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# Regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase mRNA levels in rat liver

(cholestyramine/mevinolin/mevalonolactone/*in vitro* translation/immunoprecipitation)

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ABSTRACT **Addition of cholestyramine or cholestyramine plus mevinolin to the diet has been reported to increase the activity and mass of rat liver 3-hydroxy-3-methylglutaryl-CoA reductase. The present data show that these same dietary manipulations cause an induction of functional reductase mRNA. RNA was isolated from rat livers and added to an *in vitro* translation system, and the reductase was immunoprecipitated and analyzed by polyacrylamide gel electrophoresis under denaturing conditions. One protein was specifically immunoprecipitated and was found to have a $M_r$ of 90,000 on 0.5 M urea/sodium dodecyl sulfate/polyacrylamide gels and a $M_r$ of 94,000 on 8 M urea/sodium dodecyl sulfate/polyacrylamide gels. In animals fed rat chow supplemented with 5% cholestyramine and 0.1% mevinolin, reductase mRNA levels were 5.7-fold higher than in animals fed rat chow with 5% cholestyramine and were 16-fold higher than in animals fed rat chow with 5% cholestyramine and given mevalonolactone by stomach intubation. RNA isolated from animals fed a normal diet and killed at the nadir of the diurnal cycle of enzyme activity contained no detectable amounts of reductase mRNA as determined by this assay.**

The microsomal enzyme 3-hydroxy-3-methylglutaryl-CoA reductase is a highly regulated enzyme (reviewed in refs. 1–3). The enzyme produces mevalonate, an intermediate in the biosynthesis of cholesterol, ubiquinone, dolichol, and isopentenyl-tRNA (1). The enzyme exhibits a diurnal rhythm of activity in rat liver (2, 3), and the activity can be induced 50- to 250-fold after addition of cholestyramine or cholestyramine and mevinolin to the diet (4). The activity of the enzyme declines rapidly after administration of mevalonolactone to rats (5). The mechanisms that cause these changes in enzyme activity are not known.

Utilizing antibody prepared to purified reductase, we have determined the effect of cholestyramine, mevinolin, and mevalonolactone on the cellular level of reductase mRNA. We demonstrate that the enzyme polypeptide has $M_r$ of 94,000 and that the levels of reductase mRNA are highly regulated. We propose that proteolysis of the enzyme occurs during normal purification and leads to an enzymatically active peptide of $M_r$ < 60,000.

## MATERIALS AND METHODS

**Materials.** $^{14}$C-Labeled $M_r$ standards and [$^{35}$S]methionine were purchased from Amersham. Nuclease from *Staphylococcus aureus* was purchased from Boehringer Mannheim; Pansorbin (*S. aureus* bearing protein A) was from Calbiochem; *N*-lauroylsarcosine, aprotinin, and leupeptin were from Sigma; bovine serum albumin was from Pentex (Kankakee, IL); and oligo(dT)-cellulose (type 3) was from Collaborative Research (Waltham, MA). Mevinolin was a gift from A. Alberts. The sources of all other materials have been given (6).

**Antibody Preparation.** Reductase was purified from rats fed a diet supplemented with cholestyramine and mevinolin (4), and antibody was prepared in rabbits as described (6). The antiserum obtained after three injections of pure protein (80 μg per injection) was used without further purification. This antiserum inactivated the reductase in *in vitro* assays (unpublished data). IgG was purified from normal rabbit serum as described (7).

**RNA Preparation.** Male Sprague–Dawley rats were maintained on a 12 hr light/12 hr dark cycle and received powdered rat chow supplemented with 5% cholestyramine (Questran) or 5% cholestyramine plus 0.1% mevinolin according to the procedure of Tanaka *et al.* (4). To designated animals receiving rat chow with 5% cholestyramine, mevalonolactone (1.0 mg/g rat body weight) was administered by stomach intubation at the time indicated below. At specific times during the light/dark cycle, animals were killed and RNA was extracted from 1 g of liver tissue according to the procedure of Chirgwin *et al.* (8). Poly(A)$^+$ RNA was isolated as described (8).

