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Estrogen-Induced Ribonuclease Activity in *Xenopus* Liver†

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ABSTRACT: Estrogen administration to male *Xenopus* causes the cytoplasmic destabilization of the hepatic serum protein coding mRNAs, most notably, albumin, yet has little effect on mRNAs encoding intracellular proteins such as ferritin. This report describes an estrogen-inducible ribonuclease activity found in liver polysomes that degrades albumin mRNA 4 times faster in vitro than it degrades ferritin mRNA. This differential rate of degradation was observed upon incubation of polysome extract with free liver RNA, isolated liver mRNPs, or transcripts from plasmid vectors. A cleavage fragment consisting of a doublet of approximately 194 nucleotides in length was consistently observed upon digestion of transcripts for the full length or 5′ half of albumin mRNA. The generation of this cleavage fragment was used as an assay to study properties of the polysome nuclease activity. The 194 doublet is produced by the action of a Mg^{2+}-independent endonuclease. This distinguishes the *Xenopus* liver enzyme from the enzymes that degrade histone or c-*myc* mRNA in vitro. It is inactivated by 400 mM NaCl or heating at 90 °C, but not by placental ribonuclease inhibitor or *N*-ethylmaleimide. Finally, the polysomal nuclease activity does not degrade double-stranded RNA. We believe the estrogen-induced nuclease activity contains an enzyme(s) that may mediate hormone-regulated changes in mRNA stability in this tissue.

The stabilization or destabilization of mRNAs within a cell effect significant changes in gene expression [reviewed in Brawerman (1988)]. Altering mRNA stability is the major mechanism regulating such diverse mRNAs as those encoding oncogenes [c-*myc* (Brewer & Ross, 1988; Pei & Calame, 1988), c-*fos* (Wilson & Treisman, 1988; Shyu et al., 1989)], translation elongation factors [eEf-TU (Rao & Slobin, 1988)], histones (Ross et al., 1986), tubulin (Pachter et al., 1987; Yen et al., 1988), and homeobox proteins (Brown & Harland, 1990), to name a few. The determinants of stability (or instability) for a number of RNAs have been identified and in many cases consist of a discrete nucleic acid sequence (e.g.,

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c-*fos*) or structure (e.g., histone H4), usually in the 3′-untranslated region (UTR). In the case of tubulin, mRNA instability appears to result from the interaction of the first 4 amino acid residues of the nascent peptide chain with its paired tubulin monomer (Yen et al., 1988). In spite of the progress in identifying both the numbers of RNAs regulated by changes in stability and the sequences that guide their ultimate degradation, little is known about the enzymes responsible for this process. Ross et al. (1987) have shown that a Mg^{2+}-dependent exonuclease present in polysome extracts from K562 cells is responsible for the selective degradation of histone H4 mRNA in vitro. This enzyme is not inhibited by placental ribonuclease inhibitor and does not depend on nucleoside triphosphates for activity. A nuclease that degrades c-*myc* mRNA in vitro shares many of the same properties of the enzyme that degrades histone H4 (Brewer & Ross, 1988; Pei & Calame, 1988). Recently, Bandyopadhyay et al. (1990) have identified a nuclease activity present in tight association with a variety of mRNPs which might be responsible for differences in the stability of a number of mammalian mRNAs. With the exception of the histone exonuclease, little is known about the biochemistry or regulation of the nucleases responsible for the modulation of mRNA turnover.

A major site of action of steroid hormones is at the level of mRNA stability [reviewed in Nielsen and Shapiro (1990)]. In *Xenopus* liver estrogen administration causes serum albumin mRNA to disappear from the cytoplasm at the same time that it induces vitellogenin (Riegel et al., 1986; Schoenberg et al., 1989). However, estrogen has little effect on the transcription of the albumin genes (Riegel et al., 1986) or on the nuclear levels of the primary or mature albumin transcripts (Schoenberg et al., 1989). From these data we and others (Wolffe et al., 1985; Kazmaier et al., 1985) concluded that the primary mechanism regulating albumin gene expression by estrogen is the cytoplasmic destabilization of albumin mRNA. mRNA destabilization is a generalized mechanism for reprogramming the translational apparatus of the liver cell during vitellogenesis. In addition to albumin, the mRNAs encoding γ-fibrinogen, transferrin, the second protein of inter-α-trypsin inhibitor, and serum protein 12B (which has no mammalian homologue) are coordinately regulated by destabilization (Pastori et al., 1991). Together, these mRNAs encompass greater than 85% of the secreted proteins synthesized by the liver of untreated male *Xenopus*. In contrast, estrogen has no effect on actin, poly(A)-binding protein, or ferritin mRNA.

The most common approach used to study the stabilization or destabilization of a given mRNA is to perform transfections of plasmids bearing the desired constructs under the control of a constitutive promoter. However, no estrogen-responsive *Xenopus* liver cell line exists, and only recently have we succeeded in reproducibly transfecting primary *Xenopus* hepatocyte cultures. We therefore sought to develop an in vitro system to study the degradation of albumin mRNA. The present paper describes this system and the identification of an estrogen-induced ribonuclease activity which may be responsible for the hormone-regulated instability of a number of mRNAs.[1]

[1] The experiments reported herein were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, Department of Health and Human Services Publication No. (NIH) 78-23. All recombinant organisms and molecules were handled under conditions of the NIH guidelines for recombinant DNA research.

Experimental Procedures

Subcellular Fractionation of Xenopus Liver. Two frogs were typically used for each experiment. Male *Xenopus* were injected with either 0.1 mL of injection vehicle (propylene glycol/DMSO, 9/1) or vehicle containing 10 mg/mL 17β-estradiol. The animals were killed 48 h after injection, and all subsequent steps were performed on ice. Livers were perfused with sterile 1 × SSC to remove blood, following which they were excised and placed into homogenization buffer [0.25 M sucrose, 30 mM Tris-HCl, pH 7.4, 2 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 180 KIU/mL trasylol (aprotinin)]. The livers were diced into 10-mm fragments and homogenized with 10 strokes of a Teflon–glass homogenizer in 2.5 mL of ice-cold homogenization buffer/g of tissue. The homogenates was filtered through a polyamide nylon mesh (Nitex 27621, Tetko, 0.5 mm) and centrifuged at $1000g_{max}$ for 5 min in a Sorvall HS4 rotor. The supernatant was then centrifuged for 15 min at $15000g_{max}$ in polyallomer tubes in a Beckman SW50.1 rotor to generate the postmitochondrial extract. The supernatant was removed with a syringe, taking care to avoid contamination with the lipid layer. In the initial experiments (Figure 1) the postmitochondrial extract was fractionated further on a linear 15–40% sucrose gradient layered over a 1.5-mL cushion of 70% sucrose. The gradient was prepared in 30 mM Tris-HCl, pH 7.4, and 2 mM dithiothreitol. Up to 1 mL of extract was loaded onto each gradient. The samples were centrifuged for 3.5 h at $225000g_{max}$ in a Beckman SW41 rotor. The gradients were fractionated through a UV monitor, and 0.45-mL fractions were collected on ice. Although it is customary to include Mg^{2+} in such gradients to maintain ribosome integrity, we found no difference in sedimentation profiles whether the divalent cation was present or absent. However, polysomes were disrupted by addition of EDTA to the buffer. Fractions corresponding to 20–80S mRNP particles and polysomes were pooled separately and stored at −70 °C. The experiment shown in Figure 1A used 1 mL of each subcellular fraction containing 60 μg of RNA by OD_{260}. The experiment shown in Figure 1B used 1 μL of the polysome preparation (containing 60 ng of RNA) added to 10 μg of free liver RNA.

