Reconstitution of the Biosynthetic Pathway of Selenocysteine tRNAs in *Xenopus* Oocytes†

In Soon Choi,‡ Alan M. Diamond,§ Pamela F. Crain,∥ James D. Kolker,§ James A. McCloskey,∥ and Dolph L. Hatfield*,‡

Laboratory of Experimental Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, Department of Radiation and Cellular Oncology, University of Chicago, Chicago, Illinois 60637, and Department of Medicinal Chemistry, University of Utah, Salt Lake City, Utah 84112

Received September 15, 1993⊛

ABSTRACT: Selenocysteine is cotranslationally introduced into a growing polypeptide in response to certain UGA codons in selenoprotein mRNAs. The biosynthesis of this amino acid initiates by aminoacylation of specific tRNAs (designated $tRNA^{[Ser]Sec}$) with serine and subsequent conversion of the serine moiety to selenocysteine. The resulting selenocysteyl-tRNA then donates selenocysteine to protein. In most higher vertebrate cells and tissues examined, multiple selenocysteine isoacceptors have been described. Two of these have been determined to differ by only a single modified residue in the wobble position of the anticodon. In addition, the steady-state levels and relative distributions of these isoacceptors have been shown to be influenced by the presence of selenium. In order to gain a better understanding of the relationship between these tRNAs and how they are regulated, both the *Xenopus* selenocysteine tRNA gene and an *in vitro* synthesized RNA have each been injected into *Xenopus* oocytes and their maturation analyzed. In this system, selenium enhanced RNA stability and altered the distribution of isoacceptors that differ by a single ribose methylation. Interestingly, the biosynthesis of one of these modified nucleosides (5-methylcarboxymethyl-2′-*O*-methyluridine), which has been identified only in the wobble position of selenocysteine tRNA, also occurs in oocytes. Examination of the modified residues in both the naturally occurring *Xenopus* selenocyteine tRNA and the products generated from exogenous templates in oocytes demonstrated the faithful reconstruction of the biosynthetic pathway for these tRNAs.

The UGA codeword can serve as a signal for either the termination of translation or the incorporation of selenocysteine into protein (Böck et al., 1991; Hatfield et al., 1992; Hatfield & Diamond, 1993). The biosynthesis of selenocysteine proceeds following aminoacylation of a unique tRNA with serine and subsequent conversion to selenocysteine (Böck et al., 1991; Hatfield et al., 1992). This tRNA, referred to as $tRNA^{[Ser]Sec}$, then denoates selenocysteine to the elongating peptide in response to an appropriate UGA codon. Higher vertebrates, including *Xenopus*, contain multiple $tRNA^{[Ser]Sec}$ isoacceptors which are transcribed from a single-copy gene (Hatfield et al., 1990; Lee et al., 1990). It has been established previously that the steady-state levels and relative distributions of mammalian selenocysteine tRNAs are influenced by the presence of selenium in the culture media (Hatfield et al., 1991). Recently, these studies were expanded to show that the same phenomenon occurs in whole animals when tRNA levels in a variety of organs from selenium-deprived rats were compared to those of selenium-fed rats (Diamond et al., 1993). In this same study, two $tRNA^{[Ser]Sec}$ isoacceptors were characterized from rat liver and found to differ by 2′-O-methylation of the ribose of 5-methylcarboxymethyluridine (mcm^5U) in the wobble position of the anticodon. The methylated version of this modified residue, originally described as 2′-*O*-methylcytidine on the basis of two-dimensional chromatography, has recently been identified as 5-methylcarboxymethyl-2′-*O*-methyluridine (mcm^5Um; i.e., the 2′-O-methylated derivative of mcm^5U) by combined high-performance liquid chromatography/mass spectrometry (Diamond et al., 1993). The relative amount of the mcm^5Um isoacceptor increases with selenium availability in these systems as does the total amount of the selenocysteine tRNA population (Hatfield et al., 1991; Diamond et al., 1993). The mechanism by which selenium exerts these effects on selenocysteine tRNAs has not been determined.