***In Vitro* Translation of Poly(A)$^+$ RNA.** Rabbit reticulocyte lysates were prepared under RNase-free conditions as described (9) and treated with *S. aureus* nuclease to render them mRNA-dependent (10). *In vitro* translations were carried out as described by Pelham and Jackson (10) except that addition of unlabeled amino acids was omitted and incubations were performed at 37°C for 60 min. For translating both the reductase peptide and other high molecular weight peptides, we have found this system to be much more efficient than commercially available rabbit reticulocyte lysates. A typical *in vitro* translation mixture contained 50 μl of treated rabbit reticulocyte lysate, 6 μl of [$^{35}$S]methionine (specific activity, >1,000 Ci/mmol; 1 Ci = 3.7 × $10^{10}$ Bq) and rat liver poly(A)$^+$ RNA. *In vitro* translations were stopped by the addition of 6 vol of ice-cold phosphate-buffered saline (0.137 M NaCl/2.68 mM KCl/1.47 mM $KH_2PO_4$, 8.1 mM $Na_2HPO_4$) containing 0.1% *N*-lauroylsarcosine, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, aprotinin at 2.9 μg/ml, and bovine serum albumin at 10 mg/ml. Total incorporation of [$^{35}$S]methionine into trichloroacetic acid-precipitable material was determined as described (11). Only functional mRNAs are detected by this assay, and quantitation of relative percentage of one species will be affected by the number of methionine residues present in the synthesized protein. Comparisons of reductase mRNA levels have been made on the assumption that the efficiency of translation is the same among reductase mRNAs isolated from different rats.

**Immunoprecipitation of [$^{35}$S]Methionine-Labeled Reductase.** *In vitro* translation mixtures were treated with 40 μl of Pansorbin [10% (vol/vol) in phosphate-buffered saline/1% bo-

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vine serum albumin/0.1% *N*-lauroylsarcosine]. All incubations with Pansorbin were carried out at 20°C for 1 hr and were followed by centrifugation at 12,500 × *g* for 2–5 min. Normal rabbit serum IgG (5.2 μg) or anti-reductase antiserum (4 μl) was added to the supernatant. Incubations with normal rabbit serum IgG were carried out at 4°C for 2 hr. Incubations with anti-reductase were carried out at 4°C overnight. Pansorbin was added (50 μl/4.0 μl of antiserum) and, after 60 min, was removed by centrifugation. The pellets were washed three times with phosphate-buffered saline/0.1% *N*-lauroylsarcosine and either stored at −20°C or processed directly for electrophoresis. We have observed that sequential immunoprecipitations (normal rabbit serum IgG and anti-reductase immunoprecipitates obtained from one sample) give the same results as direct immunoprecipitations (nonimmune and anti-reductase immunoprecipitates obtained from duplicate samples). The results shown here are from sequential immunoprecipitations.

**$NaDodSO_4$ Gel Electrophoresis.** Immunoprecipitates were analyzed on two types of $NaDodSO_4$/polyacrylamide gels (12) as indicated. The Pansorbin immunocomplex was resuspended in 70 μl of buffer A (2.025% $NaDodSO_4$/5% 2-mercaptoethanol/100 mM dithiothreitol/2 M urea/31 mM Tris·HCl, pH 6.8), heated for 5 min at 100°C, and centrifuged; the supernatant was added to 7 μl of solution B [50% (vol/vol) glycerol/0.01% bromophenol blue] and then applied to a 20-cm 0.5 M urea/5–20% polyacrylamide gel with a 0.5 M urea/4.5% polyacrylamide stacking gel. Alternatively, the Pansorbin was resuspended in 50 μl of buffer C (15% $NaDodSO_4$/10% 2-mercaptoethanol/62.5 mM Tris·HCl, pH 6.8) and incubated at 25°C for 40 min. After centrifugation, the supernatant was added to 8 μl of solution B, solid urea was added to a final concentration of 8.0 M, and the mixture was heated to 100°C for 5 min and applied to an 8.0 M urea/7.5% polyacrylamide gel with an 8 M urea/5% polyacrylamide stacking gel.

