Reactivation of Apolipoprotein II Gene Transcription by Cycloheximide Reveals Two Steps in the Deactivation of Estrogen Receptor-Mediated Transcription

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In this report, we describe apolipoprotein II (apoII) gene expression in cell lines derived by stable expression of the chicken estrogen receptor in LMH chicken hepatoma cells. In cell lines expressing high levels of receptor (LMH/2A), apoII gene expression is increased by estrogen 300-fold compared with levels in the receptor-deficient parent LMH line. LMH/2A cells show apoII mRNA induction and turnover kinetics similar to those in chicken liver. Inhibition of protein synthesis with cycloheximide (CHX) or puromycin following estrogen withdrawal superinduces apoII mRNA without affecting apoII mRNA stability. Superinduction is due to an estrogen-independent reactivation of apoII gene transcription. The apoII gene can be reactivated by CHX for up to 24 h following hormone withdrawal, suggesting that the gene is in a repressed yet transcriptionally competent state. These results reveal two distinct events necessary for termination of estrogen receptor-mediated transcription. The first event, removal of hormone, is sufficient to stop transcription when translation is ongoing. The second event is revealed by the CHX-induced superinduction of apoII mRNA following hormone withdrawal. This superinduction suggests that deactivation of estrogen receptor-mediated transcription requires a labile protein. Furthermore, reactivation of apoII gene expression by CHX and estrogen is additive, suggesting that estrogen is unable to overcome repression completely. Thus, a labile protein may act to repress estrogen receptor-mediated transcription of the apoII gene.

In avian liver, estrogens regulate the expression of a number of egg yolk precursor proteins required for the transport of nutrients to the developing oocyte (5). Among the proteins regulated by estrogen are vitellogenin I (VTGI), VTGII, and VTGIII (63, 64), apolipoprotein B (apoB) (17) and apoII (18) of very low density lipoprotein, and riboflavin- and biotin-binding proteins (16, 66). The primary action of estrogen is to increase transcription of the genes encoding these proteins via binding to the estrogen receptor. In addition to regulating transcription, estrogen also alters the cytoplasmic stability of apoII and VTGII mRNAs (30, 71). Analysis of the mechanisms of transcriptional regulation of these genes as well as the study of mRNA turnover in avian liver cells has been hampered by the absence of a suitable homologous cell line. Studies of gene activation and estrogen-dependent alterations in chromatin structure and DNA methylation patterns have been restricted to animal studies or tissue homogenates (2, 3, 14, 15, 38, 42, 67, 68). Similarly, transcriptional analysis with gene transfer techniques has been limited to transient assays in primary liver cultures or heterologous cell lines (4, 13, 56). The LMH cell line is a chicken hepatoma that expresses many hepatocyte properties (41) and may provide a homologous cell line for studies of avian liver gene expression. We showed previously that the endogenous apoII gene was expressed in

LMH cells in response to estrogen; however, the level of response was only 0.1% of that seen in rooster liver (9). A transfected apoII gene also was expressed in LMH cells, but its expression was dependent on cotransfection with an estrogen receptor expression vector. This finding suggested that the estrogen responsiveness of the LMH cell may be limited by the content of functional estrogen receptor. Consistent with this possibility, receptor binding activity was very low in LMH cells but was detected following transient transfection with vector encoding the receptor. Although the LMH cell has proved useful for promoter analysis of transfected VTGII and apoII genes (6, 9), the low level of endogenous apoII mRNA expression limits its usefulness for analyzing various aspects of hormone-dependent gene activation or for studies of mRNA turnover.

In this report, we describe cell lines derived from the LMH line by stable expression of the chicken estrogen receptor. In cell lines expressing high levels of receptor, expression of the endogenous apoII gene is induced strongly by estrogen. Analysis of the LMH/2A line shows mRNA induction and turnover kinetics similar to those in chicken liver. The LMH/2A line was used to study the effects of inhibiting protein synthesis on apoII gene expression and mRNA turnover following hormone withdrawal. Inhibition of protein synthesis following hormone withdrawal superinduced apoII mRNA by an estrogen-independent reactivation of apoII gene transcription without affecting apoII mRNA stability. These results suggest that removal of hormone is not itself sufficient to terminate expression of the apoII gene. Deactivation of hormone-induced expression may involve a labile protein factor that represses gene expression when estrogen is withdrawn. Thus, these

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results define two steps in the deactivation of estrogen receptor-mediated transcription of the apoII gene.

MATERIALS AND METHODS

Cell culture. LMH cells (41) were maintained in Waymouth's MB 752/1 without phenol red supplemented with penicillin (100 U/ml), streptomycin sulfate (100 µg/ml), and 10% fetal bovine serum (complete medium). Rich Waymouth's medium for transfection experiments was prepared by adding 10% LMH conditioned medium, 5% chicken serum, and 1× minimal essential medium-vitamin mix to complete Waymouth's media. Cells were grown in T-75 flasks or 60-mm-diameter dishes precoated with 0.1% gelatin. Typically, cells were cultured for 7 days following plating; medium was changed on day 4, and hormone was added on day 7. 17β-Estradiol and moxestrol [R2858; 11β-methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol; Roussel-UCLAF; obtained from New England Nuclear] were dissolved in ethanol. For hormone withdrawal, medium was aspirated, cells were rinsed with phosphate-buffered saline, and complete hormone-free medium was added. For inhibition of protein synthesis, cells were treated for the indicated times with either cycloheximide (CHX; 2.5 µg/ml) or puromycin (100 µg/ml) dissolved in Waymouth's medium.

