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Glucocorticoids Enhance Stability of Human Growth Hormone mRNA

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We have studied the control of expression of the human growth hormone (hGH) gene introduced into the chromosomes of mouse fibroblasts. Cell lines transformed with the hGH gene expressed low levels of intact hGH mRNA and secreted hGH protein into the medium. Although the level of expression of hGH mRNA was low, the gene remained responsive to induction by glucocorticoid hormones. To localize the sequences responsible for induction and to determine the mechanism by which these *cis*-acting sequences enhance gene expression, we have constructed a series of fusion genes between the hGH gene and the herpes simplex virus (HSV) thymidine kinase (*tk*) gene. We have demonstrated that a fusion gene in which hGH cDNA is flanked at its 5′ terminus by an HSV *tk* promoter and is flanked at its 3′ terminus by 3′ HSV *tk* DNA remains inducible by glucocorticoids. Our studies indicate that the hGH exons contain sequences which are responsible for glucocorticoid hormone induction. Pulse-chase experiments, in vitro nuclear transcription, and approach to steady-state measurements indicate that the mechanisms responsible for induction of the hGH cDNA fusion gene operate posttranscriptionally to enhance the stability of hGH mRNA. Moreover, this increased stability was associated with an increase in the length of the 3′ poly(A) tail on hGH mRNA.

Steroid hormones regulate gene expression by associating with receptor proteins which then interact with specific DNA sequences in the chromosome (for a review, see reference 60). The glucocorticoid receptor, for example, binds to specific sites in several hormone-responsive genes, and this interaction presumably results in enhanced transcription upon exposure to glucocorticoid hormones (for reviews, see references 41 and 59). However, the effects of steroid hormones on gene expression are not restricted to transcriptional events (6, 20, 35, 51). Estrogen, for example, results in a 1,000-fold increase in the transcription of the vitellogenin gene in *Xenopus laevis* liver but also exerts a profound effect on the stability of vitellogenin mRNA (6). Given the specificity with which estrogen enhances the stability of vitellogenin mRNA, it is likely that sequences reside within this mRNA which are responsive to the hormone and increase the expression of this gene via posttranscriptional mechanisms.

Expression of the growth hormone (GH) genes is restricted to somatotrophs of the anterior pituitary (26) and is regulated by the hypothalamic growth hormone releasing factor (3, 4, 22) and glucocorticoid and thyroid hormones. In cultures of rat pituitary cells (GH_3 and GC [2, 61]), either glucocorticoid or thyroid hormone increases the level of the GH mRNAs and a synergism is observed when these two hormones are added together (32, 45, 47, 50, 53). Thyroid hormone increases the rate of transcription of the rat GH gene in pituitary cell lines (12, 14, 19, 38, 49, 58), and the regulatory element responsive to thyroid hormone has been identified within the 5′ flanking sequences of the rat GH gene (8, 12). Both transcriptional (15, 19, 38, 49) and posttranscriptional (14, 49, 58) mechanisms have been implicated in the glucocorticoid regulation of GH. The glucocorticoid receptor binds to specific sequences in the first intron of the human GH gene in vitro (36, 48), and this interaction is thought to be responsible for the induction of transcription. However, the level of transcriptional activation in the presence of glucocorticoids observed in vitro in nuclear transcription assays is significantly lower than the steady-state level of induction of GH mRNA in the rat pituitary cells, suggesting that posttranscriptional events, mediated by steroids, may also be operative (49).

In previous studies, we have introduced the human GH (hGH) gene into murine fibroblasts and demonstrated that hGH mRNA is inducible by glucocorticoids (42). To localize the sequences responsible for induction and to determine the mechanism by which these *cis*-acting sequences enhance gene expression, we have now constructed a series of fusion genes between hGH and the herpes simplex virus (HSV) thymidine kinase (*tk*) gene. We observed that a gene consisting of hGH cDNA sequences, driven by a *tk* promoter and flanked by 3′ *tk* DNA, is inducible in transformed fibroblasts. Further, this induction results at least in part from changes in mRNA stability, suggesting that glucocorticoids regulate hGH expression at the level of RNA stability as well as RNA synthesis.

MATERIALS AND METHODS

Cell culture and hormonal induction. Mouse Ltk^- $aprt^-$ cells were maintained as described previously (56), and Ltk^- $aprt^+$ transformants were selected and maintained in Dulbecco modified Eagle (DME) medium containing 10% calf serum (Gibco Laboratories), 4 μg of azaserine per ml, and 15 μg of adenine per ml (DME plus 10% calf serum plus AzaAd) (56).

Induction was performed on subconfluent cell cultures maintained for 4 days in medium containing 10% calf serum which had been rendered hypothyroid and hypoglucocorticoid by using AG 1-X8 anion exchange resin and activated charcoal (15, 40, 44). Cells were induced in 10^{-6} M dexamethasone (Sigma Chemical Co.) for various times up to 72 h.

In experiments to discern the effect of protein synthesis

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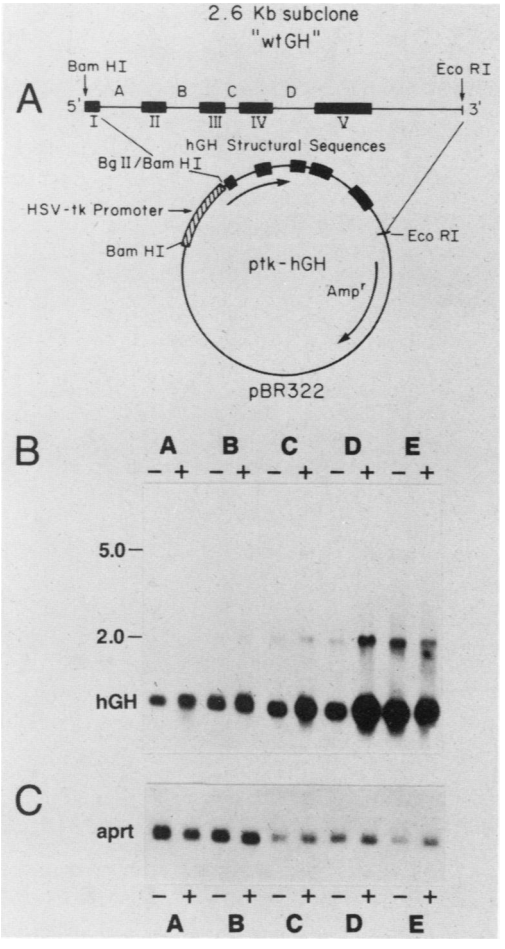

FIG. 1. Induction of tk-hGH by glucocorticoid. (A) tk-hGH contains an 825-bp *Bam*HI-*Bgl*II fragment of HSV *tk* which includes the promoter (▨), ligated to the *Bam*HI site within the 5′ untranslated region of the hGH gene (13), replacing the hGH promoter sequences. The remaining hGH DNA includes the entire structural sequences and 525 bp of 3′ flanking sequences (▬). (B, C) Total poly(A)$^+$ RNA was isolated from cells transformed with ptk-hGH and grown in the absence (−) or presence (+) of glucocorticoid for 72 h and was electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde and transferred onto a nitrocellulose filter. The filter was hybridized with ^{32}P-labeled hGH cDNA or hamster *aprt* cDNA probes. Five independent transformants (A to E) were analyzed. Poly(A)$^+$ RNA (5 μg) was loaded in each lane, except in lanes A and E, which each received 3 μg of poly(A)$^+$ RNA.

inhibitors on induction, cycloheximide (Sigma) was added to 2 μg/ml 1 h before the addition of dexamethasone.

DNA transformation of Ltk$^-$ aprt$^-$ cells. Calcium phosphate precipitates were prepared as described previously (55), by using 1 ng of pHaprt (hamster adenine phosphoribosyltransferase [*aprt*]) (31), 1 μg of plasmids containing fusion genes described in the text (tk-hGH, tk-chGH A$^+$, tk-chGH-tk3′, or other hybrid genes), and 19 μg of high-molecular-weight carrier DNA from Ltk$^-$ aprt$^-$ cells per 10^6 cells. The L cells were placed under *aprt* selection (DME plus 10% calf serum plus AzaAd) on the following day to select Ltk$^-$ aprt$^+$ transformants.