Preparation of Polysome Extracts. A more rapid procedure was developed once the enzyme activity was localized to the polysomes. Postmitochondrial extract prepared from 3 frogs (see above) was centrifuged for 50 min, at 2 °C, at $165000g_{max}$ in a Beckman TL 100.3 rotor and TL100 ultracentrifugation. The pellet obtained from this procedure was carefully resuspended in 1 mL of homogenization buffer using a B pestle of a Dounce homogenizer. This material was loaded onto a 15–40% sucrose gradient (30 mM Tris-HCl, pH 7.4, and 2 mM dithiothreitol, without a cushion) and centrifuged for 3.5 h at $225000g_{max}$. The pellet was resuspended in 2 mL of 30 mM Tris-HCl, pH 7.4, 2 mM dithiothreitol, and 12% (v/v) glycerol. The solution was adjusted to 0.4 M NaCl followed by 30 min of gentle extraction. The extract was centrifuged for 50 min at $165000g_{max}$ in the TL100.3 rotor and the resulting supernatant dialyzed against several hundred volumes of 30 mM Tris-HCl, pH 7.4, 2 mM dithiothreitol, 12% (v/v) glycerol, and 30 mM NaCl and stored in aliquots at −70 °C. Extracts prepared in this manner typically have a protein concentration of 0.25–1 mg/mL.

Assay for Ribonuclease Activity. The standard reaction was performed in a 30-μL volume in 30 mM Tris-HCl, pH 7.4, 2 mM dithiothreitol, and 1 mM $MgCl_2$ containing 10 μg of free liver RNA. Where indicated, 100 ng of radiolabeled transcript (prepared from pBluescript vectors with T3 or T7

RNA polymerase) was added. All components (minus polysomes or polysome extract) were assembled on ice. Between 1 and 5 μL of polysome extract (1 mg/mL of protein) was added, followed by incubation at 22 °C. Reactions were terminated by addition of 100 μL of 0.5% SDS. They were deproteinized by sequential extraction with equal volumes of phenol and $HCCl_3$, and the RNA was precipitated with ethanol. The action of the polysomal nuclease on free liver RNA was determined by northern blot as described previously (Schoenberg et al., 1989). The degradation of radiolabeled transcripts was assessed by autoradiography of dried agarose or acrylamide/urea gels. The albumin and ferritin cDNA clones used for hybridization analysis or preparation of transcripts were isolated from a liver library prepared in λZAP (Pastori et al., 1990). The ferritin clone described previously (Moskaitis et al., 1990) was found to contain an additional 160 bp on the 5′ end that is not present in ferritin mRNA. Consequently, a new clone with the appropriate 5′ end was isolated from the library for use in this study.

Preparation of Transcripts. Plasmids containing albumin or ferritin cDNAs were linearized by digestion with the restriction enzyme which cleaved closest to the terminus of the cloned poly(A). Transcription reactions were performed for 45 min at 37 °C in a mixture of 1 μg of DNA template, 40 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 12 mM dithiothreitol, 10 units of placental ribonuclease inhibitor (RNasin), 0.4 mM each ATP, CTP, GTP, and UTP, 10 μCi of [α-^{32}P]UTP, and 10 units of T7 or T3 RNA polymerase. The reaction was stopped by the addition of 1 unit of RNase-free DNase and incubation for 20 min at 37 °C. RNA was extracted with phenol/$HCCl_3$/isoamyl alcohol and recovered by precipitation with ethanol. The resultant pellet was washed three times with 70% ethanol, dried, and dissolved in water. Capped transcripts were prepared by inclusion of 0.4 mM $m^7G(5')ppp(5')A$, and GTP was reduced to 0.04 mM. The presence of the 5′ cap had no effect on the degradation of albumin or ferritin transcripts.

Preparation of Double-Stranded RNA. One hundred nanograms of radiolabeled 5′ albumin transcript prepared as above was incubated overnight at 52 °C in 80% formamide, 0.4 M NaCl, and 50 mM PIPES, pH 6.5, with 0, 100, 300, or 500 ng of unlabeled antisense transcript prepared from the same vector in a total volume of 20 μL. The RNA products were extracted with phenol/$HCCl_3$/isoamyl alcohol and precipitated with ethanol. The products were dissolved in H_2O and incubated with polysome extract as described.

Results

Identification of an Estrogen-Regulated Nuclease on Polysomes. A number of reports have demonstrated that a ribonuclease present in polysomes can selectively degrade mRNAs in vitro (Pei & Calame, 1988; Ross et al., 1987). Therefore, the initial experiments examined the degradation of albumin mRNA by subcellular fractions of *Xenopus* liver. A liver homogenate from untreated male *Xenopus* was fractionated into a postmitochondrial extract, followed by sucrose density gradient separation into 20–80S mRNP particles and polysomes. These fractions (each containing 60 μg of RNA) were incubated at 0 and 22 °C to examine the degradation of endogenous albumin mRNA present in each fraction (Figure 1A). After the indicated times the reactions were deproteinized and the surviving RNA was analyzed by northern blot. Only 10% of the endogenous albumin mRNA present in either the postmitochondrial extracts or the mRNP complexes was degraded after 60 min at 22 °C (Figure 1A, lanes 3 and 5). In contrast, 75% of the polysome-associated