**Other Assays.** Fluorography was performed as described by Chamberlain (13). The position of a band on a polyacrylamide gel was determined from the fluorogram, and the radioactivity was quantitated by excision of the band, addition of 1.0 ml of 30% (wt/vol) $H_2O_2$, and incubation overnight at 70°C followed by the addition of Aquasol (14). Liver microsomes were prepared by homogenizing liver in 10 vol of 0.25 M sucrose and centrifuging at 16,000 × *g* for 15 min (G. Ness, personal communication). The supernatant was recentrifuged under the same conditions before isolation of the microsomal pellet (100,000 × *g* for 60 min). The pellet was resuspended in the sucrose and recentrifuged. The reductase specific activity in these membranes was equal to or greater than that in microsomes prepared from the same liver by a standard procedure (15) (unpublished data). The microsomal protein and assay of reductase activity were as described (15). Duplicate reductase assays varied by less than 5%.

## RESULTS

**Reductase mRNA Directs Synthesis of $M_r$ 90,000 Polypeptide.** Poly(A)$^+$ RNA from rat liver was used to direct a rabbit reticulocyte *in vitro* translation system supplemented with [$^{35}$S]methionine. Analyses of the immunoprecipitates on 0.5 M urea/$NaDodSO_4$/polyacrylamide gels showed that the anti-reductase specifically precipitated one polypeptide of $M_r$ 90,000 (Fig. 1, lanes 1 and 2). The immunoprecipitation of the $M_r$ 90,000 polypeptide was specifically inhibited by the addition of 4.75 μg of purified reductase (Fig. 1, lane 3) even though the purified enzyme has been shown to be composed of two polypeptides of $M_r$ 52,000 and 54,000 (4). A number of other polypeptides were precipitated nonspecifically; they were observed after use of antireductase antiserum (Fig. 1, lanes 1–3) or normal rabbit sera (Fig. 1, lane 4) and their intensities were not decreased after addition of pure enzyme (Fig. 1, lane 3). We propose that the newly synthesized reductase polypeptide has a $M_r$ of 90,000 when determined on this denaturing gel system. In agreement with this proposal, we have found that, when rat hepatocytes were incubated with [$^{35}$S]methionine and the radiolabeled reductase was immunoprecipitated and analyzed on this same gel system, a single polypeptide of $M_r$ 90,000 was observed (unpublished data). Occasionally, reductase aggregates ($M_r$ ≐ 180,000, and higher) were also observed on this gel system (data not shown). Such aggregates were either not observed or were significantly decreased when the immunoprecipitates obtained after *in vitro* translation of rat liver mRNA or after radiolabeling of rat hepatocytes with [$^{35}$S]methionine, were analyzed on 8 M urea/$NaDodSO_4$/polyacrylamide gels (Fig. 2). In this gel system, the reductase polypeptide migrated with an apparent $M_r$ of 94,000. We concluded that the reductase polypeptide was denatured more fully in the presence of 8 M urea.

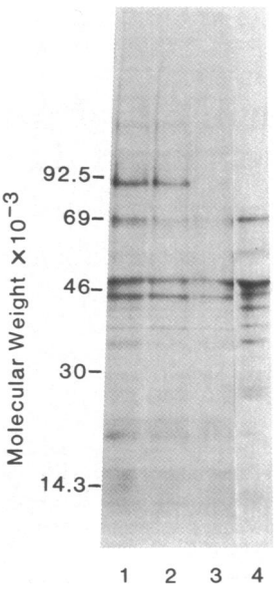


FIG. 1. $^{35}$S-Labeled translation products immunoprecipitated in the presence and absence of purified reductase. Rat liver poly(A)$^+$ RNA was isolated from rats fed diets supplemented with cholestyramine and mevinolin and killed at hour 10 of the daily 12-hr dark period (4). The RNA was translated at a final concentration of 100 μg/ml in a rabbit reticulocyte lysate system supplemented with [$^{35}$S]methionine (1.14 mCi/ml). The total translation products were immunoprecipitated and subjected to 0.5 M urea/$NaDodSO_4$/polyacrylamide gel electrophoresis. Lanes: 1, 0.5 μl of anti-reductase antiserum; 2, 0.5 μl of anti-reductase antiserum plus 100 μl of buffer D (1 mM CoA/4 mM EDTA/125 mM KCl/5 mM dithiothreitol/10 mM potassium phosphate, pH 6.8); 3, 0.5 μl of anti-reductase antiserum plus 100 μl of purified reductase (4.75 μg in buffer D); 4, 5.25 μg of normal rabbit serum IgG. Fluorograms were exposed for 1 week at −70°C. $M_r$ standards are indicated.

