MOLECULAR AND CELLULAR BIOLOGY, July 1986, p. 2334–2346
0270-7306/86/072334-13$02.00/0

Vol. 6, No. 7

Nucleotide Sequences Required for the Regulation of a Rat α_{2u}-Globulin Gene by Glucocorticoids

WILLIAM R. ADDISON AND DAVID T. KURTZ*

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Received 24 January 1986/Accepted 3 April 1986

α_{2u}-Globulin is a rat protein of as yet unknown function whose synthesis can be induced by glucocorticoids and several other hormones. Induction by glucocorticoids is a secondary response to the hormone: protein synthesis is required before the hormone can exert its stimulatory effect on α_{2u}-globulin transcription. We have used the linker-scanning mutagenesis procedure, followed by transfer of the mutant genes into mouse L-cells for analysis of their phenotype, to determine sequences within a cloned α_{2u}-globulin promoter that are required for its regulation by glucocorticoids. Mutations between positions −115 and −160 abolish or greatly reduce the inducibility of α_{2u}-globulin by the hormone. Mutations just upstream from this region, between positions −177 and −220, have an opposite effect; they increase induction two- to fourfold.

Steroid hormones are important regulators of gene expression in many organisms. In general, these hormones exert their effects at the transcriptional level, inducing or repressing the synthesis of specific mRNAs (reviewed in references 1, 61). These effects can be either direct or indirect. A direct or primary response is characterized by insensitivity of mRNA induction to inhibitors of protein synthesis and by a rapid increase in the transcription of the inducible gene (62). In the best-studied example of a primary response, the induction of mouse mammary tumor virus (MMTV) transcription by glucocorticoids, the hormone receptor complex binds to specific DNA sequences within the provirus and, by a still unknown mechanism, stimulates transcription from the viral promoter (reviewed in references 47, 48).

Indirect or secondary responses to steroid hormones require protein synthesis for transcription of the induced RNA and usually shows a lag between administration of the hormone and initiation of the response. The classical example of such a response is the induction by ecdysterone of the late puffs on the chromosomes of *Drosophila* salivary glands (reviewed in reference 46). The late puffs, unlike the early puffs, do not appear if protein synthesis is inhibited when the glands are first treated with the hormone. It seems likely that during the lag period a regulatory protein(s) is synthesized in response to the hormone. This protein is required for the expression of the secondary response (2, 13). This scheme, though reasonable, remains a conjecture. In no system have the regulatory proteins been purified or their genes cloned. A first step towards isolating such a regulatory protein would be the identification of sequences associated with the gene which are essential for hormonal regulation. These sequences are likely to be binding sites for the hormone-induced regulatory protein(s). In this paper we report the identification of DNA sequences required for the induction of transcription from a rat α_{2u}-globulin promoter, a promoter indirectly regulated by glucocorticoids.

α_{2u}-Globulin is a small protein of unknown function abundant in the urine of adult male rats but absent from that of females. Urinary α_{2u}-globulin is made in the liver. The hormonal regulation of hepatic α_{2u}-globulin synthesis has been studied in great detail (reviewed in references 15, 31, 49). Androgens, glucocorticoids, growth hormone, and insulin are required for α_{2u}-globulin synthesis in vivo, while estrogens strongly inhibit synthesis. Runoff transcription experiments with isolated rat liver nuclei show that the steroid hormones regulate synthesis at the level of transcription (27). Protein synthesis is required for the induction of α_{2u}-globulin by glucocorticoids (11). Recently, α_{2u}-globulin has been found in a number of secretory organs of the rat including the salivary, lachrymal, mammary, and preputial glands (21; 33; J. J. MacInnes, E. S. Nozik, and D. T. Kurtz, submitted for publication). Each organ is characterized by a unique pattern of α_{2u}-globulin isoelectric variants and mode of hormonal regulation, likely the result of different α_{2u}-globulin gene sets being expressed in each tissue.

The α_{2u}-globulins are encoded by a family of 20 to 25 genes clustered on chromosome 5. A number of these genes have been isolated (28). Two of the genes, each flanked by several kilobase pairs of noncoding sequence, were shown to retain their responsiveness to glucocorticoids when transfected into mouse L cells (29). Inducibility was not lost when the flanking sequences of one of the genes, designated 91, were trimmed to 235 base pairs (bp) at the 5′ end and 400 bp at the 3′ end. Fusion of the first 400 bp of α_{2u}-globulin coding sequence together with 500 bp of 5′-flanking sequence to the herpes simplex virus thymidine kinase gene rendered the kinase inducible by glucocorticoids (32). Thus, the nucleotide sequences required for the regulation of α_{2u}-globulin by glucocorticoids have been restricted to 235 bp of 5′-flanking sequence. The rat organs in which gene 91 is normally expressed is not yet known. Its sequence and the protein isoform for which it encodes are most similar to α_{2u}-globulin genes expressed in the salivary gland (unpublished results).

In the present study we have mutagenized this 235-bp region by the linker-scanning strategy developed by McKnight and Kingsbury (37). The effect of these mutations on the induction of α_{2u}-globulin mRNA was assayed in L cells stably transfected with the mutant genes. Mutations between −115 and −160 (relative to the transcription initiation site) abolish or significantly reduce induction by the hormone. We propose that these sequences are the binding site for a regulatory protein induced by glucocorticoid treatment of the cells. Mutations just upstream from this region, between −177 and about −200, have an opposite effect; they increase the gene's inducibility by the hormone two- to

* Corresponding author.

fourfold. We believe this to be the first time sequences essential for the secondary response of a gene to a steroid hormone have been identified.

In the course of our experiments we noticed a peculiar sensitivity of α_{2u}-globulin induction to inhibition by cycloheximide. Concentrations of cycloheximide that almost completely abolish α_{2u}-globulin induction had little effect on total cellular protein synthesis. This effect was not observed when protein synthesis was inhibited by emetine. Inhibition of α_{2u}-globulin induction by emetine closely paralleled its effect on overall protein synthesis.

MATERIALS AND METHODS

Materials. Unless otherwise indicated, all enzymes were purchased from New England Biolabs. Sigma Chemical Co. supplied hormones, cycloheximide, and emetine. Tissue culture medium, bovine calf serum, and Geneticin were supplied by GIBCO Laboratories. Guanidinium thiocyanate was obtained from Fluka AG. Radiolabeled compounds were purchased from Amersham Corp. SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), TBE (90 mM Tris-borate [pH 8.0], 2 mM EDTA), and Denhardt solution were prepared as described in Maniatis et al. (35). RU486 was the generous gift of Roussel Uclaf SA.

Mutagenesis. An outline of the mutagenesis procedure is shown in Fig. 3. A library of deletions, extending into the promoter from the α_{2u}-globulin structural gene (3′ deletions), was made by Bal 31 nuclease digestion from the *Sal*I site of plasmid p91FHS. A 10-μg portion of plasmid DNA was digested with SalI. The DNA was precipitated with ethanol and dissolved in 300 μl of buffer containing 12 mM $CaCl_2$, 12 mM $MgCl_2$, 20 mM Tris hydrochloride (pH 8), 1 mM EDTA, 0.6 M NaCl, and 3 U of Bal 31 nuclease and incubated at 15°C. After incubation for 5, 10, and 15 min, 100-μl samples of the mixture were added to a tube containing 50 μl of phenol-$CHCl_3$ (1:1) and 10 μl of 0.25 M EDTA, and the tube was vortexed and then centrifuged briefly. The organic phase from each tube was discarded, the aqueous phase was extracted three times with $CHCl_3$, and the DNA was precipitated with ethanol. To ensure that the ends of the DNA fragments were blunt, the DNA from each time point was dissolved in 60 μl of 10 mM Tris hydrochloride (pH 7.5), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 60 mM NaCl, 0.1 mM concentrations of each of the four deoxynucleoside triphosphates, and 5 U of the Klenow fragment of DNA polymerase I and incubated for 30 min at 37°C. *Xho*I linkers were added to the DNA fragments, and the plasmids were circularized as described by Maniatis et al. (35). Several sets of deletions were made in the course of this work, some with an *Xho*I linker with the sequence CCTCGAGG (Amersham) and the remainder with the sequence TCTCGAGA (Worthington Diagnostics). Analogously, deletions from the opposite end of the promoter region (5′ deletions) were prepared by Bal 31 nuclease digestion from the *Xba*I site of plasmid p91ZBK (see Fig. 3).

The plasmids were transformed into the DH1 strain of *Escherichia coli* as described by Hanahan (22). Single colonies were picked, 100-ml cultures were grown, and plasmid DNA was isolated by the boiling method (35). The endpoint of each deletion was determined by sequencing the promoter DNA adjacent to the *Xho*I linker by the method of Maxam and Gilbert (36).

Plasmids carrying linker-scanning mutations of the α_{2u}-globulin promoter were assembled from three fragments of DNA (see Fig. 3): the approximately 1.2-kilobase *Pvu*I-*Xho*I fragment of the appropriate 3′ deletion, containing part of the β-lactamase gene and the distal portion of the α_{2u}-globulin promoter; the approximately 4-kilobase *Xho*I-*Bam*HI fragment of the desired 5′ deletion, containing the proximal portion of the promoter and the α_{2u}-globulin structural gene; and the 1.5-kilobase *Pvu*I-*Bam*HI fragment of the vector pOH203-33 (a derivative of pAT153 [55]) containing the complementary portion of the β-lactamase gene. A 1-μg amount of each of the parental plasmids was digested with the appropriate restriction enzymes, and the resulting DNA fragments were separated by agarose gel electrophoresis. The desired fragments were extracted from the gel and combined in 50 μl of a buffer containing 50 mM Tris hydrochloride (pH 7.4), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM spermidine, 1 mM ATP, and 400 U of T4 DNA ligase. After incubation at 15°C for 16 h the ligation products were transformed into DH1 as described above. Ampicillin-resistant colonies were picked and plasmid DNA was isolated from 100-ml cultures. The structure of each mutant was checked by restriction enzyme analysis. An intact copy of α_{2u}-globulin gene 91 (28), from the *Hin*dIII site 500 bp upstream from the gene to the *Bam*HI site 300 bp downstream, was inserted in the same vector to serve as an intact control (plasmid WT91).