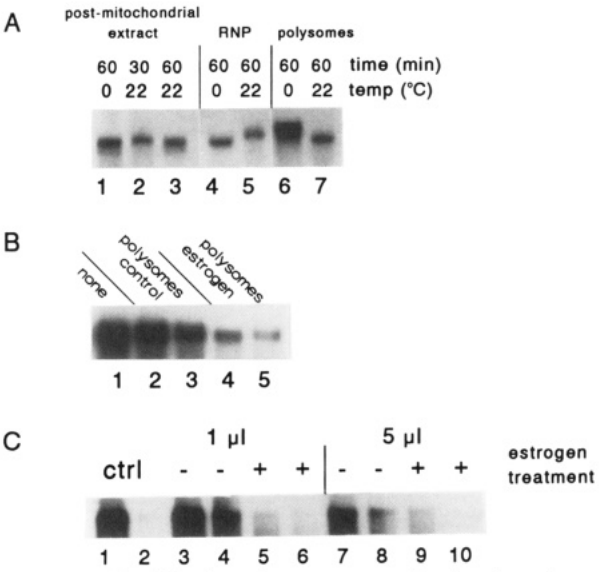

FIGURE 1: Identification of an estrogen-stimulated nuclease in polysomes. (A) A liver homogenate from untreated male *Xenopus* was separated into a postmitochondrial extract (lanes 1–3) which was further fractionated on a linear sucrose gradient into 20–80S mRNP complexes (lanes 4, 5) and polysomes (lanes 6, 7). One-milliliter fractions (containing 60 μg of RNA by OD_{260}) were incubated for the indicated times and temperatures as described under Experimental Procedures. The endogenous albumin mRNA remaining in each fraction was analyzed by northern blot. (B) Two animals were injected with vehicle and another two animals were injected with 1 mg of estradiol 48 h prior to death. Polysomes were prepared from each animal separately. In lane 1, 10 μg of free liver RNA was incubated with no additions for 30 min at 22 °C. One microliter of polysomes (containing 60 ng of RNA by OD_{260}) from each of the control animals (−, lanes 2 and 3) or each of the estrogen-treated animals (+, lanes 4 and 5) was added to 10 μg of free liver RNA and incubated as in lane 1. Surviving albumin mRNA was extracted and analyzed by northern blot. The relative amounts of albumin RNA in lanes 2 and 3 (control animals) are 0.9 and 0.6, and in lanes 4 and 5 (estrogen-treated animals) are 0.17 and 0.07, that of lane 1 (untreated RNA). (C) Polysomes were prepared separately from groups of 2 animals that received either vehicle (−) or 1 mg of estradiol (+) as in (B). They were extracted with 0.4 M NaCl as described under Experimental Procedures and dialyzed to remove excess salt. The preparations were adjusted to each contain 0.25 μg of protein/μL. One microliter (lanes 3–6) and five microliters (lanes 7–10) of each preparation was added to a mixture containing a full-length ^{32}P-labeled albumin transcript plus 10 μg of free liver RNA. RNA was extracted after a 30-min incubation at 22 °C and electrophoresed on a 1% agarose gel. Surviving albumin RNA was detected by autoradiography of the dried gel. The relative amounts of albumin RNA (with respect to the control in lane 1) in each of the duplicate treatment groups are as follows: lanes 3 and 4, 1.02 and 0.75; lanes 5 and 6, 0.07 and 0; lanes 7 and 8, 0.58 and 0.41; lanes 9 and 10, 0.08 and 0.

mRNA was degraded after 60 min (lane 7). These data suggested that mRNA-degrading enzymes might be associated with polysomes. The relative stability of albumin mRNA in the postmitochondrial extract is due to the presence of an endogenous ribonuclease inhibitor (see below).

Albumin mRNA disappears from both RNPs and polysomes following estrogen treatment (Moskaitis et al., 1991; Pastori et al., 1991). This complicates attempts to study degradation of polysomal RNA in vitro following estrogen treatment in vivo. We therefore chose to add polysomes from control and estrogen-treated animals to an excess of free liver RNA (from control animals) to look for hormone-induced changes in nuclease activity. In the experiment shown in Figure 1B groups of 2 animals each were injected with vehicle (lanes 2 and 3) or estradiol (lanes 4 and 5) 48 h prior to death. Polysomes were isolated separately from each animal as above, and a portion containing 60 ng of RNA (by OD_{250}) was added

to 10 μg of free liver RNA. The mixture was incubated for 30 min at 22 °C, following which surviving albumin RNA was extracted and assayed by northern blot. In contrast to the experiment in Figure 1A, in which endogenous albumin mRNA was extensively degraded on polysomes from untreated animals, only 10–20% of the albumin mRNA present in an excess of total RNA disappeared following the exogenous addition of a small amount of polysomes from control animals (lanes 2 and 3). However, addition of the same amount of polysomes prepared separately from 2 estrogen-treated animals resulted in the disappearance of greater than 90% of albumin mRNA present in the same preparation of free liver RNA (lanes 4 and 5). The amount of endogenous albumin mRNA present on the added polysomes is negligible compared to that present in 10 μg of free liver RNA. These data indicate that estrogen induces a polysomal nuclease activity that extensively degrades albumin mRNA.

For biochemical studies it is helpful if an enzymatic activity can be separated from a subcellular organelle. The nuclease that degrades histone (Ross et al., 1987) and c-*myc* mRNA (Pei & Calame, 1988) is salt-extractable. We therefore examined whether the estrogen-induced liver polysomal nuclease could be extracted with elevated salt. In the experiment shown in Figure 1C polysomes prepared separately from livers of 2 animals each that received either injection vehicle or estradiol 48 h prior to death were extracted with 0.4 M NaCl as described under Experimental Procedures. Excess salt was removed by dialysis. Preparations containing the same amount of polysome protein extract (0.25 μg/μL) from each animal were incubated with 10 μg of free liver RNA as in Figure 1B, to which was added a full-length radiolabeled 74-kDa albumin transcript as tracer. RNA extracted from the reaction mixture was electrophoresed on a 1% agarose gel, which was then dried and autoradiographed. The data in lanes 3–6 [in which 1 μL of extract was used] indicate that there is substantially more nuclease activity in extracts prepared from each of the estrogen-treated animals than in extracts from control animals. Increasing the amount of extract added from control animals 5-fold resulted in the disappearance of 60% of albumin mRNA (lanes 7 and 8). The extraction procedure was quantitative; no nuclease activity remained on polysomes following salt extraction (data not shown). The subsequent experiments were all performed with the salt extract from polysomes of estrogen-treated animals.

Increased Nuclease Activity Is Not Due to Suppression of Its Endogenous Inhibitor. Three general mechanisms might account for the induction of nuclease activity observed after estrogen administration: (1) increased synthesis, (2) post-translational activation of a preexisting enzyme, or (3) suppression of the endogenous inhibitor present in postmitochondrial extracts. Until antibodies and/or cDNA clones to the ribonuclease are available, it is not possible to differentiate between the first two. However, the question of hormonal regulation of the endogenous inhibitor can be addressed with the available in vitro assay. In the experiment shown in Figure 2, 10 μg of free liver RNA was incubated with an excess of polysome extract (10 μL) to ensure complete digestion of albumin RNA within 30 min (lanes 1 and 2). To this were added increasing amounts of either postmitochondrial extract or S100 from control or estrogen-treated animals. Surviving albumin RNA was assayed by northern blot. The data show first that the endogenous nuclease inhibitor is a soluble molecule as it remains soluble in the S100 preparation. Second, estrogen treatment does not suppress inhibitor activity. Rather there appears to be somewhat more inhibitor activity

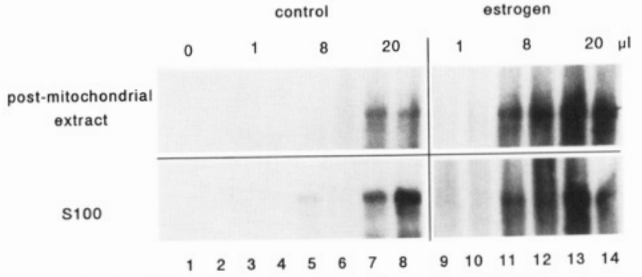

FIGURE 2: Activity of an endogenous ribonuclease inhibitor is not suppressed by estrogen. Ten micrograms of free liver RNA was incubated as in Figure 1C with 10 times more polysome extract to ensure complete degradation of albumin RNA (lanes 1 and 2). To this reaction mixture were added increasing amounts of either postmitochondrial extract (upper panels) or S100 prepared from this extract (lower panels) adjusted to a protein concentration of 10 μg/mL. Reactions were performed in duplicate with extracts from control (lanes 3–8) or 24-h estrogen-treated (lanes 9–14) animals. Surviving albumin RNA was assayed by northern blot. The signal in lanes 13 and 14 represents almost complete protection of albumin RNA.