Transcription of $tRNA^{[Ser]Sec}$ is unique among tRNAs in that it begins at the first nucleotide within the gene (Lee et al., 1987). Expression is also governed by a number of regulatory elements which are typically associated with RNA polymerase II (Lee et al., 1989; Carbon & Krol, 1992; Myslinski et al., 1992) even though the $tRNA^{[Ser]Sec}$ gene is exclusively transcribed by RNA polymerase III (Lee et al., 1989). The usefulness of *Xenopus* oocytes to define these unusual transcriptional features as well as the fact that oocytes synthesized modified nucleosides in tRNA (Melton et al., 1980; Nishikura & DeRobertis, 1981; Droogmans et al., 1986; Haumont et al., 1987; Droogmans & Grosjean, 1991) suggests that this system would be generally applicable to investigating the biochemistry and regulation of $tRNA^{[Ser]Sec}$. In this paper, we have introduced either the *Xenopus* selenocysteine $tRNA^{[Ser]Sec}$ gene or an *in vitro* synthesized precursor tRNA lacking nucleoside modification into *Xenopus* oocytes by microinjection and analyzed the intermediates in tRNA maturation. By comparing the results obtained using both sources of tRNA, it was determined that selenium affected

† This work was supported by National Institutes of Health Grants R01 CA54364 to A.M.D. and R01 GM29812 to J.A.M.

* Correspondence should be addressed to this author at the Laboratory of Experimental Carcinogenesis, National Cancer Institute, Building 37, Room 3C23, National Institutes of Health, Bethesda, MD 20892. Telephone: (301) 496-9263. Fax: (301) 496-0734.

‡ National Cancer Institute, National Institutes of Health.

§ University of Chicago.

∥ University of Utah.

⊛ Abstract published in *Advance ACS Abstracts,* December 15, 1993.

0006-2960/94/0433-601$04.50/0

both $tRNA^{[Ser]Sec}$ stability and methylation. In addition, the biosynthesis of each modified nucleoside identified in *Xenopus* liver selenocysteine tRNA in this study was found to occur in oocytes.

EXPERIMENTAL PROCEDURES

Materials. *Xenopus laevis* females were purchased from Nasco (Fort Atkinson, WI), nuclease P1 was from Boehringer-Mannheim, phosphodiesterase I (Sigma type VII) and bacterial alkaline phosphatase (Sigma type III-N) were from Sigma Chemical Co., and α-^{32}P-labeled nucleoside triphosphates (specific activity, approximately 3000 Ci/mmol) were from Amersham. *Xenopus* liver was the generous gift of Dr. Robert M. Benbow, Iowa State University, Ames, IA. The 5S RNA maxigene construct was obtained from A. Wolffe, National Institutes of Health, Bethesda, MD.

Isolation, Chromatography, and Purification of tRNA. Transfer RNA was extracted from frozen *Xenopus* liver (800 g), bulk tRNA was isolated from a DE-52 column, $tRNA^{[Ser]Sec}$ was fractionated from bulk tRNA on a BD-cellulose column, and $tRNA^{[Ser]Sec}$ was further purified initially on a Sepharose 4B column (Holmes et al., 1975) and finally on a reversed-phase chromatographic column (RPC-5) (Kelmers & Heatherly, 1971) as described (Diamond et al., 1993). The only variation in this procedure from that used previously (Diamond et al., 1993) was the introduction of Sepharose 4B column chromatography. Preparation of transcription products and precursor and mature tRNAs from *Xenopus oocytes*, separation of precursor and mature tRNAs on polyacrylamide gels, and fractionation of isoacceptors on RPC-5 columns were carried out as described (Lee et al., 1987, 1989; Diamond et al., 1993).

Directly-Combined High-Performance Liquid Chromatography/Mass Spectrometry (LC/MS) of tRNA Hydrolysates. Chromatographically-purified tRNA was digested to nucleosides with 1 unit of nuclease P1, 1 milliunit of phosphodiesterase I, and 0.5 unit of bacterial alkaline phosphatase (Crain, 1990). The digest was fractionated by reversed-phase chromatography [0.25 M ammonium acetate (pH 6.0)/40% aqueous acetonitrile buffer] and analyzed by LC/MS. The instrument, procedures, and interpretation of LC/MS data have been described elsewhere (Pomerantz & McCloskey, 1990).

Preparation of a $tRNA^{[Ser]Sec}$ Expression Construct. A $tRNA^{[Ser]Sec}$ expression construct was prepared by PCR amplification of the bovine $tRNA^{[Ser]Sec}$ gene (Diamond et al., 1990) with a 5′ primer containing a *Pst*I site, a T7 promoter, and complementarity to the 5′ end of the tRNA gene (5′-ATCTGCAGTAATACGACTCACTATAGCCCG-GATGACCCTC-3′) and a second primer containing the 3′ end of the tRNA gene, a *Nsi*I site, and an *Eco*RI site (5′-GCGAATTCATGCATGGCGCCCGAAAGGTGG-3′). The amplification product was cloned into the pCR 1000 cloning vector (InVitrogen Corp.), removed by digestion with *Pst*I and *Eco*RI, and subcloned into the multiple cloning site of pSP65, and the tRNA gene sequence was then confirmed by sequencing. RNA was generated using T7 RNA polymerase (Promega Corp.) and [α-^{32}P]GTP following *Nsi*I digestion (assuring a CCA terminus).

