# Estrogen Regulation of *Xenopus laevis* γ-Fibrinogen Gene Expression[†,‡]

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ABSTRACT: Albumin gene expression in *Xenopus* is regulated by estrogen through changes in the stability of its mRNA. The goal of the present study was to determine whether a similar pathway regulates γ-fibrinogen. Degenerate oligonucleotides directed to conserved regions of the carboxyl-terminal half (domain D) of human and lamprey γ-fibrinogen were used to isolate a full-length cDNA clone for *Xenopus* γ-fibrinogen. Analysis of codon utilization from the DNA sequence of this clone revealed that *Xenopus* γ-fibrinogen mRNA shows the same bias against CG dinucleotides as present in human, but not lamprey, fibrinogen mRNA. Features of the protein shared with the human homologue include all of the cysteine residues, an N-linked glycosylation site at amino acid 50, and 75% sequence identity in domain D. Much of the same region is conserved in lamprey γ-fibrinogen. There is only a single size mRNA encoding γ-fibrinogen in *Xenopus*, unlike rats where two mRNAs of different length are generated by alternate splicing. Administration of estrogen to male *Xenopus* results in the disappearance of γ-fibrinogen mRNA from the cytoplasm, with no effect on steady-state levels in the nucleus. This process can be blocked by prior treatment with anti-estrogen, indicating that, like the regulation of serum albumin mRNA, γ-fibrinogen is regulated posttranscriptionally through an estrogen receptor dependent mechanism. It is postulated that a consensus sequence flanking the AAUAAA polyadenylation signal in γ-fibrinogen and the 68- and 74-kDa albumin mRNAs, but not vitellogenin or β-globin mRNA, may play a role in the hormonal regulation of mRNA stability.

The liver of the South African frog *Xenopus laevis* is a major target for the female sex hormone 17β-estradiol. Estrogen administration to male or female animals results in the induction of the estrogen receptor and its cognate mRNA as well as the genes encoding retinol-binding protein (Barton & Shapiro, 1988) and the yolk protein precursor vitellogenin (Wahli & Ryffel, 1985). The induction of vitellogenin has been extensively studied as a major model for the trans activation of transcription by steroid hormone receptors. In addition, the continuous presence of estradiol causes the stabilization of vitellogenin mRNA in the cytoplasm, resulting in a half-life of 500 h (Brock & Shapiro, 1983).

Early studies (Follett & Redshaw, 1974) demonstrated that the induction of vitellogenin was associated with the disappearance of many of the proteins found in the serum of nonestrogenized animals. We have focused on serum albumin gene expression as a model for the repression of gene expression by steroid hormones. A single injection of 17β-estradiol results in the loss of 70% of albumin-coding sequences from total liver RNA (Riegel et al., 1986), with little effect on albumin gene transcription. From these data, we and others (Wolffe et al., 1985; Kazmaier et al., 1985) concluded that albumin gene expression is regulated predominantly by changes in mRNA stability. Experiments utilizing the estrogen receptor antagonist 4-hydroxytamoxifen indicate that the action of the estrogen receptor is required for albumin mRNA destabilization (Riegel et al., 1987). Finally, recent experiments from this laboratory have shown that the destabilization of albumin mRNA occurs in the cytoplasm and estrogen has no effect on splicing or polyadenylation (Schoenberg et al., 1989).

Wangh (1982) has reported that the synthesis and secretion of the normal complement of serum proteins (especially albumin and the fibrinogens) in primary cultures of *Xenopus* hepatocytes require the continuous presence of dexamethasone and thyroid hormone. Addition of estrogen to this mixture then results in the cessation of albumin and fibrinogen synthesis. It was of interest, therefore, to determine if fibrinogen is regulated in a manner similar to albumin in *Xenopus*.

The vertebrate fibrinogens consist of two heterotrimeric subunits (α, β, γ) joined by intrastrand disulfide linkages [reviewed in Doolittle (1984)]. Clot formation is initiated by the proteolytic action of thrombin resulting in the formation of the insoluble fibrin polymer. The functional roles of each portion of the fibrinogen complex have been dissected both by protein chemistry and more recently by molecular biological approaches consequent to the cloning of the corresponding cDNAs and genes. A particularly useful approach has been to examine fibrinogen peptides present in lower vertebrates, as such data shed light on both the function and evolutionary conservation of important structural features (Strong et al., 1985). The present study describes the cloning of γ-fibrinogen mRNA from *Xenopus laevis*, its relationship to other vertebrate homologues, and the regulation of its expression by estradiol.[1]

## Experimental Procedures

*Experimental Animals.* Laboratory-bred male *Xenopus laevis* were obtained from Nasco, Inc. (Fort Atkinson, WI), and kept in Plexiglas aquaria at 20 °C with a 12-h light, 12-h

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[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 (NIH) 78-23. All recombinant organisms and molecules were handled under conditions of the NIH guidelines for recombinant DNA research. The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

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dark cycle. They were fed frog brittle twice weekly. Injections of 1 mg of estradiol were performed in 0.1 mL of 5% (v/v) dimethyl sulfoxide in propylene glycol in the dorsal lymph sac. Control animals received injection vehicle 24 h prior to death. Before being killed, animals were anesthetized by immersion in a solution of tricaine methanesulfonate (Finquel, Ayerst, Rouses Point, NY). Livers were perfused of blood with sterile 1× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate), rinsed in cold 1× SSC, and used immediately for the isolation of RNA.