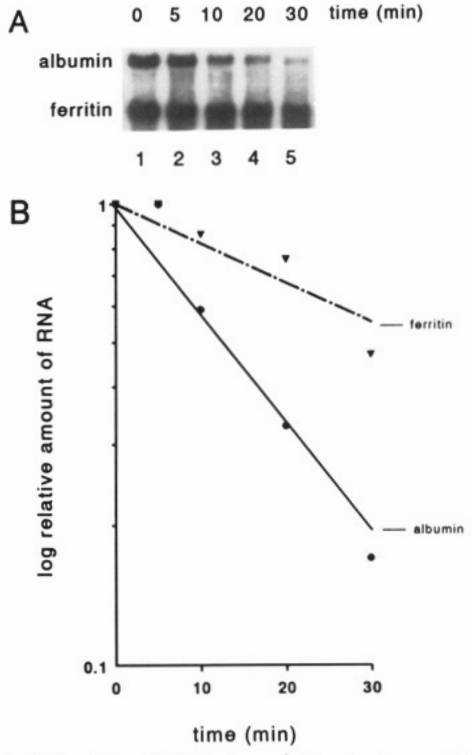

FIGURE 3: Selectivity of RNA degradation in vitro. (A) Ten micrograms of free liver RNA was incubated with 1 μL of polysome extract (containing 0.25 μg of protein) for 0–30 min at 22 °C. The deproteinized RNA extracted at each time point was electrophoresed on an agarose gel, blotted onto nylon membrane, and hybridized to albumin and ferritin cDNA. (B) The relative change in the quantity of each RNA was determined by scanning densitometry of autoradiograms and direct counting of the blot on a Betagen β camera.

present after estrogen administration. Therefore, the induction of nuclease activity observed in Figure 1 is due to either increased synthesis or activation of the polysomal nuclease.

RNA Specificity of the Inducible Nuclease Activity. A previous study from this laboratory demonstrated that estrogen treatment has no effect on steady-state levels of ferritin mRNA (Pastori et al., 1991). This observation was used as the basis for a kinetic analysis of in vitro mRNA degradation to examine the substrate specificity of the extractable nuclease. In the experiment shown in Figure 3, free liver RNA was incubated with polysome extract over a 30-min time course, and the surviving RNA was analyzed by northern blot. Free RNA

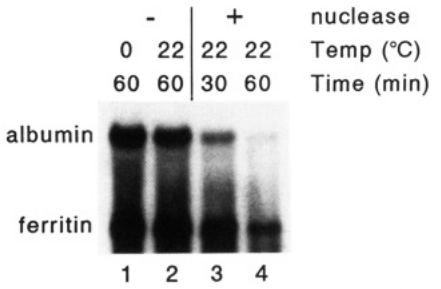

FIGURE 4: Selectivity of degradation in mRNP complexes. 20–80S mRNP complexes isolated by sucrose gradient fractionation of *Xenopus* liver were incubated for 30 or 60 min under the same conditions as in Figure 2 without (−, lanes 1 and 2) or with (+, lanes 3 and 4) 1 μL of polysome extract. The surviving RNA was electrophoresed on an agarose gel, blotted onto nylon membrane, and hybridized to a mixed probe of albumin and ferritin cDNA.

was used to ensure that no extraneous sequences (such as polylinker regions from the cDNA expression plasmids) were present that might influence the relative rate of degradation. The blot was hybridized to a mixed probe of albumin and ferritin cDNA to avoid artifacts from stripping and rehybridization (Figure 3A). Surviving mRNA was quantified both by scanning densitometry of the autoradiogram and by direct counting of the blot on a β camera. The quantified data are presented graphically in Figure 3B. Albumin mRNA has a half-life in vitro of 11 min, whereas the extrapolated half-life of ferritin mRNA is 44 min. These data indicate that the estrogen-inducible nuclease activity present in polysome extracts shows differential substrate specificity in vitro that mirrors the stability of these mRNAs in vivo.

The experiments shown in Figures 1–3 utilized free RNA to assay for nuclease activity. Because mRNA is always present in RNA–protein complexes within a cell it was incumbent to demonstrate that such complexes serve as substrates for the polysomal nuclease activity. In the experiment shown in Figure 4 the 20–80S mRNP-containing fraction of *Xenopus* liver isolated by sucrose density centrifugation was incubated with or without nuclease extracted from polysomes of estrogen-treated animals. The surviving albumin and ferritin mRNA was then analyzed by northern blot. In the absence of added nuclease, ferritin mRNA remained intact (lanes 1 and 2) as did 90% of the endogenous albumin mRNA. Addition of the nuclease activity resulted in the disappearance of 75% of albumin mRNA by 30 min and 96% by 60 min. In contrast, only 37% of the ferritin mRNA disappeared by 30 min and 70% by 60 min. The results observed at 30 min are very similar to the degree of degradation of each RNA observed at this time point with naked RNA (Figure 3). The differential degradation of albumin versus ferritin in mRNPs indicates that the estrogen-inducible nuclease activity retains substrate specificity regardless of whether the RNA is free or complexed in mRNPs. These data do not rule out the possibility that the free RNA used in the preceding experiments assembles into RNP complexes in vitro; however, this is unlikely in light of the small amount of protein (1 μg) employed.

