottom). Thus, T_3-dependent expression of MBP was regulated by the selenium content of the medium, when C6 cells expressed the *opal* mutant of the TR, while changes in selenium availability had no effect on T_3-dependent MBP expression in cells containing the wild-type TR.

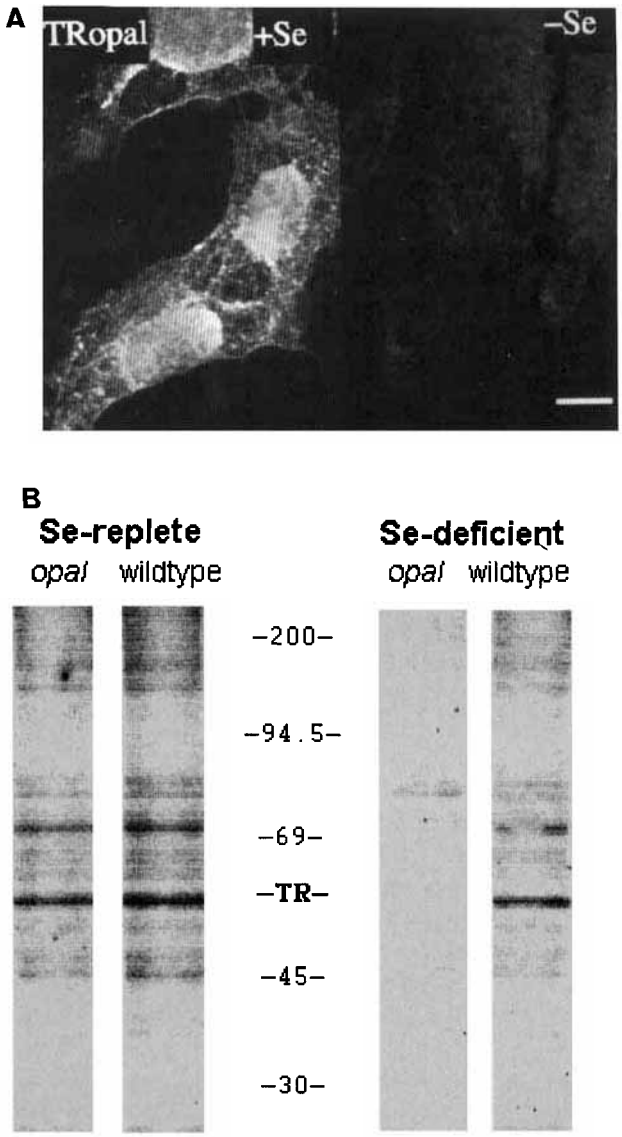


Fig. 5. **A:** Selenium-dependent nuclear localization of TR*opal*. Clone 9 cells were grown on poly-*l*-lysine coated cover slips as described in Experimental Procedures in the absence or presence of 40 nM selenium. TR was visualized using a rabbit anti-TRβ1 antisera, and immune complexes identified with a goat antirabbit IgG conjugated to the fluorochrome Texas Red. Bar = 10 μm. **B:** Selenium-dependence of nuclear TR expression in clone 9 cells. Clone 9 and C6TRwt cells were grown in triplicate in 25-cm^2 flasks to ~80% confluence. Culture medium was changed to serum-free DMEM/F12 ±40 nM Se for 2 days. At 6 h before harvest the medium was changed to serum-free DMEM/F12 containing 10 μCi/ml ^{35}S-methionine. Cell nuclei were prepared as described in Experimental Procedures, TR extracted with 0.3 M KCl, and immunoprecipitated with anti-TRβ1 IgG [32]. Immunoprecipitates were resolved by SDS–PAGE under reducing conditions and autoradiography performed.

DISCUSSION

Selenium-dependent translation of the circulating form of the rat growth hormone receptor and the human TRβ1 was conferred by conservative replacement of selenocysteine for cysteine using mutation of a UGA (encoding cysteine) to a UGA (encoding selenocysteine or termination), then fusion of the mutated coding region(s) to a 3′UTR containing the STE from GPX1, a ubiquitous selenoprotein gene. The resultant *opal* rGHRm56-STE and *opal* hTRm290-STE fusion constructs programmed the selenium-dependent expression of each receptor, and the selenoprotein products maintained normal ligand binding and signal transduction capabilities. Importantly, both *opal* mutants were processed