*Isolation of Total, Nuclear, and Extranuclear RNA Fractions.* The procedures for the separation of cytoplasmic and nuclear RNA from *Xenopus* liver, as well as the preparation of total RNA, have been described in detail elsewhere (Schoenberg et al., 1989). These procedures effectively provide preparations of cytoplasmic RNA free from contamination with nuclear RNA, and vice versa.

*Hybridization to DNA and RNA Probes.* Vitellogenin, albumin, and globin cDNA inserts were isolated from their respective plasmids and radiolabeled by priming with random hexamers (Feinberg & Vogelstein, 1983). The RNA blots were prehybridized for 10 min at 65 °C in a solution containing 1% bovine serum albumin (BSA), 7% SDS, 0.5 M sodium phosphate, pH 7.0, and 1 mM EDTA (Church & Gilbert, 1984). The prehybridization solution was removed and replaced with the same solution containing the denatured DNA or RNA probes. Concentrations of probe were chosen to achieve 10× $C_0t$ in 18-h hybridization. The blots were hybridized overnight at 65 °C. Following hybridization, the blots were washed twice for 15 min/wash under the following conditions: (1) 10× SSC + 1% SDS, 25 °C; (2) 1× SSC + 1% SDS, 37 °C; (3) 0.1× SSC + 1% SDS, 65 °C. Specific hybridization was detected by autoradiography on Kodak X-OMat AR XAR-5 film with Dupont Cronex Lightning Plus intensifying screens. The hybridized probe was removed from selected blots by heating for 15 min at 90 °C in 0.1× SSC + 1% SDS.

*Plasmid Clones.* Cloned DNA inserts for hybridization to RNA blots were isolated from the albumin cDNA clone pSPxa14/64 and the vitellogenin cDNA clone pXlvc10 by digestion with *Eco*RI and *Hin*dIII. The vitellogenin clone was a gift from Prof. Walter Wahli (University of Lausanne, Switzerland). The *Xenopus* β-globin clone was a gift from Dr. Roger Patient (Kings College, London). A liver cDNA library was prepared from poly(A) RNA of a single male *Xenopus* by the method of Gubler and Hoffman (1983) and cloned Moloney murine leukemia virus reverse transcriptase. Double-stranded cDNA was methylated, ligated to *Eco*RI linkers, and cloned into the *Eco*RI site of λZAP. *Xenopus* γ-fibrinogen cDNA was isolated by screening the cDNA library with a pair of degenerate oligonucleotides to a region of the protein conserved in both lamprey and rat γ-fibrinogen (see Results).

*DNA Sequence Analysis.* The cloned cDNA was rescued from λZAP with f1 helper phage and maintained as plasmid in Bluescript. Double-stranded DNA sequencing was performed by a modification of the method of Chen and Seeburg (1985) using an oligonucleotide primer complementary to the promoters of the cloning vectors, modified T7 DNA polymerase (Sequenase), and [$^{35}$S]dATP. To bridge gaps in the sequence, the same procedure was used with oligonucleotides derived from the preceding sequence. Both strands were sequenced, and computer analysis were performed with the GEL, SEQ, and PEP facilities of the Bionet resource and the IBI Pustell DNA sequence analysis programs.

*Enzymes, Isotopes, and Reagents.* λZAP was obtained from Stratagene. Modified T7 DNA polymerase was obtained from United States Biochemical Corp. Other enzymes used in these experiments as well as ultrapure guanidine isothiocyanate, sucrose, and CsCl were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, MD). [α-$^{32}$P]dCTP (4000 Ci/mmol) and [α-$^{35}$S]dATP (300 Ci/mmol) were obtained from New England Nuclear. All other reagents and salts were the highest quality available from Sigma Chemical Co. (St. Louis, MO).

## Results

*Sequence of Xenopus γ-Fibrinogen cDNA.* From the derived peptide sequence of lamprey γ-fibrinogen cDNA, Doolittle and co-workers (Strong et al., 1985) determined that a high degree of sequence conservation exists between the C-terminal half (which forms part of the D domain; Doolittle et al., 1979) of all γ-fibrinogens. We reasoned, therefore, that similar sequence conservation may be found in the *Xenopus* homologue. Two degenerate oligonucleotides were prepared to the sequence encoding DFGDD and GIIWA (nucleotides 1093–1106 and 1309–1322, respectively, of rat γ-fibrinogen). Chung et al. (1983) found that there is a bias against CG dinucleotides in the coding sequence of human γ-fibrinogen, a feature not present in the lamprey sequence (Strong et al., 1985). Since there was no reason to believe that the same bias against CG dinucleotides occurred in the coding sequence of *Xenopus* γ-fibrinogen, we designed the oligonucleotides with full degeneracy.