**Optimizing the *in Vitro* Translation System.** In order to use an *in vitro* translation system to quantitate the reductase mRNA levels, several conditions were optimized. The immunoprecipitation of the $M_r$ 90,000 polypeptide was maximal at 64 μl of anti-reductase antiserum per ml of translation mixture (data not shown). Two methods were used to dissociate the reductase polypeptide from the Pansorbin pellet prior to $NaDodSO_4$/polyacrylamide gel electrophoresis. Both methods released all of the reductase protein from the Pansorbin (data not shown).

We also determined whether the RNA concentration at which

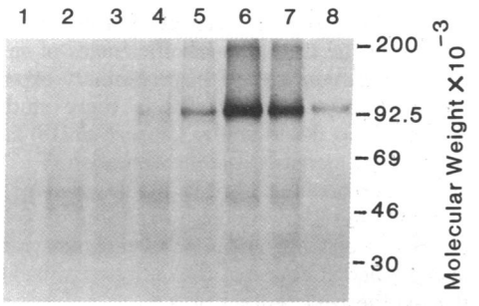


FIG. 2. Comparison of rat liver reductase mRNA levels in animals receiving diets that affect reductase activity. Rat liver poly(A)$^+$ RNA at a final concentration of 30 $\mu$g/ml was translated in a rabbit reticulocyte lysate system supplemented with [$^{35}$S]methionine at 1.39 mCi/ml. Total radioactivity incorporated into protein was determined as described and aliquots containing the same number of cpm (lanes 1–7, $4.97 \times 10^6$ cpm in a volume of 0.465 ml) were immunoprecipitated with 6.0 $\mu$g of normal rabbit serum IgG (lane 1) or 4.0 $\mu$l of anti-reductase antiserum (lanes 2–7). Immunoprecipitates were subjected to 8 M urea/NaDodSO$_4$/polyacrylamide gel electrophoresis. Lanes: 1 and 5, rat chow with 5% cholestyramine for 4 days, killed at hour 6, dark; 2 and 3, normal rat chow, killed at hour 6, light; 4, rat chow with 5% cholestyramine plus mevalonolactone given by stomach intubation 3 hr prior to killing at hour 6, dark; 6 and 7, rat chow with 5% cholestyramine and 0.1% mevinolin (4), killed at hours 8 and 10, dark, respectively. RNA was pooled from four animals (lanes 1, 4, 5, and 7) or two animals (lane 6) or prepared from individual animals (lanes 2 and 3). Lane 8 is an immunoprecipitate from $^{35}$S-labeled hepatocytes. Rat hepatocytes ($1.1 \times 10^6$) were prepared (6), incubated for 2 hr with [$^{35}$S]methionine, and lysed in 1.0 ml of 0.1% NaDodSO$_4$/0.5% deoxycholate/1% Triton X-100/5 mM EDTA/0.1 M NaCl/0.01 M phosphate, pH 7.5/1.0 mM leupeptin containing 0.3 $\mu$g of aprotinin. The sample was centrifuged at 100,000 $\times$ *g* for 1 hr, and the enzyme was immunoprecipitated from the supernatant essentially as described for the immunoprecipitation of the *in vitro* synthesized enzyme. The fluorogram was exposed for 24 hr at −70°C. $M_r$ standards are indicated.

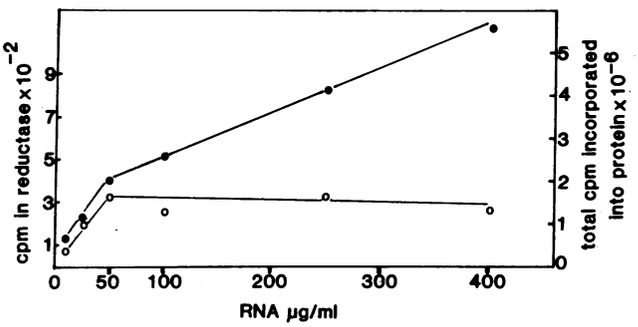


FIG. 3. Quantitation of reductase mRNA as a function of RNA concentration in the *in vitro* translation system. Rat liver poly(A)$^+$ RNA (isolated as in Fig. 1) at the final concentration shown was translated in a rabbit reticulocyte lysate system supplemented with [$^{35}$S]methionine at 1.19 mCi/ml. Total radioactivity incorporated into protein (●) was determined by trichloroacetic acid precipitation of two equal aliquots. The remainder of the translation mixture was immunoprecipitated with anti-reductase antiserum and subjected to 0.5 M urea/NaDodSO$_4$/polyacrylamide gel electrophoresis and fluorography. The fluorogram was used to localize the band migrating at $M_r$ 90,000 and the radioactivity in this band (○) was determined. No polypeptides of $M_r > 90{,}000$ were visible on the fluorogram.