Stable transfection with chicken estrogen receptor. Chicken estrogen receptor plasmids pCEO (43) and pRSVCEO (12a), driven by the simian virus 40 promoter and the Rous sarcoma virus promoter, respectively, were cotransfected with plasmid pRSVNEO (31), which confers G418 resistance. Stably transfected lines were established by the modified calcium phosphate method (19). Transfection was performed 1 day after plating, using 3 µg of selection plasmid and 27 µg of estrogen receptor plasmid. Following overnight incubation in 3% CO₂-97% air, cells were transferred to rich medium and incubated in an atmosphere of 5% CO₂-95% air for 3 days prior to selection in rich medium containing 100 µg of G418 per ml. Colonies were visible after 14 days in selection. Cell lines were then maintained in complete Waymouth's medium without G418.

RNA isolation and analysis. Total RNA was isolated by the guanidine isothiocyanate method (20) followed by extraction with phenol. For some experiments, RNA was isolated by the single-step acid-guanidinium thiocyanate-phenol method (21), either as originally described or with RNASSTAT (Tel-Test "B", Inc., Friendswood, Tex.). Nuclear and cytoplasmic RNAs were prepared as described previously (29) except that cell pellets were lysed in 4 ml of buffer (0.5% Nonidet P-40, 10 mM NaCl, 3 mM MgCl₂, 10 mM Tris-HCl [pH 7.4]), incubated on ice for 10 min, and centrifuged for 5 min at 1,000 × g. The supernatant was transferred to a sterile tube, and vanadylribonucleoside complex (10 mM) was added prior to isolation of cytoplasmic RNA. The nuclear pellet was washed with lysis buffer to remove contaminating cellular debris, and nuclear RNA was isolated by guanidinium isothiocyanate extraction as described above. For Northern (RNA) blot analysis, RNA was fractionated through a 1.2% agarose-6% formaldehyde gel, electrophoretically transferred to Nytran (Schleicher & Schuell), and cross-linked with UV light (Stratalinker; Stratagene). RNA gels were stained with ethidium bromide to ensure that samples contained equal amounts of RNA. Blots were hybridized with random-primed probes (Prime-It; Stratagene) prepared from the genomic chicken apoII clone pApo107 (12a), the chicken transferrin cDNA clone pCon64 (23), the chicken glyceraldehyde phosphate dehydrogenase (GAPDH) clone pGAD28

(27), and the chicken estrogen receptor clone pCEO as described in the figure legends, by using standard conditions.

Nuclear run-on transcription assays were carried out essentially as described previously (33). For each treatment, nuclei were pooled from dishes of cells cultured as described above. The final radiolabeled RNA pellet was resuspended in 2.0 ml of *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer (10 mM EDTA, 0.2% sodium dodecyl sulfate [SDS], 10 mM TES [pH 7.4]), and duplicate 5-µl aliquots were precipitated in 7.5% trichloroacetic acid for 15 min on ice. Precipitated RNA was collected onto glass fiber filters (Whatman), and incorporated radionucleotide was quantified by scintillation counting. Equivalent counts of radiolabeled RNA (see figure legends) from each treatment group were hybridized to Nytran strips containing dot blots of linearized and sodium hydroxide-denatured plasmid DNA containing the gene or cDNA of interest. Ethidium bromide (0.5 µg/ml) was added to DNA for dot blots, and bound DNA was visualized by UV light to ensure that equivalent amounts of DNA were blotted. Blotted DNA was cross-linked with UV light (Stratalinker) and dried for 15 min at 65°C. Blots were incubated in TES-NaCl (300 mM NaCl, 10 mM EDTA, 0.2% SDS, 10 mM TES [pH 7.4]) for 2 h at 65°C and hybridized with radiolabeled RNA in TES-NaCl at 65°C for 40 h. Samples were washed in 5 ml of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 1 h at 65°C, treated with RNase A (10 µg/ml) for 30 min at 37°C, and washed in 2× SSC at 65°C for 1 h. Plasmid DNAs used for detection of specific RNAs included pApo107, pUC8, pGAD28, pCon64, and pA1 containing the chicken β-actin cDNA (22).

DNA-excess solution hybridization was carried out as described previously (30, 69), using a probe containing nucleotides 210 to 449 of the apoII cDNA (57). RNA probe-excess solution hybridization assays used plasmid pBSapoII.240, containing the same fragment subcloned into Bluescript SK/KS+ (Stratagene). ³²P-labeled antisense apoII RNA was transcribed in vitro by T7 RNA polymerase on a *Bam*HI-linearized pBSapoII.240 template. For use as a standard curve, ³H-labeled sense strand was synthesized by T3 RNA polymerase on a *Hind*III-linearized template. Hybridization was carried out in 0.3 M NaCl-30 mM sodium citrate (pH 7.0) for 18 h at 68°C. Samples were treated with RNase T₁ (500 U/ml) and RNase A (40 µg/ml) at 55°C for 1 h, precipitated with trichloroacetic acid (7.5%), collected on glass fiber filters, and counted by liquid scintillation spectrometry. Data from solution hybridization assays are expressed as picograms of apoII mRNA per microgram of total RNA.