Construction of recombinant plasmids. Plasmid tk-hGH (Fig. 1A) was constructed by first subcloning the 2.6-kilobase (kb) *Eco*RI fragment containing the entire hGH gene (42) into the *Eco*RI site of pBR322 vector in an orientation such that the hGH promoter sequences bound by *Eco*RI (−500) and *Bam*HI (+3) were proximal to the unique *Bam*HI site of pBR322. The resulting phGH322 recombinant was digested with *Bam*HI to remove all essential gGH promoter sequences. The larger *Bam*HI fragment (6.6 kb) containing the 2.1-kb hGH structural sequences, 525-base-pair (bp) hGH 3′ flanking sequences, and 4.0-kb remaining pBR322 sequences was gel isolated. Plasmid tk harboring the HSV *tk* gene (33) was digested with *Bam*HI and *Bgl*II, and the 825-bp *Bam*HI-*Bgl*II fragment containing the HSV *tk* promoter sequences was gel purified. This 825-bp *Bam*HI-*Bgl*II fragment of the HSV *tk* gene was ligated to the 6.6-kb *Bam*HI fragment containing the hGH structural sequences lacking its own promoter. A recombinant plasmid which contains the *Bam*HI-*Bgl*II-bound HSV *tk* promoter sequences in a correct orientation with respect to the hGH translational reading frame was named ptk-hGH (Fig. 1A). The *Bgl*II site of the HSV *tk* fragment, including the *tk* promoter sequences, is 56 nucleotides beyond the transcriptional initiation site of the HSV *tk* gene (33). The *Bam*HI site of the hGH fragment is three nucleotides 3′ to the transcriptional initiation site of the hGH gene (13). The translation initiator codon AUG of the hGH gene is located 61 nucleotides 3′ to the *Bam*HI site (13). The 5′ untranslated region of the resulting tk-hGH fusion gene is 114 nucleotides long, 53 nucleotides longer than the intact, wild-type hGH gene ("wtGH" in references 42 and 46).

Plasmid tk-chGH A$^+$ (Fig. 2A) was constructed in two steps. The 0.8-kb (791-bp) *Bam*HI-*Hin*dIII fragment containing the hGH cDNA sequences [from the position +35 to the poly(A) sequences] was ligated to ptk(delta)5′-118 (34) digested with *Bgl*II and *Hin*dIII, removing all essential HSV *tk* structural sequences. In the plasmid containing the hGH cDNA, the *Bam*HI site was regenerated during cDNA cloning at position +35 within the 5′ untranslated region of the hGH gene. ptk(delta)5′-118 is a 5′ deletion mutant derivative of ptk(delta)3′-1.13 (34) and retains 118 nucleotide sequences 5′ to the transcriptional start site. These nucleotides are sufficient for essential promoter activity of the *tk* gene (34). The *Bgl*II site of ptk(delta)5′-118 is 56 nucleotides 3′ to the transcriptional initiation site of the *tk* gene (33). The *Bgl*II restriction site of ptk(delta)5′-118 was inactivated as a result of ligation to the isoschizomeric *Bam*HI site of the fragment containing hGH cDNA sequences. The resulting plasmid, ptk-chGH A$^-$, was digested with *Bgl*II and *Hin*dIII to remove the 271-bp-long *Bgl*II-*Hin*dIII fragment of the hGH cDNA sequences, spanning position +535 within the fifth exon to the 3′ end of the hGH cDNA sequences at the *Hin*dIII linker site. This fragment of the hGH cDNA sequences, was replaced with a 1.8-kb *Bgl*II-*Hin*dIII fragment derived from pwtGH (42). pwtGH contains a 2.6-kb *Eco*RI fragment consisting of the entire hGH gene subcloned into pBR325 (42; see map at the top of Fig. 2A). This 1.8-kb fragment consists of most of the fifth exon, the 525-bp hGH 3′ flanking sequences, and 1-kb pBR325 vector sequences. The resulting fusion gene tk-chGH A$^+$ consists of hGH cDNA sequences flanked by the HSV *tk* promoter and 525-bp hGH 3′ flanking sequences.

Plasmid tk-chGH-tk 3′ (Fig. 3A), consisting of HSV *tk* promoter and hGH cDNA sequences and the HSV *tk* 3′ flanking sequences, was constructed as follows. ptk(delta)5′-118 was digested with *Bgl*II and *Sma*I, removing essentially all the *tk* structural sequences. The *Bgl*II site is again 56 nucleotides 3′ to the transcriptional initiation site of the HSV *tk* gene. The resulting ptk(delta)5′-118/*Bgl*II-*Sma*I fragment

retains the HSV *tk* promoter sequences and the poly(A) addition signal sequences. A *Bam*HI-*Sma*I fragment containing hGH cDNA sequences from position +35 to position +727 was ligated into ptk(delta)5′-118 digested with *Bgl*II and *Sma*I. The *Sma*I site of the hGH cDNA sequences is located six nucleotides downstream from the translational stop codon (TAG), and the *Sma*I site of the ptk(delta)5′-118 resides 20 nucleotides 5′ to the TGA putative translational stop codon, which is in turn located about 70 nucleotides 5′ from the HSV *tk* poly(A) addition site. These two TGAs are 26 nucleotides apart and therefore are not in frame. The poly(A) addition sequences of this fusion gene are derived from the HSV *tk* 3′ DNA sequences.

Northern blot analysis. Total RNA was isolated from cells by homogenization in 4 M guanidinium isothiocyanate followed by ultracentrifugation through a 5.7 M CsCl cushion (11). Poly(A)$^+$ RNA selection was done by using oligo(dT)-cellulose chromatography (Collaborative Research, Inc.) as described by Aviv and Leder (1). Cytoplasmic RNA was isolated from cells lysed with 0.3% Nonidet P-40 by adjusting the postnuclear supernatant to 10 mM EDTA, 0.5% sodium dodecyl sulfate, and 10 μg of proteinase K (Boehringer Mannheim Biochemicals) and incubating at 42°C for 15 min.

Northern blot hybridization was done according to procedures described by Goldberg (24) for nitrocellulose filters (Schleicher & Schuell, Inc.) or according to instructions supplied by the manufacturer for Gene Screen transfer membranes (New England Nuclear Corp.). DNA probes were prepared from the hGH and hamster *aprt* cDNA sequences (generously provided by I. Lowy) by nick translating to specific activities of 0.5×10^9 to 1.0×10^9 cpm/μg (54). The plasmids used as templates for the synthesis of the complementary strand SP6 probes were constructed by inserting the hGH and human gamma-actin cDNA sequences (generously provided by P. Gunning and L. Kedes) into appropriate sites of pSP6-4 (Promega Biotec). The transcrip-