Transfection and induction procedures. Ltk^- $aprt^-$ cells (59) were transfected by the method of Wigler et al. (59). The day before the transfection confluent L cells were split 1:20 in Dulbecco modified minimum essential medium plus 10% calf serum. Each 100-mm plate was treated with a calcium phosphate precipitate containing 10 μg of linker-scanning mutant plasmid DNA, 1 μg of the neomycin resistance plasmid pKOneo (D. Hanahan, Cold Spring Harbor Laboratory), and 20 μg of L-cell carrier DNA. The precipitate was incubated with the cells for 12 h. The medium was replaced and the cells were incubated for a further 24 h. They were then refed with selective medium containing 800 μg of Geneticin (50% G418 sulfate) per ml. Clones of resistant cells could be seen in 2 weeks, usually 5 to 20 per plate. The efficiency of transfection could be increased 5- to 10-fold by treating the cells with Dulbecco modified minimum essential medium-glycerol (3:1) for 1 min immediately after removal of the precipitate. After each plate was washed twice with Dulbecco modified minimum essential medium, they were treated as above. After 3 weeks of selection the clones on each plate were trypsinized, replated, and grown to confluence. Each plate was split 1:8 and incubated for 2 days before hormone treatment.

Four of the eight plates were incubated in medium containing 2 μM dexamethasone and 1 μg of bovine insulin per ml. The other four plates served as controls. The plates were refed 2, 5, and 7 days later. After 9 days, total cellular RNA was prepared from three plates of hormone-treated cells and three plates of untreated cells. Cellular DNA was prepared from the one plate of treated cells, and the cells from the remaining untreated plate were stored frozen in liquid nitrogen.

Preparation and analysis of cellular RNA and DNA. Total cellular RNA was prepared by a slight modification of the guanidinium thiocyanate-CsCl procedure described in Maniatis et al. (35). Cellular DNA was prepared as described by Wigler et al. (60). The copy number of α_{2u}-globulin genes in DNA from each transfected cell line was determined by Southern blot analysis (35).

$Poly(A)^+$ RNA was prepared from total cellular RNA as described in Maniatis et al. (35). Northern blots were done by the formaldehyde method as described by the same

authors except that a synthetic oligodeoxynucleotide (24 bases long, complementary to positions +52 to +75 of α_{2u}-globulin mRNA) was labeled and used as a probe. The blot was prehybridized at 54°C for 2 h in 50 ml of buffer containing 6× SSC, 10× Denhardt solution, and 50 μg of yeast tRNA per ml. Probe was added (20 pmol, 5×10^7 cpm/μg) and incubation was continued at 54°C for 12 h. The filter was washed with 6× SSC at 25°C to remove excess probe and then at 60°C in 6× SSC–1 mM sodium pyrophosphate for 5 min.

S1 nuclease assay. An S1 nuclease assay was used to quantitate the α_{2u}-globulin mRNA sequences present in total RNA from the transfected cell lines. The probe was a 40-base synthetic oligodeoxynucleotide with the same sequence as the coding strand of α_{2u}-globulin gene 91 between positions −8 and +32. The probe was labeled with [^{32}P]PO_4 in the following manner. A 35-pmol portion of the oligonucleotide was dissolved in 7 μl of H_2O and heated to 70°C for 2 min. A 1-μl amount of a buffer containing 0.5 M Tris hydrochloride (pH 7.6), 0.1 M $MgCl_2$, 1 mM spermidine, and 1 mM EDTA, 1 μl of 50 mM dithiothreitol, and 1 μl of T4 polynucleotide kinase (10 U; Pharmacia Fine Chemicals, Inc.) were added to the oligonucleotide solution, and this mixture was added to a tube in which 600 μCi of [γ-^{32}P]ATP (>5,000 Ci/mmol) had been dried. After incubation at 37°C for 30 min the reaction mixture was heated to 70°C for 2 min and cooled, 1 μl of kinase was added, and the mixture was incubated for 30 min more at 37°C. A 5-μl portion of 0.1% sodium dodecyl sulfate and 35 μl of H_2O were added to the tube. The labeled probe was separated from ATP by two successive rounds of "spin dialysis" (26) through Bio-Gel P30. The probe was diluted to 0.065 pmol/μl in 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA and stored at 4°C. The specific activity of the probe was 3×10^8 to 5×10^8 dpm/μg.

For each S1 protection assay 25 μg of RNA was dried in a Microfuge tube (Beckman Instruments, Inc.). The RNA was dissolved in 20 μl of buffer containing 10 mM Tris hydrochloride (pH 7.4), 0.4 M NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 0.125 pmol of oligonucleotide probe. After heating to 90°C for 2 min the tubes were transferred to a 65°C heating block and incubated for 1 h. Each tube was taken from the heating block, and 0.25 ml of ice-cold buffer containing 30 mM sodium acetate (pH 4.8), 0.6 M NaCl, 1 mM $ZnSO_4$, 5% glycerol, 20 μg of denatured calf thymus DNA per ml, and 200 U of S1 nuclease (Pharmacia) was added. After incubation at 25°C for 1 h 75 μl of a stop mix containing 2 M NH_4 acetate, 50 mM EDTA, and 100 μg of calf thymus DNA per ml was added to the nuclease digest, followed by 1 ml of ethanol. The DNA was precipitated at −20°C, collected by centrifugation, washed with 1 ml of ethanol, and dried. Each sample was dissolved in 7 μl of loading buffer (7 M urea, 0.01% xylene cyanol, 0.01% bromophenol blue in TBE), heated to 65°C for 2 min, and applied to a denaturing gel containing 15% polyacrylamide (15 by 20 by 0.05 cm). When the bromophenol blue reached the bottom, electrophoresis was stopped and the gel was autoradiographed overnight at −70°C with an intensifying screen. With the autoradiogram as a guide, the fragment of gel containing the probe protected from nuclease digestion by α_{2u}-globulin mRNA was excised and added to 3 ml of Aquasol (New England Nuclear Corp.), and its radioactivity was determined in a scintillation counter.

S1 digestion of α_{2u}-globulin mRNA-probe hybrids produced a cluster of protected fragments rather than a single discrete band (e.g., see Fig. 4, lane a). Similar clusters are often seen with larger probes but are less apparent because of the lower resolution of the gels used. This imprecision in S1 nuclease digestion was not reduced by altering the enzyme concentration, temperature of digestion, or ionic strength or by including polyamines in the digestion buffer.

Inhibition of protein synthesis. The effect of cycloheximide and emetine on α_{2u}-globulin mRNA induction was determined by treating two subconfluent plates of WT(91)-1 cells with hormones and the inhibitor at the indicated concentration. Incubation was for 3 days with the medium changed daily. Cultures incubated with <0.2 μg of cycloheximide or 0.1 μg of emetine per ml became confluent within this time. Cellular RNA was prepared and assayed for α_{2u}-globulin transcripts as described above.

To assay the effect of the inhibitors on protein synthesis, plates of WT(91)-1 cells were prepared as above but after 60 h of incubation 300 μCi of [^{35}S]methionine (1,115 Ci/mmol) was added to each plate. Twelve hours later the plates were washed three times with cold phosphate-buffered saline and the cells were removed by scraping. The cells were suspended in 1 ml of a buffer containing 10 mM Tris hydrochloride (pH 7.4), 25 mM NaCl, and 5 mM $MgCl_2$. A 100-μl portion of 2% Triton X-100–2% deoxycholate solution was added to each sample, and the cells were broken by sonication. The cell extracts were centrifuged for 30 min at 140,000 × *g*. A 15-μl amount of the supernatant was spotted onto small squares of Whatman 3MM paper. The squares were dropped into cold 15% trichloroacetic acid (TCA), washed in three changes of TCA, and then heated in TCA to 100°C for 5 min. The squares were washed twice with cold TCA and twice with ethanol and dried before scintillation counting.

RESULTS

α_{2u}-Globulin gene expression in transfected L cells. In earlier experiments we showed that transfected α_{2u}-globulin genes were expressed and regulated by glucocorticoids in mouse L cells (29). A number of experiments were done to determine how well the induction of α_{2u}-globulin by glucocorticoids in L cells mimicked this induction in vivo and in a rat cell line of hepatic origin. One cell line, WT(91)-1, was used in these experiments. This line was derived from a mass culture of L cells transfected with plasmid WT91, a plasmid carrying an intact copy of α_{2u}-globulin gene 91. WT(91)-1 contains an average of four to five copies of the α_{2u}-globulin gene per cell.

The dependence of α_{2u}-globulin mRNA accumulation in WT(91)-1 cells upon dexamethasone concentration is shown in Fig. 1A. At 10^{-9} M dexamethasone there is no detectable induction of α_{2u}-globulin RNA. As the concentration of hormone was raised, the degree of induction steadily increased. Even at very high concentrations (10^{-5} M) inducibility had only begun to plateau. At these high glucocorticoid concentrations, however, the growth of the L cells was greatly reduced. Consequently, the concentration of dexamethasone was fixed at 2×10^{-6} M in subsequent experiments. Insulin was included in the medium of cells treated with dexamethasone to reduce the latter's toxic effect on L cells. Insulin was not required for the induction of α_{2u}-globulin mRNA in transfected cells (data not shown). In this respect α_{2u}-globulin expression in L cells differs from its expression in more physiologically relevant cells. In rat liver (50) and A49 cells, a hepatoma cell line that synthesizes α_{2u}-globulin (58), full α_{2u}-globulin induction is dependent on insulin.

The kinetics of α_{2u}-globulin mRNA induction by dexamethasone in cell line WT(91)-1 is shown in Fig. 1B.

