Xenopus Liver Ferritin H Subunit: cDNA Sequence and mRNA Production in the Liver following Estrogen Treatment†,‡

Lenē J. Holland,*,§ Amy A. Wall, and Anuradha Bhattacharya‖

Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242

Received September 5, 1990; Revised Manuscript Received November 2, 1990

ABSTRACT: In vitro translation of liver mRNA from estrogen-treated *Xenopus* frogs yields two abundant polypeptides in the range of 20 kDa. DNA clones for one of these translation products were isolated and shown to be complementary to mRNA for the heavy subunit of ferritin. The predicted *Xenopus* amino acid sequence shares about 86% identity with the ferritin heavy chain from bullfrogs and about 70% identity with the comparable mammalian and avian proteins. Clone identity was confirmed by hybridization selection followed by in vitro translation into translation products of 19.5–20 kDa. The nearly full-length cDNA clone, termed XlferH1, comprises 868 nucleotides plus 22 adenosines of the poly(A) tail, including 134 nucleotides of the 5′-untranslated region, a 528-base coding region for 176 amino acids, and a 206-nucleotide 3′-untranslated region. The clone lacks 22 nucleotides from the 5′ end of the mRNA. The level of ferritin mRNA in the liver of estrogen-treated frogs was determined over time. The amount of this mRNA relative to total RNA decreased about 3-fold 14 days after estradiol-17β was administered. However, the hormone also elevated total RNA in the liver about 24-fold. Hence, the total ferritin mRNA content of the liver increased to about 8 times its initial amount. This pattern of gene expression was very similar to that for serum retinol binding protein. The estrogen induction of these two mRNAs appeared to parallel the overall stimulation of hepatic RNA synthesis. In contrast, albumin mRNA was suppressed about 25-fold relative to total RNA such that a fairly constant total amount of albumin mRNA was maintained in the liver under these conditions of estrogen treatment.

Estrogen treatment of *Xenopus laevis* frogs causes multiple changes in gene expression in the liver. The most dramatic response is induction of the messenger RNA coding for vitellogenin (Ryffel et al., 1977; Baker & Shapiro, 1977), the precursor of egg yolk proteins, which is screted from the liver and taken up by developing oocytes in the female (Clemens, 1974). Another secreted protein, of unknown function, called Ep45 has a pattern of induction very similar to vitellogenin (Holland & Wangh, 1987). In addition to these effects on specific genes, synthesis of total RNA, which consists mainly of ribosomal RNA, is dramatically stimulated (Holland & Wangh, 1987). The rough endoplasmic reticulum also increases substantially in the liver cells (Lewis et al., 1976; Bergink et al., 1977; Skipper & Hamilton, 1977), consistent with the increase in ribosomal RNA and the greater production of secreted proteins by the liver. Simultaneously with these positive effects, estrogens also negatively regulate synthesis of other proteins, most notably albumin (Wangh et al., 1979; May et al., 1982). Finally, the most complex event is the mitogenic response to the hormone. Estrogens cause a 5-fold increase in the number of liver parenchymal cells within 2 weeks after a single in vivo injection of hormone (Spolski et al., 1985).

As part of our overall effort to characterize estrogen effects in *Xenopus* liver, we undertook in the present study to identify a small messenger RNA, distinct from vitellogenin and Ep45 mRNA, that is abundant in the frog liver following estrogen administration. We showed previously that translation in vitro of liver RNA from an estrogen-stimulated frog yields 2 major translation products in the range of 20 000 daltons, both of which are encoded by mRNAs about 1000 nucleotides in length (Holland & Wangh, 1987). Unlike vitellogenin and Ep45 mRNAs which appear de novo in response to estrogen, the mRNAs coding for the 20-kDa proteins are present in both normal and estrogen-induced liver. By isolating and sequencing cDNA clones, we show here that one of the abundant small mRNAs in estrogen-stimulated *Xenopus* liver codes for the heavy subunit of ferritin.

Intracellular iron is stored as a ferritin complex, which is a large structure consisting of a shell of 24 protein subunits with up to 4500 iron atoms sequestered in the center (Theil, 1987). In most species, there are two forms of the protein subunits with molecular weights of about 21 000 for the heavy (H) chain and 19 000 for the light (L) subunit (Theil, 1987). In bullfrogs, an additional subunit (M) of intermediate molecular weight has been identified (Dickey et al., 1987). Ferritin is very abundant in the liver. The L subunit predominates in mammalian liver (Arosio et al., 1978), but Dickey et al. (1987) showed that M-subunit mRNA predominates in *Rana* liver and suggested that in mammals a comparable form may exist that has been mistakenly designated L. Complementary DNA clones for both the H and L subunits have been characterized from several species (Brown et al., 1983; Leibold et al., 1984; Dorner et al., 1985; Boyd et al., 1985; Dickey et al., 1987). The two subunits are synthesized from mRNAs

† This work was supported in part by a Basil O'Connor Starter Scholar Research Award from the March of Dimes Birth Defects Foundation (5-620), by a grant from the National Institutes of Health (R01-HL39095), by funds from the Lindsay Trust awarded through the University of Iowa College of Medicine, and by the Diabetes and Endocrinology Research Center, University of Iowa College of Medicine (NIH Grant DK25295).

‡ The nucleotide sequence reported in this paper has been submitted to GenBank under Accession Number M55010.

* To whom correspondence should be addressed.

§ Present address: Department of Physiology, University of Missouri School of Medicine, Columbia, MO 65212.

‖ Present address: Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030.

0006-2960/91/0430-1965$02.50/0

about 1000 nucleotides in length and are encoded by evolutionarily related gene families (Jain et al., 1985; Costanzo et al., 1986; Santoro et al., 1986; Stevens et al., 1987; Leibold & Munro, 1987).

Changes in ferritin mRNA levels occur in many cell types during development (Dickey et al., 1987) and differentiation (Chou et al., 1986), and in response to a variety of agents (Torti et al., 1988; Cox et al., 1988). In addition to regulation at the mRNA level, translational utilization is a major means of control of ferritin production. Iron itself increases the availability of ferritin mRNA for translation in both liver and red blood cells (Zahringer et al., 1976; Shull & Theil, 1982, 1983; Aziz & Munro, 1986).

Materials and Methods

Construction of cDNA Clone Libraries from Xenopus Liver and Other DNA Procedures. Complementary DNA was synthesized from male *Xenopus* liver poly(A+) RNA by modification of the procedures of Prochownik et al. (1983). Double-stranded cDNA was size-fractionated on a 1% agarose/Tris–acetate (Holland & Wangh, 1983) gel, and molecules greater than 300–400 base pairs were collected onto a DEAE membrane (NA-45, Schleicher & Schuell). The DNA was eluted from the membrane by treatment at 65 °C for 15 min with 6.7 M guanidine hydrochloride, 19 mM sodium acetate, and 19 mM EDTA, pH 5.2, plus 20 μg of tRNA followed by treatment at 65 °C for 15 min with 6.3 M guanidine hydrochloride/50 mM arginine (free base) and was precipitated with 2 volumes of ethanol. C-Tailed cDNA (Michelson & Orkin, 1982) was annealed with G-tailed, *Pst*I-cut pBR322 (New England Nuclear) and transformed into DH1 cells (Hanahan, 1983).