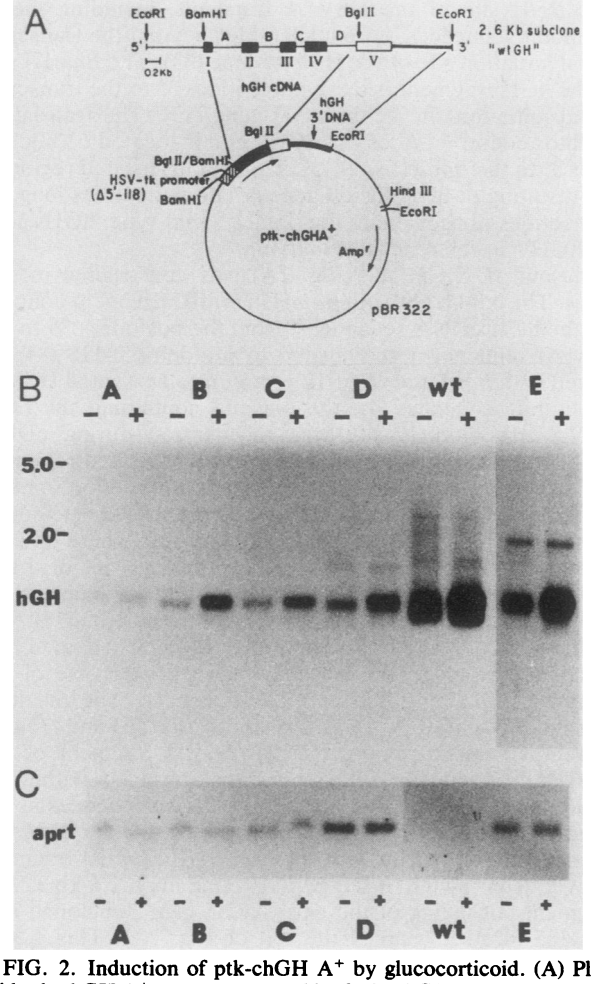

FIG. 2. Induction of ptk-chGH A$^+$ by glucocorticoid. (A) Plasmid ptk-chGH A$^+$ was constructed by fusing hGH cDNA sequences (▬, ▭) to the HSV *tk* promoter (▨) at its 5′ end and 525-bp hGH 3′ flanking sequences at its 3′ end (▬). (B, C) Transformants containing ptk-chGH A$^+$ fusion gene were grown in the absence (−) or presence (+) of glucocorticoid for 72 h, and 4 μg of total poly(A)$^+$ RNA from five independent transformants (A to E) were analyzed on a 1% agarose-formaldehyde gel by Northern blotting with ^{32}P-labeled hGH cDNA or hamster *aprt* probes. Total poly(A)$^+$ RNA (8 μg) was also analyzed from an Ltk$^+$ aprt$^-$ transformant (lane wt) containing the entire hGH gene (2.6-kb *Eco*RI fragment containing wtGH subcloned into pBR325). This line is aprt$^-$ since *ptk* was a cotransforming selectable marker (panel C).

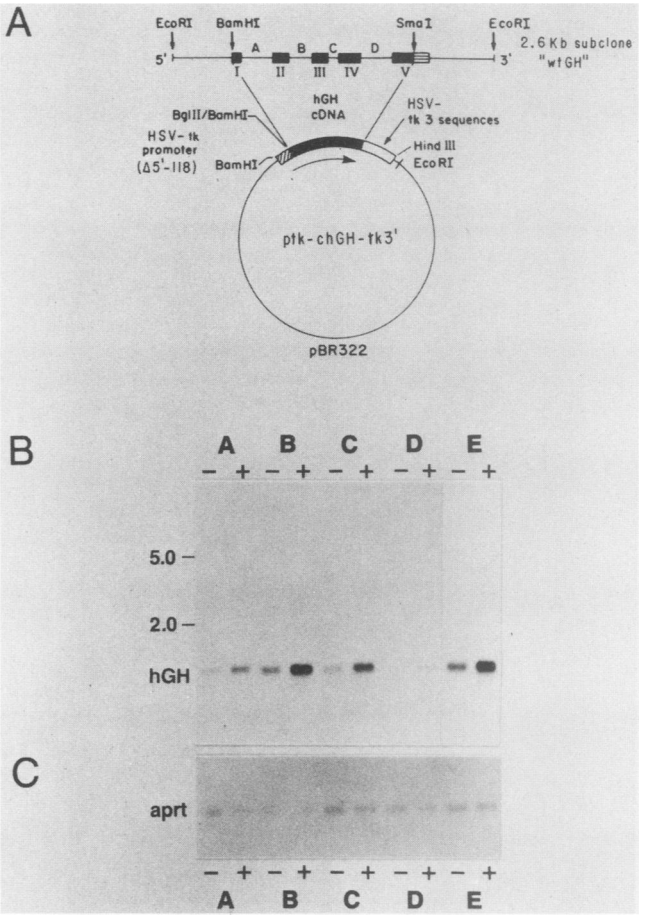

FIG. 3. Induction of tk-chGH-tk3′ by glucocorticoids. (A) Plasmid ptk-chGH-tk3′ was constructed by fusing the hGH cDNA sequences (▬) with HSV *tk* promoter sequences at the 5′ terminus (▨) and *tk* poly(A) addition site and 3′ flanking DNA at the 3′ terminus (▭). (B, C) Poly(A)$^+$ RNA (8 μg) from cells transformed with ptk-chGH-tk3′, grown in the absence (−) or presence (+) of dexamethasone, was analyzed by Northern blot analysis with ^{32}P-labeled hGH cDNA and hamster *aprt* cDNA sequences. Five independent transformants (A to E) were analyzed.

tion reaction with SP6 polymerase and RNA purification were done as described by Zinn et al. (62), except that [α-^{32}P]UTP (600 Ci/mmol) was used as the labeled nucleotide. When ^{32}P-labeled single-stranded RNA probes were used, the hybridization stringency was changed to 0.6 M NaCl and 50% formamide at 60°C and blots were washed with 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate) at 68°C.

RNase H experiment. The reactions were done essentially as described by Berkower et al. (5) and Donis-Keller (17). Briefly, total cytoplasmic RNA (20 μg) was heated to 85°C for 5 min and hybridized to 5 μg of oligo(dT)$_{12-18}$ (Pharmacia) in a 20-μl solution containing 40 mM Tris hydrochloride (pH 7.9), 4 mM $MgCl_2$, 1 mM dithiothreitol, and 30 ng of bovine serum albumin per ml. After hybridization for 10 min at 42°C, 1 U of *E. coli* RNase H was added and the solution was incubated at 37°C for 30 min. The reaction was terminated by phenol-chloroform extraction followed by precipitation with ethanol. The products of digestion were sized on a 1.5% agarose gel containing 2.2 M formaldehyde.

Pulse-chase analysis. We used a modification of the pulse-chase conditions of Levis and Penman (30). Subconfluent transformed L cells (tk-chGH A$^+$) were cultured for 4 days in DME plus 10% hormone-depleted calf serum plus AzaAd. Cells were induced for 48 h by adding 10^{-6} M dexamethasone to allow for the expression of putative regulatory factors required for hormonal induction and then labeled for 5 h with 150 μCi of [5,6-^{3}H]uridine (43.1 Ci/mmol, New England Nuclear) per ml in medium containing 10^{-6} M dexamethasone. Uninduced samples were similarly labeled in medium lacking dexamethasone. The labeling medium was removed, and the chase was initiated by adding medium containing 5 mM each of uridine and cytidine in the presence or absence of 10^{-6} M dexamethasone. The chase was continued for various times up to 70 h. At indicated times, cells were harvested and total cytoplasmic RNA was isolated as described above. The fraction of labeled hGH and actin mRNA was measured by DNA excess filter hybridization. Linearized plasmid DNAs (5 μg per filter) were immobilized on nitrocellulose filters (BA85, 0.45-μm pore size; Schleicher & Schuell) according to procedures of Kafatos et al. (27), as modified by Gasser et al. (21). Filters were prehybridized 6 to 12 h at 65°C in hybridization buffer, as described previously (58).

Duplicate samples of ^{3}H-labeled RNA (50 to 100 μl) containing 5×10^7 to 10×10^7 were denatured at 85°C for 5 min, cooled on ice, and hybridized to filters containing hGH cDNA, human gamma-actin cDNA, and pBR322 in 1-ml Nunc plastic tubes for 2 to 4 days at 65°C. After hybridization, the filters were washed with three changes of 2× SSC and 0.1% sodium dodecyl sulfate for 10 min each at 42°C and then washed with two changes for 45 min each of 0.2× SSC and 0.1% sodium dodecyl sulfate at 65°C. Filters were incubated in scintillation microvials with 200 μl of 40 mM NaOH or incubated in 1 ml of 2-methoxyethanol (Eastman Kodak Co.) at room temperature for 5 min to dissolve nitrocellulose filters and counted with 10 ml of Aquasol-2 (New England Nuclear) three times for 20 min each in a liquid scintillation counter (1219 Rackbeta; LKB Instruments, Inc.). The data shown in Fig. 6 represent the average of three 20-min countings. The background as determined by hybridization to pBR322 DNA ranged about 10^{-6} to 10^{-7} of input counts per minute.