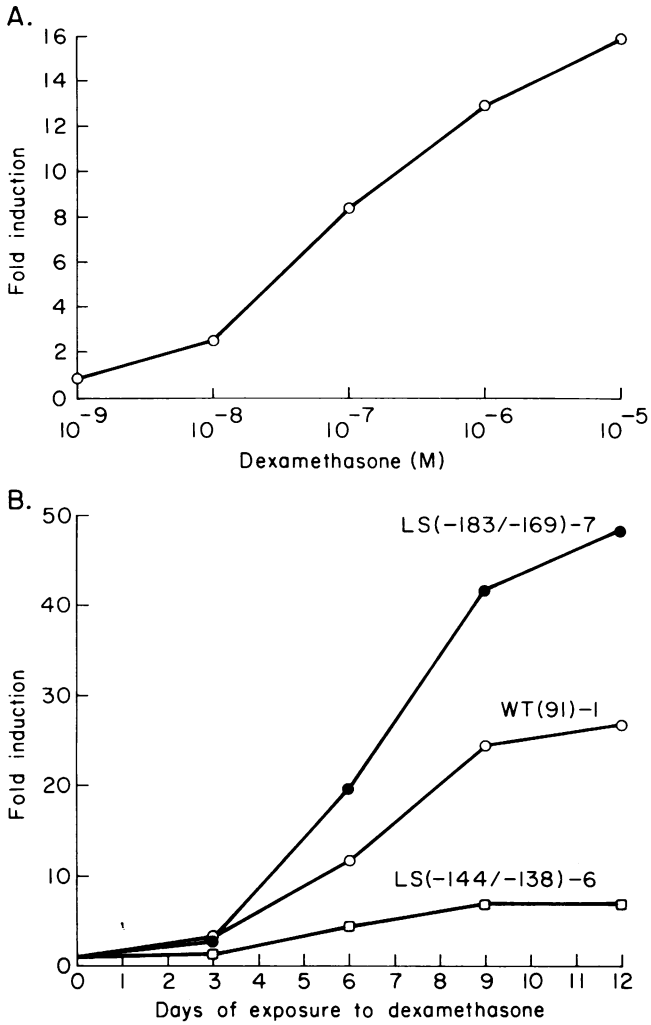

FIG. 1. Induction of α_{2u}-globulin mRNA in transfected mouse L cells. (A) Dose-response curve for α_{2u}-globulin mRNA induction by dexamethasone. Cells of mass culture (WT(91)-1 were treated with the indicated concentrations of dexamethasone for 9 days. Total RNA was isolated from the cells and assayed for α_{2u}-globulin transcripts by the S1 nuclease protection procedure (see text). The induction of α_{2u}-globulin mRNA is expressed as the ratio of α_{2u}-globulin transcripts present in 25 μg of RNA from hormone-treated cells to the transcripts present in 25 μg of RNA from untreated cells. (B) Kinetics of α_{2u}-globulin induction. Lines of transfected L cells containing wild-type α_{2u}-globulin genes [WT(91)-1], mutant genes of reduced inducibility [LS(-144/-138)-6], and hyperinducible mutant genes [LS(-183/-169)-7] were treated with 2×10^{-6} M dexamethasone. After 3, 6, 9, and 12 days of induction, total cellular RNA was prepared from each cell line, assayed for its α_{2u}-globulin mRNA content, and compared with the α_{2u}-globulin mRNA content of cells not treated with the hormone.

The induction is very slow. For the first 2 to 3 days after hormonal stimulation little or no α_{2u}-globulin mRNA accumulates above the basal level. This is an exceptionally long lag period. The lag periods for 16 proteins inducible by steroids have been tabulated by Palmiter et al. (41). Only one, β-glucuronidase of mouse kidney, has a lag time (36 h) comparable to that of α_{2u}-globulin; for the others the lags are much shorter. After the lag period α_{2u}-globulin mRNA accumulates at a linear rate until a steady-state level is achieved after 9 to 12 days of induction. The level of α_{2u}-globulin mRNA attained in fully induced WT(91)-1 cells is about one-tenth that found in adult male rat liver (see Fig. 5). From the induction curve, the half-time for the accumulation of α_{2u}-globulin RNA was determined to be about 3.5 days.

Cycloheximide is a potent inhibitor of α_{2u}-globulin mRNA accumulation. In cultured rat hepatocytes (11) and in A49 cells (58) induction of α_{2u}-globulin by glucocorticoids requires ongoing protein synthesis. We wished to confirm this requirement in L cells. In the course of these experiments it became clear that α_{2u}-globulin mRNA induction is extraordinarily sensitive to inhibition by cycloheximide. Because of the induction's long lag period the transfected cells had to be cultured for 3 days with the hormone before induction was detectable. Three days of exposure to cycloheximide at the concentrations usually used for short-term studies (2 to 20 μg/ml) proved toxic to the cells. We sought, therefore, to determine the lowest cycloheximide concentration that would inhibit the induction. Figure 2A shows the effect of cycloheximide concentration on α_{2u}-globulin mRNA accumulation in WT(91)-1 cells. At 0.01 μg of cycloheximide per ml the induction was reduced to half the control value; by 0.1 μg/ml induction had fallen to a low plateau level. General protein synthesis, as measured by [^{35}S]methionine incorporation into acid-precipitable material, was about five times less sensitive to cycloheximide. A 0.05-μg/ml concentration of cycloheximide was required to inhibit protein synthesis by 50%. The shape of the inhibition curve, however, suggests that a small part of the protein synthesis capacity of L cells may be more sensitive to cycloheximide than the remainder. Further work is required to explore this interesting possibility.

The high sensitivity of α_{2u}-globulin induction to inhibition by cycloheximide seems to be a specific property of this inhibitor. When protein synthesis was inhibited by increasing concentrations of emetine, also an inhibitor of peptide chain elongation, the decline of α_{2u}-globulin mRNA accumulation matched the inhibition of general protein synthesis (Fig. 2B).

Two other features of Fig. 2A should be noted. First, the basal level of α_{2u}-globulin mRNA as well as the induced level were reduced by low concentrations of cycloheximide. This suggests that some factor involved in both basal and induced transcription is unusually sensitive to cycloheximide. This factor is unlikely to be the glucocorticoid-inducible regulatory protein because a promoter mutant (LS-137/−125) that abolishes induction, presumably by destroying the regulatory protein's binding site, does not reduce basal expression (see Fig. 4, lanes e and f) or alter the sensitivity of basal expression to inhibition by cycloheximide (not shown). Second, even at high cycloheximide concentrations (0.2 μg/ml) a very weak induction by dexamethasone can still be detected. This could represent stabilization of α_{2u}-globulin transcripts in hormone-treated cells or stimulation of transcription by the hormone-receptor complex alone, in the absence of the hypothetical transcription factor. We think the latter possibility unlikely because the potent antiglucocorticoid RU486 (8), at concentrations sufficient to prevent induction by dexamethasone, has no effect on the basal level of α_{2u}-globulin expression (data not shown).

Protein synthesis is required for the maintenance of α_{2u}-globulin induction as well as its initiation. Addition of cycloheximide to an induced culture of WT(91)-1 cells resulted in an immediate, exponential decline in its α_{2u}-globulin mRNA content (Fig. 2C). The level of α_{2u}-globulin mRNA in uninduced cells also declined after cycloheximide

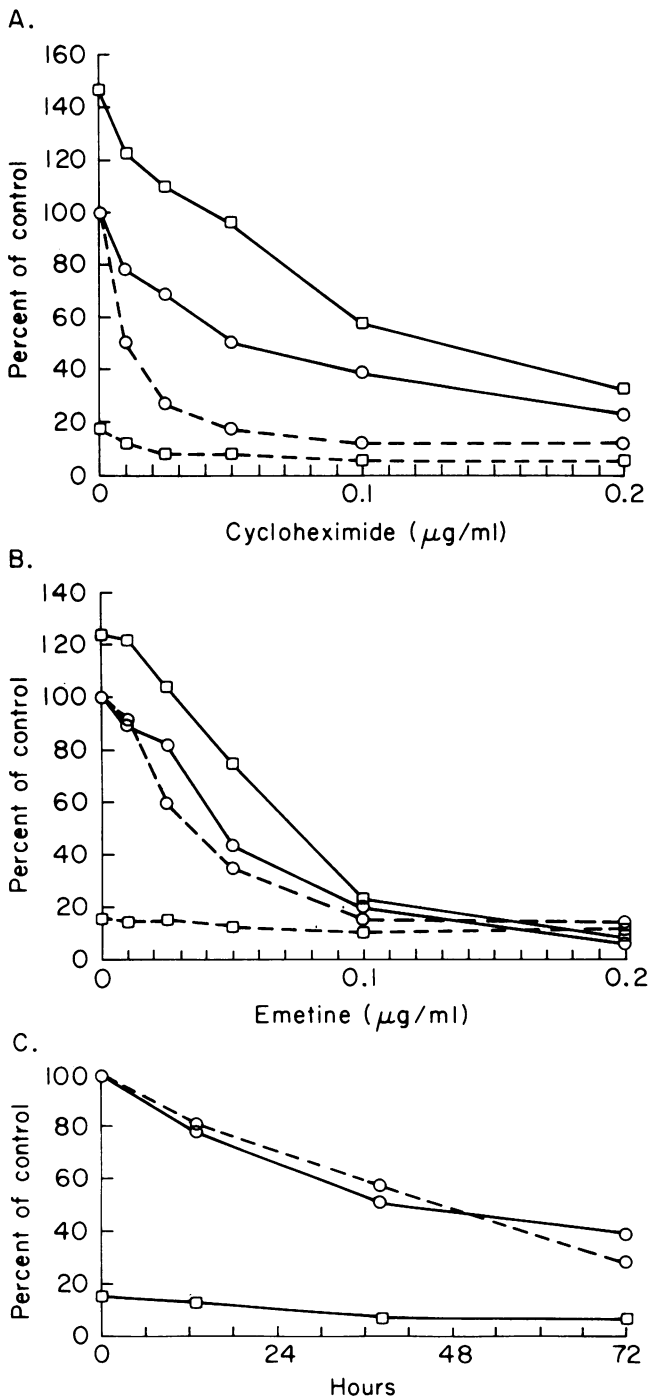

FIG. 2. Inhibition of α_{2u}-globulin induction by cycloheximide and emetine. (A) Inhibition by cycloheximide. Cultures of WT(91)-1 cells, either treated (○) or not treated (□) with 2×10^{-6} M dexamethasone, were incubated in medium containing the indicated concentrations of cycloheximide. After 3 days the cells were assayed for their ability to incorporate [^{35}S]methionine into acid-precipitable material (solid lines) and for their α_{2u}-globulin mRNA content (dashed lines) as described in Materials and Methods. Protein synthesis and α_{2u}-globulin mRNA accumulation are expressed as a percentage of the levels shown by hormone-treated cells in the absence of the protein synthesis inhibitor. (B) Inhibition by emetine. Cultures of WT(91)-1 cells were treated as above except that protein synthesis was inhibited by emetine rather than cycloheximide. (C) Deinduction of α_{2u}-globulin mRNA. WT(91)-1 cells were incubated for 8 days in the presence (○) or absence (□) of 2×10^{-6} M dexamethasone. At time zero, either the cells were washed free of the hormone (dashed line) or cycloheximide (0.1 μg/ml) was added to the medium. At the indicated times total cellular RNA was isolated and assayed for α_{2u}-globulin mRNA by the S1 nuclease protection precedure (see text). The levels of α_{2u}-globulin mRNA are expressed as a percentage of that present in hormone-treated cells at time zero.