The sequence of *Xenopus* γ-fibrinogen cDNA (pXfib-γ) is shown in Figure 1 with the location of the degenerate oligonucleotides underlined. The cloned cDNA includes most of the 5′ untranslated region and the coding sequence and terminates at poly(A). The sequence surrounding the translation initiation site CCATAATG fits the Kozak (1987) consensus sequence for the eucaryotic translation start site of CCA(G)CCAUG with a purine at position −3, however, there is only one pyrimidine in the next two positions unlike the consensus with two pyrimidines. By analogy to mammalian γ-fibrinogen, we have assigned the first hydrophobic stretch of 25 amino acids (MTRLPKQGLLLLQSLALLSSAFGNI) to the signal peptide sequence. There is a single open reading frame, and the consensus polyadenylation signal AATAAA is found 20 nt upstream from poly(A). Examination of codon utilization indicates that, unlike lamprey, *Xenopus* γ-fibrinogen mRNA retains the bias against CG dinucleotides found in the mammalian homologues (data not shown).

Comparison between the sequence of the mature *Xenopus* γ-fibrinogen peptide and human and lamprey γ-fibrinogens is shown in Figure 2. The cysteine residues that play a critical role in maintenance of fibrinogen structure are conserved throughout between human and *Xenopus* (A) and in the C-terminus (D domain) between lamprey and *Xenopus* (B) (shaded residues). The amino termini of human and *Xenopus* γ-fibrinogens are highly homologous; however, the amphibian protein has no tyrosines until 17 residues past the putative cleavage site. Mammalian γ-fibrinogen invariably has tyrosine at residue 1. Lamprey γ-fibrinogen has cyclized glutamine at the amino terminus of the mature protein. There are several glutamine residues in the putative signal peptide sequence; however, none of these reside near the junction between the hydrophobic leader and the putative mature amino terminus. The major fibrinogen domains are joined by sets of intrastrand disulfides termed "disulfide rings", and this feature is conserved between human and *Xenopus*. Following the conserved amino terminus, there is considerable divergence over the "coiled coil"

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                                                                    79
  GGAATTCGGCAGGGACATAGGACCTGCCATAATGACCAGACTTCCCAAGCAAGGATTACTGCTTCTCCAGTCCTTAGCC
                                      MetThrArgLeuProLysGlnGlyLeuLeuLeuLeuGlnSerLeuAla
                                                                   160
CTCCTCTCTAGTGCCTTTGGTAATATTATACCAAACACAGACAACTGCTGCATCTTAGATGGACGTTTTGGGGAGTACTGC
LeuLeuSerSerAlaPheGlyAsnIleIleProAsnThrAspAsnCysCysIleLeuAspGlyArgPheGlyGluTyrCys
                                                                   241
CCTACAACATGCGGCATTTCCGATTTCCTGAACAGATACCAAGAAAATGTTGACACAGACTTGCAGTACCTGGAAAACCTT
ProThrThrCysGlyIleSerAspPheLeuAsnArgTyrGlnGluAsnValAspThrAspLeuGlnTyrLeuGluAsnLeu
                                                                   322
TTAACTCAAATCAGCAACTCCACAAGTGGAACTACCATAATAGTAGAACATTTAATAGACTCTGGGAAAAAACCTGCGACA
LeuThrGlnIleSerAsnSerThrSerGlyThrThrIleIleValGluHisLeuIleAspSerGlyLysLysProAlaThr
                                                                   403
TCTCCACAAACAGCCATAGACCCTATGACTCAGAAATCAAAAACATGTTGGATGAAATTAACAGATATGAAAAACTATTAT
SerProGlnThrAlaIleAspProMetThrGlnLysSerLysThrCysTrpMetLysLeuThrAspMetLysAsnTyrTyr
                                                                   484
CAGTATGAAGAAAATATACTATACCTGCAAGAAGTATATTCTTCAAATCAAAATAAGATTTTCCTGCTTAAACAGAAAATA
GlnTyrGluGluAsnIleLeuTyrLeuGlnGluValTyrSerSerAsnGlnAsnLysIlePheLeuLeuLysGlnLysIle
                                                                   565
GCAAATCTGGAATTACAATGCCAGCAGCCTTGCCGAGATACAGTTCAGATACAGGAGTTCACAGGAAAAGACTGTCAAGAA
AlaAsnLeuGluLeuGlnCysGlnGlnProCysArgAspThrValGlnIleGlnGluPheThrGlyLysAspCysGlnGlu
                                                                   646
GTTGCTAATAAGGGAGCAAGGCTTAGCGGACTCTACTACATCAAGCCTCTAAAAGCCAAACAGCAGTTCCTGGTTTACTGT
ValAlaAsnLysGlyAlaArgLeuSerGlyLeuTyrTyrIleLysProLeuLysAlaLysGlnGlnPheLeuValTyrCys
                                                                   727
GAAATTGAACCATCTGGCAGTGCATGGACTGTTATTCAAAGAAGACTTGATGGCAGTGTGAATTTCCATAAGAACTGGGTC
GluIleGluProSerGlySerAlaTrpThrValIleGlnArgArgLeuAspGlySerValAsnPheHisLysAsnTrpVal
                                                                   808
CAGTATAGAGAAGGTTTTGGATATCTGTCACCAAACGACAAGACTGAGTTCTGGCTTGGGAATGAAAAAATACATCTACTA
GlnTyrArgGluGlyPheGlyTyrLeuSerProAsnAspLysThrGluPheTrpLeuGlyAsnGluLysIleHisLeuLeu
                                                                   889
AGCACCCAATCTACCATCCCATATGTTATGAGAATTGAGTTAGAAGACTGGAGTAATCAAAAGAGCACAGCAGACTATTCC
SerThrGlnSerThrIleProTyrValMetArgIleGluLeuGluAspTrpSerAsnGlnLysSerThrAlaAspTyrSer
                                                                   970
ACTTTCAGACTGGGTTCAGAAAAGGATAATTATCGTTTTACTTACGCATACTTCATTGGTGGTGATGCCGGTGATGCCTTT
ThrPheArgLeuGlySerGluLysAspAsnTyrArgPheThrTyrAlaTyrPheIleGlyGlyAspAlaGlyAspAlaPhe
                                                                  1051
GATGGGTTTGACTTTGGAGATGATCCAAGTGATAAATTCTACACATCTCACAATGGAATGCAGTTCAGTACTTTTGATAAA
AspGlyPheAspPheGlyAspAspProSerAspLysPheTyrThrSerHisAsnGlyMetGlnPheSerThrPheAspLys
                                                                  1132
GACAATGACAAGTTTGACGGAAACTGTGCTGAGCAAGATGGCTCTGGGTGGTGGATGAACCGATGCCATGCAGCCCACCTC
AspAsnAspLysPheAspGlyAsnCysAlaGluGlnAspGlySerGlyTrpTrpMetAsnArgCysHisAlaAlaHisLeu
                                                                  1213
AATGGCAAATACTATCAAGGAGGTACATACAGTGAGGCGGACAGTGGACCTAGCGGCTATGATAATGGTATTATCTGGGCT
AsnGlyLysTyrTyrGlnGlyGlyThrTyrSerGluAlaAspSerGlyProSerGlyTyrAspAsnGlyIleIleTrpAla
                                                                  1294
ACCTGGAGACGCCGGTGGTACTCCATGAAGTCAGTTACAATGAAAATCATGCCCCTTAACAGATATGGAGCAGAAGGACAG
ThrTrpArgArgArgTrpTyrSerMetLysSerValThrMetLysIleMetProLeuAsnArgTyrGlyAlaGluGlyGln
                                                                  1375
CAGACTTTGGGCGGCTCAAAGAAGTCTGACTTTGAAAACAGAGGAGACTTTTAAGAATCCAGCTTTTGGATTATTATTTTA
GlnThrLeuGlyGlySerLysLysSerAspPheGluAsnArgGlyAspPhe
                                                        1441
CACTTTATTGTCTTGCTTTATAAATTGTATTCACAACTTGTTTTTAAATAAAGATACACCATTGTG(A)n
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FIGURE 1: Sequence of *Xenopus* γ-fibrinogen cDNA. The sequence of γ-fibrinogen is shown, with the derived peptide sequence below. The locations of the two degenerate oligonucleotides used to isolate the clone from the cDNA library are underlined.