In vitro nuclear runoff transcription analysis. Transformed L cells were cultured for 4 days in DME medium supplemented with hormone-depleted serum. Cells were harvested after various times of incubation with 10^{-6} M dexamethasone and were lysed with 0.3% Nonidet P-40, and nuclei were prepared as described by Groudine et al. (25). The postnuclear supernatant fraction was processed as above (see Northern blot analysis) for the analysis of total cytoplasmic RNA. Isolated nuclei were incubated in vitro in the presence of heparin and ammonium sulfate with [^{32}P]UTP and cold GTP, CTP, and ATP, and the labeled RNA was purified as described by Leff et al. (29). The plasmid DNAs were immobilized on nitrocellulose filters as described above. Typically, labeled RNA containing 10^8 cpm was hybridized in duplicate filters against linearized plasmids containing hGH cDNA and human gamma-actin cDNA, and pBR322 control sequences for 2 to 4 days at 42°C. An equal amount of labeled RNA was added to hybridization tubes for each time point. Hybridization and filter washing conditions were as described by Leff et al. (29).

Approach to steady-state measurements. The approach to steady-state measurements were performed essentially as described by Cabrera et al. (7). Briefly, transformed cells (tk-chGH A$^+$), cultured for 4 days in DME plus 10% hormone-depleted calf serum plus AzaAd, were exposed to 10^{-6} M dexamethasone for 48 h to allow for the expression of putative regulatory factors required for hormonal induction. At this point, cells were continuously labeled with [5,6-^{3}H]uridine (43.1 Ci/mmol, New England Nuclear) at a final concentration of 9 to 10 μM (0.4 mCi/ml). No unlabeled uridine was added. At various times after the initiation of labeling, samples of 1×10^7 to 2×10^7 cells were harvested and total cytoplasmic RNA was isolated as described above. The amounts of labeled cytoplasmic hGH mRNA and actin mRNA were determined by hybridization to an excess of hGH cDNA, human gamma-actin cDNA, and pBR322 control DNA bound to nitrocellulose filters as described above. Portions of the labeled cells (1/20 to 1/40) were also withdrawn at times indicated for determination of the specific activity of the internal UTP pool by the polymerase method (7).

We used the computer program MESSAG (7) to estimate the relative rate constants of entry into the cytoplasm (picograms per minute per cell) and the relative rate constants of decay (minute^{-1}) from the measured values of the specific activity of the UTP pool and the amount of labeled hGH mRNA and actin mRNA as a function of time.

RESULTS

Glucocorticoid-responsive elements in the hGH structural gene. The hGH gene encodes an 825-bp mRNA which is interrupted by four introns (13, 43). This gene has been isolated within a 2.6-kb *Eco*RI fragment such that the coding sequences and introns are flanked by 500 bp of 5′ DNA and 525 bp of 3′ flanking DNA (13, 46). The administration of glucocorticoid hormone to mouse fibroblast (Ltk$^-$ aprt$^-$) cells transformed with this fragment of hGH DNA results in a two- to fivefold induction of hGH mRNA and a similar induction in secreted protein (42). To determine the location of sequences responsible for induction, we constructed a fusion gene consisting of the HSV*tk* promoter fused to position +3 of the hGH gene (Fig. 1A). This tk-hGH fusion gene, along with the selectable hamster *aprt* gene (31), was introduced into mouse fibroblasts by cotransformation, and the inducibility of the hGH mRNA synthesized by these cotransformants was analyzed by Northern blot analysis (Fig. 1B). These transformants synthesized an 880-bp tk-

hGH mRNA slightly larger than the native hGH mRNA, reflecting the presence of 56 nucleotides of *tk* 5′ untranslated RNA. In four of five transformants (Fig. 1B, lanes A to D) that we examined, we observed induction of this fusion mRNA species by glucocorticoid hormone. The level of induction ranged from 1.5- to 4-fold, with maximum induction observed for clone D (Fig. 1B, lane D), which was used in subsequent studies. No induction was observed for the endogenous actin or exogenous *aprt* gene (Fig. 1C). These experiments indicate that there are sequences within the structural region of the hGH gene, including both introns and exons, which are responsive to glucocorticoid induction. These results are consistent with previous studies which demonstrate that sequences within the first intron of hGH bind the glucocorticoid hormone receptor complex (36, 48) and appear to be capable of rendering heterologous promoters responsive to steroids (48).

We next asked whether the sequences within the structural gene responsible for induction may also reside within the coding sequences of the hGH gene. A fusion gene (tk-chGH A^+) was therefore constructed which contains the HSV *tk* promoter fused to position +35 of the hGH cDNA, which was then fused to sequences containing 525 bp of hGH 3′ flanking DNA to provide a poly(A) addition site (Fig. 2A). Thus, in this construct, all introns were deleted from the hGH structural gene. Northern blot analysis demonstrated that this fusion gene is inducible in all five transformed lines examined (Fig. 2B, lanes A to E). The tk-chGH A^+ gene directs the synthesis of an 820-bp fusion mRNA which is induced threefold in the presence of added glucocorticoids. In control experiments, we demonstrated that neither the endogenous actin gene nor the exogenously introduced *aprt* gene undergoes induction upon exposure to hormone (Fig. 2C). These experiments demonstrate that an element responsive to hormones resides either within the exon sequences themselves or within 3′ flanking DNA.

To further localize the sequences responsible for induction, we have replaced the hGH 3′ flanking DNA with 3′ flanking sequences derived from the HSV *tk* gene (Fig. 3A). The hGH sequences in this construct, therefore, derive solely from the hGH cDNA. We again observed induction of a fusion hGH mRNA in each of five transformed cell lines examined (Fig. 3B, lanes A to E). The level of induction ranged from two- to threefold as determined by densitometry. Controls with hamster *aprt* showed that this exogenously introduced cotransformed gene is unaffected by exposure to glucocorticoids (Fig. 3C). Moreover, no induction was observed when 3′ flanking DNA from the hGH gene was fused 3′ to the *tk* gene (data not shown). These experiments demonstrate that a regulatory element which is responsive to glucocorticoid induction resides within the exons of the hGH.

We tested the possibility that the glucocorticoid-responsive sequences within the hGH exons may function as an enhancer element (for reviews, see references 23 and 28) in a manner analogous to the glucocorticoid-responsive element of mouse mammary tumor virus (10). We have fused hGH cDNA in either a direct or an inverted orientation 118 nucleotides 5′ to the HSV *tk* gene (34). We observed no changes in the *tk* mRNA expression upon the addition of glucocorticoids to transformants bearing these constructs (data not shown). These data suggest that the regulatory sequences within the hGH exons do not function as a glucocorticoid-responsive enhancer element, activating transcription of the hGH genes.

Kinetics of induction of hGH mRNA. In initial experiments to discern the mechanism of glucocorticoid induction of the hGH cDNA fusion gene, we examined the kinetics of induction of hGH mRNA in two transformants in detail: one transformant containing the fusion gene tk-chGH A^+ (Fig. 2B, lane E) consisting of HSV *tk* 5′ flanking sequences, hGH cDNA sequences, and the hGH 3′ flanking sequences; and a second transformant containing the fusion gene tk-hGH (Fig. 1B, lane D) consisting of HSV *tk* promoter fused to the structural sequences of the hGH at position +3 within the 5′ untranslated region. The fusion gene tk-chGH A^+ (Fig. 2A) differs from tk-hGH (Fig. 1A) in that it lacks the four introns as well as 34 bp of 5′ untranslated hGH DNA.

Cells transformed with these fusion genes were exposed to 10^{-6} M dexamethasone, and the level of hGH mRNA was determined by Northern blot analysis at various times up to 72 h after induction. The kinetics of induction of the hGH cDNA fusion gene (tk-chGH A^+) revealed a long lag (Fig. 4A). Increased levels of steady-state hGH mRNA were not apparent until 12 to 24 h after the addition of glucocorticoid, and the hGH mRNA level was still increasing after 72 h of induction. In contrast, the induction of the tk-hGH gene occurred more rapidly (Fig. 4B). Two- to threefold induction was observed within 24 h of incubation with glucocorticoid, but maximum levels were not achieved until 60 h (see Fig. 5B for 60-h time point). Maintenance of an increased level of hGH required the continued presence of glucocorticoids, since upon removal of hormone, the hGH mRNA levels returned to that of the uninduced cells within 24 h (data not shown).