Modified Base Analysis. Samples representing each isolated peak were completely digested with nuclease P1, and the resulting nucleotide 5′-monophosphates were identified by two-dimensional chromatography on cellulose TLC plates (J.T. Baker Co.) using solvents A and C of Silberklang *et al.* (1979). The position of each radioactive spot on the autoradiograms of the developed TLC plates was compared to the mobility of pN standards (Sigma Chemical Co.) and the known R_f of modified nucleotides (Silberklang et al., 1979; Diamond et al., 1993).

Maintenance and Microinjection of Xenopus Oocytes. Preparation of *Xenopus* oocytes and microinjection of plasmids, tRNA, and labeled nucleoside triphosphates into *Xenopus* oocytes were carried out as previously described (Zasloff, 1983; Lee et al., 1987, 1989). The *Xenopus* $tRNA^{[Ser]Sec}$ gene and labeled RNA generated from the $tRNA^{[Ser]Sec}$ expression construct were microinjected into oocyte nuclei, and labeled tRNA isolated from oocytes was injected into oocyte cytoplasm. Oocytes were incubated for 16 h following microinjection unless otherwise noted in the figures or figure legends. Sodium selenite (5 μM) was added to the oocyte incubation medium in experiments which contained supplemental selenium.

RESULTS AND DISCUSSION

Xenopus $tRNA^{[Ser]Sec}$. The experimental approach to reconstitute the biosynthetic pathway of $tRNA^{[Ser]Sec}$ in oocytes requires adequate information regarding the structure of the mature tRNA. The primary sequence of the *Xenopus* $tRNA^{[Ser]Sec}$ gene has been reported (Lee et al., 1990) and differs at only a single base (a pyrimidine transition at position 11) from the analogous gene in humans (Hatfield et al., 1992), rabbits (Hatfield et al., 1992), and cows (Diamond et al., 1990). Recently, the rat $tRNA^{[Ser]Sec}$ isoacceptors have been characterized and shown to contain only four modified nucleosides each (Diamond et al., 1993). These include 1-methyladenosine (m^1A) at position 58, pseudouridine (Ψ) at position 55, N^6-isopentenyladenosine (i^6A) at position 37, and either mcm^5U or mcm^5Um in the wobble position of the anticodon. Although selenocysteine tRNA contains two extra nucleotides in the 5′ half of the molecule as compared to other tRNAs, we adopted the numbering system of Sprinzl et al. (1987) for identifying nucleoside positions in tRNA. In order to determine whether the same residues were present in the *Xenopus* tRNA, the modified nucleoside content of *Xenopus* $tRNA^{[Ser]Sec}$ was determined. Total tRNA was prepared from *Xenopus* liver, and the more hydrophobic $tRNA^{[Ser]Sec}$ isoacceptor was purified by column chromatography. A hydrolysate of this tRNA was prepared and analyzed by combined high-performance liquid chromatography–mass spectrometry (LC/MS). The chromatogram is presented in Figure 1. The retention times and thermospray mass spectra (not shown) of the resulting peaks identify the presence of the same modified residues as observed for the comparable rat isoacceptor (Diamond et al., 1993).

Selenium Affects the Stability of $tRNA^{[Ser]Sec}$. In order to gain a better understanding of how selenium influences the biosynthesis of $tRNA^{[Ser]Sec}$ isoacceptors, we sought to establish whether selenium would affect either the transcription or the stability of $tRNA^{[Ser]Sec}$ in oocytes. A recombinant plasmid containing 800 bp of *Xenopus* DNA encoding the selenocysteine $tRNA^{[Ser]Sec}$ gene (Lee et al., 1990) was microinjected into *Xenopus* oocytes along with a 5S RNA maxigene and ^{32}P-labeled dGTP. The 5S RNA maxigene serves as a control to ensure that any changes in steady-state levels of $tRNA^{[Ser]Sec}$ are specific for that tRNA and not a general effect of the presence of selenium. The injected oocytes were then incubated either with or without supplemental selenium, and the resulting radiolabeled RNA was extracted and analyzed by polyacrylamide gel electrophoresis (Figure 2). As shown in the figure, more tRNA was present in oocytes incubated