Plasmid DNA was extracted by alkaline lysis as described by Just et al. (1983), substituting potassium acetate for sodium acetate. The DNA was treated with 50 μg/mL heat-treated (Bhattacharya et al., 1990b) RNase A for 30 min at 37 °C, extracted with phenol and chloroform, and ethanol-precipitated. RNA was removed by precipitation twice with final concentrations of 10% PEG 8000/0.5 M NaCl. The pellet was collected, after at least 60 min on ice, by centrifugation at 16000*g* for 20 min. DNA fragments were purified on agarose gels containing 1 μg/mL ethidium bromide in Tris–acetate buffer, illuminated with 366-nm UV light, electroeluted in dialysis bags in 0.1× TBE (Maniatis et al., 1982), and passed over a NACS column (Bethesda Research Labs).

DNA Sequencing, Oligonucleotide Synthesis, and Primer Extension. The XlferH1 cDNA insert was transferred to the vector Bluescript SK− (Stratagene) and subjected to unidirectional deletion (Promega protocols) and transformation into DH5α-competent cells (Bethesda Research Labs). Purified plasmid DNA (2 μg) was denatured in alkali (Chen & Seeburg, 1985), mixed with 10 ng of primer [either T7 or T3 primers (Stratagene) or specific oligonucleotides], and precipitated from 0.45 M sodium acetate, pH 4.5, with 3.8 volumes of ethanol. Sequencing by the dideoxy termination method was carried out with [α-^{35}S]dATP and the enzyme Sequenase (U.S. Biochemicals protocol). DNA was resolved on a gel containing 8.3 M urea/6% polyacrylamide in 1× TBE (Maniatis et al., 1982). The gels were fixed with 10% acetic acid/10% methanol, and dried gels were exposed to Kodak XAR5 film at −70 °C. GenBank was searched with the sequence analysis software package of the Genetics Computer Group (Devereux et al., 1984). Protein molecular weight was calculated by a PC Gene program from Intelligenetics. Comparison of ferritin sequences used computer programs described previously (Bhattacharya et al., 1990a).

Antisense oligonucleotides were synthesized (Beaucage & Caruthers, 1981) by the DNA Facility at the University of Iowa. The *Xenopus* ferritin oligonucleotide is the complement of bases 465–497 in Figure 5. The SRBP oligonucleotide is complementary to residues 501–533 of the cDNA sequence (McKearin et al., 1987), and the albumin oligonucleotide is complementary to nucleotides 1–33 of the mRNA coding region of the 74-kDa albumin gene (Schorpp et al., 1988). Primer extension reactions were carried out as described (Bhattacharya et al., 1990b).

Animals and Hormone Treatment. For the RNA fractionation shown in Figure 1, the hormone treatment of a female frog has been described previously (Holland & Wangh, 1987). The liver RNA samples for the experiments shown in Figure 7 were prepared from adult male *Xenopus laevis* (Nasco) weighing 50–70 g at the time of sacrifice. Each animal received a single 100-μL injection of 20 mg/mL estradiol-17β (Holland & Wangh, 1987).

mRNA Selection. The purified cDNA insert of XlferH1 was used to select complementary mRNA as described (Bhattacharya et al., 1990b) with total RNA from the liver of a female frog estrogen-treated as in Figure 1. The [^{35}S]-methionine-labeled translation products were analyzed by electrophoresis (Wangh et al., 1983) on a 21% acrylamide gel [200:1 acrylamide:bis(acrylamide) ratio] containing 0.75 M Tris-HCl, pH 8.9, and 0.1% SDS, with a running buffer of 50 mM Tris base, 39 mM glycine, and 0.1% SDS. Treatment of the gel with Enhance and autoradiography were as before (Bhattacharya et al., 1990b).

Radioactive Labeling of DNA. Random-priming (Feinberg & Vogelstein, 1983, 1984) and nick-translation (Holland & Wangh, 1984) were performed essentially as described. 5′-End labeling was accomplished by incubation at 37 °C for 45 min in a 20-μL reaction containing 100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM DTT or 10 mM β-mercaptoethanol (βME),[1] 50 μCi [γ-^{32}P]ATP (4500 Ci/mmol, ICN), 0.003 ODU oligodeoxynucleotide, and 10 units of T_4 kinase (New England Biolabs). Labeling reactions were adjusted to 20 mM EDTA, 100 μg of carrier yeast RNA (Sigma, phenol/$CHCl_3$ extracted) was added, the sample was extracted with $CHCl_3$, and unincorporated nucleotides were removed by two cycles of ethanol precipitation with incubation on dry ice for 15 min.

RNA Purification, Fractionation, Gel Electrophoresis, and Molecular Hybridization. Purification of total RNA and two cycles of poly(A+) RNA selection were carried out as before (Holland & Wangh, 1983). High-resolution mRNA size-fractionation is described elsewhere (Holland & Wangh, 1983, 1984). Fraction 4 in Figure 1 corresponds to fraction 26 in an earlier report (Holland & Wangh, 1987). RNA was resolved in 1.2% agarose gels containing formaldehyde with sample buffer as described (Holland et al., 1990) and was transferred to Genescreen (Holland & Wangh, 1984). In some cases, the membrane was exposed to 254-nm UV light (580 mW/cm^2, 15 cm for 3 min) and baked under vacuum for 2 h at 80 °C. Ribosomal RNA (Holland & Wangh, 1984) and RNA molecular weight markers (Bethesda Research Labs) were stained on the membrane with methylene blue (Maniatis et al., 1982).

For quantitative analysis, RNA samples were bound to a Zetaprobe (Bio-Rad) membrane through a slot-blotter (Schleicher & Schuell). Each RNA was applied in four amounts, 0.1, 0.2, 0.5, and 1 μg in 25 mM sodium phosphate,

[1] Abbreviations: 1× SSC, 0.15 M NaCl/0.015 M sodium citrate; SRBP, serum retinol binding protein; RBC, red blood cell(s); DTT, dithiothreitol; βME, β-mercaptoethanol.

pH 6.5, and the membranes were baked under vacuum for 2–3 h at 80 °C. Denaturation of the RNA with formaldehyde or boiling did not improve binding or hybridization. Control experiments indicated that the capacity of the Zetaprobe was exceeded when more than 1 μg of RNA was loaded per slot.