Measurement of mRNA half-life. ApoII mRNA half-life was determined during hormone induction by approach-to-steady-state analysis (7, 65). ApoII mRNA half-life in the absence of hormone was determined directly by semilogarithmic analysis of apoII mRNA decay in hormone-withdrawn cells. Half-life (*t*_{1/2}) was calculated from the slope of the regression line, using the equation $t_{1/2} = \ln 2/k$, where slope = $-k$.

Measurement of estrogen receptor. Estrogen receptor binding site concentration and equilibrium dissociation constants were determined for nuclear, cytoplasmic, microsomal, and whole cell extracts of LMH/2A cells by Scatchard analysis of ligand binding data. Ligand binding assays were carried out as described previously (45) except that homogenization buffer was that used by Green et al. (32). Labeling with [ring-³H]tamoxifen aziridine was carried out as described previously (40).

FIG. 1. Induction of endogenous apoII mRNA in LMH cells stably transfected with the chicken estrogen receptor. (A) Northern analysis of estrogen receptor mRNA in stable cell lines. Total RNA (10 μ g) was probed for estrogen receptor with an *Eco*RI fragment of pCEO as described in Materials and Methods. Cell lines resulting from transfection with pCEO, pRSVCEO, and pUC8 (negative control) are designated by 1, 2, and 3, respectively. Transfected (2.3-kb) and endogenous (7.5-kb) receptor mRNAs are indicated by arrows. (B) Induction of apoII mRNA in LMH stable cell lines. Cells were harvested following incubation for 24 h in medium containing 50 nM 17 β -estradiol or ethanol. ApoII mRNA was quantified by DNA-excess solution hybridization. Each value represents the average of duplicate samples. Cell lines correspond to those in panel A. Two lines not represented in panel A, 2E and 2F, are also included. Relative levels of estrogen receptor mRNA determined by Northern analysis in panel A are indicated by one or more plus signs for cell lines in panel B.

RESULTS

Establishment of cell lines that stably express functional chicken estrogen receptor. LMH cells were transfected with pRSVNEO together with either pCEO or pRSVCEO as described in Materials and Methods. Colonies resistant to G418 were expanded into independent lines and screened for expression of chicken estrogen receptor. Transfection of cells with pCEO and pRSVNEO resulted in 31 G418-resistant lines, and 14 clones were positive for expression of chicken estrogen receptor mRNA as determined by Northern blot analysis. Transfection of cells with pRSVCEO and pRSVNEO yielded seven G418-resistant lines, and four lines were positive for estrogen receptor mRNA. Representative clones are shown in Fig. 1A. Relative abundance of estrogen receptor mRNA was greater for the LMH/2 lines transfected with pRSVCEO than for the LMH/1 lines transfected with pCEO (compare lanes 2A and 2B with lanes 1H to 1V). Clones positive for expression of estrogen receptor mRNA were assayed for apoII mRNA by

probe-excess solution hybridization following treatment with 50 nM 17 β -estradiol. Levels of apoII mRNA and estrogen receptor mRNA correlated roughly (Fig. 1B), confirming the dependence of apoII expression on functional estrogen receptor. Clonal lines LMH/2A, LMH/2B, LMH/2E, and LMH/2F expressed apoII mRNA at levels of 20 to 35 pg/ μ g (Fig. 1B). In additional experiments carried out under optimal induction conditions, the estrogen-induced LMH/2A and LMH/2E lines expressed apoII mRNA at levels of 30 to 40 and 60 to 80 pg/ μ g, respectively. These levels are 10 to 20% of the apoII mRNA level in estrogen-induced rooster liver. Furthermore, this induction is more than 300-fold higher than the maximal level (0.065 pg/ μ g) observed in the parent LMH line (data not shown). One clonal line, LMH/2A, was selected for further investigation.

Estrogen receptor level in LMH/2A cells. Estrogen receptor levels in extracts from LMH/2A cells treated with 17 β -estradiol were determined by a ligand binding assay as described in

FIG. 2. ApoII mRNA half-life following hormonal induction. LMH/2A cells were cultured as described in Materials and Methods. Cells were treated with 50 nM moxestrol for 0 to 72 h, and apoII mRNA was analyzed by DNA-excess solution hybridization. (A) ApoII mRNA expression versus time. Each point represents a pool of two dishes. (B) Approach-to-steady-state analysis. The ascending portion of the curve in panel A was replotted by the method of Watson et al. (65). Half-life was calculated from the slope of the regression line, using the equation $t_{1/2} = \ln 2/k$, where slope = $-k$.