Differential Sensitivity of 5′ versus 3′ Albumin Sequences to Digestion by the Polysomal Nuclease. The degradation of a number of mRNAs both in vivo and in vitro is initiated by endo- or exonucleolytic digestion beginning at the 3′ end of the molecule (Bernstein & Ross, 1989). We therefore sought to determine whether portions of albumin mRNA showed differential susceptibility to degradation by the polysomal nuclease. Figure 5A shows a cartoon of albumin cDNA present in our pBluescript vectors. Digestion with *Hin*cII

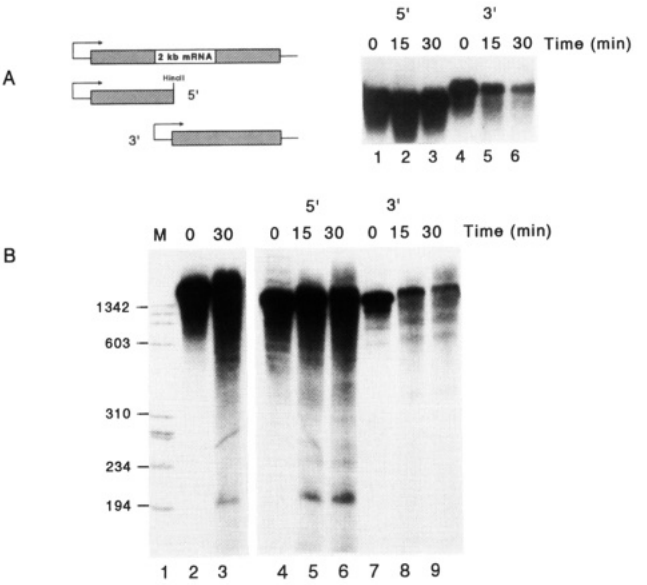

FIGURE 5: Differential degradation of albumin 5′ and 3′ transcripts. (A) Albumin cDNA in the pBluescript SK(−) was cleaved with *Hin*cII to generate vectors that make a 900-nucleotide transcript corresponding to the 5′ half of albumin mRNA and a 1100-nucleotide transcript corresponding to the 3′ half. Uniformly labeled transcripts were incubated for 0–30 min at 22 °C with polysome extract plus 10 μg of free liver RNA. The surviving RNA was assayed by electrophoresis on a 1% agarose gel which was subsequently dried and autoradiographed. Reactions using the 5′ transcript were loaded in lanes 1–3, and reactions using the 3′ transcript were loaded in lanes 4–6. (B) The products from the reaction in (A) were loaded in the same order in lanes 4–9 of a 6% polyacrylamide/urea gel. Lane 2 is a full-length transcript incubated for 30 min in the absence of polysome extract, and lane 3 is the same full-length transcript incubated for 30 min with polysome extract. Lane 1 contains end-labeled *Hae*III φX174 DNA as a size marker (M).

cleaves the template into portions that will generate transcripts of 900 (5′) and 1100 (3′) nucleotides in length. The degradation of uniformly labeled transcripts from these subclones by the nuclease present polysome extract is shown next to the cartoon in Figure 5A. In this experiment agarose gel electrophoresis was used to follow the disappearance of intact transcripts. Greater than 90% of the 3′ transcript is degraded by 60 min of incubation with polysome extract (lanes 4–6), whereas the 5′ transcript appears substantially intact (lanes 1–3). These data indicate that the 3′ half of albumin mRNA is more susceptible to degradation than the 5′.

The reaction mixtures from Figure 5A were also electrophoresed on a 6% polyacrylamide/urea gel to detect small degradation fragments (Figure 5B, lanes 4–9). Under these more stringent conditions of gel electrophoresis it is clear that the 5′ transcript is degraded with the resultant generation of a distinct fragment of approximately 194 nucleotides (lanes 5 and 6). Subsequent experiments show that the material migrating at this position is in fact a doublet (see below). While some faint bands could be observed at the top of the gel for the 3′ transcripts, the general absence of small fragments seen throughout indicates it is more extensively degraded than the 5′. In fact, we have yet to identify with any certainty any degradation fragments from the 3′ half of albumin mRNA either in vitro or by S1 assay in vivo (data not shown). Lane 2 of Figure 5B shows the product of 30 min of digestion of a full-length albumin transcript with polysome extract. Only a single product is clearly identifiable which is identical to the 194 degradation product from the 5′ transcript seen in lanes 5 and 6. We conclude that there is differential susceptibility of the 5′ versus the 3′ half of albumin mRNA to the polysomal nuclease and that the degradation products observed with

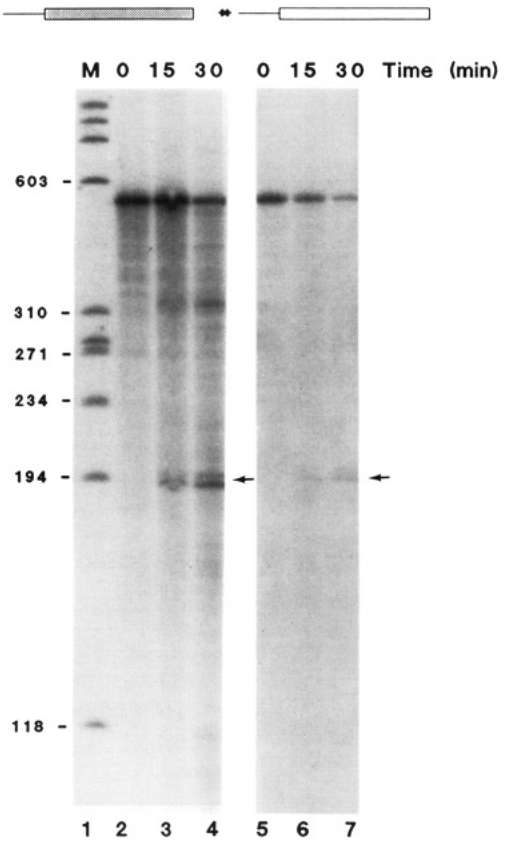

FIGURE 6: Identification of endonuclease activity in polysome extract. Albumin cDNA was digested with *Sau*I to generate 523-nucleotide transcripts corresponding to the 5′ end of albumin RNA. These were either labeled uniformly with [α-^{32}P]UTP (shaded figure, lanes 2–4) or 5′-end-labeled by transcription in the presence of [γ-^{32}P]GTP (open figure, lanes 5–7). The transcripts were incubated at 22 °C as in Figure 4, and the products were visualized by autoradiography after electrophoresis on a 6% acrylamide/urea gel. The position of the major 194-nucleotide cleavage fragment is shown by the arrow. The size markers (lane 1, M) consist of end-labeled *Hae*III restriction fragments of ϕX174 DNA.