Molecular hybridization and autoradiography were carried out under our standard conditions (Bhattacharya et al., 1990b) with 0.2× SSC for nick-translated or random-primed DNAs and 1× SSC for oligonucleotide probes. Oligonucleotide probes were used in the slot-blots for all three mRNAs so that identical hybridization conditions could be used. The ferritin oligonucleotide had exactly the same hybridization pattern as the cDNA probe shown in Figure 1C. The albumin oligonucleotide hybridized to a single 2000-base RNA (data not shown), establishing that it could be used in slot-blots without background hybridization to other RNAs. Densitometric scanning of slot-blots was carried out with an LKB Ultroscan XL. The peak heights were obtained from autoradiograms within the linear range of the film, as determined by obtaining a straight line through the values for the range of RNA amounts. RNA amounts were expressed as densitometric units per microgram of total RNA. The mean and standard error of the mean were calculated, and the data were converted to the percent of the maximum value for each specific mRNA.

Results

Isolation of cDNA Clones Complementary to an Abundant 1000-Base mRNA in Liver of Estrogen-Treated Frogs. We have previously shown that, in addition to vitellogenin, there are three other abundant translation products from RNA of estrogen-stimulated frog liver, which are shown in Figure 1A as pre-Ep45 and two bands labeled pre-Ep20. High-resolution size-fractionation of liver RNA showed that the 2 pre-Ep20 proteins were synthesized from mRNAs about 1000 nucleotides in length (Holland & Wangh, 1987). The particular RNA fractions that translated into the pre-Ep20 polypeptides are shown in Figure 1B. Both pre-Ep20 translation products were coded by mRNAs present mainly in fraction 4.

In order to identify and characterize the 1000-base mRNA that was abundant after estrogen treatment, we constructed complementary clones from size-fractionated RNA enriched for 1000-nucleotide species. We identified a clone of approximately 400 base pairs, designated Ep20/70, which recognized exclusively a single RNA species of about 1000 bases when hybridized to total frog liver mRNA (Figure 1C). Furthermore, when this clone was hybridized to the fractionated RNA (Figure 1D), it hybridized primarily to fraction 4, exactly the same major fraction that translated into the pre-Ep20 polypeptides. Ep20/70 was then used to screen the total liver cDNA library, and a larger clone, termed XlferH1, was isolated.

Confirmation of cDNA Clone Identity by Messenger RNA Selection. To confirm that clone XlferH1 is complementary to mRNA coding for one of the previously identified abundant Ep20 polypeptides, we carried out messenger RNA selection followed by in vitro translation. The translation products were separated on a high-percentage acrylamide gel, which gave greater resolution in the 20-kDa range compared to the gradient gels used in Figure 1. As a control, the translation reaction was carried out with no added RNA, and no radioactive proteins were visible (Figure 2, lane B). We have demonstrated elsewhere that no translation products were detectable when mRNA selection was carried out with pBR322 DNA on the filter (Bhattacharya et al., 1990a). Figure 2, lane C, shows that clone XlferH1 specifically selected a messenger RNA that coded for one of the major proteins

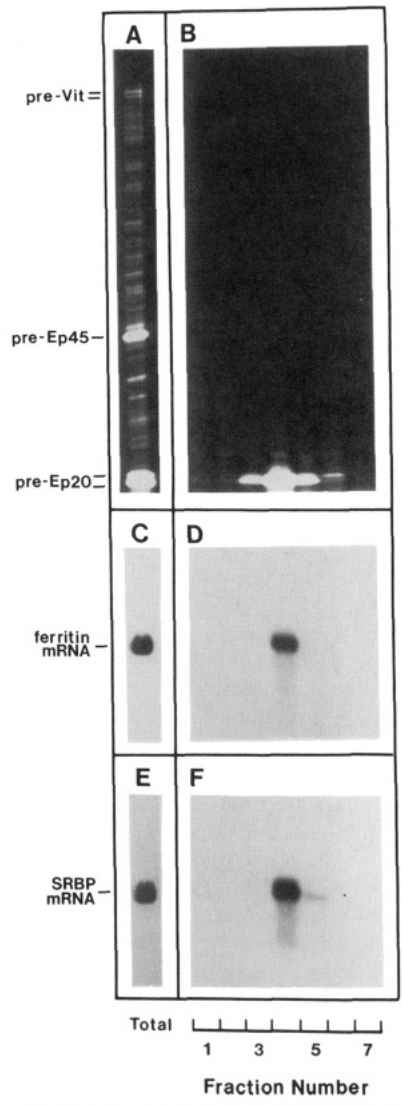

FIGURE 1: Characterization of pre-Ep20 translation products and ferritin and SRBP messenger RNAs in liver RNA from estrogen-treated *Xenopus*. (A) Poly(A+) RNA from a female frog that received multiple estrogen injections was translated in vitro, and the products were resolved by SDS–polyacrylamide gel electrophoresis. The results are shown in a negative image of the autoradiogram. Pre-vit (precursor of vitellogenin), pre-Ep45, and pre-Ep20 indicate the positions of migration of major translation products. (B) Poly(A+) RNA from the animal described in (A) was first subjected to high-resolution size-fractionation. Then individual fractions were translated and analyzed as in (A). Only the fractions with translation products in the region of the pre-Ep20 polypeptides are shown. (C and E) Total liver RNA from the animal described in (A) was resolved on a formaldehyde-containing agarose gel, transferred to Genescreen, and hybridized with either ^{32}P-labeled Ep20/70 cDNA (C) or SRBP oligonucleotide (E). (D and F) The RNA fractions described in (B) were run on a formaldehyde–agarose gel, transferred to Genescreen, and hybridized with either ^{32}P-labeled Ep20/70 cDNA (D) or SRBP oligonucleotide (F).

of approximately 20 kDa, and under these conditions, this protein appeared to be separated into two bands with apparent sizes of 19.5 and 20 kDa. These closely migrating bands were more clearly resolved in the translation products of total liver RNA (Figure 2, lane A). These data suggest either that two translation products were made from a single mRNA or that two similar mRNAs were selected by the XlferH1 clone. We have no other evidence for two species of XlferH1 mRNA since eight independently isolated clones had the same restriction map as XlferH1. The 19.5–20-kDa size of the proteins translated from XlferH1-selected mRNA corresponds to the smaller form of pre-Ep20 in Figure 1A. Among the

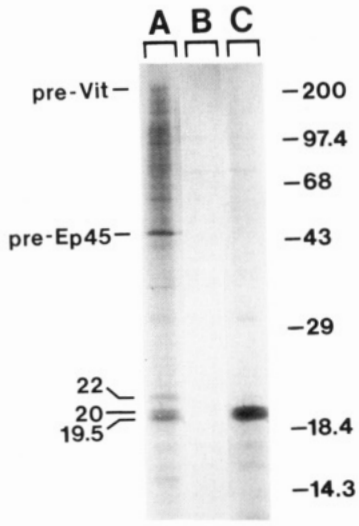

FIGURE 2: In vitro translation of mRNA hybrid-selected by clone XlferH1. Bound RNA was eluted and translated in vitro in the presence of [^{35}S]methionine (lane C). Translation reactions containing 1 μg of the same total liver RNA (lane A) or no added RNA (lane B) were also carried out. Molecular weight markers (×10^{-3}) (Bethesda Research Labs) are indicated by the numbers on the right.

