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SPECIFIC ANTISENSE RNA INHIBITION OF GROWTH HORMONE PRODUCTION IN DIFFERENTIATED RAT PITUITARY TUMOUR CELLS

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An expression vector that carried an inverted 800 base pair insert of the rat growth hormone (rGH) cDNA downstream of the SV40 promotor was used to transfect two different growth hormone (GH) producing rat pituitary cell strains, GH_12C_1 and GH_3. This resulted in a specific transient inhibition of growth hormone production up to 75 percent in the course of 72 hours. GH synthesis reduction occurred parallel to a decrease of GH cytoplasmic mRNA levels. Levels of ß-actin and guanine nucleotide-binding regulatory protein (G protein) mRNAs were unaltered, but PRL mRNA levels were increased. Transfection with a control vector did not affect GH production.

Transcriptional units that generate antisense RNAs have been introduced to many cells by DNA transfection and shown to inhibit expression of cellular genes (1,2,3). The use of antisense RNA expression has been proposed as a general method for specific inhibition of gene expression (1,2). Antisense RNA molecules contain sequences complementary to a portion or all of the target mRNA. The mechanism of inhibition may be due to the formation of RNA:RNA hybrids that might result in blocked RNA translation (4), the failure of RNA transport to the cytoplasm (3), an increased RNA turnover (5) or a combination of these events.

The GH cell system is a versatile system for studies of hormone synthesis and secretion (6,7) and hormone regulation of gene expression (8,9,10). GH cells have retained a large number of the receptors and signal transduction pathways found in pituitary tissue and are a valuable system to study signal transduction and second messenger cascades (11,12,13,14).

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For the present study we constructed an expression vector producing antisense rGH RNA for transfection of GH cells by the method of electroporation (15,16,17). We have previously established the optimal conditions for DNA transfer to GH cells using this method (Paulssen et al., unpublished). The results demonstrate that GH production can be strongly and specifically inhibited by antisense RNA techniques.

MATERIALS AND METHODS

Plasmids: p(as)GHneo and the control plasmid pLTneo used for transfections were constructed from pBSVneo (kindly provided by Dr. J. Sambrook, Cold Spring Harbour Laboratory, Cold Spring Harbour, USA), prGH-1 (18) and pKGE-73 (KabiGen AB, Sweden) as shown in figure 1. pGEM4rGH used for probe synthesis was made by ligating the Hind III rGH cDNA fragment into pGEM4 (Promega Biotec). Plasmid preparations were carried out as described by Maniatis et al.(19).

Cell culture: Rat pituitary tumour cells, GH_12C_1 and GH_3 (20,21), were grown as monolayer cultures as previously described (22). Experiments were carried out using cells in the exponential phase of growth.

Electroporation: The cells were harvested with trypsin (23) and washed twice with ice cold electroporation buffer (272 mM sucrose, 8 mM N-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES) pH 7.4, then resuspended at a concentration of about 1.0 x 10^6 cells/ml in the same buffer containing 62.5 µg/ml supercoiled plasmid DNA of different construction. The samples were transferred to an electroporation cuvette and warmed briefly to room temperature before receiving an electropulse. The electroporation was carried out using the Bio-Rad Gene Pulser™ apparatus and presterilized disposable cuvettes (interelectrode distance 0.4 cm). Gene Pulser settings were 25 µF, 0.3 kV for GH_12C_1 and 0.24 kV for GH_3 cells. The electroporated cells were divided into three equal portions, and each were diluted with an appropriate volume of equilibrated medium and plated in 9 cm petri dishes (Falcon). The optimal transfection conditions resulted in approximately 50 percent cell death.

Radioimmunoassay (RIA): rPRL and rGH was measured in the culture medium with specific radioimmunoassay as described previously (24,25).

RNA preparations: Total RNA was isolated by the guanidine-isothiocyanate/CsCl method (26).

Protein measurements: The cell protein levels were determined by the method of Lowry et al. (27) using bovine serum albumin as standard.