the *in vitro* translation was performed affected the efficiency of translation of the mRNA encoding reductase (Fig. 3). At RNA concentrations <50 $\mu$g/ml, the radioactive content of the reductase polypeptide was a constant proportion (0.016%) of the radioactivity found in the total protein translation products. However, at RNA concentrations >50 $\mu$g/ml, the relative efficiency of translation of the reductase mRNA decreased relative to that of total liver mRNA. Consequently, in all further studies, rat liver mRNA was translated at a concentration of 30 $\mu$g/ml.

**Regulation of Reductase mRNA.** Rats were fed a normal diet and killed at the nadir of the circadian rhythm of reductase activity or were fed diets supplemented with cholestyramine or cholestyramine plus mevinolin to induce the enzyme activity to high levels (4) (Table 1). Translation of the liver mRNA from these same animals and analysis of the immunoprecipitated reductase showed that no functional mRNA for reductase was detectable in rats fed the normal diet (Fig. 2, lanes 2 and 3). The amount of reductase mRNA was greatest in animals having the highest levels of enzyme activity—i.e., those fed cholestyramine plus mevinolin (Fig. 2, lanes 6 and 7; Table 1). Intermediate levels of both reductase mRNA and reductase enzyme activity were observed in rats fed the diet supplemented with cholestyramine (Fig. 2, lane 5; Table 1). Administration of mevalonolactone to rats fed the cholestyramine-supplemented diet decreased the microsomal reductase activity and also decreased the amount of reductase mRNA (Fig. 2, lanes 4 and 5; Table 1).

In order to quantitate the levels of reductase mRNA, the radioactivity present in the $M_r$ 94,000 polypeptides shown in Fig. 2 was compared to the activity of the microsomal enzyme assayed from the same livers (Table 1). For purposes of comparison, the values obtained from rats fed diets supplemented with cholestyramine plus mevinolin and killed at hour 8 of the dark period (4) were given a value of 100%. In these animals, 0.07% (3,373 cpm) of the *in vitro* synthesized proteins were recovered in the $M_r$ 94,000 reductase. The relative decrease in enzyme activity observed in rats fed cholestyramine or cho-

Table 1. Quantitation of specific activities and functional mRNA levels of reductase

| Additions to diet | Time killed* | Radioactivity in reductase cpm† | Radioactivity in reductase %‡ | Reductase activity nmol MVA/min/mg proteins§ | Reductase activity %‡ |
|---|---|---|---|---|---|
| Cholestyramine/mevinolin | D8 | 3,373 | 100 | 33.6 ± 3.1 | 100 |
| Cholestyramine/mevinolin | D10 | 1,613 | 48 | 50 ± 3.3 | 149 |
| Cholestyramine | D6 | 591 | 18 | 7.9 ± 3.9 | 24 |
| Cholestyramine/mevalonolactone | D6 | 212 | 6 | 0.57 ± 0.25 | 1.7 |
| None | L6 | ND | — | 0.092¶ | 0.3 |

*Letters refer to the daily 12-hr dark (D) or light (L) schedule; numerals refer to the hour within designated period.

†The radioactivity in the $M_r$ 94,000 peptides in Fig. 2. A background of 217 cpm (determined from Fig. 2, lane 1) has been subtracted from all values. ND, not detectable.

‡The values obtained with rats fed cholestyramine and mevinolin and killed at D8 have been set at 100%.

§MVA, mevalonolactone. Activities are given as the mean ± SD.

¶Mean from two separate animals.

lestyramine plus mevalonolactone was paralleled by a similar decrease in reductase mRNA (Table 1). The lowest level of enzyme activity was observed in rats fed the normal diet. Under these conditions, reductase mRNA levels were undetectable.