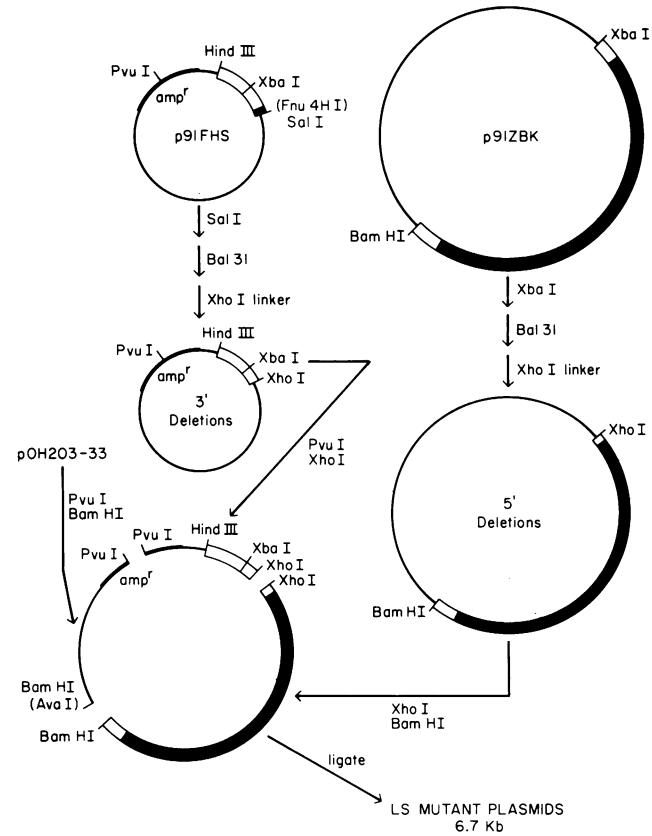

FIG. 3. Construction of linker-scanning mutants of α_{2u}-globulin gene 91. Bal 31 nuclease was used to generate two nested series of deletions, one that originated from a SalI site within the coding sequence (3′ deletions) and a second that originated from an *Xba*I site upstream to the promoter (5′ deletions). 5′ and 3′ deletions whose endpoints were separated by approximately 8 bp were joined at their *Xho*I sites (8 bp) to generate a series of linker-scanning mutations in the α_{2u}-globulin promoter. The dark box represents α_{2u}-globulin coding sequence; the open boxes, α_{2u}-globulin flanking sequence; thin lines, plasmid vector sequences; and heavy lines, the β-lactamase gene of pBR322. Restriction sites that have been changed to sites with a new enzyme specificity are shown in parentheses. Kb, kilobases.

treatment. Cycloheximide could cause the observed decline in the steady-state level of α_{2u}-globulin mRNA in two ways: by inhibiting transcription of the α_{2u}-globulin gene or by promoting the degradation of α_{2u}-globulin mRNA. The latter possibility is unlikely because the level of α_{2u}-globulin mRNA declined at the same rate when induced WT(91)-1 cultures were either treated with cycloheximide or deprived of dexamethasone (Fig. 2C).

Mutagenesis. The linker-scanning method (37) was used to mutagenize 235 bp of sequence upstream to the α_{2u}-globulin transcription start site. This method was chosen because,

TABLE 1. Properties of the linker-scanning mutants

Mutant	Linker sequence[a]	Net deletion or insertion (bp)
LS-38/-31	A	0
LS-44/-39	A	+2
LS-44	A	+7
LS-58/-51	C	0
LS-70/-61	A	−2
LS-84/-61	A	−16
LS-101/-98	B	+4
LS-116/-107	B	−2
LS-137/-125	A	−5
LS-144/-138	B	+1
LS-152/-144	B	−1
LS-158/-156	A	+5
LS-176/-169	B	0
LS-183/-162	B	−14
LS-183/-169	B	−7
LS-183/-173	A	−3
LS-197/-189	A	−1
LS-222/-214	B	−1
LS-235/-214	B	−14

[a] The linker sequences are: A, CCTCGAGG; B, CCTCGAGA; C, TCTCGAGA.

unlike point mutagenesis, fairly long sequences can be efficiently saturated with mutants. In addition, linker-scanning mutants, unlike deletions, retain the spacing of promoter elements found in the "wild-type" sequence.

The protocol used to generate the mutants is illustrated in Fig. 3. Briefly, two deletion mutants of the promoter, one lacking distal sequences (5′ deletion) and the other lacking proximal sequences (3′ deletion) are joined by an 8-bp *Xho*I linker. In the ideal case the parental deletions would be chosen such that the linker exactly replaces 8 bp of promoter sequence. In most cases, however, the two deletions were greater or less than 8 bp apart, creating linker-scanning mutants carrying small deletions or insertions. The properties of the mutants used in this study are presented in Table 1.

S1 nuclease protection assay. The determination of α_{2u}-globulin mRNA levels in total RNA from many different transfected cell lines required a rapid, simple, and sensitive assay. A modification of the S1 nuclease protection assay (7) best satisfied these criteria. The only departure from standard procedures was the use of a short synthetic oligodeoxynucleotide as a probe. The S1 assay provided both a qualitative and a quantitative estimate of the amount of α_{2u}-globulin mRNA present in samples of total cellular RNA. The results of a typical assay are shown in Fig. 4. The fragment of the probe protected from nuclease digestion by α_{2u}-globulin was located on the electrophoresis gel; the piece of gel containing the protected fragment was excised, and its radioactivity was determined. When total RNA from male rat liver (about 0.01% α_{2u}-globulin mRNA) was assayed, the amount of probe protected was linearly dependent on the amount of RNA over the range 0.04 to 25 μg of RNA (data not shown). There is no protection of the probe by RNA present in untransfected L cells (Fig. 4, lane b).

Unexpectedly, total RNA from L cells transfected with α_{2u}-globulin genes contained transcripts that protected the entire oligonucleotide probe from nuclease digestion (Fig. 4). The amount of this RNA is considerably greater than the amount of correctly initiated α_{2u}-globulin transcripts, is little affected by treatment of the cells with glucocorticoids, and bears no fixed relationship to the amount of correctly initiated mRNA. The structure of the aberrant transcripts is unknown. They may initiate at sites in the transfected DNA flanking the α_{2u}-globulin genes and "run through" the α_{2u}-globulin promoter. The Northern blot shown in Fig. 5 is consistent with this suggestion. Male rat liver poly(A)$^+$ RNA (lane a) contains a single, abundant species of α_{2u}-globulin mRNA. An RNA of the same size is present in poly(A)$^+$ RNA from cell line WT(91)-1. However, the transfected cell RNA also contains a large, heterogeneous population of RNAs longer than the authentic α_{2u}-globulin messenger. These RNAs are present both before and after hormone treatment (lanes b and c) and can be detected in total cellular RNA (lanes d and e).

Effects of linker-scanning mutations on induction of α_{2u}-globulin expression by dexamethasone. Plasmid DNA from each linker-scanning mutant and a plasmid conferring resistance to the antibiotic G418 were cotransfected into mouse L cells. Mass cultures of cells resistant to the antibiotic (10 to 20 clones per culture in early experiments, 50 to 200 clones per culture later) were prepared and treated with dexamethasone for 9 days. Total cellular RNA was isolated from induced and uninduced cells and assayed for α_{2u}-globulin mRNA as described earlier. The autoradiogram resulting from a typical set of assays is shown in Fig. 4. For each pool of transfectants, the ratio of α_{2u}-globulin mRNA present in 25 μg of total RNA isolated from hormone-treated cells to that present in 25 μg of RNA from untreated control cells defines the induction displayed by that cell line.

It should be noted that expression of genes transfected

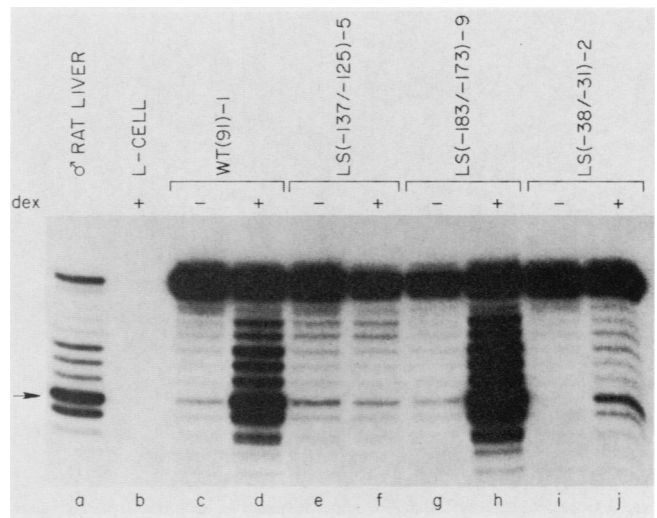

FIG. 4. S1 nuclease assay of α_{2u}-globulin mRNA induction in cell lines containing wild-type or mutant α_{2u}-globulin genes. Total cellular RNA from male rat liver (lane a, 1.7 μg of liver RNA, 25 μg of L-cell RNA), dexamethasone-treated L cells (lane b, 25 μg), and from untreated and hormone-treated cultures of the indicated transfected cell lines (lanes c to j, 25 μg) was assayed for α_{2u}-globulin mRNA as described in Materials and Methods. The period of induction was 9 days. The arrow marks the position of the 32-base fragment of the probe that is protected from nuclease by correctly initiated α_{2u}-globulin mRNA. The dark band at the top of the autoradiogram is a fraction of the 40-base probe that is completely protected, probably by aberrant transcripts that run through the α_{2u}-globulin promoter. The degree of induction observed in each cell line is as follows: WT(91)-1, 24-fold; LS(-137/-125)-9, 0.7-fold; LS(-183/-173)-9, 58-fold; LS(-38/-31)-2, 8-fold. The concentration of α_{2u}-globulin mRNA in male rat liver RNA is about seven times higher than in RNA from fully induced WT(91)-1 cells.

into tissue culture cells is strongly influenced by their site of integration into the host chromosome (19). It is often difficult to distinguish these "position effects" from the effect of interest, that of the mutations on α_{2u}-globulin gene expression. We tried to average the position effects by using mass cultures of transfected cells, each a pool of many independent clones, and by analyzing α_{2u}-globulin induction in a number (usually four to five) of mass cultures containing each mutant.