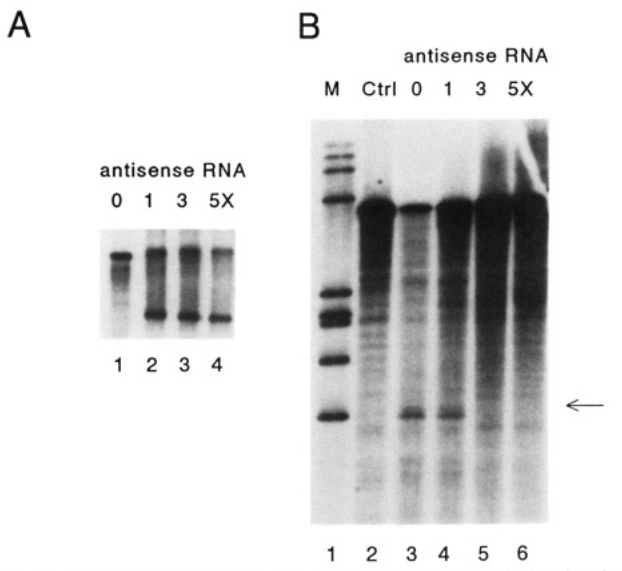

FIGURE 7: Double-stranded RNA is resistant to degradation by the polysomal nuclease activity. One hundred nanograms of uniformly labeled 5′ transcript prepared as in Figure 5 was hybridized in duplicate overnight with 0, 100, 300, and 500 ng of unlabeled antisense RNA transcribed from the same cDNA in the opposite direction. (A) One set of reactions was electrophoresed on a 6% nondenaturing polyacrylamide gel to determine the degree of hybrid formation. Lane 1 contains the radiolabeled transcript alone. Lanes 2, 3, and 4 contain the hybrids formed by incubation with a 1-, 3-, and 5-fold excess of unlabeled antisense transcript prior to electrophoresis. (B) The other set of hybrid transcripts were incubated for 30 min at 22 °C with 3 μL of polysome extract after which the reaction products were electrophoresed on a denaturing 6% polyacrylamide/urea gel. Lanes 2 and 3 contain the single-stranded radiolabeled transcript incubated for 30 min at 22 °C without and with polysome extract, respectively. In lanes 4–6 double-stranded substrate prepared as in (A) was incubated with polysome extract. *Hae*III ϕX174 size markers (M) are in lane 1. The position of the characteristic 194-nucleotide fragment is shown by the arrow. The use of somewhat more polysome extract in this experiment resulted in greater degradation of the 5′ transcript (lane 3) than was observed in Figure 4.

either half of the molecule accurately reflect those generated from the parental transcript.

Characteristics of the Liver Ribonuclease. Since the only identifiable degradation fragment came from the 5′ end of albumin RNA, we used the production of this fragment to determine whether degradation involves endonucleolytic or exonucleolytic activity. A unique fragment can be generated either by pausing of an exonuclease or cleavage by an endonuclease. In the experiment shown in Figure 6 a pair of 523-nucleotide transcripts were made from the 5′ end of albumin cDNA. These RNAs were either labeled uniformly by transcription in the presence of [α-^{32}P]UTP (hatched box) or 5′-end-labeled by transcription in the presence of [γ-^{32}P]-GTP (open box). Both transcripts were incubated for 0, 15, or 30 min with polysome extract followed by electrophoresis of the digestion products. Digestion of the uniformly labeled RNA for 15 or 30 min (lanes 3 and 4) generated a doublet just below the 194 marker. The same doublet was generated by digestion of the end-labeled transcript (lanes 6 and 7). If the 194 fragment was generated by pausing of a 5′–3′ exonuclease, we would have expected to see no fragments generated with the end-labeled substrate. Conversely, if it was generated by pausing of a 3′–5′ exonuclease, we would have expected to see a progressive decrease in the size of the substrate band in lanes 6 and 7 down to the 194 fragment. Instead, the production of a discrete 194-nucleotide fragment with no change in the size of the remaining substrate indicates that the fragment is generated by an endonucleolytic cleavage event. Furthermore, the inverse change in signal intensity for the substrate and 194-nucleotide fragment indicates a precursor–product relationship. To maintain consistency, all subsequent experiments were performed with the 523-nucleotide 5′ transcript as substrate.

Next we sought to determine whether there was specificity for cleavage of single-stranded versus double-stranded RNA. To address this, 100 ng of uniformly labeled 5′ transcript was hybridized overnight with 1, 3, or 5 times more unlabeled antisense transcript prepared from the same vector. Figure 7A shows a nondenaturing polyacrylamide gel of the hybridized products. In all cases a substantial amount of the radiolabeled transcript was shifted into a duplex form that migrated faster on the gel. The single-stranded and duplexed substrates were incubated with polysomal nuclease in the presence of 10 μg of free liver RNA as in the preceding experiments. The digestion of duplexed transcripts by polysomal nuclease is shown Figure 7B. In this experiment and subsequent ones, 3 times more polysome extract was used to obtain significant degradation of the substrate transcript as well as the production of the 194-nucleotide fragment. An equimolar amount of antisense transcript (lane 4) had little effect on the generation of the 194-nucleotide fragment. However, the degradation of the substrate transcript was significantly reduced. The hybrids prepared with 3- and 5-fold excess antisense transcript show greater resistance to the nuclease, with complete inhibition of fragment production and degradation

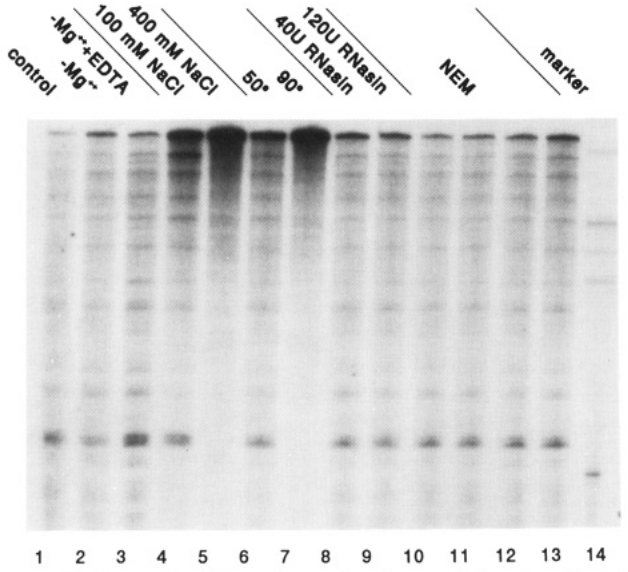

FIGURE 8: Properties of the polysomal nuclease activity. A uniformly labeled 5′ transcript was used as in Figures 5 and 6 as substrate for the polysomal nuclease. In this experiment the control consisted of polysome extract in which nuclease activity was inactivated by heating at 90 °C for 5 min (lane 7). Lane 1 is a reaction performed under the same conditions as employed in Figure 6. The buffer for these reactions contained 1 mM $MgCl_2$. Lanes 2 and 3 contain reaction products generated in buffers lacking Mg^{2+} (lane 2) or lacking Mg^{2+} and supplemented with 10 mM EDTA (lane 3). In lanes 4 and 5 the reactions were performed in buffers containing 100 and 400 mM NaCl, respectively. In lanes 6 and 7 the polysome extract was heated for 5 min at 50 and 90 °C, respectively. The reactions shown in lanes 8 and 9 contained 40 and 120 units of placental ribonuclease inhibitor (RNasin). Lane 10 contains a control reaction like lane 1. In lanes 11–13 polysome extract was incubated on ice with 10 mM *N*-ethylmaleimide for 10–30 min prior to the beginning of the experiment. The size marker in lane 14 consists of *Hpa*II fragments of M13mp9 RF. This particular experiment used a nuclease preparation that was partially purified by chromatography on Mono Q.

of substrate. The observed change in substrate degradation could not be due to titration of the nuclease with added RNA since these reactions were performed in the presence of a large excess (10 μg) of free liver RNA. These data indicate that the polysomal nuclease activity preferentially degrades single-stranded versus double-stranded RNA. Furthermore, they indicate that the site of cleavage that generates the 194 fragment is likely in a single-stranded region of the RNA.

