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Introduction of rat growth hormone gene into mouse fibroblasts via a retroviral DNA vector: Expression and regulation

(murine sarcoma virus DNA/pituitary hormone/gene transfer/glucocorticoid induction)

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ABSTRACT **We have introduced the rat growth hormone gene into mouse fibroblasts via a retroviral DNA vector. The ability of the viral DNA to induce foci in the recipient cells was used as a dominant selection marker. Several copies of rat growth hormone DNA were integrated in the mouse cells. The transformed mouse cells expressed rat growth hormone-specific mRNA and secreted mature rat growth hormone. In rat cells, the expression of this gene is regulated by glucocorticoids. We demonstrate that hormone-dependent regulation transfers with the clone and thus appears to be an intrinsic property of the gene or its RNA products.**

Introduction of purified eukaryotic genes into heterologous host cells provides a powerful means for studying the expression of specific genes outside their usual cellular environment (1). Specific genes have been introduced into mammalian cells in tissue culture by cotransfer with a marker gene for which there is dominant selection (1). The herpes simplex virus thymidine kinase gene has been used as a selectable marker for introduction of the genes for rabbit β-globin (2), chicken ovalbumin (3), and adenine phosphoribosyltransferase (4) into cells. Stable expression of each gene was achieved but in a nonregulated fashion. Recently, it has been shown that cloned genes transfected into heterologous cells can be expressed and induced by glucocorticoids (5–7).

We are interested in identifying the mechanisms and sites involved in the expression and regulation of eukaryotic genes. The rat growth hormone (rGH) gene provides a model for these studies because its expression can be studied *in vivo*, in primary pituitary cell cultures, and *in vitro* in a stable clonal cell line (8). The use of cultured cell lines permits direct analysis of hormonal regulation of genes not possible *in vivo* or using organ cultures. Growth hormone (GH) production in these cells has previously been shown to be induced by both glucocorticoids and thyroxine (9–11). We have used pulse-labeling analysis to identify the primary transcript of this gene, and recently Dobner *et al.* (8, 12) have provided evidence that glucocorticoids act transcriptionally to induce this RNA. The complete nucleotide sequence of this gene and the organization of its flanking chromosomal regions have been defined (13, 14). Here we report the use of DNA-mediated gene transfer to assess the functional significance of sequences involved in the expression and regulation of this gene. We have introduced the rGH gene into mouse 3T3 cells, using a plasmid vector that contains the entire genome of Moloney mouse sarcoma virus (Mo-MSV) (15). Mo-MSV is able to transform fibroblasts *in vitro* and thus provides a selection criterion. We have introduced a 7.6-kilobase pair (kb) *Bam*HI fragment of rat genomic DNA into NIH/3T3 mouse fibroblasts by inserting it into Mo-MSV DNA.

Several cellular clones were isolated after growth in soft agar and analyzed for expression of rGH. The results indicate that (*i*) DNA isolated from mouse NIH/3T3 cells propagated from an isolated focus (gh-2) contained both rGH-specific and Mo-MSV_{src} (v-mos^{Mo})-specific sequences, (*ii*) total poly(A)$^+$RNA isolated from one-focus gh-2/3T3 cells reacted to rGH-specific cDNA, (*iii*) gh-2/3T3 cells containing the rGH gene secreted immunoreactive rGH into the culture media at up to 900 ng per 10^6 cells per 150 hr, (*iv*) the secreted rGH cochromatographed with *bona fide* rGH, and finally (*v*) addition of dexamethasone to gh-2/3T3 cells showed enhanced production of rGH.

MATERIALS AND METHODS

Molecular Cloning. About 3×10^5 plaques of a rat chromosomal DNA library constructed by partial *Eco*RI cleavage (16) were screened, and five plaques reacting to labeled rGH cDNA probe (pGH-1) (8) were obtained. One positive plaque, λRGH5, was purified by successive screening and analyzed extensively (unpublished results). A 7.6-kb *Bam*HI fragment of λRGH5 containing the entire rGH gene was further subcloned into plasmid pBR322 (unpublished). The construction of the plasmid pMSV-12 containing the entire Mo-MSV DNA has been described (15).