Materials and Methods. Receptor was detected in cytosolic and microsomal fractions (697 and 725 fmol of receptor per mg of protein, respectively), but the highest concentration was found in nuclear extracts. The concentration of nuclear estrogen receptor in LMH/2A cells was sixfold higher than the concentration in estrogen-induced immature chick liver nuclei assayed simultaneously (6,200 versus 1,000 fmol of receptor per mg of protein). These LMH/2A estrogen receptor levels are 150-fold greater than levels in LMH parent cells (<40 fmol/mg of protein, nuclear; <5 fmol/mg of protein, cytosolic) and 25-fold greater than levels in LMH cells transiently transfected with pCEO (260 fmol/mg of protein, nuclear; 36 fmol/mg of protein, cytosolic), as described previously (9). Scatchard analysis predicted a dissociation constant of 2.36 nM for nuclear receptors (data not shown). Nuclear estrogen receptor, examined by SDS-polyacrylamide gel electrophoresis after [3 H]tamoxifen aziridine labeling of nuclear extracts, migrated with the expected molecular size of 64 kDa (data not shown). Thus, LMH/2A cells express functional estrogen receptor at levels markedly greater than those in estrogen-treated rooster liver and LMH parent cells.

ApoII mRNA induction and turnover in LMH/2A cells. LMH/2A cells were induced with the synthetic estrogen moxestrol in order to characterize apoII mRNA expression. Moxestrol was chosen because of its slower metabolism compared with 17 β -estradiol (51). Treatment of cells with 50 nM moxestrol for 24 h yielded maximal (30 to 40 pg/ μ g) apoII mRNA levels (data not shown). Time courses of apoII mRNA induc-

FIG. 3. ApoII mRNA half-life following hormone withdrawal. LMH/2A cells were cultured as described in Materials and Methods. Moxestrol (50 nM) was added for 24 h, and cells were withdrawn from hormone for 0 to 72 h. ApoII mRNA was analyzed by DNA-excess solution hybridization. Each point represents a pool of two dishes. Half-life was calculated from the slope of the regression line, using the equation $t_{1/2} = \ln 2/k$, where slope = $-k$.

tion and withdrawal were carried out to determine apoII mRNA half-life in LMH/2A cells. Half-life during hormone induction from 0 to 72 h was determined by approach-to-steady-state analysis (7, 65). This analysis yielded a first-order decay constant of 0.082 h^{-1} and a $t_{1/2}$ of 8.4 h (Fig. 2). This decay rate is consistent with but slightly faster than the half-life of 12 h reported for apoII mRNA in rooster liver (30).

ApoII mRNA half-life following withdrawal from hormone was determined directly by analysis of apoII mRNA remaining at various times after induction and removal of hormone. Under these conditions, apoII mRNA decayed after an initial lag period of approximately 8 h following hormone withdrawal (Fig. 3). Therefore, half-life was determined in the 8- to 72-h time interval, yielding a value of 10.7 h. In subsequent experiments, apoII mRNA half-life varied between 9 and 11 h. These results are consistent with the 8.4-h half-life determined by the approach-to-steady-state analysis.

To determine whether the lag time in apoII mRNA decay was due to residual transcription, the estrogen receptor antagonist 4-hydroxytamoxifen was added to cells during hormone withdrawal. In avian cells, tamoxifen and its metabolite 4-hydroxytamoxifen are potent antagonists of estrogen action that act by competitively inhibiting the binding of estrogens to the estrogen receptor (11, 60, 61). Tamoxifen also rapidly reverses the estrogen-dependent induction of apoII synthesis and apoII mRNA in chick liver (10, 30). For the current experiments, the concentration of 4-hydroxytamoxifen used (1 μ M) was sufficient to block moxestrol-induced apoII mRNA expression. Addition of 4-hydroxytamoxifen had little or no effect on apoII half-life in several experiments and did not affect the apparent lag time for mRNA decay (data not shown). Thus, residual transcription of the apoII gene is unlikely to account for the lag period. This conclusion is supported by the finding that both intron A-containing nuclear apoII mRNA precursors and transcription of the apoII gene are near background levels by 8 h after hormone withdrawal (see Fig. 6 and 7).

The data presented above suggest that apoII mRNA expression in the LMH/2A cell line is similar to that observed in estrogen-treated liver. The kinetics of induction and decay are similar but slightly faster than those reported in rooster liver. Thus, this cell line provides a useful model for investigating mechanisms involved in apoII mRNA turnover.

Effect of inhibiting protein synthesis on apoII mRNA turnover. Inhibition of protein synthesis has been shown to either

FIG. 4. Effects of CHX and puromycin on apoII mRNA turnover. LMH/2A cells were cultured as described in Materials and Methods. Moxestrol (50 nM) was added for 24 h, and cells were withdrawn from hormone (time zero). ApoII mRNA was analyzed by RNA probe-excess solution hybridization. (A) CHX (2.5 μ g/ml) was added 8 h after withdrawal. Cells were harvested at the indicated times for control (filled circles) and CHX-treated (open circles) samples. (B) Puromycin (100 μ g/ml) was added 8 h after withdrawal. Cells were harvested at the indicated times for control (filled circles) and puromycin-treated (open circles) samples. Each point represents the mean $\pm$ standard deviation of three replicate samples. Note that for small standard deviations, error bars are indistinguishable from datum points.