portion of the molecule (Doolittle, 1984). Both the human (residue 51) and *Xenopus* (residue 50) proteins bear an N-linked glycosylation signal (arrowheads) at residue 52 within the coiled coil structure, a feature absent from the lamprey protein.

Strong et al. (1985) found that the highest degree of sequence homology between human and lamprey γ-fibrinogens residues in the carboxyl-terminal half, which contributes substantially to the D domain of the fibrinogen complex. This portion of the molecule is essential for calcium binding, platelet aggregation, and clumping of staphylococcal bacteria (Doolittle, 1984). Not surprisingly, this domain is also highly conserved in the *Xenopus* peptide. Whereas the overall sequence identity is 67% between the human and amphibian peptides, the region between amino acids 133 and 407 shows 75% identity. Furthermore, the sequences chosen for preparation of degenerate oligonucleotides (DFGDD, GIIWA) are completely conserved in the human, frog, and lamprey peptides. Two final sequence features are noteworthy. First, all three γ-fibrinogens have paired glutamine residues near the carboxyl terminus that are likely to be important for cross-linking of polymerized fibrinogen. Second, the *Xenopus* and lamprey, but not human, peptides contain the sequence arginine-glycine-aspartic acid (RGD) one residue from the carboxyl terminus. This sequence has been identified as the recognition motif for a number of molecules which bind to cell surface receptors (e.g., fibronectin).

*Estrogen Regulates Steady-State Levels of γ-Fibrinogen mRNA.* The first lane of Figure 3A is a Northern blot of total *Xenopus* liver RNA from male animals which received injection vehicle 24 h prior to death. γ-Fibrinogen mRNA migrates as a single species of 1440 nt, a size identical with that of the sequenced cDNA. A larger fibrinogen mRNA generated in rats by alternate splicing of the primary transcript (Crabtree & Kant, 1982) is not observed, even upon prolonged exposure of the autoradiogram. Lane 2 contains an equal amount of total RNA from animals which received 1 mg of estradiol 24 h prior to death. The amount of fibrinogen