The observation that induction of the hGH cDNA fusion gene (tk-chGH A^+) occured after a long lag and was apparent only after 24 h of exposure to hormone suggests that the induction of hGH mRNA in this transformant may not be a primary event. Induction may not result from the direct transcriptional activation of the fusion hGH gene by the activated glucocorticoid-receptor complex but may require the participation of additional factors which do not preexist within the uninduced cell but must be synthesized de novo upon the addition of steroid before induction of the fusion mRNA is observed.

We tested this possibility by analyzing the kinetics of induction of hGH cDNA fusion gene (tk-chGH A^+; Fig. 2A) in the presence of the protein synthesis inhibitor, cycloheximide. Cells were incubated with cycloheximide (2 μg/ml) 1 h before the addition of steroid, and induction was continued for 24 or 48 h in the presence of both cycloheximide and dexamethasone. The concentration of cycloheximide used in this experiment inhibited protein synthesis by 95% as determined by incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material. The cDNA fusion gene, tk-chGH A^+, was induced 1.5-fold at 24 h and about 3-fold at 48 h in the absence of cycloheximide (Fig. 5A). In the presence of cycloheximide, induction was completely blocked at both time points.

In a transformant containing the tk-hGH fusion gene, the response to glucocorticoids was more rapid, with threefold induction observed within 24 h (Fig. 5B). Exposure of these transformants to cycloheximide and dexamethasone resulted in a 70% inhibition of induction at 24 and 44 h after the addition of steroid. Control experiments measuring the level of actin mRNA in identical mRNA samples showed no effect of cycloheximide on the steady-state level of actin mRNA sequences (Fig. 5B; data not shown for Fig. 5A).

These studies suggest that the mechanism of induction of the hGH cDNA fusion gene (tk-chGH A^+) requires continuing protein synthesis and perhaps the de novo synthesis of

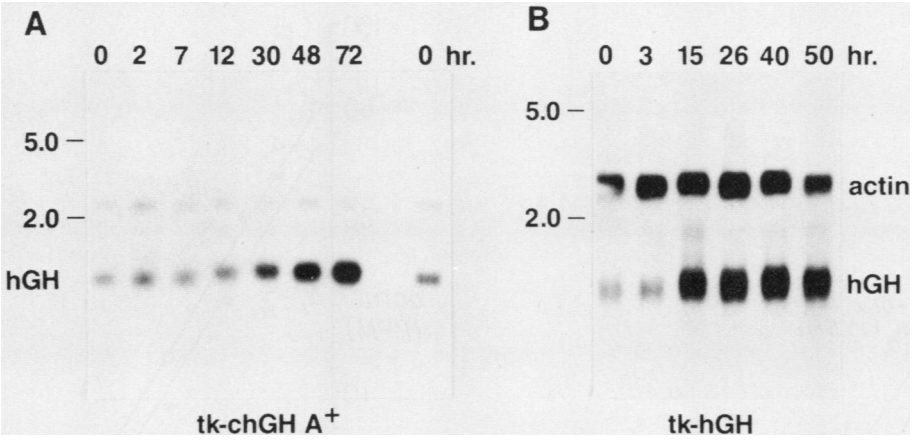

FIG. 4. Kinetics of induction of hGH mRNA. Total cytoplasmic RNA (20 μg) was isolated from a cell line expressing the tk-chGH A^+ fusion gene (transformant E in Fig. 2B) at various times after exposure to 10^{-6} M dexamethasone, electrophoresed through a 1.5% agarose gel containing 2.2 M formaldehyde, and transferred onto a Gene Screen transfer membrane. A ^{32}P-labeled, single-stranded RNA probe synthesized from a recombinant pSP6-4 plasmid harboring 0.8 kb of hGH cDNA was used for hybridization. Numbers at the top of the figure indicate the incubation time (in hours) with dexamethasone. The size markers are indicated at the left and are internal mouse ribosomal 28S and 18S RNAs. The origin of the upper bands (3 kb) is unknown, and they do not appear to be inducible. (B) Total cytoplasmic RNA (20 μg) isolated from cells transformed with the tk-hGH fusion gene (transformant D in Fig. 1B) was similarly analyzed by using ^{32}P-labeled, single-stranded RNA probes synthesized from the hGH cDNA and human gamma-actin cDNA.

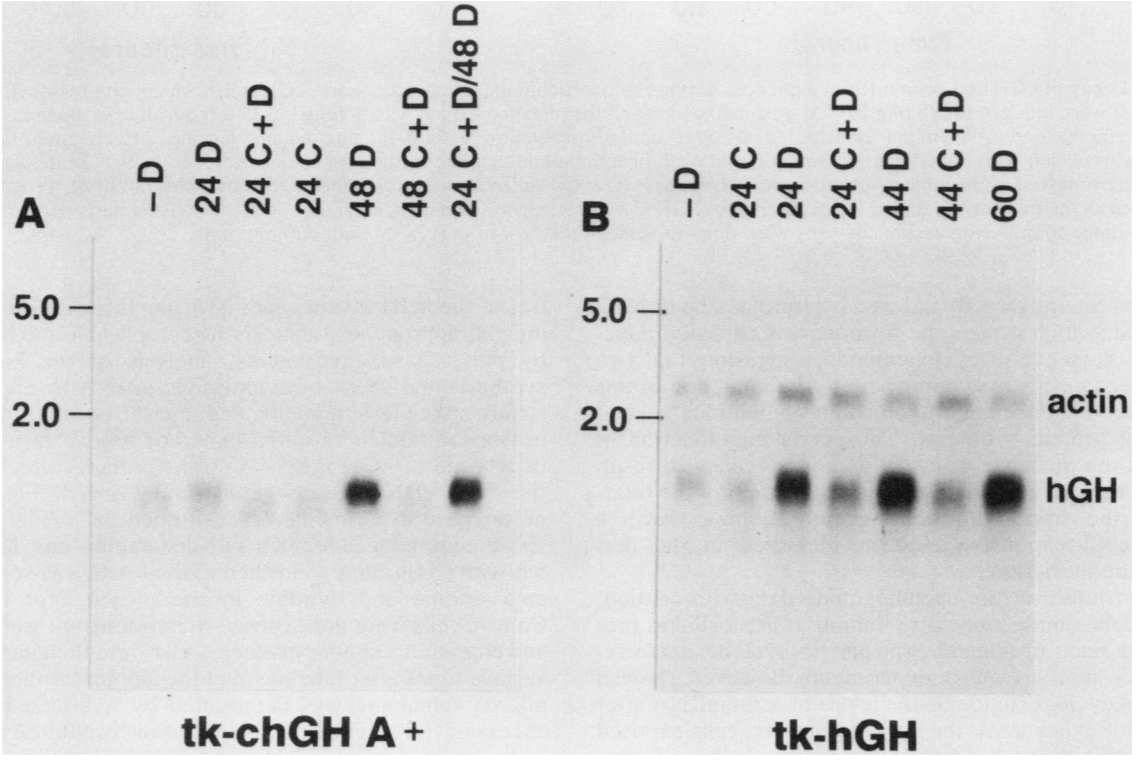

FIG. 5. Kinetics of induction of hGH fusion mRNA in the presence of a protein synthesis inhibitor. (A) Induction of hGH fusion mRNA (tk-chGH A^+) is abolished by cycloheximide. A transformant containing the tk-chGH A^+ fusion gene (lane E in Fig. 2B) was incubated in 10^{-6} M dexamethasone (D) in the presence or absence of 2 μg of cycloheximide (C) per ml, and at 24 or 48 h total cytoplasmic RNA was isolated. Control cells were maintained in medium lacking dexamethasone in the absence or presence of cycloheximide. Cycloheximide was added 1 h before the addition of dexamethasone in each case. Total RNA (20 μg) was electrophoresed through a 1.5% agarose-formaldehyde gel and subjected to Northern blot analysis by using ^{32}P-labeled, single-stranded RNA probe synthesized from the hGH cDNA. (B) Induction of tk-hGH fusion mRNA was significantly reduced by cycloheximide. A transformant containing the tk-hGH fusion gene (lane D in Fig. 1B) was incubated with cycloheximide, dexamethasone, or both, as described above. Total cytoplasmic RNA (20 μg) was analyzed on the Northern blot by using ^{32}P-labeled single-stranded probes specific for hGH cDNA and human gamma-actin cDNA sequences.