The enzyme that has been shown to degrade histone mRNA in vitro is a Mg^{2+}-dependent exonuclease whose activity is inhibited by EDTA (Ross et al., 1987). On the basis of this we included 1 mM $MgCl_2$ in the buffer in the first experiments. In Figure 8 the 5′ albumin transcript was incubated with nuclease in buffer containing 1 mM $MgCl_2$ (lane 1), in buffer lacking Mg^{2+} (lane 2), or in buffer lacking Mg^{2+} but containing 10 mM EDTA (lane 3). In this particular experiment the control consisted of transcript incubated with heat-inactivated nuclease (lane 7). Removing Mg^{2+} from the buffer had no effect on the cleavage of the substrate transcript into the characteristic 194-nucleotide fragments. Next the reaction was carried out in buffer containing 100 and 400 mM NaCl to determine the effect of increasing the salt concentration. A 100 mM concentration of NaCl substantially reduced the overall degradation of the substrate transcript (lane 4), with somewhat less effect on the generation of the 194-nucleotide cleavage fragment. This differential effect could be due to inactivation of another nuclease in the extract or a salt-induced change in the secondary structure of the substrate or product. Increasing NaCl to 400 mM abolished nuclease activity (lane 5). It is noted above that the nuclease is inactivated by heating for 5 min at 90 °C (lane 7). However, it is stable to heating at 50 °C (lane 6).

The properties described above are similar to those of the phosphotransferase type of RNase typified by pancreatic RNase A. A notable property shared by these enzymes is their inhibition by complex formation with placental ribonuclease inhibitor. In the experiment shown in lanes 8 and 9, 40 and 120 units of inhibitor (RNasin) were included in the reaction mixture. Neither concentration had any effect on the digestion of the substrate transcript. Another commercially available inhibitor (InhibitAce) was similarly ineffective (data not shown). Finally, we examined the effect of the sulfhydryl reagent *N*-ethylmaleimide (NEM) on nuclease activity. The enzyme preparation was incubated without NEM for 30 min at 0 °C (lane 10) or with 10 mM NEM for 10–30 min prior to the addition of substrate (lanes 11–13). NEM had no effect on the cleavage of the substrate transcript.

Discussion

In *Xenopus* liver the de novo induction of vitellogenin by estrogen is accompanied by the posttranscriptional disappearance of albumin mRNA from the cytoplasm (Schoenberg et al., 1989). This study describes the nuclease activity that may be a candidate enzyme responsible at least in part for regulated mRNA instability. Nuclease activity is present in low levels in polysomes from control animals (Figure 1). Upon estrogen treatment there is a significant increase in activity. An endogenous inhibitor of this nuclease present in postmitochondrial extract of *Xenopus* liver blocks albumin RNA degradation in these crude extracts. The inhibitor remains soluble in S100 extracts whereas the nuclease sediments in the polysome fraction. This was fortuitous, since cofractionation would have prevented the identification of the nuclease in the first place. The presence of such an inhibitor raised the possibility that hormonal stimulation of nuclease activity could be effected by decreased inhibitor activity. The experiment in Figure 2 disputes this notion. Rather, estrogen appears to increase somewhat the amount of inhibitor activity.

The nuclease activity demonstrates the same substrate specificity (Figures 3 and 4) against either naked albumin and ferritin mRNA or RNPs as observed following estrogen administration either in vivo or to liver explant cultures (Pastori et al., 1991). Although there is much lower activity, the same substrate specificity is observed with polysome extract from control animals (unpublished). Therefore, the induction by estradiol represents an increase in the amount or activity of an enzyme(s) present in untreated animals rather than the appearance of a previously unexpressed gene product. This seems quite reasonable and is in keeping with results reported by Binder et al. (1989) on apolipoprotein II mRNA stability. Abrupt withdrawal of estrogen from hormone-stimulated chicks causes a change in half-life of apoII mRNA from 13 to 1.5 h. Binder et al. (1989) demonstrated that cleavage of apoII mRNA in vivo occurred at the same sites regardless of hormonal status. From these and other data they concluded that the destabilization of apoII mRNA is not mediated through a new pathway but rather by increased targeting by a preexisting mechanism. The increased activity (induction) of a preexisting ribonuclease as described in this report is entirely consistent with their findings.

The polysomal nuclease degrades the 3′ half of albumin RNA so rapidly in vitro that we were unable to reproducibly detect specific cleavage fragments. However, a distinct cleavage fragment of approximately 194 nucleotides (based on proximity of its mobility to the *Hae*III ϕX174 DNA marker of this size) was generated from both full-length transcripts

and transcripts of the 5′ half of albumin mRNA. Comparison of fragments generated by digestion of a uniformly labeled versus a 5′-end-labeled transcript showed the 194 fragment results from endonucleolytic cleavage (Figure 6). The actual product is a doublet that most likely results from cleavage at sites 1 nucleotide apart. Enzymatic RNA sequencing experiments are in progress to map the exact site of cleavage.

The generation of the 194-nucleotide cleavage fragment was used as an assay to characterize some of the properties of the polysomal nuclease activity. Both the generation of the 194 fragment and the disappearance of the substrate transcript are inhibited by hybridization of the 5′ substrate transcript to an antisense RNA generated by transcription of the same plasmid in the opposite direction with T3 polymerase (Figure 7). These data indicate that the polysomal endonuclease preferentially digested single-stranded RNA. It is therefore possible that the relatively stable 194 fragment is generated by cleavage at a single-stranded site adjacent to a region with a relatively stable secondary structure. This will be addressed in future experiments by enzymatic mapping of albumin secondary structure.

In contrast to the exonuclease described by Ross et al. (1987) or the enzyme that degrades c-*myc* in vitro, the polysomal nuclease does not require Mg^{2+} for activity. In fact, it works as well in the presence of an excess of EDTA as in the presence of Mg^{2+}. Like the histone exonuclease, the *Xenopus* liver enzyme activity is inhibited by elevated salt and is resistant to inhibition by placental ribonuclease inhibitor. It is fairly temperature stable, retaining activity after 5 min at 50 °C but not 90 °C. The latter treatment can be used to inactivate the enzyme for control incubations. The enzyme activity is resistant to inactivation by *N*-ethylmaleimide, indicating that exposed sulfhydryl groups are unlikely to play a role in its activity. In addition, the polysomal nuclease is active over a range of pH from 6.0 to 8.8 and is unaffected by the presence or absence of ATP in the reaction mixture (data not shown).