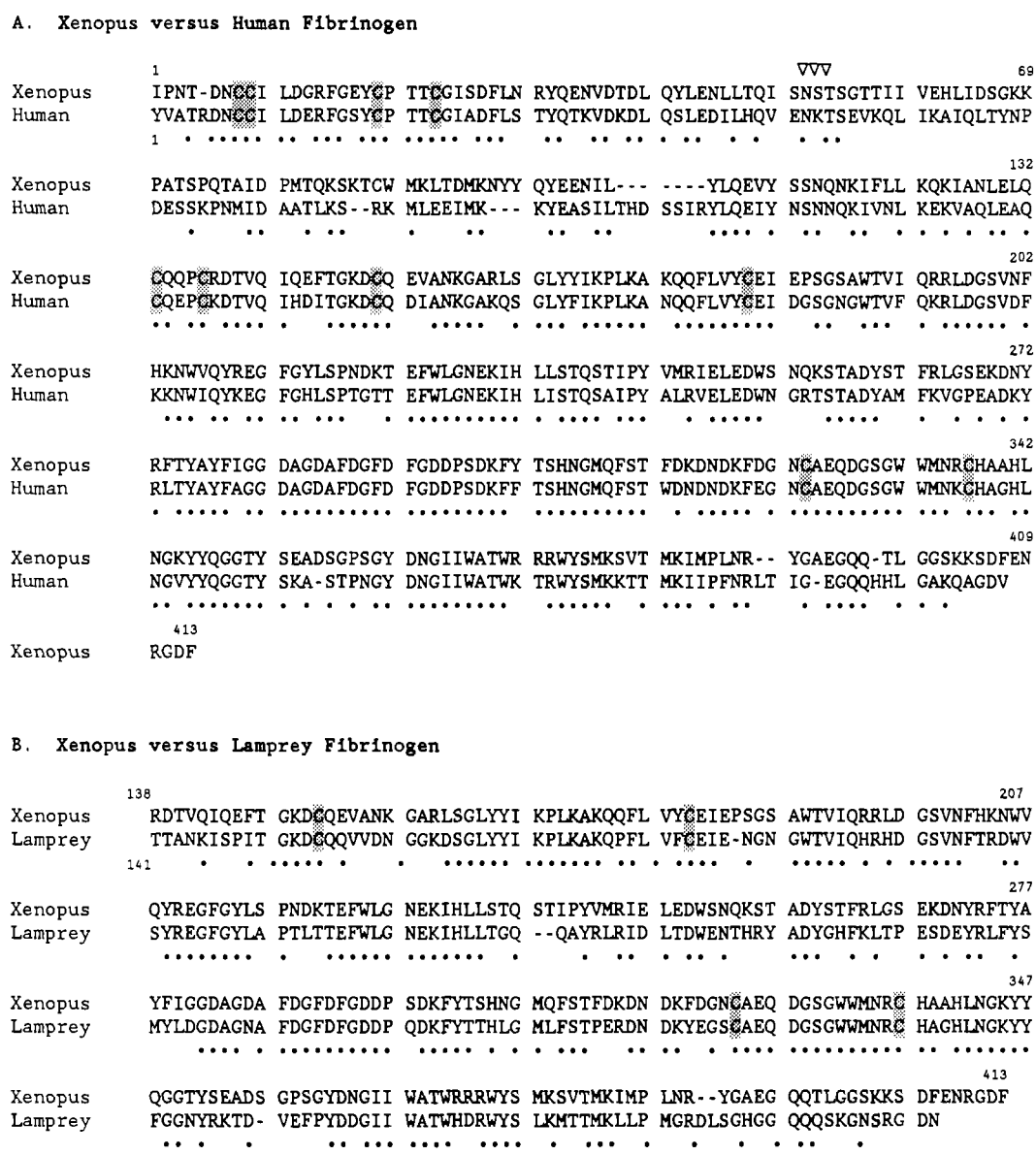


FIGURE 2: Comparison of *Xenopus* human and lamprey γ-fibrinogens. The sequence of the mature *Xenopus* γ-fibrinogen peptide was aligned with human (A) and lamprey (B) fibrinogens. Conserved cysteine residues are shaded, identical residues are shown with a dot. The arrowheads in (A) identify an N-linked glycosylation signal (Asn-X-Thr) present in both the *Xenopus* and human peptides. Only the highest degree of sequence conservation between *Xenopus* and lamprey fibrinogens (which is localized to the carboxyl-terminal half of the molecules) is shown.

mRNA was halved by this treatment. The induction of vitellogenin mRNA serves as a control for the effectiveness of hormone administration. That equivalent amounts of RNA were loaded is shown in the second panel where the blot was stripped and reprobed with β-globin cDNA, which we have previously demonstrated to be a constitutive control (Martin et al., 1986).

We have previously demonstrated that the destabilization of serum albumin mRNA by estrogen proceeds by an estrogen receptor dependent mechanism (Riegel et al., 1987). This conclusion was drawn by the ability to block the disappearance of message by the anti-estrogen 4-hydroxytamoxifen (OHT). We therefore administered anti-estrogen or vehicle control 24 h prior to injection of estradiol, and total RNA was isolated 24 h later for analysis by Northern blot. The data in Figure 3B show that anti-estrogen blocked the disappearance of γ-fibrinogen mRNA. Therefore, the suppression of fibrinogen mRNA, like albumin mRNA, proceeds through an estrogen receptor dependent mechanism.