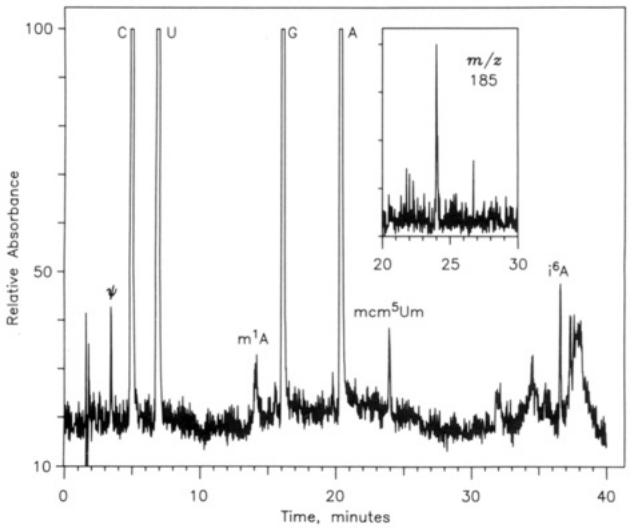

FIGURE 1: LC/MS analysis of a hydrolysate of purified *Xenopus* tRNA$^{[Ser]Sec}$. Chromatogram from reversed-phase fractionation; UV detection at 254 nm. Inset: time vs intensity profile for m/z 185, the characteristic BH_2^+ ion (Diamond et al., 1993; Pomerantz & McCloskey, 1990) from mcm^{5}Um.

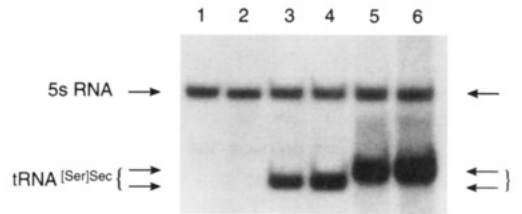

FIGURE 2: Effect of selenium on the levels of selenocysteine tRNA$^{[Ser]Sec}$ isoacceptors. The *Xenopus* tRNA$^{[Ser]Sec}$ gene (Lee et al., 1990), a 5S maxigene, and [α-^{32}P]GTP- or [α-^{32}P]GTP-labeled RNA generated from an expression vector and the 5S RNA maxigene were microinjected into oocytes, and after incubation, the RNA was extracted and electrophoresed on a polyacrylamide gel as given (Lee et al., 1987, 1989). Lanes show the following: RNA extracted from oocytes which contained the 5S RNA maxigene and incubated without (lane 1) and with (lane 2) supplemental selenium, the *Xenopus* gene, and the 5S RNA maxigene from oocytes incubated without (lane 3) and with (lane 4) additional selenium, and the *in vitro* generated RNA and the 5S RNA maxigene from oocytes incubated without (lane 5) and with (lane 6) additional selenium. *Xenopus* tRNA$^{[Ser]Sec}$ migrates slightly faster on polyacrylamide gels than mammalian tRNA$^{[Ser]Sec}$ due to a pyrimidine transition at position 11 (Lee et al., 1989). Bands were cut out and counted, yielding the following cpm: lane 1, 5S RNA, 3213; lane 2, 5S RNA, 3185; lane 3, 5S RNA, 3516, and tRNA$^{[Ser]Sec}$, 1887; lane 4, 5S RNA, 3242, and tRNA$^{[Ser]Sec}$, 2697; lane 5, 5S RNA, 3265, and tRNA$^{[Ser]Sec}$, 3233; and lane 6, 5S RNA, 3156, and tRNA$^{[Ser]Sec}$, 5026. Percent of cpm in tRNA$^{[Ser]Sec}$ (i.e., the number of cpm in 5S RNA divided by those in tRNA$^{[Ser]Sec}$) in each lane is as follows: lane 3, 54%; lane 4, 83%; lane 5, 99%; lane 6, 159%; these data demonstrate that 1.55× more tRNA$^{[Ser]Sec}$ was recovered in the presence of selenium from oocytes injected with the *Xenopus* gene, while 1.59× more was recovered from oocytes injected with RNA.

with supplemental selenium (lane 4) than in oocytes without additional selenium (lane 3). No tRNA was generated in the absence of the exogenous tRNA construct (lanes 1 and 2). The effect of additional selenium on the steady-state levels of tRNA$^{[Ser]Sec}$ was not a general effect on Pol III transcription as levels of control 5S RNA were similar under both experimental conditions. Because this approach cannot, by itself, distinguish between a selenium effect on transcription rate or RNA stability, we generated a tRNA expression construct which could be used to produce an authentic labeled tRNA$^{[Ser]Sec}$ precursor *in vitro*. This precursor can be aminoacylated with serine with a similar efficiency as *in vivo* produced tRNA (data not shown). This RNA, along with