In rats fed cholestyramine and mevinolin, the enzyme activity was 1.5-fold greater in animals killed at hour 10 of the dark period compared to those killed at hour 8 (Table 1). However, the reductase mRNA levels do not show this same relationship. Further study is required to determine whether a rhythm of reductase mRNA levels precedes the diurnal cycle of reductase activity (4).

## DISCUSSION

Recent reports indicate that purified rat liver reductase is composed of two polypeptides of $M_r$ 52,000 and 54,000 (4) or $M_r$ 57,500 and 58,500 (16). The possibility that these enzymatically active polypeptides may be generated by proteolytic cleavage of a larger peptide was originally suggested by Ness *et al.* (17). In this report, we demonstrate that antibody raised to purified reductase specifically precipitates a polypeptide of $M_r$ 94,000 from radiolabeled rat hepatocytes or after *in vitro* translation of rat liver poly(A)$^+$ RNA. The $M_r$ varies from 90,000 to 94,000, depending on the urea concentration in the polyacrylamide gel. This difference presumably results from different degrees of denaturation of the polypeptide. These results indicate that the endogenous rat liver reductase polypeptide has a $M_r$ of 94,000 and that active fragments of $M_r < 60,000$ can be purified from the larger polypeptide. Chin *et al.* (18) recently proposed that the reductase polypeptide of Chinese hamster ovary cells has a $M_r$ of 90,000 and that this is proteolytically cleaved to polypeptides of $M_r$ 68,000 and 53,000. It appears that rat liver (Figs. 1 and 2), Chinese hamster ovary fibroblasts (18), and chicken myeloblasts (unpublished data) all synthesize a reductase polypeptide with a $M_r$ of approximately 94,000.

We report here the effect of diet and drugs on the relative concentration of functional rat liver mRNA for 3-hydroxy-3-methylglutaryl-CoA reductase. Diets supplemented with either cholestyramine or cholestyramine and mevinolin were shown to increase both enzyme activity and reductase mRNA levels. These levels were highest in animals fed mevinolin together with cholestyramine (Table 1). Further investigation is required to assess whether the effects of mevinolin and cholestyramine are additive. Mevalonolactone administration decreased both enzyme activity and reductase mRNA. Further studies are required to determine whether the decreased enzyme activity following mevalonolactone administration is due solely to decreased synthesis of the enzyme resulting from a lower mRNA concentration or whether changes in the rate of enzyme degradation or post-translational modification also are important. Reductase mRNA was undetectable in rats fed a normal diet and killed at the nadir of the diurnal cycle (Fig. 2; Table 1). This is analogous to the recent finding that reductase mRNA levels were undetectable in normal Chinese hamster ovary cells (18).

The *in vitro* translation of reductase mRNA is sensitive to competition by other mRNAs (Fig. 3). Although the concentration of RNA was kept within the range of optimal efficiency (30 $\mu$g/ml), it is possible that a poor efficiency of translation of reductase mRNA would mask the actual relative percentage of this species. Chin *et al.* (18) observed that *in vitro* translations of poly(A)$^+$ RNA obtained from a Chinese hamster ovary clone and treated with $CH_3HgOH$ gave rise to significantly greater amounts of reductase than did *in vitro* translations of the same RNA not treated with $CH_3HgOH$. This agent has been shown to increase the translational efficiencies of mRNA for ovalbumin and conalbumin (19). In preliminary experiments, we treated the poly(A)$^+$ RNA used in these studies with $CH_3HgOH$ exactly as described by Chin *et al.* (18) and found that such treatment increased the incorporation of [$^{35}$S]methionine into trichloroacetic acid-precipitable proteins approximately 2-fold and into the reductase peptide 2- to 6-fold (data not shown). RNA isolated from animals fed cholestyramine and mevinolin and killed at hour 8 of the dark period had 0.16% of the *in vitro* synthesized protein present as reductase. A 50% decrease from this level was observed in animals fed only cholestyramine and a 90% decrease was observed in animals fed cholestyramine and receiving mevalonolactone (data not shown). These decreases are similar in magnitude to those observed with untreated mRNA (Table 1). RNA isolated from untreated animals had no detectable reductase peptide even after $CH_3HgOH$ pretreatment (data not shown).

In the studies reported here, we determined the functional level of reductase mRNA by assay in an *in vitro* translation system. Further studies will be required to determine whether the changes we report in the levels of functional mRNA are paralleled by changes in total reductase mRNA.

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