The original conclusion that albumin mRNA is regulated posttranscriptionally came from experiments which demonstrated that changes in steady-state levels of mRNA did not correspond to changes in transcriptional activity as measured by the in vitro run-on assay (Riegel et al., 1986). We were unable to extend this correlation to γ-fibrinogen because of the substantially lower transcription rate for this gene (see Discussion). However, we have developed a method to quantitatively separate nuclear and cytoplasmic RNA from *Xenopus* liver and used this to demonstrate that the relative steady-state level of albumin mRNA in the nucleus accurately reflects the transcriptional status of the albumin genes (Schoenberg et al., 1989). This approach was therefore used to address the subcellular location and mechanism responsible for the decrease of γ-fibrinogen mRNA.

Figure 4A is a time course following the steady-state level of cytoplasmic γ-fibrinogen mRNA after a single injection of estradiol (shown are pooled samples from two frogs per point). It is evident from these data that estrogen has little effect for the first 12 h, after which γ-fibrinogen mRNA declines to almost undetectable levels by 48 h. Hybridization of the same samples to vitellogenin cDNA confirms the efficacy of hormone treatment, and the equivalency of these

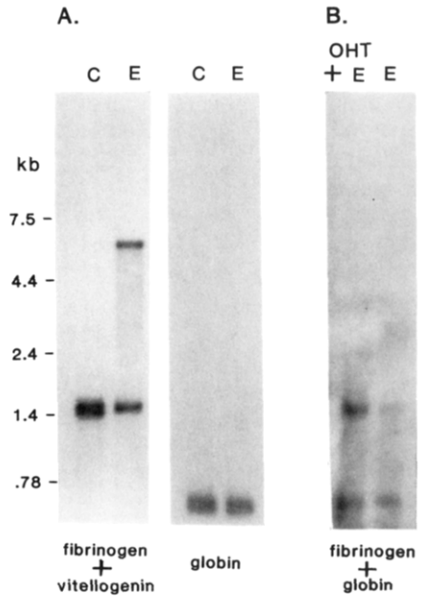


FIGURE 3: Northern blot analysis of γ-fibrinogen and its regulation by 17β-estradiol. (A) Ten micrograms of total liver RNA from control animals (C) and animals which received 1 mg of estradiol 24 h prior to death (E) was electrophoresed on a 1% agarose gel, blotted onto nylon membrane, and hybridized to a mixture of γ-fibrinogen and vitellogenin cDNAs. The blot was stripped and subsequently hybridized with β-globin cDNA as a constitutive control. (B) Male *Xenopus* were injected with 1 mg of 4-hydroxytamoxifen (OHT) or vehicle, followed 24 h later by 1 mg of estradiol (E). Total RNA isolated 24 h later was electrophoresed on a 1% agarose gel, blotted onto a nylon membrane, and hybridized to a mixture of γ-fibrinogen and β-globin cDNAs.

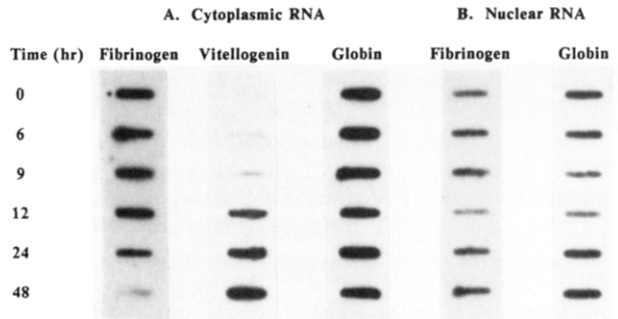


FIGURE 4: Cytoplasmic regulation of γ-fibrinogen mRNA. Male *Xenopus* were injected with 1 mg of estradiol at $t = 0$, and liver cytoplasmic and nuclear RNAs were prepared at the indicated times. (A) Five-microgram samples of cytoplasmic RNA pooled from two frogs per point were applied in replicate to a nylon membrane using a slot blot apparatus. The membranes were hybridized to γ-fibrinogen, vitellogenin, and β-globin cDNAs. (B) Five-microgram samples of nuclear RNA were applied to a nylon membrane as in (A) and hybridized to γ-fibrinogen and β-globin cDNA.

samples is demonstrated by hybridization to β-globin cDNA. In contrast, the data in Figure 4B indicate that estrogen has no effect on the steady-state level of γ-fibrinogen mRNA in the nucleus. (The variation in signal intensity on this autoradiogram is due to variations in sample loading as shown for the β-globin signal.) We conclude from these data that, like albumin, γ-fibrinogen gene expression is regulated by estrogen through changes in the steady-state levels of its mRNA in the cytoplasm.