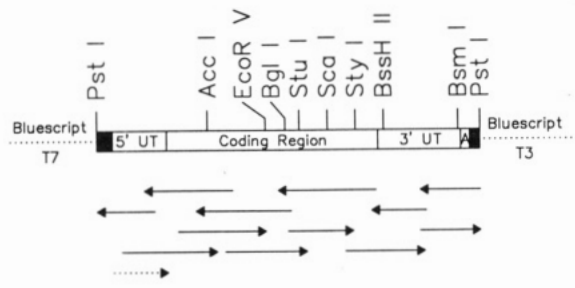

FIGURE 3: Restriction enzyme cleavage map and sequencing strategy for the clone XlferH1. The top line depicts the restriction map of XlferH1 and the orientation in Bluescript. The black boxes indicate the G/C tails: 39 residues on the 5′ end and 26 on the 3′ end. The arrows show the length and direction of sequence determination. A portion of the sequence was confirmed from an independently isolated phage clone in the vector λgt10, which is indicated by the dotted arrow at the lower left.

total translation products (Figure 2, lane A), there was also a protein of 22 kDa, which is probably the same as the larger pre-Ep20 polypeptide in Figure 1A.

Determination of the Nucleotide Sequence of XlferH1. The restriction enzyme cleavage map of XlferH1 is shown in Figure 3. The arrows in the lower portion of the figure indicate the sequencing strategy, and Figure 4 shows the complete nucleotide sequence. There is an open reading frame of 176 amino acids with a calculated molecular weight of about 20.5K, in good agreement with the 19.5–20-kDa size estimated by gel electrophoresis. The deduced amino acid sequence shares about 86% identity with the ferritin heavy-chain subunit from the bullfrog *Rana catesbeiana*, providing strong evidence that XlferH1 represents the same protein from *Xenopus laevis*. The sizes of the *Xenopus* ferritin mRNA and protein are very similar to those found in other animals (Brown et al., 1983; Costanzo et al., 1984; Theil, 1987).

At the 3′ end, XlferH1 contains an untranslated region of 206 nucleotides, 22 adenosines of the poly(A) tail of the mRNA, and the polyadenylation signal AAUAAA (Proudfoot & Brownlee, 1976) 17–21 bases upstream of the A stretch. XlferH1 includes 134 nucleotides of the 5′-untranslated region. The AACGCCATC immediately upstream of the initiation codon conforms to the consensus sequence found in other eukaryotic mRNAs (Kozak, 1987), and the CTTTTG at positions −29 to −34 matches the 18S ribosomal RNA recognition site (Baralle & Brownlee, 1978). In addition to these common elements, the 5′-untranslated region contains a sequence homologous to the highly conserved 28-nucleotide stretch, the iron response element, found in other ferritin H- and L-subunit mRNAs, which plays a role in the regulation of translation of ferritin mRNA in response to iron (Leibold & Munro, 1987; Aziz & Munro, 1987; Hentze et al., 1987).

We determined the full length of ferritin mRNA at the 5′ end by primer extension. Two different antisense 33-mers, complementary to nucleotides 10–42 and 16–48 of XlferH1, were hybridized with total frog liver RNA, extended up to the 5′ end of the RNA, and analyzed by gel electrophoresis (Figure 5). For oligonucleotide 10–42, there were 2 major bands of 63 and 64 nucleotides, showing that the mRNA is 21 or 22 (63 or 64 minus 42) bases longer at the 5′ end than the XlferH1 clone. The same conclusion was reached with the oligonucleotide 16–48, since the primer extension products were 69 and 70 bases long. Doublet bands differing by one nucleotide are found frequently in primer extension reactions and have been attributed to premature termination when reverse transcriptase reaches the 5′-terminal 7-methylguanosine cap (Benyajati et al., 1983; Shelness & Williams, 1984; Didsbury et al., 1986). Hence, the full length of the mRNA complementary to XlferH1, not including the poly(A) tail, is 890 bases: 868 in established sequence and 22 additional bases at the 5′ end, 21 which are coded for in the genome and 1 which is added posttranscriptionally. The mRNA size of about 1000 bases determined by gel electrophoresis suggests a poly(A) tail length of approximately 100 bases.

The sequence we report here is similar to but not identical with a *Xenopus* ferritin cDNA sequence recently published by Moskaitis et al. (1990). Within the coding region, there are five nucleotide differences, at our positions 151, 176, 177, 178, and 604, which result in three amino acid differences. In addition, our amino acid 158 is missing from the previously published sequence. The amino acids we report have a higher degree of identity with ferritin from other species (Figure 6). Moskaitis et al. placed a block of 15 bases in the wrong position in the nucleotide sequence, but the order was correct in the amino acid sequence. In the 5′-untranslated region, several nucleotides are different between the two sequences. As indicated in Figure 3, 100% of our sequence was determined unambiguously in both directions. The major difference between the two *Xenopus* ferritin cDNAs is the additional 160 nucleotides found by Moskaitis et al. at the 5′ end. The primer extension data presented above indicate that ferritin mRNA is only 22 nucleotides longer than clone XlferH1 at the 5′ end. Although premature termination could have occurred in that experiment, we have used the same protocol to obtain reasonable size estimates of several other *Xenopus* liver mRNAs. The 5′-end structure we report is more similar to all other ferritin cDNA sequences.