Cell Culture. Cells were maintained in Dulbecco's modified Eagle's minimal essential (DME) medium/heat-inactivated 10% calf serum (Irvine Scientific) supplemented with penicillin at 100 units/ml and streptomycin at 100 μg/ml. To decrease nonspecific backgrounds, cells were grown in serum-substituted medium when assayed for GH production. The attachment of transformed cells in the synthetic medium is aided by incubating the culture plates overnight with polylysine (20 μg/ml) prior to use.

Transfection. MSV DNA containing the GH gene was cleaved from plasmid pMSV-GH-1 by using restriction enzyme *Cla* I, which removes 81 nucleotides from the Mo-MSV DNA, and *Bam*HI, which leaves 0.3 kb of pBR322 sequences (see Fig. 1). The insert DNA was separated from the plasmid by preparative agarose gel chromatography and DNA was recovered by elution with glass powder. Transfections were carried out essentially as described (17, 18). Briefly, 1 μg of purified

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Abbreviations: GH, growth hormone; rGH, rat growth hormone; Mo-MSV, Moloney mouse sarcoma virus; kb, kilobase pair(s); DME medium, Dulbecco's modified Eagle's medium.

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MSV–GH DNA in 3.3 μl of 10 mM Tris/0.1 mM EDTA was diluted into 246.7 μl of Hepes-buffered saline (137 mM NaCl/6 mM dextrose/5 mM KCl/0.7 mM Na_2HPO_4/20 mM Hepes adjusted to pH 6.98 with 0.5 M NaOH) and 25 μg of NIH/3T3 DNA/ml as carrier was added. The DNA was precipitated by addition of 13.2 μl of 2.5 M $CaCl_2$ (final concentration, 125 mM), and the mixture was incubated at room temperature for 25 min and then added to 3×10^5 NIH/3T3 cells in a 3-cm Petri dish. After 4 hr of incubation, the cells were treated with 15% glycerol (vol/vol) in DME medium for 2 min and then washed twice with DME medium. After overnight incubation in DME medium/10% calf serum, the cells were trypsinized and seeded into three 3-cm Petri dishes. After 4 days, the cultures were transferred into 0.25% soft agar. Colonies visible 3 weeks after transfer into soft agar were picked at about 6 weeks and grown in mass cultures ($>10^7$ cells) for further study.

Nucleic Acid Analysis. High molecular weight DNA from cells was prepared and analyzed by the Southern blot transfer procedure (19). Cytoplasmic RNA was isolated after lysis with 0.5% Nonidet P-40 in isotonic/high pH buffer as described (8). RNA was isolated from pituitaries that were pulverized at −70°C. The RNA was analyzed as described (20).

Radioimmunoassay. GH levels in culture media were assayed by radioimmunoassay using materials provided by the Rat Pituitary Hormone Distribution Program of the National Institute for Arthritis, Diabetes, and Digestive and Kidney Diseases. Separation of bound from free tracer was achieved by precipitation of antiserum with fixed *Staphylococcus aureus*. The abilities of samples and standard rGH to compete with iodinated rGH for binding to specific monkey anti-rat GH serum have been compared.

RESULTS

Construction of the Transforming Vector. We have isolated an 18-kb genomic clone containing the rGH gene from the rat chromosomal library constructed by Sargent *et al.* (16). For screening the genomic library, a cDNA clone, pGH-1, containing portions of rGH structural sequences, as confirmed by hybridization-translation analysis (21), was used. The genomic sequences that bear sequence homology to pGH-1 are contained in a 7.6-kb *Bam*HI restriction fragment (Fig. 1). Direct DNA sequence analysis shows the precise organization of the gene and suggests that the primary transcript should be ≈2 kb long (13, 14). Pulse-labeling and RNA blot analysis have identified a primary transcript ≈3.0 kb long (8). Although the nature of this discrepancy is unclear, it seems reasonable to assume that the 7.6-kb *Bam*HI restriction fragment contained the entire functional gene. This fragment has been subcloned into pBR322 and used as a source for GH chromosomal sequences. The isolation and cloning of full-length murine sarcoma viral DNA has been described (15). This is a defective retrovirus that can cause neoplasia in animals and can transform mouse fibroblasts *in vitro*. The cloned viral DNA has been shown to induce focus formation in an uninfected mouse NIH/3T3 fibroblast cell line (15).