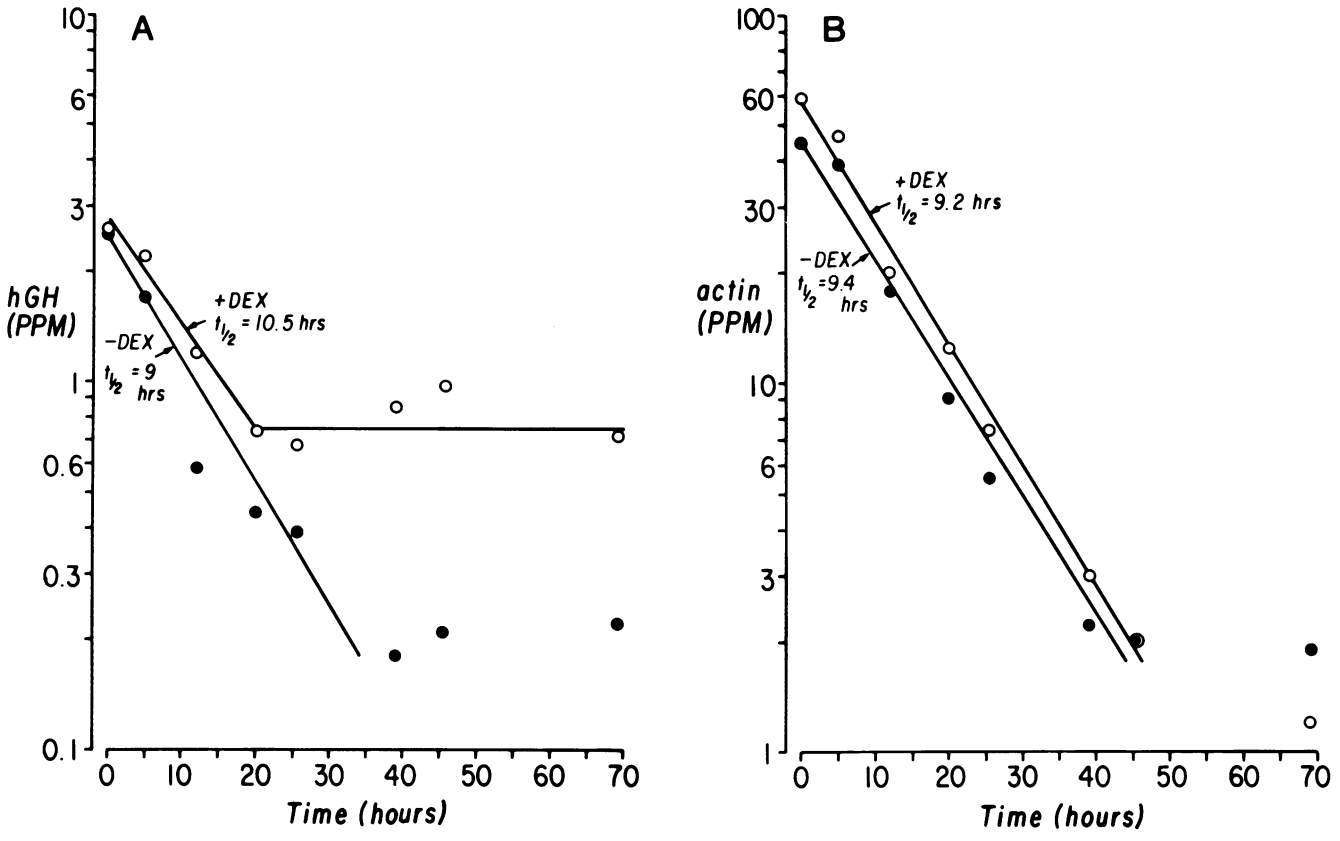

FIG. 6. Decay of hGH and actin mRNA sequences during glucocorticoid induction. Ltk$^-$ aprt$^+$ cells transformed with ptk-chGH A$^+$ (lane E in Fig. 2B) were induced for 48 h in 10^{-6} M dexamethasone and then labeled for 5 h with 150 μCi of [5,6-^{3}H]uridine in medium containing 10^{-6} M dexamethasone (○). Uninduced samples (●) were similarly labeled in medium lacking dexamethasone. At zero time, the labeling medium was replaced with a medium containing 5 mM each of uridine and cytidine in the absence or presence of 10^{-6} M dexamethasone. Samples were removed at the indicated times, and DNA excess filter hybridizations with ^{3}H-labeled total cytoplasmic RNA were done. Data are presented as the fraction of labeled RNA specific for hGH cDNA sequences (A) and actin cDNA sequences (B) in parts per million (PPM) of the total input counts. Regression analyses were done to achieve the best-fit curves through datum points.

regulatory proteins directly induced by steroid. The tk-hGH fusion gene, which retains the introns, was partially inducible in the presence of cycloheximide, suggesting that two distinct mechanisms may contribute to the induction of this fusion gene, one dependent upon and a second independent of ongoing protein synthesis. The persistent induction of tk-hGH in the presence of cycloheximide is likely to result from direct transcriptional activation mediated by the interaction of the steroid hormone-receptor complex with a glucocorticoid-responsive element identified in the first intron of the hGH gene.

We must interpret the cycloheximide data with caution, since cycloheximide may also inhibit other cellular processes and since prolonged exposure to cycloheximide reduces cell viability. Control experiments, however, showed no effect of cycloheximide on the levels of actin mRNA after 24 or 48 h of exposure to the drug. Moreover, cells exposed to cycloheximide and dexamethasone for 24 h and then washed free of cycloheximide exhibited induction of the hGH mRNA with kinetics identical to that of fresh cells never exposed to the drug (Fig. 5A, lane 24C + D/48 D). Thus, these studies suggest that the maximal induction of our fusion genes (tk-chGH A$^+$ and tk-hGH) is dependent upon continued protein synthesis.

Mechanisms of hormonal induction. We attempted to distinguish between transcriptional and posttranscriptional control of the hGH fusion genes by using three different experimental approaches: analysis of cytoplasmic mRNA decay by pulse-chase experiments, analysis of the kinetics of synthesis and decay of cytoplasmic mRNA by approach to steady-state measurements, and nuclear runoff transcription in isolated nuclei. In initial experiments, we measured the decay rate of hGH mRNA in transformants which contain the hGH cDNA fusion gene (tk-chGH A$^+$, Fig. 2A) by pulse-chase experiments. Transformed cells (Fig. 2B, lane E) were preinduced for 48 h with dexamethasone, labeled for 5 h with [^{3}H]uridine, and then chased with a vast excess of cold uridine and cytidine in the presence of hormone. Control cells were not exposed to hormone but were labeled and chased in a similar manner. Cells were then harvested at various times after labeling, and the amount of the [^{3}H]hGH mRNA remaining was determined by hybridization to an excess of the hGH cDNA bound to nitrocellulose filters.