Figure 6 summarizes the effects of the linker-scanning mutants on the induction of α_{2u}-globulin mRNA by dexamethasone. Hormone treatment increased expression of the intact α_{2u}-globulin gene about 20-fold (Fig. 5, lanes c and d). This induction was abolished in cells transfected with the LS-137/-125 mutant gene. Five of the seven mass cultures containing this mutant show no induction at all (Fig. 5, lanes e and f); in the remaining cultures induction was very weak (two- to threefold). The basal level of transcription is unaffected in cells bearing this mutant. This is an important observation because we wish to distinguish mutants that alter the gene's hormonal regulation from mutants that reduce its basic promoter activity. All of the transfected cells contain a low but detectable amount of α_{2u}-globulin mRNA in the absence of added hormone (Fig. 4, lanes c, e, g, and i). Only one mutation, LS-38/-31, reduced this basal level of expression. The other mutants had no consistent effect on the basal level of α_{2u}-globulin mRNA accumulation, although it is difficult to be certain of this because there were considerable differences in basal transcription among cell lines, probably the result of the position effects discussed above. We conclude that, with one exception, mutants altering α_{2u}-globulin induction do so by altering the gene's regulatory sequences, not sequences required for basic promoter function.

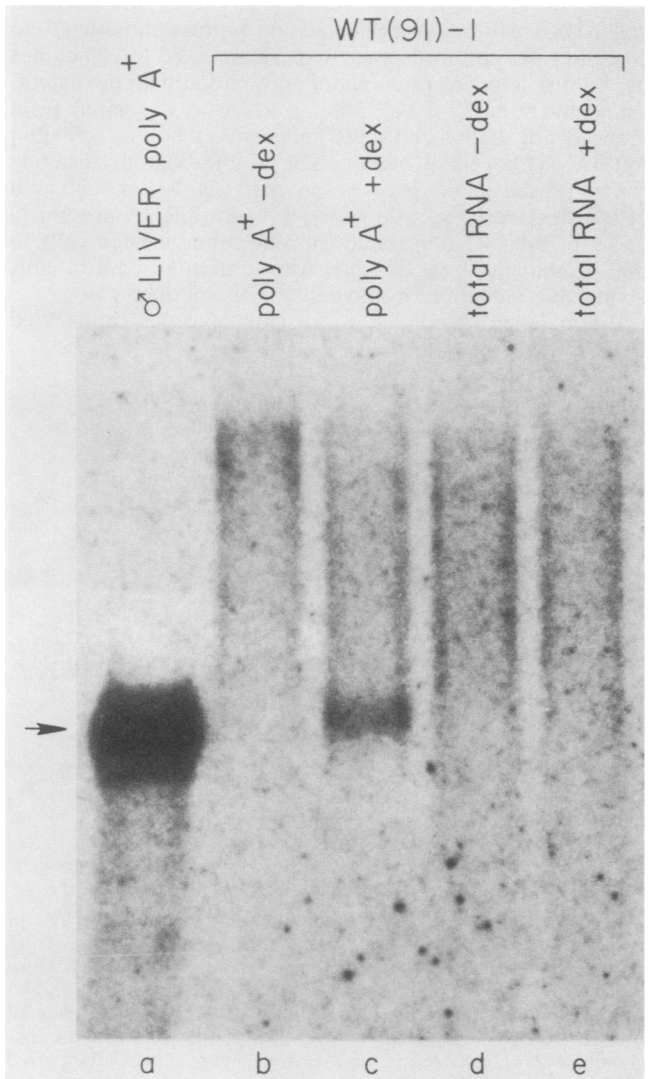

FIG. 5. Hybridization of an α_{2u}-globulin probe to RNA from rat liver and a transfected L-cell line. A 0.2-μg portion of poly(A)$^+$ male liver RNA (lane a), 5 μg of poly(A)$^+$ RNA from uninduced [lane WT(91)-1] cells (lane b), and cells induced with dexamethasone for 3.5 days (lane c), and 25 μg of total RNA from uninduced (lane d) and induced (lane e) cells were electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde, blotted onto nitrocellulose, and probed as described in Materials and Methods. The arrow marks the position of correctly initiated α_{2u}-globulin mRNA.

LS-137/-125, therefore, marks the location of sequences essential for regulation of the gene by glucocorticoids. Three contiguous mutants distal (i.e., further from the transcription start site) to the "null" mutant, LS-144/-138, LS-152/-144, and LS-158/-156, reduce inducibility to four- to sevenfold. This is significantly different from the wild-type level of inducibility (Student's t test, $P = 0.01$). Mutations in this class reduce the rate at which α_{2u}-globulin mRNA accumulates in transfected cells and lower the steady-state level of transcripts achieved after prolonged hormone treatment (Fig. 1B). Mutations in sequences further upstream are highly inducible. Therefore, we place the distal margin of the regulatory region at about −160. The proximal margin of the region is more difficult to define. The inducibility of mutation LS-116/-107, although much higher than that of the null mutant, is about half that shown by the wild-type gene. We doubt that this decrease is significant since most of the mutants between −20 and −100 are also less inducible than the wild type. We tentatively place the proximal border of the regulatory sequence between −110 and −120. This coincides with the border of a region of dyad symmetry present in the regulatory region and with the border of sequences conserved among α_{2u}-globulin promoters (see Discussion). In conclusion, the mutagenesis experiments have defined a sequence between about −115 and −160 required for α_{2u}-globulin induction by glucocorticoids. Experiments to more precisely define the borders of the glucocorticoid regulatory element are in progress.

An unexpected outcome of the mutagenesis experiments was the discovery of promoter mutants in which the inducibility by glucocorticoids is increased. This region is defined by a series of three overlapping mutants. LS-183/-162, LS-183/-169, and LS-183/-173. Cell lines containing these mutants display increased inducibility (Fig. 6). On average, the inducibility is double that of the intact gene ($P = 0.1$), but some cell lines accumulate four times the wild-type level of α_{2u}-globulin mRNA in response to dexamethasone. The S1 analysis of RNA from one "hyperinducible" cell line is shown in Fig. 4 (lanes g and h). The kinetic properties of these mutants is the converse of the down mutations discussed above: the rate of accumulation of transcripts and the final steady-state level of transcripts are both higher than those of the wild type (Fig. 1B). There is a great deal of variablity in the degree of induction displayed by cell lines transfected with these mutants. However, 12 of 18 cell lines containing these mutants were more inducible than the wild-type gene. We think, therefore, that the hyperinduc-

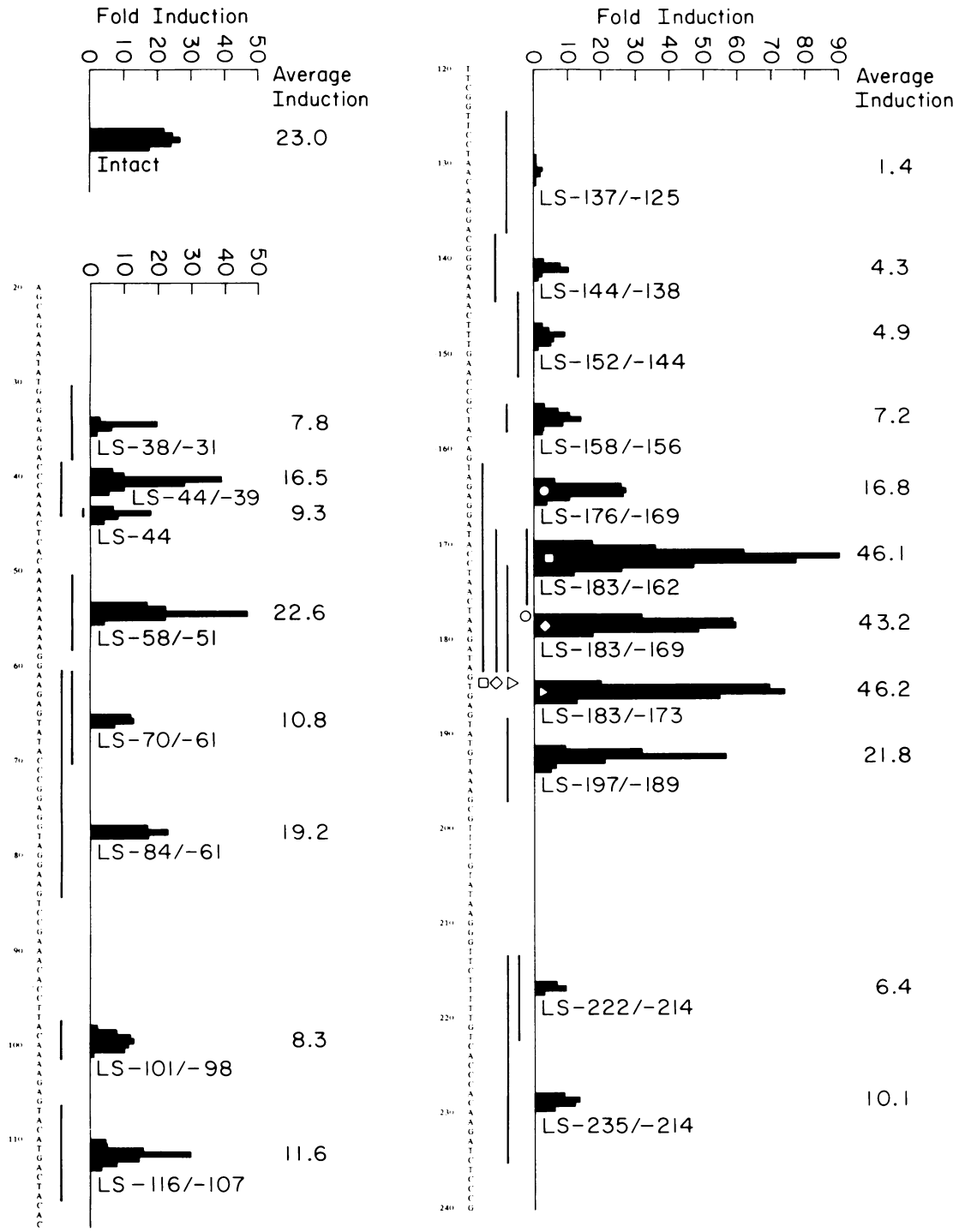

FIG. 6. Effect of linker-scanning mutations on α_{2u}-globulin induction. The DNA sequence is that of the promoter of α_{2u}-globulin gene 91 (the noncoding stand is shown). The sequence is numbered backwards from the transcription initiation site (+1). The 3′ end of the sequence is at the top. Sequences replaced by an 8-bp *Xho*I linker in the various linker-scanning mutants are marked by the adjacent vertical lines. The histograms, drawn next to sequences altered by each mutation, show the induction of α_{2u}-globulin mRNA displayed by cell lines transfected with each mutant gene. The histograms are keyed to the appropriate mutations where overlap of the mutated sequences makes the relationship ambiguous (mutants between −160 and −185). Each bar within a histogram shows the induction achieved by a single mass culture of cells transfected with a mutant α_{2u}-globulin gene after 9 days of treatment with dexamethasone. At the upper left is a histogram showing the response of mass cultures transfected with a plasmid bearing the wild-type sequence of gene 91. The average of the inductions displayed by all of the mass cultures containing each mutation is shown to the right of the corresponding histogram.