The 7.6-kb *Bam*HI restriction fragment containing the entire GH gene was inserted into the unique *Bgl* II site of pMSV-12 DNA to make a hybrid plasmid, pMSV-GH, whose organization is shown in Fig. 1. This plasmid is constructed such that the two genomes have opposing transcriptional orientations.

Transduction of rGH Genes into Mouse Fibroblasts. The insert containing the GH and Mo-MSV DNAs was excised from pMSV-GH-1 by digestion with *Cla* I/*Bam*HI and transfected onto NIH/3T3 mouse fibroblasts. To study the organization of MSV-GH-1 DNA in the transformed cells, cellular clones were grown and their DNAs were analyzed by blot analysis (19). Because the *Bam*HI sites of the GH insert were destroyed during construction of pMSV-GH-1, this enzyme can be used to determine the number of MSV–GH copies integrated into the DNA from the transformed cells. A *Bam*HI/*Kpn* I restriction fragment from the GH insert or the v-*mos*^{Mo} sequences (22) were used as probes to detect insert DNA sequences. Fig. 2

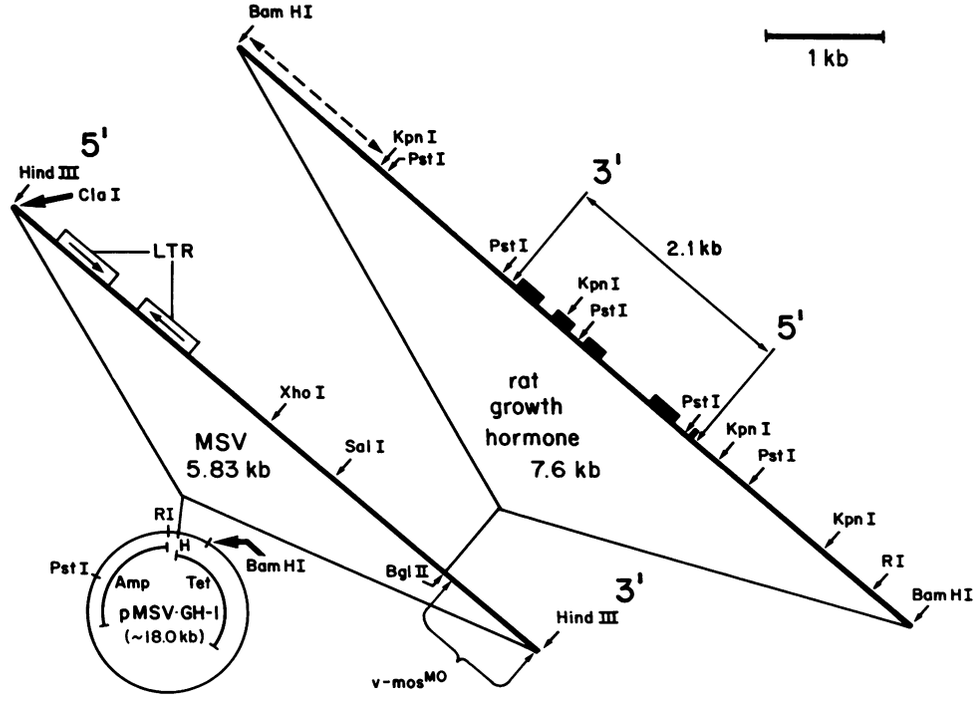

FIG. 1. Map of pMSV-GH-1. A diagram of the recombinant clone containing a *Bam*HI fragment of genomic GH DNA inserted into the unique *Bgl* II site of a MSV DNA cloned in pBR322 is shown. ----, GH fragment used for nick-translation; —, GH gene within adjacent rat cell DNA; ■, transcribed regions (exons). Insert MSV-GH-1 DNA was isolated by digestion with *Cla* I/*Bam*HI (←) and purified prior to transfection.