Analysis of hGH mRNA in uninduced cells revealed a first-order decay from which we calculated a half-life of hGH mRNA of 9 h (Fig. 6A). In the presence of hormone, however, two kinetic components were observed: one component constituting about 75% of the hGH mRNA decayed with a half-life of 9 h, similar to that observed for hGH mRNA in uninduced cells. A second component, however, which constitutes 25% of the RNA, showed no decay over the 2.5-day chase period. These data indicate that in the

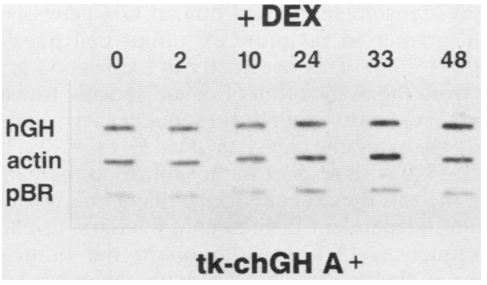

FIG. 7. Nuclear runoff transcription of the tk-chGH A^+ fusion gene. Transformant E in Fig. 2B was cultured for 4 days in medium supplemented with hormone-depleted serum. Nuclei were prepared after various times of incubation with 10^{-6} M dexamethasone (DEX), and in vitro transcription reactions were done as described in Materials and Methods. The in vitro ^{32}P-labeled runoff transcripts were hybridized to duplicate nitrocellulose filters containing linearized plasmids harboring hGH cDNA sequences, human gamma-actin cDNA sequences, and pBR322. Equal amounts (typically, 10^8 cpm) of labeled RNA were added to the hybridization tube for each time point.

presence of hormone a subpopulation of hGH mRNA is stabilized and shows no significant degradation for over 50 h. Control experiments measuring the decay rate of the endogenous actin mRNA species show a single first-order decay with a half-life of approximately 9 h both in the absence and in the presence of glucocorticoids (Fig. 6B).

These data provide an explanation for the long lag in induction of the steady-state hGH mRNA seen with the hGH cDNA constructs (Fig. 4A). The subpopulation of hGH mRNA afforded enhanced stability in the presence of hormone would not constitute a significant fraction of the newly synthesized hGH mRNA over short periods but, over time, would increasingly dominate the mRNA population.

The pulse-chase experiments demonstrate that glucocorticoids acted posttranscriptionally to prolong the half-life of a subpopulation of hGH mRNA, but these experiments do not exclude possible additional contributions from transcriptional events. We therefore performed approach-to-steady-state measurements (7) to determine the rate of entry of hGH mRNA into the cytoplasm. Briefly, cells transformed with a cDNA fusion gene were preinduced in the presence of glucocorticoids for 48 h and then were continuously labeled with [^{3}H]uridine. The amount of labeled cytoplasmic hGH mRNA at various times after the initiation of labeling was then determined by hybridizatrion to excess hGH cDNA bound to nitrocellulose filters. Given that the specific activity of the UTP pool and the amount of labeled hGH mRNA can be determined as a function of time, we were able to determine the rate constant of entry of hGH mRNA into the cytoplasm and the cytoplasmic decay constant for these sequences by using a computer program previously described (7). We observed that the kinetics of incorporation of [^{3}H]UTP into hGH mRNA was virtually identical either in the presence or absence of hormone for the 24-h labeling period (data not shown). Both induced and uninduced cells revealed an entry rate of hGH mRNA of about 260 molecules per cell per day (10^{-7} pg per cell per min), a value in accord with our estimate from steady-state levels and decay rates. Control experiments with the endogenous actin gene similarly revealed no change in the kinetic parameters of synthesis or decay in the presence or absence of hormone (data not shown).

The simplest interpretation of these data is that glucocorticoids do not significantly alter the rate of transcription of the tk-chGH A^+ fusion gene, but changes in both transcription and nuclear processing may also explain these observations. We also performed nuclear runoff transcription assays in isolated nuclei to determine the relative rates of transcription of the hGH cDNA fusion gene tk-chGH A^+ (Fig. 2A) before and after induction. Transcription reactions were done on isolated nuclei from induced and uninduced cells in the presence of [^{32}P]UTP. Newly synthesized hGH mRNA was then quantitated by hybridization to filters containing excess amounts of linearized plasmids containing hGH cDNA sequences. The level of transcription of hGH mRNA from the hGH cDNA fusion gene remained constant in nuclei from uninduced cells and from cells induced for various times in dexamethasone (Fig. 7). Control experiments with endogenous actin sequences similarly showed no changes in the level of transcription upon exposure to hormone. These experiments further suggest that the induction of the hGH

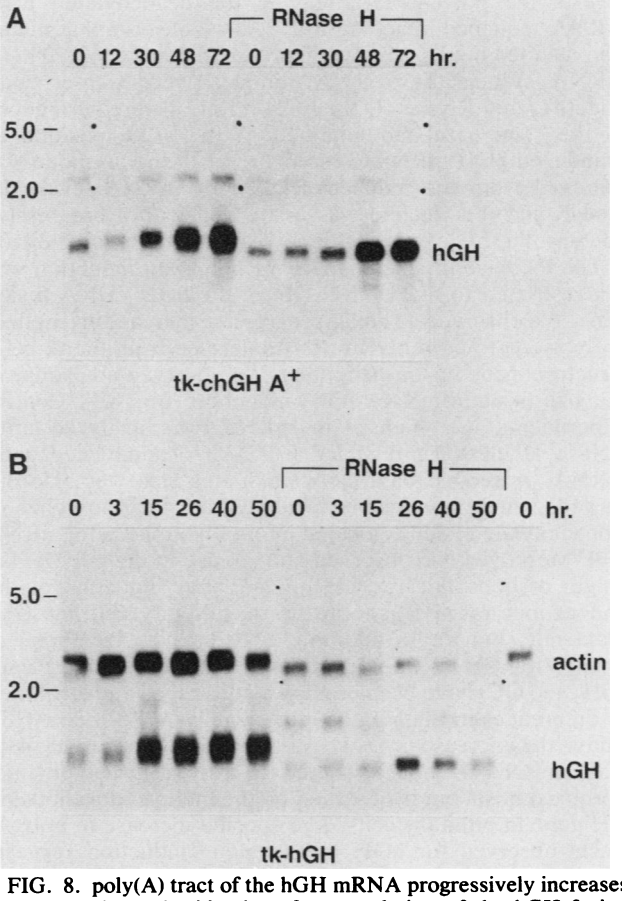

FIG. 8. poly(A) tract of the hGH mRNA progressively increases upon induction. The kinetics of accumulation of the hGH fusion messages in cells transformed with tk-chGH A^+ (A) (transformant E in Fig. 2B) and with tk-hGH (B) (transformant D in Fig. 1B) was analyzed on a 1.5% agarose gel containing 2.2 M formaldehyde. Total cytoplasmic RNA (20 μg) isolated after incubating cells with 10^{-6} M dexamethasone for indicated times (denoted by numbers on top) was blotted onto a Gene Screen transfer membrane and probed with ^{32}P-labeled, single-stranded hGH cDNA and human gamma-actin cDNA sequences. Lanes labeled RNase H are samples treated with RNase H in the presence of oligo(dT). In the RNase H lanes in panel B, 10 μg of total cytoplasmic RNA was loaded instead. The faint extra bands (1.1 kb) observed in RNase H-treated samples are derived from digestion of internal actin mRNA sequences (see the text for details).

cDNA fusion gene involves posttranscriptional rather than transcriptional regulation.

The hGH poly(A) tract increases upon induction. Our data suggest that the induction of the cDNA fusion gene is likely to be a consequence of enhanced stability of hGH mRNA. One possible explanation for the increase in stability derives from analysis of the size of hGH mRNA after induction. Northern blots with high-percentage formaldehyde-agarose gels revealed that the hGH mRNA became larger with induction (Fig. 8A). At the time of maximal induction (72 h), hGH mRNA was about 100 nucleotides longer than hGH mRNA synthesized in uninduced cells.

We performed RNase H experiments (17, 52) to demonstrate that this increase in the size of hGH mRNA upon induction is caused by an increase in the size of the poly(A) tail. Total cytoplasmic RNAs isolated at various times of induction were hybridized to oligo(dT) and digested with RNase H. Since RNase H digests only RNA which exists as an RNA-DNA hybrid (5, 17, 52), this enzyme completely digests the poly(A) tail, leaving the deadenylated hGH mRNA sequence intact. In Fig. 8A, we observe that induction resulted in a progressive increase in the size of the hGH mRNA. When these RNA samples were treated with oligo(dT) and RNase H, the hGH mRNAs were all reduced to the same size and migrated to the same position as uninduced hGH mRNA. Moreover, when the hormone was removed from the induced cells, hGH mRNA levels declined, and this decline was associated with a progressive decrease in the length of the poly(A) tail (data not shown).