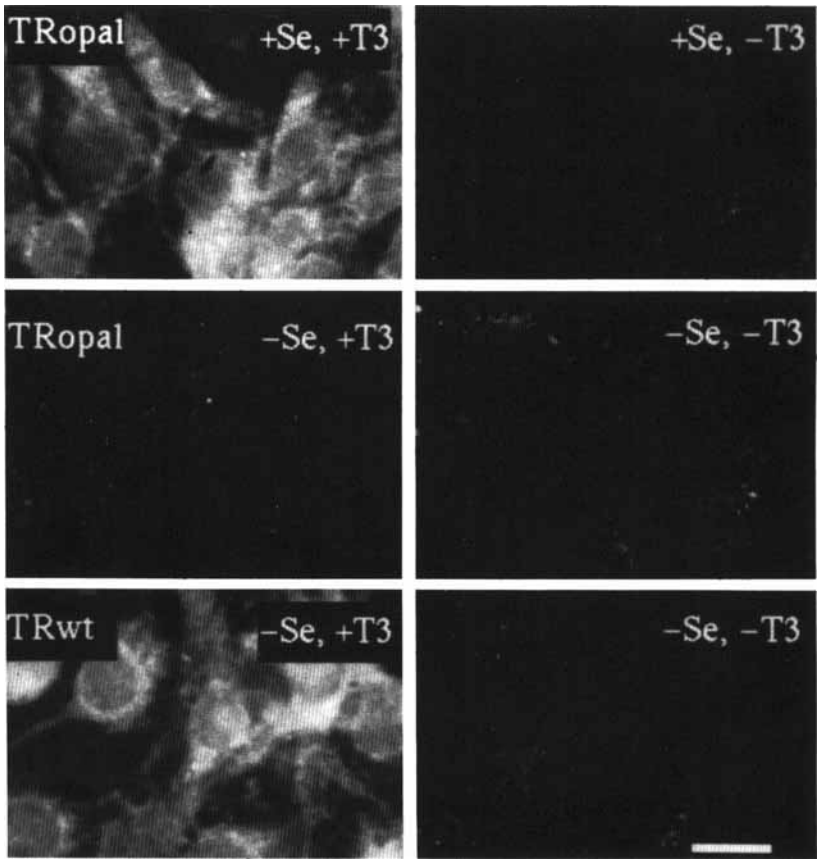


Fig. 6. Selenium-regulated expression of the *opal* mutant of TRβ1 determines the hormone-dependent expression of myelin basic protein. Clone 9 and C6TRwt cells were grown on coverslips in serum-free DMEM/F12 medium supplemented ±40 nM Se and/or ±100 nM T_3 for 2 days. Myelin basic protein was visualized by indirect immunofluorescence using anti-myelin basic protein IgG from Boheringer-Mannheim, and Texas Red-conjugated goat antirabbit IgG from Amersham. Bar = 10 μm.

in the cell normally; GHR*opal* was secreted and TR*opal* translocated to the cell nucleus. The latter polypeptide also retained its ability to regulate the expression of the native T_3-dependent gene, MBP. Thus, replacement of cysteine by selenocysteine did not disturb the fate of the mutant protein, did not alter the cellular processing of these two mutant proteins, and did not affect the functional capabilities of either receptor.

While expression of selenocysteine mutants of the rGHRs and hTRβ1 was readily demonstrated in transient transfection systems, expression levels fell well below those of the wild-type controls. Similarly, replacement of selenocysteine with cysteine in the authentic selenoprotein, type I iodothyronine deiodinase, also resulted in a substantial increase in expression of the enzyme polypeptide [35], illustrating that the differences in expression levels observed with *opal* mutants of non-selenoproteins are also observed in authentic selenoproteins. In all of these cases, the failure of selenoprotein to reach the expression levels achieved by its non-selenocysteine containing polypeptide is presumable due to the demands of multiple plasmid copy number, the strong promoters used to overexpress the introduced gene products, and the limiting supply of selenocysteine-charged $tRNA^{[Ser]SeC}$. While the limiting supply of $tRNA^{[Ser]SeC}$ provides an attractive explanation for the differences in expression levels of *opal* mutants and their wild-type parent, overexpression of this tRNA led to only a twofold increase in the expression of the authentic selenoprotein, type I iodothyronine deiodinase in transient expression assays [36], suggesting that other cellular factors limit selenoprotein synthesis in situ. Alternatively, inefficient interpretation of the UGA codon for selenocysteine incorporation may result from losses of a large fraction of the *opal* mutant mRNA due to degradation by cytoplasmic editing enzymes that eliminate RNAs containing nonsense codons [37]. Such accelerated cytoplasmic degradation of

RNAs containing an in-frame UGA, especially in the absence of selenium supplementation, would tend to amplify the differences between selenium-dependent expression levels of *opal* containing mutants of polypeptides carried in the p*OPAL* expression vector. Under selenium-deficient conditions, both translation and cytoplasmic mRNA levels of the transfected gene product will be diminished due to the interpretation of the UGA codon as a stop signal and due to the accelerated clearance of the *opal* mutant mRNA by cytoplasmic editing enzymes.

Interestingly, integration of the TR*opal* mutant-STE fusion construct into the genome overcame the problems of limited expression and equivalent quantities of TR*opal* and TRwt were found in cells carrying this introduced gene product. Analysis of the TR translation products found in the stably transfected cells revealed that only full-length TR was present and little, if any, truncated TR was found, even in cell grown in selenium-deficient medium. Since truncation of the TR at amino acid 290 in the *opal* mutant produces a polypeptide containing the DNA-binding domain, but lacking the ligand binding domain of the receptor, the truncated TR*opal* should retain the ability to bind to the TREs of T_3-responsive genes, functionally similar to that of the v-erbA oncogene product [38]. While the truncated TR*opal* mutant produced in transient expression experiments suppressed T_3-dependent reporter expression in cells grown in selenium-deficient medium, such truncated forms of the TR were not found either in the nucleus or cytoplasm of stably transfected cells. These findings suggest that the truncated TR is short-lived in the cell, presumably due to rapid clearance and such truncated products may be present only during the short times typical of transient expression assays. Since most cells rapidly eliminate truncated translation products, selenium-dependent regulation of translation is likely to exert control at three separate points in the synthetic pathway (1) mRNA stabilization; (2) polypeptide chain completion, and (3) elimination of potentially confounding truncated translation products.

The ability of transfected cells to direct the full-length expression of *opal* mutants of non-selenoproteins carrying an in-frame UGA codon definitively establishes the ability of an STE in the 3′untranslated region of an mRNA to interpret a coding region UGA codon as a signal for selenocysteine incorporation rather than for chain termination. Similarly, by conservative replacement of selected nucleophilic residues such as cysteine with selenocysteine, preservation of function in gene products can be maintained. Thus, *opal* mutants of both the GHR and TR, and presumably any polypeptide, retain normal functional properties, creating the potential for the general application of selenium-dependent translational control to the expression of a wide variety of target genes, and for site-specific heavy atom substitution or ^{75}Se radioisotopic labeling of the transfected gene products.

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