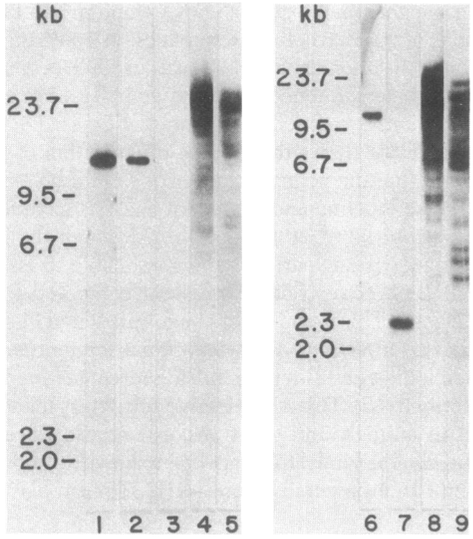

FIG. 2. Analysis of transformed cell DNA. Ten micrograms of high molecular weight DNA was digested with *Bam*HI (*A*) or *Eco*RI (*B*), and the digests were subjected to electrophoresis on 0.5% or 0.7% agarose and analyzed by Southern blotting and hybridization with probes for the GH insert (lanes 1, 3, 4, 6, and 8) or v-*mos*^{Mo} (lanes 2, 5, 7, and 9). Lanes: 1 and 2, 300 pg of MSV-GH-1 insert DNA; 3, NIH/3T3 DNA; 4, 5, 8, and 9, gh-2/3T3 DNA; 6 and 7, 300 pg of *Eco*RI-cut MSV-GH-1.

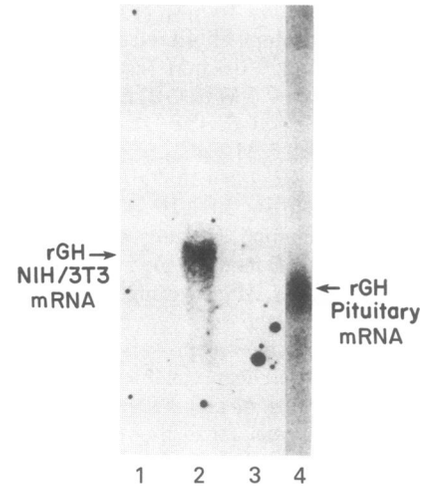

FIG. 3. Synthesis of rGH mRNA by mouse cells. Total cytoplasmic RNA from transformed cells and pituitary cells was prepared as described (8). RNAs were taken up in 10 mM NaH_2PO_4, pH 7.4/50% formamide/2.2 M formaldehyde, heated at 65°C for 5 min, and subjected to electrophoresis on a slab gel composed of 1.5% agarose in 10 mM NaH_2PO_4, pH 7.4/0.5 mM EDTA/1.1 M formaldehyde, using 10 mM NaH_2PO_4, pH 7.4/0.5 M formaldehyde. The gel was stained with acridine orange to identify rRNA markers, photographed, gently shaken for 90 min with 50 mM NaOH, and neutralized with two washes of 0.2 M NaOAc (pH 4.3). The RNA was then transferred to diazotized paper, washed in prehybridization buffer, and hybridized for 16 hr at 42°C with ^{32}P-labeled growth hormone cDNA (10^8 cpm/ml). Hybrids were washed twice at room temperature with 0.30 M NaCl/0.03 M Na citrate/0.1% $NaDodSO_4$ and twice at room temperature with 0.15 M NaCl/0.015 M Na citrate/0.1% $NaDodSO_4$ and then autoradiographed. Lanes: 1, 60 μg of 3T3 RNA; 2, 60 μg of gh-2/3T3 RNA; 3, 60 μg of RNA from 3T3 cells transformed with chimeric plasmid containing MSV–rabbit β-globin DNA; 4, 1 μg of RNA from rat anterior pituitaries.