Comparison of the Deduced Amino Acid Sequences of Ferritin from Xenopus and Several Other Species. The deduced amino acid sequence from clone XlferH1 is compared in Figure 6 with ferritin heavy and light chains from a broad range of animals, including amphibian, avian, and mammalian species. The highest percent identity (86%) was found with the ferritin H subunit from the bullfrog *Rana catesbeiana*. Slightly less identity (84%) occurred with the bullfrog ferritin M subunit. Avian and mammalian H subunits are about 70% identical, and amphibian and mammalian L chains, though less similar, share about 55–65% identity with the *Xenopus* protein. The conserved amino acids are distributed throughout

```
                                                                    AGTAGAGTTCTTGCTTCAACAGTGTTTGAACGGAACCCTCTCTGAGTCTTTTTTT55

TAGACCAAACTCTCTCTCTCGCATTACTCTCTTCTTTTTGTGACTCTTTTTTGCGCCACCAGCAAACACCGAAACGCCATC134

ATG CAA TCC CAG GTG CGC CAG AAC TTC AAC AGC GAC TGC GAA GCC GCC ATC AAC CGG ATG194
Met Gln Ser Gln Val Arg Gln Asn Phe Asn Ser Asp Cys Glu Ala Ala Ile Asn Arg Met

GTG AAC CTG GAG ATG TAT GCC TCC TAT GTC TAC CTG TCC ATG TCT TAC TAC TTC GAT CGT254
Val Asn Leu Glu Met Tyr Ala Ser Tyr Val Tyr Leu Ser Met Ser Tyr Tyr Phe Asp Arg

GAT GAC GTG GCA CTC CAT CAT GTG GCC AAG TTC TTC AAG GAG CAG AGT CAC GAG GAA AGG314
Asp Asp Val Ala Leu His His Val Ala Lys Phe Phe Lys Glu Gln Ser His Glu Glu Arg

GAG CAC GCC GAA AAG TTC CTC AAA TAC CAA AAC AAA CGT GGG GGC CGT GTC GTC CTT CAG374
Glu His Ala Glu Lys Phe Leu Lys Tyr Gln Asn Lys Arg Gly Gly Arg Val Val Leu Gln

GAT ATC AAG AAA CCA GAG CGT GAC GAA TGG AGT AAC ACC CTG GAA GCC ATG CAG GCC GCT434
Asp Ile Lys Lys Pro Glu Arg Asp Glu Trp Ser Asn Thr Leu Glu Ala Met Gln Ala Ala

CTG CAA TTG GAG AAG ACC GTG AAC CAG GCC TTG CTG GAT CTG CAC AAG CTG GCA TCC GAC494
Leu Gln Leu Glu Lys Thr Val Asn Gln Ala Leu Leu Asp Leu His Lys Leu Ala Ser Asp

AAG GTT GAT CCT CAG CTC TGT GAC TTC CTT GAA TCT GAG TAC TTG GAG GAA CAG GTG AAG554
Lys Val Asp Pro Gln Leu Cys Asp Phe Leu Glu Ser Glu Tyr Leu Glu Glu Gln Val Lys

GCC ATG AAG GAG CTT GGA GAC TAC ATC ACC AAC CTG AAG CGC CTT GGG GTG CCG CAG AAT614
Ala Met Lys Glu Leu Gly Asp Tyr Ile Thr Asn Leu Lys Arg Leu Gly Val Pro Gln Asn

GGC ATG GGC GAG TAC CTG TTC GAC AAG CAC ACC CTG GGG GAG AGT AGC TAAGCGCGCTCTCAG677
Gly Met Gly Glu Tyr Leu Phe Asp Lys His Thr Leu Gly Glu Ser Ser

GGTAGAAACAACCAGTCAGCTCTCTGTTTCAAAATACTGTCTCTTAATATCTGTGTGGTATATACCATAGCTCCCATGC756

CCATGTTCAGACCTTTTCATTTTGAGGACGATGGGAATATATCTGTGCTCTTAATCATGCAGAAGCTGCTGTGCTTGTC835

AACATGTTTTCAATAAAGTTTTTTCAGCATTCC(A)22
```

FIGURE 4: Nucleotide sequence and predicted amino sequence of cDNA clone XlferH1. The nucleotide sequence shown is that of cDNA clone XlferH1 except at nucleotide 529. XlferH1 contains T, but six independently isolated clones, otherwise identical, have C at position 529. Thus, it is most likely that XlferH1 differs because of a cloning artifact. Underlining marks the 3′-polyadenylation signal. Gray shading indicates two sequence motifs common to the 5′ end of many mRNAs, and black shading shows the highly conserved iron-responsive element.

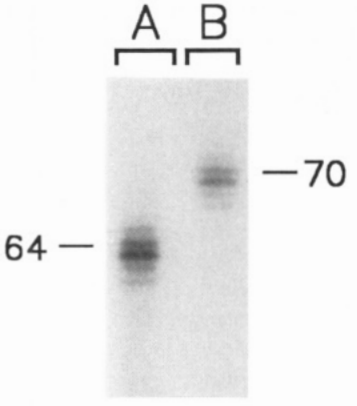

FIGURE 5: Determination of the full length of *Xenopus* ferritin H mRNA at the 5′ end. The antisense oligonucleotides complementary to residues 10–42 and 16–48 of the XlferH1 cDNA clone were used in primer extension reactions with total liver RNA from a male animal that received a 2-mg injection of estradiol-17β 8 days before sacrifice. The extended products of the 10–42 oligonucleotide are shown in lane A and those of the 16–48 oligonucleotide in lane B.

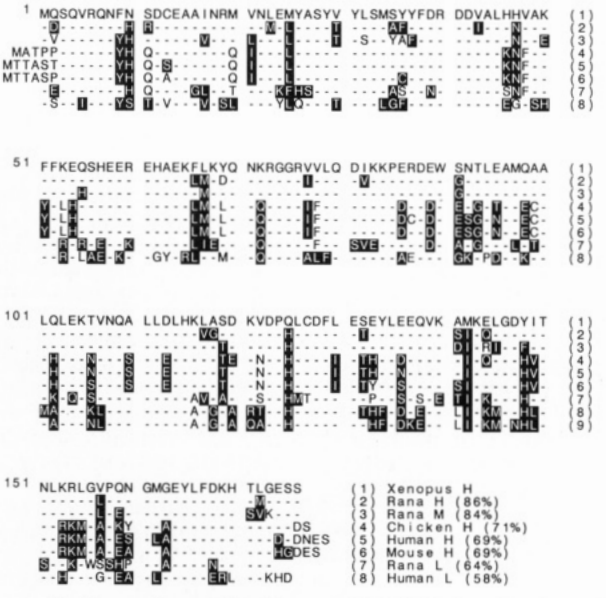

FIGURE 6: Comparison of the *Xenopus* XlferH1 deduced amino acid sequence with ferritin H and L sequences from other animals. The dashes represent residues identical with those in *Xenopus* H ferritin, and black boxes indicate amino acid replacements likely to occur over time. The superscript numbers on the left refer to the amino acid number in the *Xenopus* sequence. The percentages in parentheses show the percent identity of the *Xenopus* protein with each of the other ferritins. The deduced amino acid sequences are from the following references: *Rana* (Dickey et al., 1987); chicken (Stevens et al., 1987); human (Boyd et al., 1985); and mouse (Torti et al., 1988).

the length of the apoferritin molecule, and most of the non-identical residues are evolutionarily conservative substitutions.