increase or decrease the half-lives of many mRNAs (70). The inhibitors CHX, which blocks chain elongation (28, 35, 58), and puromycin, which causes premature chain termination (49, 50, 62), were used to determine the effects of inhibiting protein synthesis on apoII mRNA turnover in LMH/2A cells. For these experiments, inhibitors were added 8 h after hormone withdrawal at concentrations sufficient to inhibit protein synthesis by >90%. As shown in Fig. 4A, treatment of cells with CHX superinduced apoII mRNA levels well above the levels observed in both samples withdrawn from hormone for 8 h (the time of CHX addition) and moxestrol-induced samples (time zero). Treatment of cells with puromycin had a similar effect on apoII mRNA (Fig. 4B). This superinduction could reflect either an apparent induction due to decreased levels of total cellular (primarily ribosomal) RNA or an actual induction due to increased apoII transcriptional activity in the absence of hormone. To test the first hypothesis, cells were induced with moxestrol and treated with or without CHX during hormone withdrawal. Total cellular RNA in extracts was assayed by the orcinol method, which measures ribose by specific reaction with orcinol after acid hydrolysis (39). No differences were observed for total cellular RNA from CHX-treated compared with withdrawn or moxestrol-treated cells (data not shown). Thus, the superinductive effect of CHX was not due to decreased levels of bulk cellular RNA.

FIG. 5. Effect of DRB on CHX-induced superinduction of apoII mRNA. LMH/2A cells were cultured as described in Materials and Methods. Moxestrol (50 nM) was added for 24 h, and cells were withdrawn from hormone (time zero). CHX (2.5 μ g/ml) and DRB (300 μ M) were added 8 h after withdrawal. Cells were harvested at the indicated times for control (filled circles), CHX-treated (open circles), DRB-treated (filled diamonds), and DRB-CHX-treated (open diamonds) samples. ApoII mRNA was analyzed by RNA probe-excess solution hybridization. Each point represents the mean $\pm$ standard deviation of three replicate samples. Note that for small standard deviations, error bars are indistinguishable from datum points.

Several experiments were conducted to determine whether reactivation of transcription could account for superinduction of apoII mRNA following CHX treatment. First, the RNA synthesis inhibitor 5,6-dichlorobenzimidazole (DRB) was added simultaneously with CHX during hormone withdrawal. As shown in Fig. 5, apoII mRNA decayed slightly more slowly in samples treated with DRB alone compared with control samples. In the presence of both CHX and DRB, apoII mRNA decay was similar to that of DRB-treated samples. Thus, neither stabilization nor superinduction of apoII mRNA was evident. This result suggests that transcription is necessary for superinduction by CHX. Furthermore, because apoII mRNA decayed normally in the presence of CHX when transcription was blocked, apoII mRNA does not appear to be stabilized by inhibition of protein synthesis.

To determine whether superinduction by CHX might require trace levels of hormone remaining after hormone withdrawal, cells were treated 8 h after hormone withdrawal with CHX in the presence of 4-hydroxytamoxifen. Control values at 0, 8, and 16 h after hormone withdrawal were 39.2 ± 5.8 , 21.3 ± 3.9 , and 11.9 ± 3.1 pg of apoII mRNA per μ g, respectively. Sixteen-hour values for CHX-treated and CHX-4-hydroxytamoxifen-treated cells were 41.1 ± 2.5 and 51.6 ± 8.9 pg of apoII mRNA per μ g, respectively. 4-Hydroxytamoxifen did not inhibit superinduction by CHX, which suggests that the hormone-liganded form of the estrogen receptor is not required for superinduction. Nevertheless, when CHX was added without prior hormone treatment, apoII mRNA was not induced (1.4 ± 1.3 pg/ μ g) compared with the ethanol vehicle (1.0 ± 0.2 pg/ μ g) or the moxestrol control (32.6 ± 1.3 pg/ μ g). This finding indicates that hormone-induced events, perhaps assembly of the receptor-dependent transcription complex, were necessary for CHX-induced transcriptional activation.

To test further for transcriptional activation of the apoII gene, apoII mRNA precursors were assayed by Northern blotting of LMH/2A nuclear RNA by using an intron A-specific DNA probe. Multiple intron A-containing splicing intermediates ranging up to 3.2 kb were evident (Fig. 6), as previously described (24). As shown in Fig. 6, apoII mRNA precursors were abundant in moxestrol-induced samples (lane

FIG. 6. Effect of CHX on apoII nuclear mRNA precursors. LMH/2A cells were cultured as described in Materials and Methods. Moxestrol (50 nM) was added for 24 h, and cells were withdrawn from hormone. CHX (2.5 μ g/ml) was added 8 h after withdrawal. Cells were harvested, and nuclear RNA was isolated. Each lane contains 10 μ g of nuclear RNA. (A) Autoradiogram of Northern blot hybridized with an intron A-specific *Bst*EII-*Eco*RI fragment of the apoII genomic clone pApo107. (B) Ethidium bromide-stained gel. Lanes: 1, moxestrol-induced cells; 2, moxestrol-induced cells, withdrawn for 8 h; 3, moxestrol-induced cells, withdrawn for 21 h; 4, moxestrol-induced cells, withdrawn for 21 h, with CHX added 8 h after withdrawal. Positions of migration of 18S and 28S ribosomal bands and approximate molecular sizes are indicated.

1), markedly reduced in samples from cells withdrawn from hormone for 8 h (lane 2), and barely detectable in cells withdrawn from hormone for 21 h (lane 3). However, nuclear precursors were increased in cells withdrawn from hormone for 8 h and then treated with CHX for 13 h (lane 4), suggesting that apoII transcription was reactivated when CHX was added 8 h after hormone withdrawal.