shows that the transformants contain reactive sequences when screened with GH- or v-*mos*^{Mo}-specific probes. The DNA from control NIH/3T3 cells (lane 3) does not show any hybridization to the GH probe. When *Bam*HI-cleaved DNA from the transformed cells was hybridized to v-*mos*^{Mo}-specific probes, a number of bands larger than the 14.0-kb insert were also detected (lane 5). A 23.0-kb fragment identified by the v-*mos*^{Mo} probe represents the cellular homologue of the v-*mos*^{Mo} sequences. The presence of several bands in each transformant suggests that the transformed cells contain multiple copies of MSV-GH-1 DNA. It is, however, noteworthy that some of the bands observed by the GH and v-*mos*^{Mo} probes are similar, indicating that the two genes remained linked after transfection. In addition to the expected high molecular weight forms, subgenomic fragments are found in some of the lines (lanes 4 and 5), suggesting that not all integrations contain intact genomic sequences.

In an attempt to characterize further the nature of the integrated GH, the DNA was cleaved with *Eco*RI, which separates the GH sequences from the v-*mos*^{Mo} sequences (Fig. 1). Again, hybridization to the GH-specific (Fig. 2, lane 8) and v-*mos*^{Mo}-specific (lane 9) probes shows a number of bands representing individual integration sites containing GH or v-*mos*^{Mo} sequences. Unlike the *Bam*HI digestion, however, the bands hybridized to GH- and v-*mos*^{Mo}-specific probes do not correspond in size. The presence of GH- and v-*mos*^{Mo}-related bands smaller than 12.0 and 2.2 kb, respectively (lanes 6 and 7), supports the conclusion that subgenomic fragments may integrate.

Expression of rGH Gene. To test whether the integrated rGH DNA is expressed, we analyzed both the RNA and the GH secreted into the medium. Total RNA from transformed cells found to have incorporated GH sequences was analyzed for the presence of rGH-specific RNA transcripts. Cellular RNA was size fractionated on formaldehyde gels and then blotted onto diazotized paper and hybridized with a nick-translated GH cDNA clone. gh-2/3T3 cells were found to produce a cytoplasmic mRNA species that was larger than authentic rGH mRNA (Fig. 3, lane 2). No hybridization was found with mRNA from NIH/3T3 cells or from cells transformed with pMSV alone (lanes 1 and 3).

The presence of a discrete-sized GH-reactive mRNA in one of the transformants led us to consider the possibility that it could be translated to yield mature rGH polypeptide. Because this hormone precursor contains a leader or signal peptide sequence (23), an accurately translated and processed polypeptide should be secreted from the cell into the medium. Culture medium from MSV-GH-1-transformed cells was harvested after 72 hr of incubation, and a quantitative radioimmunoassay was used to show that it contained substantial levels (≈150 ng/ml) of GH. Culture media from NIH/3T3 cells or pMSV-transformed cells contained background levels (<1 ng/ml).

The identity of the secreted GH was further established by gel fractionation (Bio-Gel P-60) and immunodisplacement analysis. The results showed that the GH produced by gh-2/3T3 cells has an exclusion volume similar (within the resolution of this analysis) to that of *bona fide* GH isolated from rat pituitary extracts, with a M_r of ≈20,000 (Fig. 4). An apparent shift of one fraction is the consequence that not every fraction was analyzed separately in the column assay of the GH produced by mouse cells. Instead, fractions were pooled and then assayed for immunoreactivity.