Comparison of Xenopus Ferritin and Serum Retinol Binding Protein mRNAs. Other investigators have studied estrogen regulation of another small mRNA in *Xenopus* liver which codes for serum retinol binding protein (SRBP) (Hayward et al., 1985; McKearin et al., 1987). By hybridizing an oligonucleotide specific for SRBP mRNA to total *Xenopus* liver RNA, we found that, like ferritin mRNA, the SRBP mRNA is approximately 1000 nucleotides in length (Figure 1E). When total frog liver mRNA was subjected to high-resolution size-fractionation, both ferritin and SRBP mRNAs were recovered in precisely the same fraction, which yielded the two translation products designed pre-Ep20 (Figure 1, panels B, D, and F). We demonstrated in this paper by messenger RNA selection that ferritin mRNA codes for the smaller 20-kDa translation product. Thus, it is likely that SRBP mRNA codes for the larger pre-Ep20 polypeptide in Figure 1A,B, corresponding to the 22-kDa translation product in Figure 2. Despite the many similarities between SRBP and ferritin mRNA, the nucleotide sequences prove they are distinct. A small estrogen-induced protein of about 20000 daltons has also been detected among the proteins secreted from liver cells (Wangh et al., 1979; Hayward et al., 1985; Holland & Wangh, 1987). This secreted protein is probably SRBP since it is known to be exported from the liver, whereas ferritin is expected to be intracellular.

Quantitation of Ferritin, SRBP, and Albumin mRNA Levels following Estrogen Treatment. Since both SRBP and

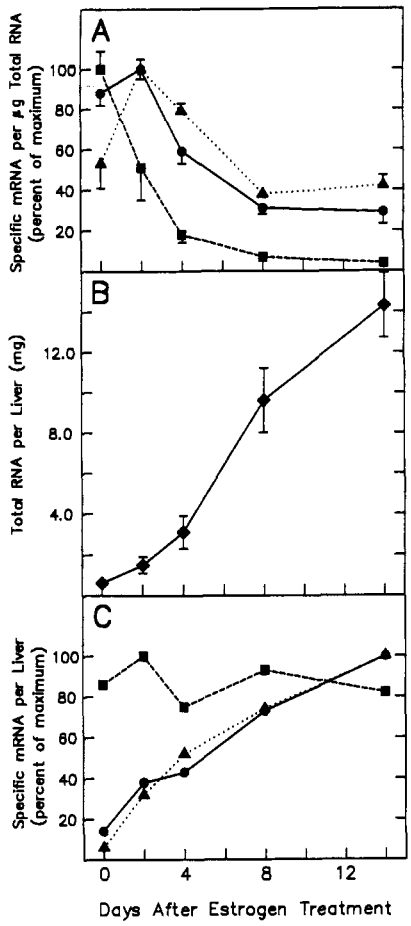

FIGURE 7: Quantitation of ferritin, SRBP, and albumin mRNA in the liver throughout the time course of estrogen treatment. (A) Total liver RNA was hybridized in a slot configuration with ^{32}P-labeled ferritin (solid line), SRBP (dotted line), or albumin (dashed line) oligonucleotide. Where error bars are not shown, they were smaller than the size of the symbol. There were four RNA samples for every time point, except at day 4 there were five samples and at day 8 and day 14 the albumin values were derived from three samples. (B) The amount of total RNA recovered from each frog was determined by A_{260}. The means and standard errors of the mean were calculated from four samples at every time point, except at day 4 there were five samples. (C) To determine the total liver amount of each specific mRNA in a given sample, the densitometric units per microgram (panel A) were multiplied by the number of micrograms of RNA recovered for that sample (panel B), yielding total densitometric units. The mean of these values for each specific mRNA was calculated and is shown as percent of maximum in panel C, with symbols as in panel A. The error bars are not included since the results in panels A and B indicate the variation in the raw data.

ferritin mRNAs were abundant after estrogen treatment, we compared their regulation throughout a time course of estrogen administration to male frogs. RNA was purified from the liver of each animal on various days after a single injection of 2 mg of estradiol-17β. The RNA was hybridized in a slot-blot to radioactively labeled probes for ferritin, SRBP, and albumin mRNAs. The amounts of each mRNA per microgram of total RNA are presented in Figure 7A. Relative to total RNA, ferritin mRNA remained the same, or increased slightly, from day 0 to day 2 and then dropped to about one-third of its peak value by day 14. SRBP mRNA increased about 2-fold within 2 days after estrogen treatment and then, like ferritin mRNA, decreased to about 40% of its maximum amount. Albumin mRNA declined steadily until its final level was about 4% of the initial quantity.

The estrogen treatment also caused total RNA in the liver to increase about 24-fold after 14 days (Figure 7B). Liver weight remained relatively constant as expected (Spolski et al., 1985; Holland & Wangh, 1987), with the following average liver weights: day 0, 2.5 g; day 2, 1.9 g; day 4, 2.7 g; day 8, 2.1 g; and day 14, 2.3 g. Because total RNA increased, an accurate assessment of the total liver content of ferritin, SRBP, and albumin mRNAs requires multiplying the amount of each specific mRNA per microgram of total RNA times micrograms of total RNA (Figure 7C). Ferritin and SRBP mRNAs had very similar overall patterns of accumulation in the liver. In spite of the drop relative to total RNA, the absolute hepatic content of ferritin mRNA increased about 8-fold and SRBP mRNA about 16-fold after 2 weeks (Figure 7C). In the early phase of the response, both ferritin mRNA and total RNA increased about 2.5–3-fold from day 0 to day 2, whereas SRBP mRNA increased about 5-fold in the same period. Although the difference between these values is small, it is possible that SRBP mRNA underwent a small specific induction by estrogen early in the response, in addition to the general transcriptional activation in the liver. The stimulation of SRBP mRNA levels appeared similar to that previously reported (McKearin et al., 1987), but in our experiments, the increase paralleled total RNA content in the liver. Total albumin mRNA levels remained relatively constant throughout the time course of hormone treatment, under the particular conditions used here. Although different methods of quantitation make different comparisons difficult, our results are in general agreement with the inhibitory effect of estrogens on albumin mRNA production reported by several other laboratories (May et al., 1982; Philipp et al., 1982; Kazmaier et al., 1985; Wolffe et al., 1985; Riegel et al., 1986).

Discussion

Rationale for the Method of RNA Quantitation. In order to make meaningful comparisons of mRNA levels under different conditions, it is essential to normalize experimental values to a standard that can be measured in all samples. Often, mRNA concentrations are expressed on a per cell basis, but in the case of *Xenopus* liver, this is not a reliable method. Since amphibian liver contains large amounts of nucleated red blood cells (RBC), counting nuclei or determining DNA content does not accurately measure the relevant cells, the liver parenchymal cells. Even after extensive in situ perfusion (Wangh et al., 1979), normal male frog liver has about 5 times more RBC than parenchymal cells. Unperfused tissue used for RNA purification would have considerably more red blood cells. The parenchymal cell number can be determined after perfusion and disaggregation of the liver, but this procedure is incompatible with rapid tissue dissociation to keep RNA intact. For the studies described above, we normalized the specific mRNA levels to total RNA and then determined their amount in the whole liver based on the quantity of total RNA recovered from the liver in each sample. This per liver instead of per cell method of quantitation was used because the total amount of RNA was experimentally determined for every sample, and therefore it was not necessary to make unverifiable assumptions regarding liver parenchymal cell number. It is also not accurate to use a single average value of parenchymal cell number because estrogen treatment causes proliferation of the liver parenchymal cells (Spolski et al., 1985). Although the tissue weight remains unchanged, the number of parenchymal cells increases for 5-fold 2 weeks after a single injection of estrogen.