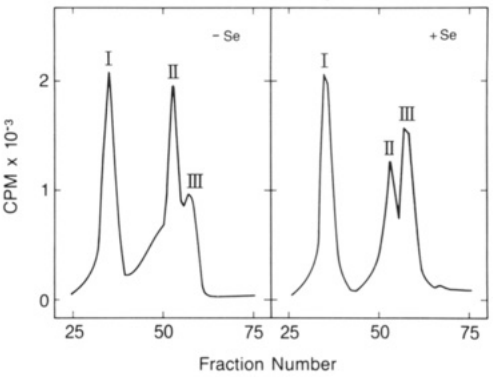

FIGURE 3: Effect of selenium on the distribution of tRNA$^{[Ser]Sec}$ isoacceptors. RPC-5 chromatographic profiles of RNA extracted from oocytes which had been incubated with and without additional selenium following microinjection of RNA generated from a tRNA$^{[Ser]Sec}$ expression vector are shown. –Se designates that this study was carried out in the absence of added (or supplemental) selenium.

the 5S maxigene control, was injected into oocytes which were incubated with and without supplemental selenium. The recovery of the labeled RNA from oocytes is shown in Figure 2, lanes 6 and 5. More tRNA was recovered from oocytes incubated with additional selenium, demonstrating that selenium influences the stability of tRNA$^{[Ser]Sec}$ in oocytes. Approximately 1.5 times more tRNA$^{[Ser]Sec}$ was recovered from oocytes injected with the *Xenopus* gene and with in vitro synthesized precursor tRNA when the oocytes were incubated in the presence of additional selenium, suggesting that it exerts its effect on RNA stability. Interestingly, the levels of enhancement of the selenocysteine tRNA population by selenium in *Xenopus* oocytes are similar to those observed in mammalian cells in culture (Hatfield et al., 1991) and in various rat tissues (Diamond et al., 1993).

Selenium Affects the Maturation of tRNA$^{[Ser]Sec}$ Isoacceptors. To evaluate whether selenium influences the distribution of the tRNA$^{[Ser]Sec}$ isoacceptors in the oocyte system, tRNA generated by *in vivo* transcription of the exogenous tRNA$^{[Ser]Sec}$ gene was extracted from oocytes and chromatographed on a RPC-5 column. Figure 3 shows that the tRNA generated in oocytes from the exogenous gene chromatographs as three peaks, designated I–III. The differences in peaks I–III are due to specific nucleoside modifications, where peaks II and III represent mature tRNAs observed in other systems (Hatfield et al., 1991; Diamond et al., 1993) and peak I is an undermodified precursor (see below). An examination of the distribution of peaks I–III with and without supplemental selenium indicates that more peak II is converted to peak III when the oocytes are incubated with additional selenium. Thus, selenium also influences the distribution of the selenocysteine isoacceptors in oocytes as observed previously in mammalian cells in tissue culture (Hatfield et al., 1991) and in certain rat organs (Diamond et al., 1993).

The precursor/product relationships of peaks I–III are illustrated in Figure 4. The *in vitro* generated tRNA was microinjected, the oocytes were incubated, and the resulting tRNA was extracted and chromatographed. After overnight incubation, approximately one-third of the RNA was converted to peak II (see the two leftmost panels). Isolation and reinjection of peak II resulted in its conversion to peak III (central panel), and more of peak II was converted to peak III in 16 h than in 8 h (upper right three panels). Reinjection of peak III did not result in any other interconversions. Clearly, peak I (or at least some portion of peak I; see below) is converted to peak II which in turn is converted to peak III. Although