The data in Figure 4 and several repetitions of this experiment were quantified by laser scanning densitometry and are shown in Figure 5 as the amount relative to the steady-state levels of cytoplasmic (open bars) and nuclear (hatched bars) γ-fibrinogen mRNA found in control animals. For the sake

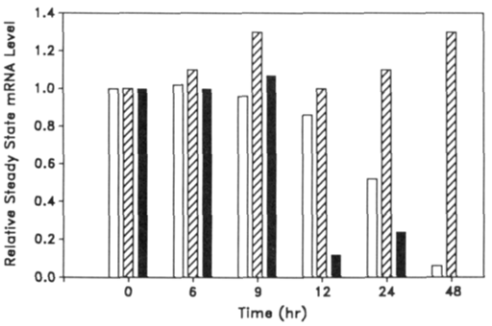


FIGURE 5: Differential degradation of γ-fibrinogen and serum albumin mRNA. The data in Figure 4 (and several repeats) were quantified by scanning laser densitometry and normalized to the relative steady-state level of each mRNA in control animals. Cytoplasmic γ-fibrinogen mRNA is shown in the open bars, and nuclear γ-fibrinogen mRNA is shown in the hatched bars. The time course for the degradation of cytoplasmic serum albumin mRNA (solid bars) is presented for the sake of comparison.

of comparison, we have included a quantitative scan of the Northern blot we have previously published for cytoplasmic albumin mRNA (solid bars) (Schoenberg et al., 1989). We have previously noted that steady-state levels of albumin mRNA are unaffected for 9 h after estrogen. However, between 9 and 12 h, greater than 90% of the cytoplasmic mRNA disappears. This is not the case for γ-fibrinogen. Rather, the steady-state level of γ-fibrinogen mRNA delines more gradually beginning at 12 h and is only 50% of the control level at 24 h. We have observed similar results for these two mRNAs in cultured liver cubes exposed to estradiol (data not shown). The significance of this difference is unclear at present.

## Discussion

The peptide sequence derived from the cloned *Xenopus* γ-fibrinogen cDNA indicates that there is a high degree of sequence conservation between the amphibian and human protein in the amino terminus of the protein constituting the disulfide ring structure, followed by a poorly conserved region equivalent to the "coiled coil" portion of all γ-fibrinogens examined to date. The presence of carbohydrate is a consistent feature of the γ-fibrinogens. *Xenopus* fibrinogen has an *N*-linked glycosylation site at position 50 (51 in human) that is absent from the same site in the lamprey homologue. The carboxyl-terminal half of the molecule (which contributes to the D domain of fibrinogen) is highly conserved throughout evolution from lamprey to human, and this same degree of conservation is evident in the *Xenopus* protein. This domain is implicated in many of the biological functions of fibrinogen including calcium binding, platelet aggregation, and clumping of staphylococcal bacteria (Doolittle, 1984). Finally, one interesting structural feature shared by the lamprey and *Xenopus* proteins is the presence of the RGD motif one amino acid from the carboxyl terminus. This sequence has been implicated as a major determinant for recognition by a number of cell surface receptors (the best characterized to date being the fibronectin receptor). It is curious that this feature is present in the proteins from these lower vertebrates, yet lacking in those from higher vertebrates.

Our interest in γ-fibrinogen mRNA stems from our research into the regulation of serum albumin gene expression by the steroid hormone 17β-estradiol. We have previously shown that estrogen regulates albumin gene expression through changes in the cytoplasmic stability of albumin mRNA. Although other cases of hormonal regulation of mRNA stability are known, this is the only one to date in which the major gene

3′ UNTRANSLATED REGIONS

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68 KDa albumin   UAAGAGUCCAUAAGAGCAAAGACCAGUCUUCAAACUCACUGAGGAACACCUUCCAUCUCUCAAACACAAGAAAAAAAA
74 KDa albumin   UAAGAGUCCAUUAGAGCAAAGGCCAGUCUUCAAACUCACUGAGGAACAUCUUCCAUCUCUCACAU    GAAAAAA
fibrinogen       UAAGAAUCCAGCUUUUGGAUUAUUAUUUUACACUUUAUUGUCUUGCUUUAUAAAUUGUAUUCACAACUUGUUUUUAAA