Our observation of a substantial increase in total RNA in the liver following estrogen treatment is consistent with reports from several laboratories regarding the effect of the hormone on the rate of synthesis of total RNA (Witliff et al., 1972; Tata

& Baker 1975; Brock & Shapiro, 1983a; Martin et al., 1986). In spite of elevated total RNA synthesis, previous reports of the amount of RNA recovered from total *Xenopus* liver tissue indicated only a modest increase of about 2-fold following estrogen treatment (Baker & Shapiro; 1977; Philipp et al., 1982). Red blood cell DNA present in those samples could, however, have masked a greater increase in RNA quantity. It is noteworthy in those earlier studies that very shortly after estrogen treatment, the amount of total liver RNA as determined by A_{260} was very similar to the amount of DNA in the same weight of tissue (Baker & Shapiro, 1977). Our RNA purification procedure may be more effective at eliminating DNA (Chirgwin et al., 1979; Cox, 1968; Deeley et al., 1977) than the older methods. Although RBC can contribute a significant amount of DNA to a nucleic acid preparation from the liver, they do not have a significant amount of RNA. We have recovered only about 0.1 μg of total RNA from 10^8 RBC.

Mechanisms of Estrogen Action. The data reported here show that ferritin and SRBP mRNAs are relatively abundant in *Xenopus* liver and increase approximately in parallel with the rise in total hepatic RNA following in vivo administration of estrogen. In contrast, the mRNAs encoding vitellogenin (Ryffel et al., 1977; Baker & Shapiro, 1977) and the secreted protein Ep45 (Holland & Wangh, 1987) are undetectable in untreated frog liver and are very dramatically induced by estrogens. The elevated levels of vitellogenin mRNA are due primarily to selective stimulation of vitallogenin gene transcription (Brock & Shapiro, 1983a; Martin et al., 1986), but the increased stability of this mRNA at high estrogen doses (Brock & Shapiro, 1983b; McKenzie & Knowland, 1990) and the increased rate of total RNA synthesis (Brock & Shapiro, 1983a; Martin et al., 1986) also contribute to the response. Not all mRNAs in the liver increase following estrogen treatment. Albumin mRNA, which is abundant in normal animals, decreases substantially relative to total RNA. In the present study, total albumin mRNA in the liver remained relatively constant at the hormone dosage used. At higher estrogen concentrations, secreted albumin and albumin mRNA can be suppressed even further to very low levels (Wangh, 1982, and our unpublished experiments). Thus, it appears that stimulation of ferritin and SRBP mRNA levels occurs as part of the more general phenomenon of estrogen-induced total transcriptional activation and RNA accumulation, which is distinctly different from the specific mechanisms regulating expression of the vitellogenin and Ep45 genes. We have optimized procedures for introducing cloned DNA into primary *Xenopus* liver cells by transfection, so that the multiple mechanisms by which estrogens act on different genes can be analyzed in more detail.

ACKNOWLEDGMENTS

We appreciate very much the generosity of Dr. Edward Prochownik in giving advice on cDNA cloning and of Drs. Lawrence Wangh (Brandeis University) and Stuart Orkin (Harvard Medical School), in whose laboratories the initial construction of the *Xenopus* liver cDNA library was carried out. We thank Diane Ochs in the Diabetes and Endocrinology Research Center, University of Iowa College of Medicine, for help with DNA sequencing and sequence database searches, Dr. Joe Forrester for help with computer programs, Allan Shepard for conducting the primer extension experiments, and Dr. Lewis Roberts for valuable assistance with computer programs.

REFERENCES

Arosio, P., Adelman, T. G., & Drysdale, J. W. (1978) *J. Biol. Chem. 253*, 4451–4458.

Aziz, N., & Munro, H. N. (1986) *Nucleic Acids Res. 14*, 915–927.

Aziz, N., & Munro, H. N. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8478–8482.

Baker, H. J., & Shapiro, D. J. (1977) *J. Biol. Chem. 252*, 8428–8434.

Baralle, F. E., & Brownlee, G. G. (1978) *Nature 274*, 84–87.

Beaucage, S. L., & Caruthers, M. H. (1981) *Tetrahedron Lett. 22*, 1859–1862.

Benyajati, C., Spoerel, N., Haymerle, H., & Ashburner, M. (1983) *Cell 33*, 125–133.

Bergink, E. W., Tseng, M. T., & Wittliff, J. L. (1977) *Cytobiologie 14*, 362–377.

Bhattacharya, A., Shepard, A. R., Moser, D. R., & Holland, L. J. (1990a) *Mol. Cell. Endocrinol. 72*, 213–220.

Bhattacharya, A., Shepard, A. R., Moser, D. R., Roberts, L. R., & Holland, L. J. (1990b) *Mol. Cell. Endocrinol.* (in press).

Boyd, D., Vecoli, C., Belcher, D. M., Jain, S. K., & Drysdale, J. W. (1985) *J. Biol. Chem. 260*, 11755–11761.

Brock, M. L., & Shapiro, D. J. (1983a) *J. Biol. Chem. 258*, 5449–5455.

Brock, M. L., & Shapiro, D. J. (1983b) *Cell 34*, 207–214.

Brown, A. J. P., Leibold, E. A., & Munro, H. N. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1265–1269.

Chen, E. Y., & Seeburg, P. H. (1985) *DNA 4*, 165–170.

Chirgwin, J. M., Przbyla, A. E., MacDonald, R. J., & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5299.

Chou, C.-C., Gatti, R. A., Fuller, M. L., Concannon, P., Wang, A., Chada, S., Davis, R. C., & Salser, W. A. (1986) *Mol. Cell. Biol. 6*, 566–573.

Clemens, M. J. (1974) *Prog. Biophys. Mol. Biol. 28*, 69–108.

Costanzo, F., Santori, C., Colantuoni, V., Bensi, G., Raugei, G., Romano, V., & Cortese, R. (1984) *EMBO J. 3*, 23–27.

Costanzo, F., Colombo, M., Staempfli, S., Santoro, C., Marone, M., Frank, R., Delius, H., & Cortese, R. (1986) *Nucleic Acids Res. 14*, 721–736.

Cox, R. A. (1968) *Methods Enzymol. 12B*, 120–129.