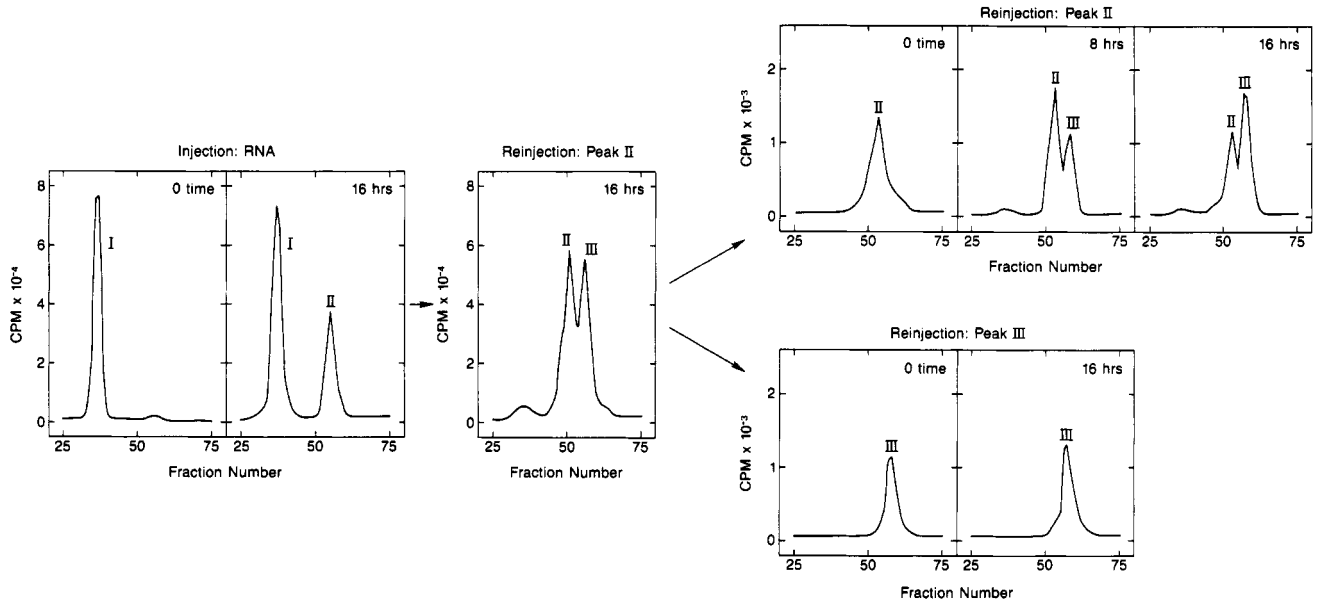

FIGURE 4: Biosynthesis of selenocysteine tRNA$^{[Ser]Sec}$ isoacceptors. The radiolabeled RNA was microinjected into oocytes, extracted at the indicated time intervals, and chromatographed on a RPC-5 column. Peak II generated in oocytes for subsequent microinjection (left-hand panel) was isolated from oocytes that were incubated without supplemental selenium. All other profiles were obtained from tRNA isolated from oocytes incubated in the presence of supplemental selenium.

it is not clear whether peak I (or some portion of peak I) is converted directly to peak III, the initial 16-h incubation of peak I in oocytes resulted in its conversion only to peak II in the experiment shown in the leftmost panel of Figure 4. This observation suggests that peak I is not converted directly to peak III, but must proceed through the peak II intermediate.

Biosynthesis of Modified Nucleosides. In order to determine the structural changes that cause the alterations in the elution properties of peaks I–III, precursor tRNA$^{[Ser]Sec}$ was synthesized *in vitro* with either [α-^{32}P]ATP, [α-^{32}P]GTP, [α-^{32}P]CTP, or [α-^{32}P]UTP and injected into oocytes. The tRNAs processed in oocytes from each labeling were isolated from column fractions. The tRNA from each peak was then digested with nuclease P1, and the modified nucleotides were identified by two-dimensional TLC. Every modified nucleoside identified in the rat tRNAs (Diamond et al., 1993), as well as in the endogenous *Xenopus* tRNA characterized in this study (Figure 1), was observed as shown in Figure 5. All three peaks contained m^{1}A (panel 5A) and Ψ (panel 5U). Only peaks II and III contain i^{6}A (panel 5A). Peak I contained approximately equal amounts of mcm^{5}U and mcm^{5}Um while peak II contained predominantly mcm^{5}U and peak III contained predominantly mcm^{5}Um (panel 5U). No modified nucleotides were detected with [α-^{32}P]CTP or [α-^{32}P]GTP. We therefore conclude that the greater hydrophobicity of peaks II and III (i.e., requiring higher NaCl concentrations for elution) is a consequence of the addition of i^{6}A. It is further concluded that isopentenylation is not required for the synthesis of either mcm^{5}U or mcm^{5}Um as evidenced by the fact that peak I was composed of tRNAs containing either mcm^{5}U or mcm^{5}Um but not i^{6}A. Clearly, that portion of peak I which contains mcm^{5}U is converted to peak II, whereas that portion of peak I which contains mcm^{5}Um is apparently not converted to peak III (see above). Thus, it may be further concluded that isopentenylation may proceed only if the wobble nucleotide is mcm^{5}U and that ribose methylation to generate a fully modified tRNA$^{[Ser]Sec}$ (peak III) follows isopentenylation. The occurrence of mcm^{5}Um in peak I does not appear to arise from the removal of i^{6}A from peak III as evidenced from the observation that overnight incubation of peak III in oocytes does not result in further interconversions (see above and lower right panel of Figure 4). Most certainly, m^{1}A, Ψ, i^{6}A, and mcm^{5}Um occur in the same nucleoside positions (i.e., 58, 55, 37, and the wobble nucleoside, respectively) in *Xenopus* tRNA$^{[Ser]Sec}$ as has been determined for the corresponding rat liver isoacceptor (Diamond et al., 1993).