68 KDa albumin   GUUUCCUCCGUCUGAAAAGGCAAUUUGCUUAGAGCAUUCAACUGUGUGUUGUAAUAAAUAAAGCAUUUAAAAAAU(A)n
74 KDa albumin   GUUUCCUCCAUCUGAAAAGAAAAUUUG UU    CAUUCAACUGUCUGUUGAAAUAAAUAAAGCGUUUAAAAUAU(A)n
fibrinogen       UAAAGAUACACCAUUGUG(A)n
```

BASE COMPOSITION - 3′ UTR

| | A | C | G | % U | A+U | G+C |
|---|---|---|---|---|---|---|
| 68 KDa albumin | 41 | 20 | 15 | 24 | 65 | 35 |
| 74 KDa albumin | 37 | 20 | 14 | 29 | 66 | 34 |
| fibrinogen | 29 | 14 | 12 | 45 | 74 | 26 |

POLY(A) ADDITION SITES

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68 KDa albumin      CAACUGUGUG UUGUAAUAA- -AUAAAGCAU U
74 KDa albumin      CAACUGUGUG UUGUAAUAA- -AUAAAGCAU U
fibrinogen          CAACU-UGUU UUUAAAUAAA GAUACACCAU U

β-globin            CGUAUCUGCU -CCUAAUAAA AAGAAAGUUU C
vitellogenin A2     UGUAAUACAU UUCGAAUAAA ACUGUGCAUU C
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FIGURE 6: Sequence of the 3′ untranslated regions of serum albumin and γ-fibrinogen mRNAs. The sequences of the 3′ UTR of the 68- and 74-kDa albumin and γ-fibrinogen mRNAs are shown beginning with the termination codon. The relative base composition is tabulated in the second panel. The bottom panel shows the alignment of the sequences surrounding the poly(A) addition signal for these mRNAs compared to β-globin and vitellogenin. Conserved nucleotides in the albumin and γ-fibrinogen mRNAs are shaded.

product of the target cell is so regulated. To date, there is no estrogen-responsive *Xenopus* liver cell line in which one may perform transfection experiments to identify sequences important to the regulation of a given gene or RNA. We therefore sought to determine whether shared sequence or structural elements might be present on another serum protein mRNA which is regulated in the same manner as albumin. Follet and Redshaw (1974) and later Wangh (1982) found that estrogen administration to either intact animals or *Xenopus* hepatocytes in primary culture resulted in cessation of the synthesis of the majority of serum proteins found in the absence of hormone. The fibrinogens were one group of proteins specifically identified in the latter study.

The data presented in Figures 3–5 indicate that, like albumin, γ-fibrinogen mRNA decreases to almost undetectable levels in the cytoplasm in response to estrogen. Furthermore, estrogen has no effect on steady-state levels of γ-fibrinogen RNA in the nucleus. From this, we conclude that the most likely mechanism regulating γ-fibrinogen gene expression is the destabilization of its mRNA. Unfortunately, the low transcription rate of the γ-fibrinogen gene made it impossible to confirm this by the transcription run-on assay we previously employed to study albumin and vitellogenin (Riegel et al., 1986). The reason for this is that the transcription rate in poikilothermic vertebrates is 20–30-fold lower than that seen in mammalian cell nuclei. At best, $10^8$ nuclei in a typical assay yield only $10^5$–$10^6$ dpm incorporated into nascent transcripts, a problem we have described in detail previously (Martin et al., 1986). This limits the utility of transcription run-on assays to only the most actively transcribed genes.

A previous study (Schoenberg et al., 1989) utilized both intron and cDNA probes to authenticate a procedure to fractionate albumin nuclear and cytoplasmic RNA from *Xenopus* liver. We found that, whereas estrogen caused albumin mRNA to disappear from the cytoplasm, nuclear steady-state levels of both the primary and mature transcript remained relatively unchanged. By analogy to albumin, we therefore conclude that the disappearance of γ-fibrinogen mRNA from the cytoplasm without concomitant changes in nuclear steady-state levels results from a posttranscriptional mechanism in which the target mRNA is destabilized. The data in Figure 5, however, indicate that the rate at which γ-fibrinogen mRNA disappears is substantially slower than that for albumin. The basis for this is unknown at present.

Finally, we have examined the sequences of both the 68- and 74-kDa albumin mRNAs (Moskaitis et al., 1989) and γ-fibrinogen mRNA for sequence or structural elements which they may share that could impart similar regulatory consequences. RNA folding programs failed to demonstrate unusual structures in the 5′ and 3′ untranslated regions (UTR) of these mRNAs. However, a comparison of the sequences present in the 3′ UTR in Figure 6 shows that they are AU-rich, ranging from 65% A + U in the 68-kDa albumin mRNA to 74% in γ-fibrinogen. There are no striking UA motifs or repeats of such sequences as the UUAUUUAU found in the 3′ UTR of the mRNAs encoding a number of inflammatory response mediators (Shaw & Kamen, 1986). These later sequences have been found to impart instability to those mRNAs, and recent studies demonstrate that a single repeat of this element is sufficient to interfere with translation in *Xenopus* oocytes (Kruys et al., 1989). Those authors suggest that such translational control may function in somatic cells and be masked by the rapid turnover of mRNAs bearing the UA motif. Beutler et al. (1989) have found that the UA dinucleotide, like CG (Nussinov, 1981), is rare in the protein coding regions of mRNAs. They hypothesize that this sequence serves as a recognition site for a UA-selective ribonuclease, hence, the scarcity of this dinucleotide in general, and the striking instability of mRNAs bearing the repeated UUAUUUAU motif. Excluding the UAA termination codons and AAUAAA polyadenylation signals, there are five UA dinucleotides in the 3′ UTR of the 68-kDa albumin, three in the 74-kDa albumin, and nine in γ-fibrinogen. Given the more rapid degradation of the albumin mRNAs, it is unlikely that the number of these dinucleotides alone specifies instability. What then could be the features these mRNAs share that

might signal their instability? The only conserved feature of all of these mRNAs is the sequence surrounding the poly(A) addition signal (Figure 6). There is no apparent symmetry to this sequence, yet it is clear that the regions of similarity extend both 5′ and 3′ to the canonical AAUAAA signal. Comparison to two other *Xenopus* liver mRNAs, vitellogenin (which is synthesized in the same cell) and β-globin (which is not), fails to demonstrate similar sequence features. It will be instructive to determine whether this sequence is conserved on other mRNAs also regulated by estrogen, and to pursue experiments to test the biological function of this sequence in the regulation of mRNA stability.

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