Bernstein et al. (1989) have demonstrated that the presence of poly(A)-binding protein modulates the in vitro degradation of c-*myc* RNA. All of the full-length and 3′ transcripts used in this study are polyadenylated. However, no poly(A)-binding protein was detectable in western blot of polysome extracts using a previously described antibody to the *Xenopus* protein (Zelus et al., 1989). We have also found that the presence or absence of a 5′ cap has no effect on in vitro degradation (data not shown).

A significant question that remains is the nature of the selectivity of degradation between albumin and ferritin mRNA in vivo and in vitro. If the polysomal nuclease activity described in this report is one of the enzymes responsible for hormone-regulated mRNA instability, protein–RNA interactions cannot serve as the sole determinant of RNA instability since substrate specificity is maintained for free liver RNA, in vitro generated transcripts, and RNA present in mRNPs. Therefore, in vitro selectivity must lie in primary sequence or structural features of these mRNAs. For consistency between experiments we focused on the degradation of the 5′ end of albumin RNA since an identifiable degradation fragment could be seen that demonstrated a precursor–product relationship with the substrate (Figure 6). However, the data in Figure 5 show that the 3′ half of albumin RNA is more extensively degraded by the polysomal nuclease activity. It is likely that a number of nucleases are extracted from liver polysomal; hence we cannot at present rule out the possibility that different enzymes are responsible for the contrast between the two halves of albumin RNA as substrate. However, a nuclease preparation purified 12-fold by chromatography on Mono Q demonstrates the same properties using a transcript from the 3′ half of albumin RNA as seen for the 5′ transcript in Figure 8 (data not shown).

A number of cleavage sites have been identified both in vivo and in vitro in mRNAs regulated by posttranscriptional processes. These include CAUG (Cochrane & Deeley, 1989) or AAU/UAA (Binder et al., 1989) in the 3′ end of apo very low density lipoprotein II, and the repeating motif ACCTA found in a homeobox mRNA which is unstable in *Xenopus* oocytes (Brown & Harland, 1990). Previous studies from this laboratory demonstrated that mRNA destabilization following estrogen administration is a generalized response in *Xenopus* liver that regulates the amount of the majority of mRNAs encoding serum proteins (Pastori et al., 1990, 1991). Transferrin and γ-fibrinogen mRNA are degraded in vitro much like albumin (Pastori and Schoenberg, unpublished). Whereas no clear-cut consensus emerges from the sequences of the unstable *Xenopus* liver mRNAs, they do have one feature in common. The 3′ ends of these mRNAs are AU-rich, but not in the motif observed in GM-CSF (Shaw & Kamen, 1986) and mRNAs encoding other inflammatory response mediators. Long U-rich stretches are found in the 3′ UTR of transferrin (UUUUUUCCUUAUCUUCUGUUGUUUUUGUUUAU) and γ-fibrinogen (UUUUGGAUUAUUAUUUUACACUUUAUUGUCUU) with shorter stretches present in the albumins (UUCAACUGUGUGUUGU). The sequence UUAUU, UUGUU, or UUAUC is found multiple times in the 3′ UTR of all regulated mRNAs. None of these features is present in the 3′ UTR of ferritin mRNA. These data suggest that a U-rich motif, while not necessarily the site of cleavage, may identify an mRNA for preferential degradation. Additional instability determinants may reside within the coding sequences in both the 5′ and 3′ portions of the RNAs. There is a precedent for this in c-*fos*, in which distinct instability determinants reside in the 3′ UTR and within the coding sequence (Shyu et al., 1989).

Finally, the question remains whether the estrogen-inducible nuclease found on *Xenopus* liver polysomes is the enzyme responsible for mRNA degradation in response to hormone. Preliminary experiments from S1 mapping of albumin degradation products resulting from estrogen administration in vivo or incubation of free liver RNA in vitro with polysome extract demonstrate a major site of cleavage at the 194 nucleotide position observed in the present study. More detailed nuclease protection and primer extension studies are in progress to verify whether the identified polysomal nuclease is indeed the enzyme that mediates resulted mRNA instability.

Added in Proof

Data obtained at press suggest that the induction of the ribonuclease described in this paper may in fact be due to an estrogen-stimulated recruitment of preexisting nuclease to the large polysomal complexes isolated as part of the first step of the extraction procedure rather than a quantitative increase in the amount of enzyme.

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Analysis of the RNA- and DNA-Dependent DNA Polymerase Activities of Point Mutants of HIV-1 Reverse Transcriptase Lacking Ribonuclease H Activity†

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ABSTRACT: The RNA- and DNA-dependent DNA polymerase activities of two point mutants of HIV-1 reverse transcriptase lacking ribonuclease H activity have been compared to the wild-type enzyme activities using substrates consisting of an oligodeoxynucleotide primer hybridized to either a RNA or a DNA template. The RNase H phenotype had a negligible effect on the steady-state kinetics and processivity of reverse transcription of a homopolymer template-primer [poly(A)·oligo(dT)]. However, analysis of the distribution of DNA products indicated that the ability of the mutants to reverse-transcribe a specifically primed 345-nucleotide heteropolymeric RNA template derived from the *gag* region of HIV-1 was impaired relative to the wild-type enzyme. Although the wild-type and mutant enzymes shared the same pause sites of synthesis along the RNA template, certain prematurely terminated nascent primer chains were poorly extended by the mutant enzymes and hence accumulated, suggesting that a catalytically functional RNase domain facilitated reinitiation of DNA synthesis at specific pause sites along a heteropolymer template. In contrast, the processivity and product distribution of DNA synthesis directed by a heteropolymer *gag* DNA template of the same nucleotide sequence were not significantly influenced by the RNase H phenotype of the mutants.

In the search for novel therapeutic strategies against acquired immune deficiency syndrome (AIDS),[1] the enzymes encoded by the causative agent, HIV-1, are under intense scrutiny as potential targets for rational drug design. The reverse transcriptase (RT) enzyme encoded by a retrovirus is solely responsible for converting the ss-RNA genome into a ds-proviral DNA form, which is the substrate for integration of the virus into the genome of the infected host cell (Panganiban & Fiore, 1988). The three RT-associated activities needed to accomplish this task are (i) RNA-dependent DNA polymerase, (ii) ribonuclease H, and (iii) DNA-dependent DNA polymerase (Goff, 1990). The experimental data bearing on the structure–function relationships in HIV-1 RT were recently reviewed by Jacobo-Molina and Arnold (1991). The linker insertion mutagenesis data of Prasad and Goff (1989) and of Hizi et al. (1990), in conjunction with the identification of a carboxy-terminal "p15" RNase H activity (Hansen et al., 1988), suggested that the domain organization of HIV-1 RT parallels that of other RT enzymes, with the RNase H and polymerase domains distinct and separate from one another

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[1] Abbreviations: AIDS, acquired immune deficiency syndrome; HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; RNase H; ribonuclease H; nt, nucleotide(s); ss, single stranded; ds, double stranded; dNTP, deoxynucleoside 5′-triphosphate; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

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