Melton et al. (1980) have shown that processing of tRNA at the 5′ and 3′ ends and syntheses of the CCA terminus, m^{1}A, and Ψ occur in the nucleus of *Xenopus* oocytes. Nishikura and de Robertis (1981) have demonstrated that isopentenylation of tRNA occurs in the cytoplasm. We have previously shown that processing, which occurs only at the 3′ end of tRNA$^{[Ser]Sec}$, and 3′-terminal CCA addition take place in *Xenopus* oocyte nuclei (Lee et al., 1987). The syntheses of m^{1}A and Ψ in tRNA$^{[Ser]Sec}$ are therefore likely to occur in the nucleus and isopentenylation in the cytoplasm in a manner similar to the studies of Melton et al. (1980) and Nishikura and de Robertis (1981) using tRNA^{Tyr}. Since methylation of the 2′-*O*-ribose of mcm^{5}U may also occur after isopentenylation, the methylation event is most likely to take place in the cytoplasm. The synthesis of mcm^{5}U is also likely to occur in the nucleus since it is the site of synthesis of most minor bases in *Xenopus* oocytes (Melton et al., 1980; Nishikura, 1981).

The faithful reconstitution of selenocysteine tRNA biosynthesis in *Xenopus* oocytes has permitted elucidation of some of the control mechanisms involved. By comparing tRNA levels generated from an expression construct (representing both expression and stability) to those observed following microinjection of *in vitro* synthesized precursor tRNA (a function of stability), it appears that selenium exerts an effect, at least in part, at the posttranscriptional level. Selenium also influenced methylation of the ribose portion of the mcm^{5}U residue at the wobble position of the anticodon as previously observed in tissue culture cells (Hatfield et al., 1991) and a number of rat organs (Diamond et al., 1993). Each of the above observations argues that the oocyte system will be useful in investigating how the biosynthesis of these tRNAs is regulated and how the cell ensures the appropriate insertion of selenocysteine into protein.