Nuclear run-on assays for apoII gene transcription were conducted to confirm this point. Figure 7 shows that apoII gene transcription was higher in moxestrol-induced cells than in cells treated with the ethanol vehicle (lane 2 versus lane 1) and was markedly decreased in cells withdrawn from hormone for 8 or 15 h (lanes 3 and 4). However, apoII transcription was increased in cells withdrawn from hormone for 8 h and then treated with CHX for 7 h (lane 5 versus lane 4). Furthermore, in comparison with the chicken β -actin gene, the effect of CHX appears to be selective for the apoII gene. We have also confirmed in another run-on experiment that transcription of the apoII gene is increased by CHX selectively in comparison with either the transferrin or GAPDH gene (data not shown).

FIG. 7. Effect of CHX on apoII gene transcription. LMH/2A cells were cultured in T-75 flasks as described in Materials and Methods. Moxestrol (50 nM) was added for 24 h. Cells were withdrawn from hormone, and CHX (2.5 μ g/ml) was added 8 h later. Samples were harvested 0, 8, and 15 h following hormone withdrawal. Nuclei were isolated, and run-on transcription assays were conducted as described in Materials and Methods. Lanes: 1, ethanol-treated cells; 2, moxestrol-induced cells; 3, moxestrol-induced cells, withdrawn for 8 h; 4, moxestrol-induced cells, withdrawn for 15 h; 5, moxestrol-induced cells, withdrawn for 15 h, with CHX added 8 h after withdrawal. Dot blots contained 5 μ g of the indicated plasmid DNA, and 3.0×10^6 cpm of labeled RNA from each transcription reaction was used for hybridization.

FIG. 8. Effects of CHX on transferrin and GAPDH mRNAs. LMH/2A cells were cultured as described in Materials and Methods. Moxestrol (50 nM) was added for 24 h, and cells were withdrawn from hormone for 8 h (lanes 1) or 21 h (lanes 2 and 3). CHX (2.5 μ g/ml) was added 8 h after withdrawal (lanes 3). Cells were harvested, and cytoplasmic RNA was isolated. Each lane contains 10 μ g of cytoplasmic RNA. (A) Northern blot probed for transferrin mRNA, using a 500-bp *Bam*HI fragment of pCon64; (B) ethidium bromide-stained gel used in panel A; (C) blot probed for GAPDH mRNA, using an 1,100-bp *Pst*I fragment of pGAD28; (D) ethidium bromide-stained gel used in panel C. Positions of migration of 18S and 28S ribosomal bands are indicated.

Puromycin was also found to increase apoII gene transcription when added 8 h after hormone withdrawal (data not shown). These findings confirmed that the protein synthesis inhibitors CHX and puromycin reactivated apoII transcription following hormone withdrawal. We also examined the steady-state levels of transferrin and GAPDH mRNAs by Northern blot analysis. Neither transferrin mRNA (Fig. 8A) nor GAPDH mRNA (Fig. 8B) was increased in cells treated with CHX following induction and withdrawal from moxestrol. Thus, rather than being a general response of transcriptionally active genes, superinduction appears to be selective for the apoII gene.

This ability of CHX to reactivate transcription suggests that a suppressed yet functional transcription complex was present on the apoII promoter following hormone withdrawal. The observation that two distinct translation-inhibiting drugs superinduce apoII mRNA suggests that a labile protein, rather than the drug itself, is responsible for the effect. If such a repressor normally is involved in blocking transcription following hormone withdrawal, then resynthesis of the repressor and reversal of superinduction should occur upon removal of CHX from cells. To test this hypothesis, CHX was removed from LMH/2A cells following 7 h of treatment. As shown in Fig. 9, apoII mRNA superinduction was blocked and apoII mRNA levels decreased upon removal of CHX for 9 h. This result supports the idea that a repressor protein is involved in the normal transcriptional regulation of the apoII gene.

To determine whether CHX could superinduce apoII mRNA at later times following hormone withdrawal, cells were treated with moxestrol, withdrawn from hormone, and treated with CHX at 24 and 40 h following hormone withdrawal. As shown in Table 1, CHX was able to superinduce apoII mRNA at 24 h following hormone withdrawal, but the response was diminished at 40 h. These data indicate that the capacity to reactivate apoII gene transcription following hormone withdrawal is a transient phenomenon.

The possibility of a repressor of apoII gene transcription raises the question of whether hormonal induction of transcription fully overcomes repression. The experiment shown in Fig. 10 was conducted to compare induction by moxestrol

FIG. 9. Reversal of cycloheximide-induced superinduction of apoII mRNA. LMH/2A cells were cultured as described in Materials and Methods. Moxestrol (50 nM) was added for 24 h. Cells were withdrawn from hormone (time zero), and CHX (2.5 μ g/ml) was added 8 h after withdrawal. Medium was changed 15 h after withdrawal (7 h after CHX addition) for two sets of CHX-treated cells in order to remove inhibitor. CHX was then added back to one set of dishes (CHX); the second set was cultured without inhibitor (CHX/REV). Control cells were withdrawn from hormone in the absence of inhibitor. Cells were harvested at the indicated times, and apoII mRNA was analyzed by RNA probe-excess solution hybridization. Each point represents the mean $\pm$ standard deviation of three replicate samples. Note that for small standard deviations, error bars are indistinguishable from datum points.

alone and in combination with CHX. Reinduction of apoII mRNA following hormone withdrawal shows similar kinetics and magnitude for treatment with either moxestrol or CHX alone. However, the induction of apoII mRNA from 16 to 28 h after hormone withdrawal was completely additive for treatment with both moxestrol and CHX compared with either treatment alone. This result suggests that moxestrol and CHX elicit apoII mRNA induction via mechanistically distinct pathways and indicates that hormone binding to the receptor is not sufficient to induce transcription maximally.