Glucocorticoid Regulation of GH Synthesis. The synthetic glucocorticoid dexamethasone has been shown to modulate expression of the GH gene in cultured rat pituitary cells (11, 12). To determine whether the GH gene displays steroid-dependent regulation in MSV-GH-1-transformed cells, cultures were examined for their rate of GH synthesis in the absence or

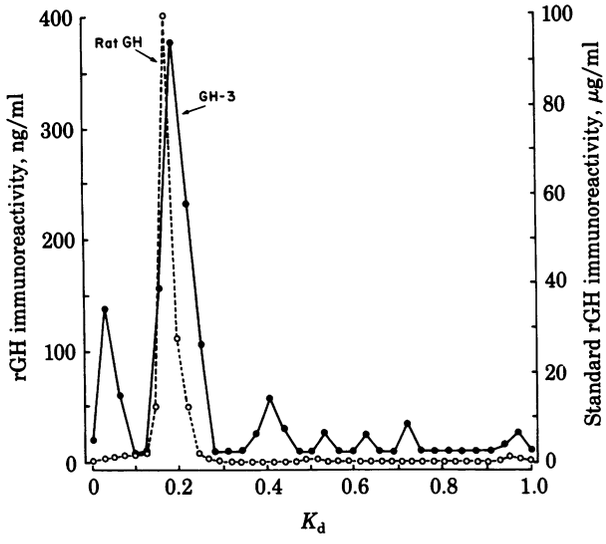

FIG. 4. Chromatography of rGH secreted by mouse cells. The size of the GH peptide secreted by gh-2/3T3 cells was examined by column chromatography as follows: About 35 ml of culture fluid was dialyzed (spectrapor-3) against 10 M NH_4OAc (pH 7.2) with three changes. The material retained was lyophilized, suspended in 0.8 ml of 0.1 M NH_4OAc (pH 7.2), and applied to a 0.7 × 50 cm column of Bio-Gel P-60 (100–200 mesh) previously coated with bovine serum albumin. The column was eluted with 0.1 M NH_4OAc (pH 7.2) at a flow rate of 1.0 ml/hr. Eluted fractions were lyophilized, suspended in 100 nM Hepes/0.1% bovine serum albumin, and assayed for GH (●). The column was standardized with dextran blue (V_0), ^{125}I-labeled rGH, and phenol red (V_T) (○).

presence of dexamethasone. In parallel, the effects of dexamethasone on the kinetics of synthesis of GH in a rat pituitary cell line (GC) were also studied. Fig. 5 shows that dexamethasone treatment of mouse transformants enhanced GH production by a factor of ≈2.5. The kinetics of induction and the augmentation of GH synthesis in rat pituitary cell lines closely mimic the patterns of mouse transformants. The absolute amount of GH produced by GC cells, however, is ≈100 times that of gh-2/3T3 cells. Normal cells or cells transformed by MSV-containing β-globin show no GH synthesis or response to steroid treatment.

DISCUSSION

The ability of molecularly cloned MSV DNA to transform mouse fibroblasts has been used as a selective marker to construct mouse cell lines containing the rGH gene. Such a selection system (i.e., focus formation or growth in soft agar) can be used as a dominant morphological marker. Introduction of the molecularly cloned rGH gene into mouse cells provides an opportunity to study its expression in a heterologous host. Analysis of the transformed cells indicated that most of the GH-related sequences are localized in high molecular weight DNA (Fig. 2). The restriction endonuclease pattern remains unchanged for several generations, suggesting that the stability of these transformants is consequential to chromosomal integration. DNA blot analysis indicated that independent transformants carry between 3–20 copies and that subgenomic fragments may be integrated.

Introduction of the rGH gene into mouse fibroblasts provides an *in vitro* system to study the functional significance of various features of DNA sequence organization. Because GH is synthesized with a leader or signal peptide that targets the protein for secretion, one can rapidly screen transformants for expression by radioimmunoassay of the culture media. This feature enhances the utility of this gene as a model for analysis of expression and regulation. We found that the rGH gene can direct the synthesis of stable, discrete-sized, and functional mRNA in mouse fibroblasts. This mRNA is ≈250 nucleotides larger than its *bona fide* rat pituitary counterpart but, nonetheless, gives rise to an apparent identical GH peptide, based on immunological and physical properties. The basis of the structural polymorphism needs to be determined but most likely represents a 5′ or 3′ extension because all splice sites have been mapped into GH coding regions (13, 14). Therefore, this variation is likely to represent the use of an alternative transcription start site or polyadenylylation site. The use of alternative poly(A) sites has been suggested to be associated with important physiological switching mechanisms in both the endocrine (24) and the immune (25) systems. Since the GH gene is both developmentally and homeostatically regulated, it is possible that its structure observed in the mouse fibroblast could have a relevant physiological correlate. Other genes, including β-globin and ovalbumin, introduced by cotransfection into mouse cells have been observed to produce mRNAs cor-