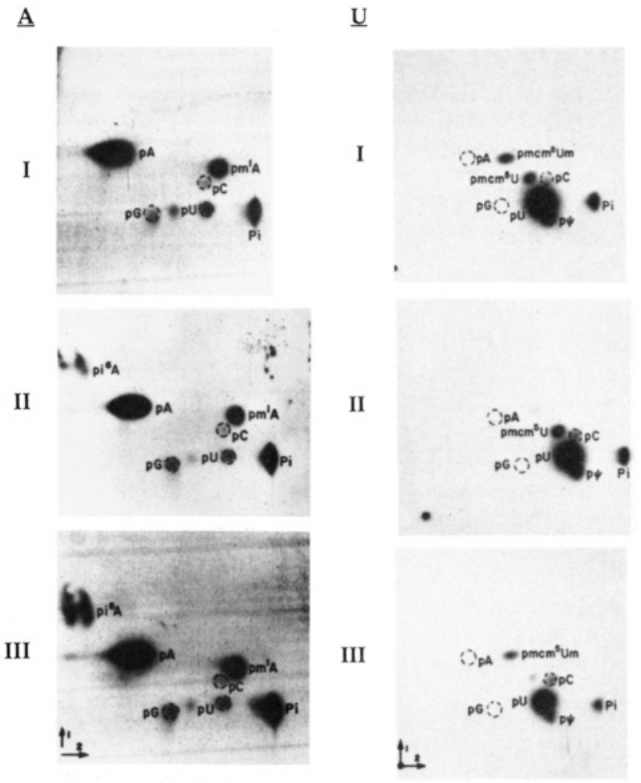

FIGURE 5: Identification of modified nucleotides in selenocysteine tRNA$^{[Ser]Sec}$ generated in *Xenopus* oocytes. RNA was prepared from the expression construct labeled individually with [α-^{32}P]ATP, [α-^{32}P]-GTP, [α-^{32}P]UTP, or [α-^{32}P]CTP, microinjected into oocytes, extracted, and chromatographed. Peak I (labeled with each individual ^{32}P-nucleotide) was isolated from an RPC-5 column and reinjected to yield substantial amounts of peaks II and III (see Figure 4). All peaks (I–III) were individually chromatographed a second time on the RPC-5 column in the absence of Mg^{2+} to further purify each isoacceptor prior to base analysis. The figure shows the autoradiograms of nucleotides generated from analysis of samples representing peaks I–III labeled with [α-^{32}P]ATP (panel A) and [α-^{32}P]UTP (panel U). No modified nucleotides were detected in experiments where the tRNA was labeled with either [α-^{32}P]GTP or [α-^{32}P]-CTP, and these data are therefore not shown. The detection of some radioactivity migrating as pNs other than that used in the particular labeling reaction was present in submolar quantities and is therefore considered an artifactual consequence of the labeling protocol.

ACKNOWLEDGMENT

We express our sincere appreciation to S. C. Pomerantz for technical assistance.

REFERENCES

Böck, A., Forchhammer, K., Heider, J., & Baron, C. (1991) *Trends Biochem. Sci. 16*, 463–467.
Carbon, P., & Krol, A. (1992) *EMBO J. 10*, 599–606.
Crain, P. F. (1990) *Methods Enzymol. 193*, 782–790.
Diamond, A. M., Montero-Puerner, Y., Lee, B. J., & Hatfield, D. (1990) *Nucleic Acids Res. 18*, 6727.
Diamond, A. M., Choi, I. S., Crain, P. F., Hashizume, T., Pomerantz, S. C., Cruz, R., Steer, C. J., Hill, K. E., Burk, R. F., McCloskey, J. A., & Hatfield, D. L. (1993) *J. Biol. Chem. 268*, 14215–14223.
Droogmans, L., & Grosjean, H. (1991) *Biochimie 73*, 1021–1025.
Droogmans, L., Haumont, E., de Henau, S., & Grosjean, H. (1986) *EMBO J. 5*, 1105–1109.
Hatfield, D., & Diamond, A. M. (1993) *Trends Genet. 9*, 69–70.
Hatfield, D., Smith, D. W. E., Lee, B. J., Worland, P. J., & Oroszlan, S. (1990) *CRC Crit. Rev. Biochem. Mol. Biol. 25*, 71–96.
Hatfield, D., Lee, B. J., Hampton, L., & Diamond, A. M. (1991) *Nucleic Acids Res. 19*, 939–943.
Hatfield, D., Choi, I. S., Lee, B. J., & Jung, J.-E. (1992) in *Transfer RNA in Protein Synthesis* (Hatfield, D., Lee, B.-J., & Pirtle, R. M., Eds.) pp 269–278, CRC Press, Boca Raton, FL.
Haumont, E., Droogmans, L., & Grosjean, H. (1987) *Eur. J. Biochem. 168*, 219–225.
Holmes, W. M., Hurd, R. E., Reid, B. R., Rimerman, R. A., & Hatfield, G. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1068–1071.
Kelmers, A. D., & Heatherly, D. E. (1971) *Anal. Biochem. 44*, 468–495.
Lee, B. J., de la Pena, P., Tobian, J., Zasloff, M., & Hatfield, D. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6384–6388.
Lee, B. J., Kang, S. G., & Hatfield, D. (1989) *J. Biol. Chem. 264*, 9696–9702.
Lee, B. J., Rajagopalan, M., Kim, Y. S., You, K., Jacobson, K. B., & Hatfield, D. (1990) *Mol. Cell. Biol. 10*, 1940–1949.
Melton, D. A., De Robertis, E. M., & Cortese, R. (1980) *Nature 284*, 143–148.
Myslinski, E., Krol, A., & Carbon, P. (1992) *Nucleic Acids Res. 20*, 203–209.
Nishikura, K., & De Robertis, E. M. (1981) *J. Mol. Biol. 145*, 405–420.
Pomerantz, S. C., & McCloskey, J. A. (1990) *Methods Enzymol. 193*, 796–824.
Silberklang, M., Gillum, A. M., & RajBhandary, U. L. (1979) *Methods Enzymol. 59*, 58–109.
Sprinzl, M., Hartmann, T., Meissner, F., Moll, J., & Vorderwülbecke, T. (1987) *Nucleic Acids Res. 15*, r53–188.
Zasloff, M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6436–6440.