Cox, F., Gestautas, J., & Rapoport, B. (1988) *J. Biol. Chem. 263*, 7060–7067.

Deeley, R. G., Gordon, J. I., Burns, A. T. H., Mullinix, K. P., Bina-Stein, M., & Goldberg, R. F. (1977) *J. Biol. Chem. 252*, 8310–8319.

Devereux, J., Haeberli, P., & Smithies, O. (1984) *Nucleic Acids Res. 12*, 387–395.

Dickey, L. F., Sreedharan, S., Theil, E. C., Didsbury, J. R., Wang, Y.-H., & Kaufman, R. E. (1987) *J. Biol. Chem. 262*, 7901–7907.

Didsbury, J. R., Theil, E. C., Kaufman, R. E., & Dickey, L. F. (1986) *J. Biol. Chem. 261*, 949–955.

Dorner, M. H., Salfeld, J., Will, H., Leibold, E. A., Vass, J. K., & Munro, H. N. (1985) *Proc. Natl. Acad Sci. U.S.A. 82*, 3139–3143.

Feinberg, A. P., & Vogelstein, B. (1983) *Anal. Biochem. 132*, 6–13.

Feinberg, A. P., & Vogelstein, B. (1984) *Anal. Biochem. 137*, 266–267.

Hanahan, D. (1983) *J. Mol. Biol. 166*, 557–580.

Hayward, M. A., Barton, M. C., & Shapiro, D. J. (1985) *Mol. Cell. Endocrinol. 39*, 91–98.

Hentze, M. W., Caughman, S. W., Rouault, T. A., Barriocanal, J. G., Daneis, A., Harford, J. B., & Klausner, R. D. (1987) *Science 238*, 1570–1573.

Holland, L. J., & Wangh, L. J. (1983) *Nucleic Acids Res. 11*, 3283–3300.

Holland, L. J., & Wangh, L. J. (1984) *Mol. Cell. Biol. 4*, 2543–2548.
Holland, L. J., & Wangh, L. J. (1987) *Mol. Cell. Endocrinol. 49*, 63–73.
Holland, L. J., Diehl, E. E., Colburn, R. A., & Schmidt, T. J. (1990) *J. Steroid Biochem. 35*, 391–398.
Jain, S. K., Barrett, K. J., Boyd, D., Favreau, M. F., Crampton, J., & Drysdale, J. W. (1985) *J. Biol. Chem. 260*, 11762–11768.
Kazmaier, M., Bruning, E., & Ryffel, G. U. (1985) *EMBO J. 4*, 1261–1266.
Kozak, M. (1987) *Nucleic Acids Res. 15*, 8125–8148.
Leibold, E. A., & Munro, H. N. (1987) *J. Biol. Chem. 262*, 7335–7341.
Leibold, E. A., Aziz, N., Brown, A. J. P., & Munro, H. N. (1984) *J. Biol. Chem. 259*, 4327–4334.
Lewis, J. A., Clemens, M. J., & Tata, J. R. (1976) *Mol. Cell. Endocrinol. 4*, 311–329.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning*, Cold Spring Harbor Larboratory, Cold Spring Harbor, NY.
Martin, M. B., Riegel, A. T., & Schoenberg, D. R. (1986) *J. Biol. Chem. 261*, 2355–2361.
May, F. E. B., Ryffel, G. U., Weber, R., & Westley, B. R. (1982) *J. Biol. Chem. 257*, 13919–13923.
McKearin, D. M., Barton, M. C., Keller, M. J., & Shapiro, D. J. (1987) *J. Biol. Chem. 262*, 4939–4942.
McKenzie, E. A., & Knowland, J. (1990) *Mol. Endocrinol. 4*, 807–811.
Michelson, A. M., & Orkin, S. H. (1982) *J. Biol. Chem. 257*, 14773–14782.
Moskaitis, J. E., Pastori, R. L., & Schoenberg, D. R. (1990) *Nucleic Acids Res. 18*, 2184.
Philipp, B. W., Morgan, E. A., & Shapiro, D. J. (1982) *J. Biol. Chem. 257*, 8496–8501.
Prochownik, E. V., Markham, A. F., & Orkin, S. H. (1983) *J. Biol. Chem. 258*, 8389–8394.
Proudfoot, N. H., & Brownlee, G. G. (1976) *Nature 263*, 211–214.
Riegel, A. T., Martin, M. B., & Schoenberg, D. R. (1986) *Mol. Cell. Endocrinol. 44*, 201–209.
Ryffel, G. U., Wahli, W., & Weber, R. (1977) *Cell 11*, 213–221.
Santoro, C., Marone, M., Ferrone, M., Costanzo, F., Colombo, M., Minganti, C., Cortese, R., & Silengo, L. (1986) *Nucleic Acids Res. 14*, 2863–2876.
Schorpp, M., Dobbeling, U., Wagner, U., & Ryffel, G. U. (1988) *J. Mol. Biol. 199*, 83–93.
Shelness, G. S., & Williams, D. L. (1984) *J. Biol. Chem. 259*, 9929–9935.
Shull, G. E., & Theil, E. C. (1982) *J. Biol. Chem. 257*, 14187–14191.
Shull, G. E., & Theil, E. C. (1983) *J. Biol. Chem. 258*, 7921–7923.
Skipper, J. K., & Hamilton, T. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2384–2388.
Spolski, R. J., Schneider, W., & Wangh, L. J. (1985) *Dev. Biol. 108*, 332–340.
Stevens, P. W., Dodgson, J. B., & Engel, J. D. (1987) *Mol. Cell. Biol. 7*, 1751–1758.
Tata, J. R., & Baker, B. (1975) *Biochem. J. 150*, 345–355.
Theil, E. C. (1987) *Annu. Rev. Biochem. 56*, 289–315.
Torti, S. V., Kwak, E. L., Miller, S. C., Miller, L. L., Ringold, G. M., Myambo, K. B., Young, A. P., & Torti, F. M. (1988) *J. Biol. Chem. 263*, 12638–12644.
Wangh, L. J. (1982) *Dev. Biol. 89*, 294–298.
Wangh, L. J., Osborne, J. A., Hentschel, C., & Tilly, R. (1979) *Dev. Biol. 70*, 479–499.
Wangh, L. J., Holland, L. J., Spolski, R. J., Aprison, B. S., & Weisel, J. W. (1983) *J. Biol. Chem. 258*, 4599–4605.
Witliff, J. L., Lee, K.-L., & Kenney, F. T. (1972) *Biochim. Biophys. Acta 269*, 493–504.
Wolffe, A. P., Glover, J. F., Martin, S. C., Tenniswood, M. P. R., Williams, J. L., & Tata, J. R. (1985) *Eur. J. Biochem. 146*, 489–496.
Zahringer, J., Baliga, B. S., & Munro, H. N. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 857–861.