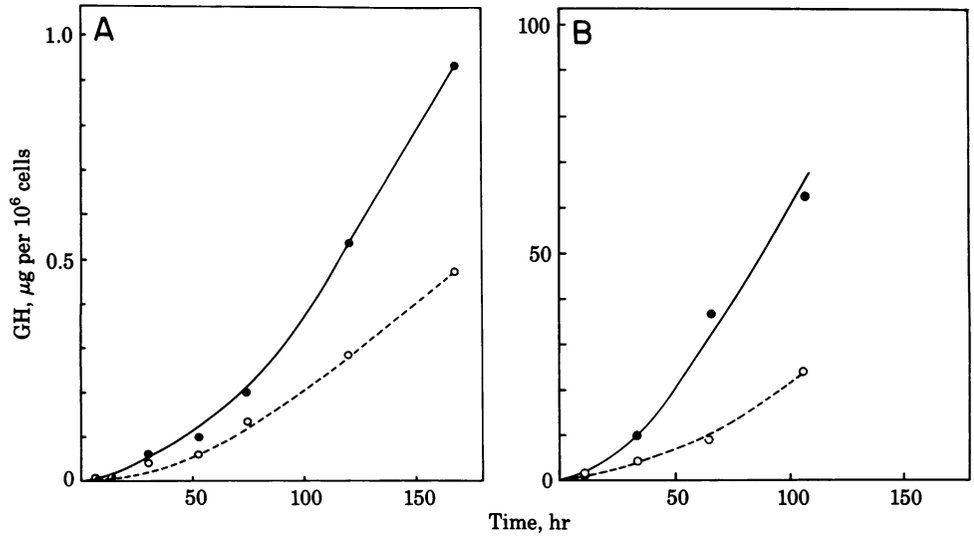

FIG. 5. Effect of dexamethasone on production of GH. gh-$2S_3$/3T3 (a subclone of gh-2/3T3) cells (*A*) and GC cells (*B*) (1 × 10^7 per 140-mm dish) in 25 ml of synthetic medium without serum were incubated without (○) and with (●) 5 μM dexamethasone, 300-μl aliquots were taken from the culture fluid, and GH levels were determined by radioimmunoassay.

rectly spliced but with 5′-terminal extensions (2, 3). Therefore, it is equally possible that mouse cells do not consistently promote correct initiation or polyadenylation of heterologous genes whose expression is normally restricted to other differentiated cell types or species.

Expression of the rGH gene in clonal rat pituitary cells has been shown to be responsive to glucocorticoids (11, 12). We reason that, if steroid induction of GH is due to an inducible control element, this regulatory site may be located near the GH gene. Since the insert MSV-GH-1 contained at least 2 kb of adjacent cellular sequences at both the 5′ and 3′ termini of the GH-coding region, it is possible that the regulatory control elements were transferred to the mouse fibroblasts along with the GH gene. Since mouse 3T3 fibroblasts contain cytoplasmic receptors for glucocorticoids (unpublished data), we examined the potential effect of the synthetic glucocorticoid dexamethasone on the production of GH, both by a subclone of gh-2/3T3 (gh-2S_3/3T3) and by rat pituitary cells. A 2.5-fold enhancement of GH released in the medium from cells treated with dexamethasone was observed in both cases (Fig. 5). Thus, it appears that a regulatory element can be transferred to heterologous host cells. However, these results do not show that dexamethasone induction is a primary transcriptional event. Other factors, such as stability of the mRNA or effect of the growth cycle (26, 27), cannot be discounted. The ability to obtain regulated expression of the rGH gene in heterologous cells suggests that hormonal responsiveness is intrinsic to the structure of the gene or its RNA products and permits further investigation into the nature of hormonally inducible control elements.

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