The increase in size of hGH mRNA upon induction was not restricted to hGH mRNA from the hGH cDNA fusion gene. We observed a similar progressive increase in length of the poly(A) tract in hGH mRNAs derived from the tk-hGH structural gene fusions (Fig. 8B). We observed no change in the size of actin mRNA upon induction (Fig. 8B). Control experiments in which actin mRNA was analyzed after RNase H cleavage revealed a 2.2-kb deadenylated actin mRNA. A second band about half this size was also observed, which presumably resulted from long stretches of riboadenylate residues located internally in the actin mRNA (39). Moreover, we observed an increase in the poly(A) tail length of bona fide rat GH mRNA after induction of the endogenous rat GH gene in the rat pituitary cell line GH_3 (data not shown). In addition, two distinct size forms of mature rat GH mRNA in cultured anterior pituitary cells (GH_3 and GC) have been previously described and attributed to differences in the lengths of their poly(A) segments (18). Thus, the increase in poly(A) tract length observed with hGH mRNA is not restricted to fusion genes or their fibroblast hosts but is observed on the intact endogenous rat GH gene in pituitary cells. The specific increase in poly(A) tracts observed for hGH mRNA upon induction suggests that sequences reside within the exons of GH which are responsible for this increase. Moreover, this change in the length of the poly(A) tail may be related to the enhanced stability of hGH mRNA in induced cells.

DISCUSSION

Expression of the GH gene is restricted to the somatotrophs of the anterior pituitary (26). Experiments to identify the *cis*-acting elements which control the expression of the GH genes have revealed two classes of regulatory sequences: sequences responsible for tissue-specific expression of GH genes and sequences which render these genes responsive to glucocorticoids or thyroid hormones. Expression of the transfected rat and human GH genes is 100- to 1,000-fold greater in rat pituitary tumor cell lines than in fibroblasts (16, 42). This specificity of expression is thought to result from the association of tissue-specific transcription factors with discrete regulatory sequences within the 235 nucleotides flanking the cap sites of rat GH (37) and between −285 and −470 with respect to the cap site of human GH (9) genes. In the rat, these sequences behave in a position- and orientation-independent fashion characteristic of classic enhancer sequences (37). In addition to the tissue-specific enhancer, a glucocorticoid-responsive element has been identified within the first intron of the human GH gene (36, 48). These sequences which bind glucocorticoid receptor in vitro render a heterologous gene inducible by steroids, albeit at low levels (48).

We have studied the control of expression of the hGH gene introduced into the chromosome of mouse fibroblasts. These transformed cell lines express low levels of intact hGH mRNA and secrete hGH proteins into the culture medium (42). Although the level of expression of hGH mRNA is low, presumably due to the absence of tissue-specific regulatory factors, the gene remains responsive to induction by glucocorticoid hormones. These cells, therefore, provide an opportunity to investigate the mechanisms of steroid hormone induction free of the pituitary-specific factors regulating basal hGH mRNA levels.

We have previously demonstrated that a fusion gene consisting of hGH 5′ flanking DNA and HSV *tk* structural gene sequences remains inducible by steroid hormone (42). In the current study, we demonstrated that a fusion gene (tk-hGH) consisting of the HSV *tk* promoter linked to the whole structural sequences of the hGH gene in the 5′ untranslated region was inducible (Fig. 1). Moreover, a second fusion gene (tk-chGH A^+) in which the hGH structural sequences are replaced with the hGH cDNA sequences, thereby eliminating all introns, remained inducible by glucocorticoids (Fig. 2). Additional experiments with hGH hybrids deleted in the 3′ hGH flanking sequences confirmed that 0.8 kb of hGH exon sequences contained sequences which were responsive to glucocorticoid hormone induction (Fig. 3). We performed a series of experiments which indicate that the mechanisms responsible for induction of the cDNA fusion gene (tk-chGH A^+; Fig. 2A) operated posttranscriptionally and therefore differ from the transcriptional induction by steroid hormones observed, for example, with the mouse mammary tumor virus genome (for reviews, see references 41 and 59). First, the kinetics of the induction of hGH mRNA synthesized from the cDNA fusion gene was slow (Fig. 4A): increased levels of hGH mRNA were not observed until 12 to 24 h, and maximal induction required at least 72 h of exposure to the steroid. Second, induction of hGH mRNA was abolished by the protein synthesis inhibitor, cycloheximide (Fig. 5A). Third, nuclear runoff transcription experiments (Fig. 7) and approach-to-steady-state measurements suggest that the rate of transcription of hGH mRNA is unaltered by exposure to hormone. Finally, pulse-chase experiments to measure the half-life of hGH mRNA revealed that a subpopulation of the hGH mRNA was afforded considerably enhanced stability in the presence of hormone (Fig. 6A).

Thus, the removal of the glucocorticoid-responsive enhancer element within the first intron of hGH and the replacement of the hGH promoter with that of HSV *tk* eliminated all known *cis*-acting transcriptional regulatory sequences and allowed us to uncover sequences which operated posttranscriptionally to enhance the stability of

hGH mRNA in the presence of glucocorticoids. Our observations are consistent with a model in which glucocorticoids induce the synthesis or maintain the expression of a second protein which either directly or indirectly interacts with hGH mRNA to increase its stability. The continued presence of glucocorticoids is required to maintain increased stability of hGH mRNA, for upon removal of hormone, hGH mRNA levels return to that of the uninduced cells within 24 h. This model may explain both the slow induction kinetics as well as the observations with protein synthesis inhibitors.

We showed that an uninduced cell maintains a steady-state level of 30 molecules of hGH mRNA per cell by synthesizing 78 molecules per day, with a half-life of 9 h. If after induction, 25% of the newly synthesized RNA (for example, approximately 20 molecules a day) is afforded a significantly longer half-life (several days for example), then after 24 h in dexamethasone, steady-state induction would only be minimal (50 molecules). By 72 h, the induction rises to over threefold (90 molecules). Such a model invoking a time-dependent accumulation of a stable subpopulation of hGH mRNA in the presence of glucocorticoid is therefore entirely consistent with the kinetic measurements of both newly synthesized and steady-state hGH mRNA.

How may glucocorticoids alter the stability of a subpopulation of specific mRNA? One possible mechanism is suggested by the observation that the poly(A) tract of hGH mRNA, but not control mRNAs, increases in length with time after induction and decreases in length when dexamethasone is removed. An increase in the length of the 3′ poly(A) tract may result from the initial addition of a longer poly(A) tract in the nucleus, a decrease in the rate of degradation of poly(A) tail, or a time-dependent addition of adenylate residues to the 3′ end of cytoplasmic mRNA. Although we cannot distinguish among these alternatives, it is tempting to assume that the increased length of 3′ poly(A) is a consequence of hormone induction and, in turn, is responsible for enhanced stability of hGH mRNA. This suggestion is consistent with the correlation between half-life and the length of the poly(A) tract observed for adenovirus mRNAs (57). It remains possible, however, that the increased length of the poly(A) tract is not the cause but the consequence of the increased stability of hGH mRNA. Whatever the mechanism responsible for maintenance of longer poly(A) tracts in induced cells, this process is specific for hGH mRNA and is not observed for control mRNAs such as actin. Moreover, this process can be observed on hGH cDNA fusion genes containing either hGH or *tk* 3′ untranslated sequences, suggesting that the exons of hGH contain sequences which render this specific mRNA more stable upon glucocorticoid induction.

These data, taken together with those of other laboratories, demonstrate that glucocorticoids play a dual role in regulating expression of the hGH gene. We have previously shown that sequences within the 5′ flanking region of the hGH were responsive to glucocorticoids (42). The glucocorticoid hormone-receptor complex was shown to interact with sequences located within the first intron of the hGH gene in vitro (36, 48). These sequences presumably enhance the rate of transcription of the hGH gene. Our data furthermore suggest a possibility that glucocorticoids also induce the synthesis of a protein which interacts with hGH exon sequences in RNA to increase its stability. By regulating two independent events in RNA metabolism, the inductive effect of glucocorticoids could become multiplicative. In this manner, small changes in transcription can be amplified considerably by concomitant changes in mRNA stability. This dual level of control affords a flexibility which may be important in the control and maintenance of mRNA levels of genes such as GH which are under multihormonal control.

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