ibility is an intrinsic property of the mutants and is not due to chromosomal position effects. Since LS-183/-173 is as effective as LS-183/-162 in producing hyperinducibility, the sequences between −162 and −173 seem irrelevant to the phenomenon. This is confirmed by the normal inducibility displayed by LS-175/-169. Therefore, the proximal boundary of the "hyperinducibility region" is around −177. The distal boundary is less well defined. LS-197/-189 seems to be mildly hyperinducible, though because of the great variability in α_{2u}-globulin expression among cell lines this is not reflected in its average induction. Its upstream neighbors, mutants LS-222/-214 and LS-235/-214, show slightly reduced inducibility. Therefore, the distal border of the hyperinducibility region lies around −200.

Promoters in higher eucaryotes usually contain a "CAAT" box, a sequence related to 5′-GGC/TCAA/TCT-3′, about 80 bp upstream from their initiation sites (6, 17, 63). In the best-studied case, the promoter of the thymidine kinase gene of herpes simplex virus, linker-scanning or point mutants that destroy the thymidine kinase CAAT sequence (present in inverse orientation in this gene) greatly reduce the promoter's efficiency (37, 38). Recently this region has been shown to bind a factor required for efficient transcription in vitro (24). The α_{2u}-globulin gene 91 promoter contains the sequence 5′-GGCCCATAT-3′, between positions −74 and −66 Fig. 6) which we assume to be its CAAT box. Mutants LS-70/-61 and LS-84/-61 lack this sequence in whole or in part but retain normal promoter activity. The basal level of α_{2u}-globulin mRNA in cell lines containing these mutants is similar to the wild type. Either the CAAT sequence is not needed for transcription of the gene under these conditions, or we have not correctly identified the CAAT sequence.

The remaining mutants merit a few comments. LS-58/-51 destroys a conspicuous run of A · T base pairs between the gene's CAAT and TATA boxes without reducing α_{2u}-globulin mRNA accumulation. LS-44/-39 has no effect on gene expression. Inducibility is reduced in the similarly positioned LS-44, perhaps because of changes in spacing attendent to replacing a single base pair by the 8-bp linker. Finally, inducibility is low in LS-38/-31. This mutant is unique in also showing a consistently low basal level of activity compared with the wild-type gene (Fig. 4, lanes i and j). This may be due to the proximity of the mutation to the gene's TATA box, the 5′-TATAAA-3′ sequence between positions −30 and −25.

DISCUSSION

In vitro mutagenesis of a cloned DNA sequence followed by transfer of the mutants into a suitable host cell for analysis of their phenotypes is a powerful technique for identifying regulatory elements associated with eucaryotic genes. We used mouse L cells as a host cell in our study of the α_{2u}-promoter because they are known to have a functional glucocorticoid receptor and because of the efficiency with which this line can be transfected. In assessing the significance of our results it is important to know how closely the induction of α_{2u}-globulin by glucocorticoids in L cells, a fibroblast line, resembles its induction in more physiologically relevant cells. α_{2u}-Globulin induction by glucocorticoids has been studied in vivo and in cultures of α_{2u}-globulin-producing cell lines. The best in vitro model of α_{2u}-globulin induction is provided by the A49 cell line isolated by Widman and Chasin (58). This is a derivative of the rat hepatoma cell line FU5-5 that synthesizes α_{2u}-globulin in response to glucocorticoids. In almost all respects α_{2u}-globulin expression in transfected L cells closely resembles its expression in A49 cells. The kinetics of α_{2u}-globulin induction in both cell lines is identical. In each case a slow, biphasic response is seen. The size of the induction is much larger in A49 cells (500-fold) due to the very low basal level of expression seen in untreated cells. A slow induction of hepatic α_{2u}-globulin is also observed in vivo when adrenalectomized rats are treated with hydrocortisone (30). The steady-state level of α_{2u}-globulin mRNA achieved in fully induced L cells is remarkably high, 10% of that found in adult male rate liver. Finally, in L cells, as in rat liver and A49 cells, protein synthesis is required for the initiation and maintenance of α_{2u}-globulin expression.

These results suggest that α_{2u}-globulin induction in mouse L cells is very similar to its induction in A49 cells and in vivo. This has the important implication that some ubiquitous mechanism of induction is operating in all of these cases. This would not be surprising if α_{2u}-globulin expression was directly regulated by the hormone-receptor complex since transfected genes usually adopt an "active" chromatin structure (57) and glucocorticoid receptors are found in almost all tissues (3). It is somewhat surprising in a secondary response to the hormone, hinting that at least some regulatory molecules induced by glucocorticoids may have a wide distribution among tissues and among organisms. Such molecules would be important in the regulation and perhaps coordination of hormone-controlled gene networks (61). If the regulatory protein does prove to be widely distributed it is, like the glucocorticoid receptor itself, unlikely to be an agent of tissue-specific gene expression.

Linker-scanning mutagenesis of the α_{2u}-globulin gene 91 promoter revealed two sequences required for normal expression in response to glucocorticoids. Mutations in the first region, between −115 and −160, abolished or significantly reduced the inducibility of the gene. We term this sequence the glucocorticoid regulatory region. We suggest that this sequence is the binding site for a factor produced in response to glucocorticoid stimulation of the cell and required for efficient transcription from the α_{2u}-globulin promoter. This site is conserved among the 14 α_{2u}-globulin promoters we have sequenced (unpublished results). The promoters, like α_{2u}-globulin structural genes (16), are over 90% homologous. In general, nucleotide differences among the promoters are randomly distributed. However, there are few changes between −90 and −115 and the sequence between −116 and −137 is absolutely conserved. The latter sequence coincides with the sequence altered in the null mutant, LS-137/-125. The sequence data complement our mutagenesis results and emphasize the unique importance of the sequences around −130 for α_{2u}-globulin regulation.

The regulatory sequence we have identified in the α_{2u}-globulin gene 91 promoter is also conserved in sequences flanking the genes for the homologous major urinary protein (MUP) of mouse. Clark et al. (12) have sequenced two highly homologous MUP genes, one of which is a pseudogene. The α_{2u}-globulin sequence most closely resembles the MUP pseudogene showing 80% homology to 400 bp of sequence flanking the 5′ end of the MUP gene. The divergence between the rat and mouse promoters is due to scattered base pair substitutions and small insertions and deletions. The glucocorticoid regulatory region we have identified in the α_{2u}-globulin promoter is conserved in both MUP genes (90% homology). However, the "hypersensitivity" region is only 50% homologous and the TATA box is quite different in the rat and mouse genes.

The phenotype of the null mutant LS-137/-125 is striking and indicates that the α_{2u}-globulin promoter is organized quite differently from that of MMTV, a promoter directly regulated by the glucocorticoid receptor. Yamamoto's laboratory has shown that the hormone-receptor complex binds to at least five sites in the MMTV promoter (42). A nested series of deletions only gradually reduced MMTV induction as the binding sites were successively lost (23). Similarly, linker-scanning mutants that disrupted individual sites prevented receptor from binding to the mutated site but did not abolish induction, although in some cases induction was reduced (14). Thus the MMTV regulatory region seems to be diffuse, made up of multiple functional copies of a regulatory sequence. This architecture contrasts with the compact regulatory sequence we have identified in the α_{2u}-globulin promoter.

The α_{2u}-globulin gene 91 promoter does contain sequences similar to regulatory sequences associated with genes directly induced by glucocorticoids. Karin et al. (25) have identified a consensus sequence, 5'-$\underset{C}{T}$GGTN$\underset{A}{T}$CA$\underset{A}{T}$NTG T$\underset{C}{T}$CT-3', common to glucocorticoid receptor binding sites in the human metallothionein-II_A and MMTV promoters (25, 51). Part of this motif, 5'-TGTTCT-3', is found in other promoters regulated, either directly or indirectly, by glucocorticoids such as the human growth hormone gene (39), the chicken lysozyme gene (45), the rabbit uteroglobin gene (10), and the rat α_1-acid glycoprotein (34, 44), tryptophan oxygenase (53) and tyrosine aminotransferase genes (54). This sequence is found (on the opposite strand to that shown in Fig. 6) outside the regulatory region of gene 91 between positions −228 and −233. Mutation LS(-235/-214) removes this sequence without drastically reducing α_{2u}-globulin induction. Two sequences within the α_{2u}-globulin regulatory regions, 5'-AGAATCAA-3' between −181 and −174 and 5'-AGATGACAT-3' between −165 and −157, resemble a sequences, 5'-AGA$\underset{A}{T}$CA(G)$\underset{A}{T}$-3' (the complement to the right end of the consensus sequence), repeated many times in the receptor binding regions of MMTV (42). Glucocorticoid receptor binding studies have not yet been done with the α_{2u}-globulin promoter. In their absence it is difficult to assess the significance of these homologies. It will be important to determine if receptor binding is required for the induction of transcription from this promoter.