DISCUSSION

Expression of apoII mRNA was augmented in LMH cells stably transfected with the chicken estrogen receptor as a result of increased receptor expression. In LMH/2 cell lines, which express estrogen receptor under the control of the Rous sarcoma virus promoter, both estrogen receptor and apoII mRNAs were increased markedly compared with levels in LMH/1 cell lines, which express receptor under control of the simian virus 40 promoter. ApoII mRNA levels in LMH/2A cells were increased more than 300-fold compared with levels in the LMH parent cell line but were only 10 to 20% of the level observed in rooster liver. This lower level of induction in

FIG. 10. Additive effect of CHX and moxestrol on induction of apoII mRNA. LMH/2A cells were cultured as described in Materials and Methods. Moxestrol (Mox; 50 nM) was added for 24 h, and cells were withdrawn from hormone (time zero). CHX (2.5 μ g/ml) and moxestrol (50 nM) were added 16 h after hormone withdrawal. Samples were harvested 0 and 16 h after withdrawal (filled circles) and 4, 8, and 12 h after subsequent addition of hormone or inhibitor (open circles). ApoII mRNA was analyzed by RNA probe-excess solution hybridization. Each point represents the mean $\pm$ standard deviation of three replicate samples. Note that for small standard deviations, error bars are indistinguishable from datum points.

the LMH/2 lines does not appear to reflect an inadequate concentration of estrogen receptor because the LMH/2A line has approximately sixfold more nuclear estrogen receptor than is found in rooster liver. The lower response could reflect the absence of another factor permissive for estrogen action. Alternatively, growing LMH cells may have higher levels of the putative repressor protein than nongrowing adult hepatocytes.

Availability of the LMH/2A cell line should allow detailed study of the mechanisms of apoII mRNA turnover. In LMH/2A cells, apoII mRNA half-life was approximately 8.4 h in the presence of the synthetic estrogen moxestrol and approximately 9 to 11 h during hormone withdrawal following a 24-h induction. These values are similar to those for rooster liver following induction with 17 β -estradiol for 24 h (30). In studies to be described elsewhere, we have also determined that the pattern of apoII mRNA degradation intermediates in LMH/2A cells (56a) is identical to that seen in rooster liver (8). The LMH/2 cell lines should prove useful for the further analysis of apoII mRNA turnover.

Because ongoing translation is required for the turnover of numerous mRNAs (70), we examined the effect of inhibiting protein synthesis on apoII mRNA degradation. Following hormone withdrawal, CHX and puromycin superinduced apoII mRNA but not GAPDH or transferrin mRNA. Because superinduction occurs with both CHX and puromycin, it is likely that the observed effects are due to the inhibition of protein synthesis rather than to unrelated effects of the inhibitors. The observed superinduction could have been attributable to increased apoII gene transcription or a combination of increased transcription and mRNA stabilization. Treatment of cells with CHX together with the transcription inhibitor DRB indicated that apoII mRNA stabilization by CHX was not involved in superinduction. This result suggests that apoII mRNA degradation does not require ongoing translation and that superinduction is a transcriptional event. Increased levels of apoII mRNA nuclear precursors and increased transcription in nuclear run-on assays confirmed that superinduction resulted from reactivation of apoII gene transcription. Superinduction was not a result of hormone-induced transcription of the apoII gene because the estrogen receptor antagonist

TABLE 1. Superinduction of apoII mRNA 24 and 40 h after hormone withdrawal

Time (h) after hormone withdrawal ^a	Mean apoII mRNA concn (pg/ μ g) $\pm$ SD	
	Control	CHX ^b
0	32.6 $\pm$ 1.3	
24	7.0 $\pm$ 0.4	
40	5.3 $\pm$ 0.6	12.6 $\pm$ 0.5
56	2.7 $\pm$ 0.2	5.8 $\pm$ 0.6

^a Time zero reflects the level after 24 h of induction with moxestrol.

^b CHX (2.5 μ g/ml) was added 24 or 40 h after withdrawal, and apoII mRNA was measured 16 h later for 40- and 56-h time points, respectively.

4-hydroxytamoxifen did not prevent the effect. Nevertheless, prior induction with hormone was required for superinduction, perhaps to establish a competent transcriptional complex on the apoII promoter.

These findings indicate that at least two distinct events are necessary for the deactivation of estrogen receptor-mediated transcription of the apoII gene. The first event is removal of hormone: in the presence of ongoing translation, removal of hormone is sufficient to terminate expression of the gene. The second event is revealed in the presence of inhibitors of protein synthesis: superinduction by CHX or puromycin following hormone withdrawal suggests that deactivation of estrogen receptor-mediated transcription requires a labile protein whose concentration decreases when protein synthesis is inhibited. This labile protein could function at several levels in apoII gene transcription. First, the labile protein could be a repressor that interacts with the unligated estrogen receptor itself. Second, the labile protein could interact either with DNA elements distinct from the estrogen response elements or with other transcription factors that bind to the apoII promoter (4, 67). Third, a labile protein could be required in the reestablishment of a repressive chromatin configuration following hormone withdrawal. Although we cannot distinguish among these possibilities, several recent findings are relevant.