The α_{2u}-globulin glucocorticoid regulatory region displays imperfect dyad symmetry. A 6-bp core sequence, 5'-GCCAAG-3', is separated by 22 bp from its inverted complement. If small interruptions are allowed, the complementary regions (upper case characters) can be expanded to 12 bp each:

5'-TGAcAtcGCCAAGTTtcaaaagggcaggaacAAtcCTTGGCTTCA-3'
-162 -118

The presence of dyad symmetry in the regulatory region is reminiscent of other regulatory sequences. Dyad symmetry is often found in the binding sites for procaryotic regulatory proteins. In these systems dyad symmetry in the regulatory sequence correlates with a dimeric structure for the regulatory protein (40). Some eucaryotic regulatory proteins also bind to sequences of dyad symmetry. Examples are the GAL4 protein of yeasts (9, 20) and a protein that binds to the chicken β-globin promoter (18). Unlike these examples, the two elements of the dyad present in the α_{2u}-globulin promoter are quite far apart. Each repeat could be an independent binding site for a transcription factor. The sequence between the elements is, however, important for regulation of the gene. This is indicated by the phenotype of mutation LS-144/-138, a mutation which alters neither element and changes the spacing between them by only one nucleotide yet significantly reduces induction.

Within the regulatory region shown above (positions −131 and −136) is a hexanucleotide, 5'-GGAACA-3', found at a very similar position in the promoter of the rat α_1-acid glycoprotein gene (34, 44). Like α_{2u}-globulin induction, α_1-acid glycoprotein induction is a secondary response to glucocorticoids (5, 56). An estimation of the significance of this short sequence homology between the two promoters awaits the identification of regulatory elements associated with the α_1-acid glycoprotein gene.

The four mutations defining the glucocorticoid regulatory region show a distinct polarity in their effects on induction. The mutant nearest the transcription start site, LS-137/-125, is most severe in its effects, while the most distal mutant, LS-158/-156, is the least. The polarity may be an artifact resulting from changes in the spacing of promoter elements. Of the four mutants, the null mutant LS-137/-125 has suffered the most drastic change in sequence, a deletion of 5 bp in addition to the linker substitution. Alternatively, the polarity may reflect real differences in the importance of sequences within the regulatory region. This suggestion is supported by the sequence analysis of α_{2u}-globulin promoters mentioned above. There is significantly greater conservation of sequences around −130 than of sequences between −140 and −160. Examples of similar effects are known, even in much more highly symmetric regulatory sequences. In the *E. coli* lactose operon, *lac* repressor has higher affinity for the promoter-proximal than for the promoter-distal half of the operator sequence (4). Another example can be found in the herpesvirus thymidine kinase promoter: mutations affecting the distal half of the CAAT box dyad have a much more severe effect on promoter efficiency than mutations in the other half (38).

Just upstream to the putative regulatory factor binding site is a second regulatory sequence we term the hyperinducibility region. Mutations between −177 and about −200 increase the gene's inducibility by glucocorticoids. These mutations do not change the basal level of expression from the promoter; their effect is specific to cells in the induced state. We are unaware of other regulatory sequences that have these properties. These mutations have a phenotype opposite to deletions of sequences upstream to the human β-interferon gene that raise the basal level of expression 5- to 10-fold while not affecting the induced level (64). The existence of a class of promoter mutants more inducible than the wild-type gene hints that the function of the wild-type hyperinducibility region sequence is to set the maximum efficiency at which the promoter can function.

The mechanism by which the hyperinducibility region exerts its effects on the promoter is obscure. Simple models in which these mutations facilitate the binding of the regulatory factor, or of RNA polymerase, or reduce the binding of a constitutive repressor are not adequate because they do not explain how the mutants show increased expression in induced cells yet have no effect on expression in uninduced cells. Formulation of more complex models to explain the properties of the hyperinducible mutants is probably premature. We do not, for example, yet know if this region can

function independently of the glucocorticoid regulatory region.

The kinetics of induction of an intact α_{2u}-globulin gene, a hyperinducible mutant (LS-183/-169), and a mutant of low inducibility (LS-144/-138) are compared in Fig. 1B. Cells transfected with all three genes show the same lag period between addition of the hormone and the induction of α_{2u}-globulin mRNA. This is expected if the lag period is required for the synthesis of a *trans*-acting regulatory protein(s) in response to the hormone. After the lag, α_{2u}-globulinmRNA accumulates in induced cells according to the simple turnover kinetics originally applied to hormonally induced enzyme synthesis by Schimke et al. (52). The initial rates of accumulation differ among the three cell lines and are roughly proportional to the steady-state levels of α_{2u}-globulinmRNA achieved in each cell line. This is exactly the kinetic behavior expected of *cis*-acting regulatory mutations.

The induction of α_{2u}-globulin mRNA by glucocorticoids is a secondary response to the hormone. As expected, cycloheximide and emetine, inhibitors of protein synthesis, prevent this induction. The inhibition of induction by cycloheximide has two puzzling properties. First, induction is much more sensitive to the inhibitor than is overall protein synthesis and, second, this acute sensitivity is not shown by emetine, another inhibitor of peptide chain elongation. As yet we have no explanation for our observations. The only precedent we have found for this behavior is that reported by Palmiter et al. (41). These workers found that, at concentrations of inhibitor that reduced protein synthesis to a similar degree, cycloheximide inhibited ovalumin induction by estrogen more strongly than did emetine. It is possible that cycloheximide inhibits α_{2u}-globulin induction directly, by a mechanism independent of the drug's effect on the eucaryotic ribosome. This idea could be tested by studying the inhibition of α_{2u}-globulin induction in cells containing cycloheximide-resistant ribosomes. Mutant Chinese hamster ovary cells with such resistant ribosomes have been isolated (43).

The mutagenesis experiments described in this paper have delineated the sequences required for the regulation of α_{2u}-globulin by glucocorticoids. The task that now presents itself is the isolation and characterization of the *trans*-acting factor or factors that interact with these sequences to increase transcription from the promoter. Our knowledge of the factor's properties is meager. A promoter mutation that abolishes α_{2u}-globulin induction by glucocorticoids has no effect on the basal level of the gene's expression. This basal level of expression, unlike the induced level, is insensitive to the antiglucocorticoid RU486. These observations suggest that the factor is truly regulatory and is not absolutely required for transcription.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants 5PO1CA29569-05 to Cold Spring Harbor Laboratory and 5RO1AM26968-06 to D.T.K. from the National Institutes of Health. W.R.A. is grateful for the support of the Medical Research Council of Canada and the Robertson Research Fund of Cold Spring Harbor Laboratory.

We thank Patricia Bird for preparing the manuscript.

LITERATURE CITED

1. **Anderson, J. N.** 1983. The effect of steroid hormones on gene transcription, p. 169–212. *In* R. F. Goldberger and K. R. Yamamoto (ed.), Biological regulation and development, vol. 3b. Plenum Press, New York.
2. **Ashburner, M., C. Chibara, P. Meltzer, and G. Richards.** 1974. Temporal control of puffing activity in polytene chromosomes. Cold Spring Harbor Symp. Quant. Biol. **38**:655–662.
3. **Ballard, P. L., J. D. Baxter, S. J. Higgins, G. G. Rousseau, and G. M. Tomkins.** 1974. General presence of glucocorticoid receptors in mammalian tissues. Endocrinology **94**:998–1002.
4. **Barkley, M. D., and S. Bourgeois.** 1980. Repressor recognition of operator and effectors, p. 77–220. *In* J. H. Miller and W. S. Reznikoff (ed.), The operon, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Baumann, H., G. L. Firestone, T. L. Burgess, K. W. Gross, K. R. Yamamoto, and W. A. Held.** 1983. Dexamethasone regulation of α_1-acid glycoprotein and other acute phase reactants in rat liver and hepatoma cells. J. Biol. Chem. **258**:563–570.
6. **Benoist, C., K. O'Hare, R. Breathnach, and P. Chambon.** 1980. The ovalbumin gene-sequence of putative control regions. Nucleic Acids Res. **8**:127–142.
7. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 nuclease digested hybrids. Cell **12**:721–732.
8. **Bourgeois, S., M. Pfahl, and E.-E. Baulieu.** 1984. DNA-binding properties of glucocorticoid receptors bound to the steroid antagonist RU486. EMBO J. **3**:751–755.
9. **Bram, R. J., and R. D. Kornberg.** 1985. Specific protein binding to far upstream activating sequences in polymerase II promoters. Proc. Natl. Acad. Sci. USA **82**:43–47.
10. **Cato, A. C. B., S. Geisse, M. Wenz, H. M. Westphal, and M. Beato.** 1984. The nucleotide sequences recognized by the glucocorticoid receptor in the rabbit uteroglobin gene region are located far upstream from the initiation of transcription. EMBO J. **3**:2771–2778.
11. **Chen, C.-L. C., and P. Feigelson.** 1979. Cycloheximide inhibition of hormonal induction of α_{2u}-globulin mRNA. Proc. Natl. Acad. Sci. USA **76**:2669–2673.
12. **Clark, A. J., P. Ghazal, R. W. Bingham, D. Barrett, and J. O. Bishop.** 1985. Sequence structures of a mouse major urinary protein gene and pseudogene compared. EMBO J. **4**:3159–3165.
13. **Clever, U., and C. G. Romball.** 1966. RNA and protein synthesis in the cellular response to a hormone, ecdysone. Proc. Natl. Acad. Sci. USA **56**:1470–1476.
14. **DeFranco, D., O. Wrange, J. Merryweather, and K. R. Yammamoto.** 1985. Biological activity of a glucocorticoid regulated enhancer: DNA sequence requirements and interactions with other transcriptional enhancers. UCLA Symp. Mol. Cell. Biol. **20**:305–321.
15. **Dolan, K. P., R. Reinke, K. R. Lynch, M. McLaughlin, R. Unterman, H. L. Nakhasi, Y. Laperche, and P. Feigelson.** 1983. The application of recombinant techniques to the study of the control of α_{2u}-globulin gene expression, p. 1–22. *In* G. Litwack (ed.), Biochemical actions of hormones, vol. 10. Academic Press, Inc., New York.
16. **Dolan, K. P., R. Unterman, M. McLaughlin, H. L. Nakhasi, K. R. Lynch, and P. Feigelson.** 1982. The structure and expression of very closely related members of the α_{2u}-globulin gene family. J. Biol. Chem. **257**:13527–13534.
17. **Efstratiadis, A., J. W. Posakony, T. Maniatis, R. M. Lawn, C. O'Connell, R. A. Spritz, J. K. De Riel, B. G. Forget, S. M. Weissman, J. L. Slighton, A. E. Blechl, O. Smithies, F. E. Baralie, C. C. Shoulders, and N. Proudfoot.** 1980. The structure and evolution of the human β-globulin gene family. Cell **21**:653–668.
18. **Emerson, B. M., C. D. Lewis, and G. Felsenfeld.** 1985. Interaction of specific nuclear factors with the nuclease-hypersensitive region of the chicken adult β-globin gene: nature of the binding domain. Cell **41**:21–30.
19. **Feinstein, S. C., S. R. Ross, and K. R. Yamamoto.** 1982. Chromosomal position effects determine transcriptional potential of integrated mammary tumor virus DNA. J. Mol. Biol. **156**:549–565.