A yeast protein, SSN6, that represses the estrogen receptor has been identified recently (48). SSN6 blocks transactivation in yeast cells by the TAF1 domain of the human estrogen receptor. Furthermore, in a mutant yeast strain that lacks SSN6, basal transactivation by the TAF1 domain is increased compared with activity in wild-type cells. Thus, SSN6 appears to repress the hormone-independent activity of the TAF1 domain. These results raise the possibility of a similar repressor in avian and mammalian cells.

Several mammalian transcription factors have also been shown to repress transactivation by steroid receptors in transfection assays. In MCF-7 cells, overexpression of c-Fos and c-Jun was shown to inhibit estrogen receptor activity, and overexpression of receptor could abolish the repression by c-Fos (26). Similar findings have been reported with other steroid receptors. For example, the Jun-Fos heterodimer may repress vitamin D₃- and retinoic acid-induced transcription of the osteocalcin gene (55), and c-Fos and c-Jun may repress transcription by complexing with the glucocorticoid receptor (37, 54, 72). Together, these findings suggest that a repressor may be a common participant in the regulation of gene expression by the estrogen receptor. However, the finding that CHX does not superinduce transcription of either the *Xenopus* vitellogenin gene (34) or the human pS2 gene (12) suggests that there may be a gene-specific component to superinduction that extends beyond the estrogen receptor itself.

With regard to alterations in chromatin, a number of studies have shown estrogen-dependent changes in DNase I-hypersensitive sites (42) and dimethyl sulfate-reactive sites (67) within 300 bp upstream of the apoII transcription initiation site. These changes may result from binding of specific transcription factors, including the receptor (4, 67), but may also reflect changes in nucleosomal organization on the apoII promoter. A structural change in the chromatin organization of the mouse mammary tumor virus promoter occurs upon glucocorticoid treatment as a result of an altered interaction of promoter DNA with a specifically positioned nucleosome (52). This change appears to derepress the promoter by mediating access of transcription factors to their DNA binding sites (1). It is possible that the deactivation of receptor-mediated transcription may require reversal of such chromatin alterations. If similar events occur in the apoII promoter, reactivation of

transcription by CHX might reflect the loss of a protein that is directly or indirectly involved in establishment of a repressive chromatin configuration.

Superinduction of message levels following treatment with protein synthesis inhibitors also has been reported for 9E3 mRNA (59), beta interferon mRNA (53), metallothionein-I mRNA, (47), adenovirus early gene products (25), and CYP1A1 mRNA (36). In the case of CYP1A1 mRNA (46), superinduction by CHX required a 20-nucleotide *CYP1A1* gene promoter element that contained the core recognition sequence for the Ah receptor. However, CHX-induced alterations were not observed in either in vitro gel shift or in vivo DNA methylation interference analyses, suggesting that a CHX-sensitive factor was involved in a protein-protein interaction that repressed transcription of CYP1A1.

In the cases described above, superinduction by CHX reflects increased expression of genes that are actively transcribed. A unique feature of apoII superinduction is that CHX induces transcription up to 24 h following termination of transcription by hormone withdrawal but has little effect if added 40 h after hormone withdrawal. One explanation for this transiency is that a functional yet repressed transcription complex remains on the apoII promoter for a considerable time after removal of hormone before eventually dissociating. Another explanation is that an altered chromatin configuration necessary for apoII transcription is gradually lost following hormone withdrawal. In either case, reactivation of transcription by estrogen during this transient period following hormone withdrawal may be facilitated if it is not necessary to reestablish the complex of transcription factors or the altered chromatin configuration that occurs upon first exposure to the hormone. This model may be physiologically relevant because the egg-laying cycle and plasma estrogen concentration in the hen fluctuate with similar periodicities of 26 to 28 h (44). Transcription of the apoII gene would be expected to occur with a similar periodicity in response to plasma estrogens. Maintaining the apoII gene in a functional yet repressed mode when the estrogen concentration drops may facilitate reactivation of transcription when the estrogen concentration rises in the next cycle.

The present findings are consistent with the idea of a repressor acting to deactivate transcription of the apoII gene upon hormone withdrawal. In this model, CHX treatment leads to reactivation of transcription by blocking synthesis of the repressor. The data are also consistent with a more indirect role of CHX: CHX may lead to the loss not of a repressor but of a third component that is necessary for the regulation of repressor function. One implication of these findings is that loss or mutational inactivation of repressor function could lead to constitutive estrogen-independent expression of a gene that is normally under strict hormonal control. This may be relevant, for example, in the loss of growth control in breast cancer or in the progression to hormone-independent growth states. The LMH/2 cell lines should prove useful for the analysis of steps leading to deactivation of estrogen receptor-mediated transcription. These cell lines will also facilitate other studies of estrogen-regulated avian liver gene expression by providing a homologous cell line.

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