20. **Giniger, E., S. M. Varnum, and M. Ptashne.** 1985. Specific DNA binding of GAL4, a positive regulatory protein of yeast. Cell **40**:767–774.
21. **Gubits, R. M., K. R. Lynch, A. B. Kulkarni, K. P. Dolan, E. W. Gresik, P. Hollander, and P. Feigelson.** 1984. Differential regulation of α_{2u}-globulin gene expression in liver, lachrymal gland, and salivary gland. J. Biol. Chem. **259**:12803–12809.
22. **Hanahan, D.** 1983. Transformation of Escherichia coli with plasmids. J. Mol. Biol. **166**:557–580.
23. **Hynes, N., A. J. J. van Ooyen, N. Kennedy, P. Herrlich, H. Ponta, and B. Groner.** 1983. Subfragments of the large terminal repeat cause glucocorticoid-responsive expression of mouse mammary tumor virus and of an adjacent gene. Proc. Natl. Acad. Sci. USA **80**:3637–3641.
24. **Jones, K. A., K. R. Yamamoto, and R. Tjian.** 1985. Two distinct transcription factors bind to the HSV thymidine kinase promoter in vitro. Cell **42**:559–572.
25. **Karin, M., A. Haslinger, H. Holtgreve, R. J. Richards, P. Krauter, H. M. Westphal, and M. Beato.** 1984. Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-II_A gene. Nature (London) **308**:513–519.
26. **Kruzer, K. N., and B. M. Alberts.** 1984. Site-specific recognition of bacteriophage T4 type II DNA topoisomerase and E. coli DNA gyrase. J. Biol. Chem. **259**:5339–5346.
27. **Kulkarni, A. B., R. M. Gubits, and P. Feigelson.** 1985. Developmental and hormonal regulation of α_{2u}-globulin gene transcription. Proc. Natl. Acad. Sci. USA **82**:2579–2582.
28. **Kurtz, D. T.** 1981. Rat α_{2u}-globulin is encoded by a multigene family. J. Mol. Appl. Genet. **1**:29–38.
29. **Kurtz, D. T.** 1981. Hormonal inducibility of rat α_{2u}-globulin genes in transfected mouse cells. Nature (London) **291**:629–631.
30. **Kurtz, D. T., K.-M. Chan, and P. Feigelson.** 1978. Glucocorticoid induction of hepatic α_{2u}-globulin synthesis and messenger RNA level in castrated male rats *in vivo*. J. Biol. Chem. **253**:7886–7890.
31. **Kurtz, D. T., and P. Feigelson.** 1978. Multihormonal control of the messenger RNA for the hepatic protein α_{2u}-globulin, p. 433–455. *In* G. Litwack (ed.), Biochemical actions of hormones, vol. 5. Academic Press, Inc., New York.
32. **Kurtz, D. T., L. McCullough, D. Bishop, and M. M. Manos.** 1982. DNA sequences required for hormonal induction of rat α_{2u}-globulin genes. Cold Spring Harbor Symp. Quant. Biol. **47**:985–988.
33. **Laperche, Y., K. Lynch, K. P. Dolan, and P. Feigelson.** 1983. Tissue-specific control of α_{2u}-globulin gene expression: constitutive synthesis in the submaxillary gland. Cell **32**:453–460.
34. **Liao, Y. C., J. M. Taylor, J. L. Vannice, G. A. Clawson, and E. A. Smuckler.** 1985. Structure of the rat α_1-acid glycoprotein gene. Mol. Cell. Biol. **5**:3634–3639.
35. **Maniatis, T., E. T. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
37. **McKnight, S. L., and R. Kingsbury.** 1982. Transcriptional control signals of a eukaryotic protein coding gene. Science **217**:316–324.
38. **McKnight, S., R. Kingsbury, S. Eisenberg, F. Tufaro, S. Weinheimer, S. Triezenburg, P. Johnson, and B. Graves.** 1985. Studies on the promoter of the herpes virus thymidine kinase gene, p. 116–122. *In* Y. Gluzman (ed.), Eukaryotic transcription: the role of Cis- and transacting elements in initiation. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Moore, D. D., A. R. Marks, D. I. Buckley, G. Kapler, F. Payvar, and H. M. Goodman.** 1985. The first intron of the human growth hormone gene contains a binding site for glucocorticoid receptor. Proc. Natl. Acad. Sci. USA **82**:699–702.
40. **Pabo, C. O., and R. T. Sauer.** 1984. Protein-DNA recognition. Annu. Rev. Biochem. **53**:293–321.
41. **Palmiter, R. D., P. B. Moore, E. R. Mulvihill, and S. Emtage.** 1976. A significant lag in the induction of ovalbumin messenger RNA by steroid hormones: a receptor translocation hypothesis. Cell **8**:557–572.
42. **Payvar, F., D. DeFranco, G. L. Firestone, B. Edgar, O. Wrange, S. Okret, I.-A. Gustafsson, and K. R. Yamamoto.** 1983. Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. Cell **35**:381–392.
43. **Poche, H., I. Junghahn, E. Geissler, and H. Bielka.** 1975. Cycloheximide resistance in Chinese hamster cells. III. Characterization of cell-free protein synthesis by polysomes. Mol. Gen. Genet. **138**:173–177.
44. **Reinke, R., and P. Feigelson.** 1985. Rat α_1-acid glycoprotein: gene sequence and regulation by glucocorticoids in transfected L-cells. J. Biol. Chem. **260**:4397–4403.
45. **Renkawitz, R., G. Shutz, D. von der Ahe, and M. Beato.** 1984. Sequences in the promoter region of the chicken lysozyme gene required for steroid regulation and receptor binding. Cell **37**: 503–510.
46. **Richards, G., and M. Ashburner.** 1984. Insect hormones and the regulation of genetic activity, p. 213–253. *In* R. F. Goldberger and K. R. Yamamoto (ed.), Biological regulation and development, vol. 3b. Plenum Press, New York.
47. **Ringold, G. M.** 1983. Regulation of mouse mammary tumor virus gene expression by glucocorticoid hormones. Curr. Top. Microbiol. Immunol. **106**:79–103.
48. **Rousseau, G. G.** 1984. Control of gene expression by glucocorticoid hormones. Biochem. J. **224**:1–12.
49. **Roy, A. K.** 1979. Hormonal regulation of α_{2u}-globulin in rat liver, p. 481–517. *In* G. Litwack (ed.), Biochemical actions of hormones, vol. 6. Academic Press, Inc., New York.
50. **Roy, A. K., B. Chatterjee, M. S. K. Prasad, and N. J. Unakar.** 1980. Role of insulin in the regulation of the hepatic messenger RNA for α_{2u}-globulin in diabetic rats. J. Biol. Chem. **255**:11614–11618.
51. **Scheidereit, C., S. Geisse, H. M. Westpahl, and M. Beato.** 1983. The glucocorticoid receptor binds nucleotide sequences near the promoter of mouse mammary tumour virus. Nature (London) **304**:749–752.
52. **Schimke, R. T., E. W. Sweeney, and C. M. Berlin.** 1964. An analysis of the kinetics of rat liver tryptophan pyrolase induction: the significance of both enzyme synthesis and degradation. Biochem. Biophys. Res. Commun. **15**:214–219.
53. **Schmid, W., G. Scherer, U. Danesch, H. Zentgraf, P. Matthias, C. M. Strange, W. Rowekamp, and G. Shutz.** 1982. Isolation and characterization of the rat tryptophan oxygenase gene. EMBO J. **1**:1287–1293.
54. **Shinomiya, T., G. Scherer, W. Schmid, H. Zentgraf, and G. Shutz.** 1984. Isolation and characterization of the rat tyrosine aminotransferase gene. Proc. Natl. Acad. Sci. USA **81**:1346–1350.
55. **Twigg, A. J., and D. Sherratt.** 1980. Trans-complementable copy-number mutants of plasmid ColE1. Nature (London) **283**:216–218.
56. **Vannice, J. L., G. M. Ringold, J. W. McLean, and J. M. Taylor.** 1983. Induction of the acute-phase reactant, α_1-acid glycoprotein, by glucocorticoids in rat hepatoma cells. DNA **2**:205–212.
57. **Weintraub, H.** 1985. Assembly and propagation of repressed and derepressed chromosomal states. Cell **42**:705–711.
58. **Widman, L. E., and L. A. Chasin.** 1982. Multihormonal induction of α_{2u}-globulin in an established rat hepatoma cell line. J. Cell Physiol. **112**:316–326.
59. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. USA **76**:1373–1376.
60. **Wigler, M., R. Sweet, G.-K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel.** 1979. Transformation of mammalian cells with genes from procaryotes and eucaryotes. Cell **16**:777–785.

61. **Yamamoto, K. R.** 1985. Steroid receptor regulated transcription of specific genes and gene networks. Annu. Rev. Genet. **19**:209–252.
62. **Yamamoto, K. R., and B. M. Alberts.** 1976. Steroid receptors: elements of modulation of eukaryotic transcription. Annu. Rev. Biochem. **45**:721–746.
63. **Yaniv, M.** 1984. Regulation of eukaryotic gene expression by transactivating proteins and cis acting DNA elements. Biol. Cell. **50**:203–216.
64. **Zinn, K., D. DiMaio, and T. Maniatis.** 1983. Identification of two distinct regulatory regions adjacent to the human β-interferon gene. Cell **34**:865–879.