Northern Blot Analysis: Total RNA was separated on formaldehyde containing 1.4% agarose gels and blotted to Amersham Hybond-N nylon filters as described (19), except that 1x Tris-acetate-EDTA buffer (TAE) was used as transfer buffer (28). HindIII digested lambda DNA labelled with ^{32}P were used size markers. ^{32}P labelled sense or antisense RNA hybridization probes with a

specific activity of approx. 10^8 cpm/µg RNA were made as SP6 or T7 transcripts from the pGEM4rGH and as a SP6 transcripts from pGEM2G$_\alpha$ (29). The PRL and ß-actin probes were made by the random primer method (30) from purified cDNA inserts from the plasmids pPRL-I (31) and pAI (30) with a specific activity about 1.5 x 10^8 cpm/µg DNA. Hybridization conditions for RNA probes were 1M NaCl, 10 mM Tris ph 7.4, 1% SDS, 5x Denhart solution (19) and 60% formamide at 60°C for more than 12 hours. Filters were washed with 0.2x SSC, 0.2% SDS at 68°C. Hybridization conditions for random primed probes were 50% formamide at 42°C for more than 12 hours. Filters were washed with 0.1x SSC, 0.1% SDS at 50°C. Kodak XAR-5 x-ray film was used for autoradiography.

Scanning: Semiquantitive RNA data from autoradiograms were obtained with an universal densitometer (Vitatron TLD 100).

RESULTS

Northern blot analysis of GH cells transfected with an antisense rGH plasmid construct. In order to analyze the effect transfection with p(as)GHneo (Fig. 1) on rGH mRNA levels in $GH_1 2C_1$ and GH_3 cells, Northern blot analysis was carried out. The results are shown in Fig. 2A. In both cell lines transfected with p(as)GHneo, we observed a significant decrease of rGH mRNA levels in compari-

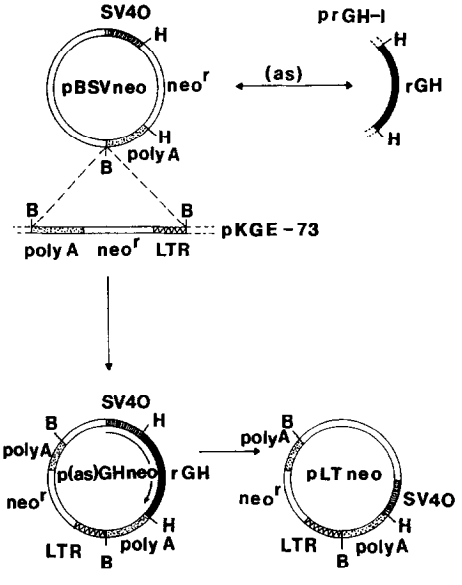
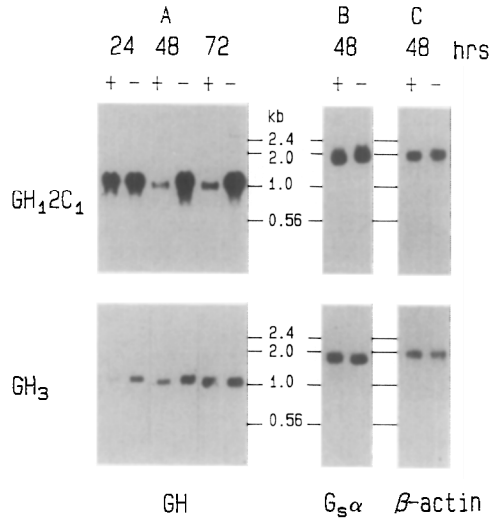


Figure 1. **Construction of the transfection vectors.**
The neo^r HindIII (H) fragment from pSVBneo was excised and replaced with the HindIII fragment of rGH cDNA (black) from prGH-1 in an antisense orientation relative to the SV40 promotor (striped). The BamHI (B) fragment from pKGE-73, including a neomycin resistance gene downstream of a LTR promotor (zig-zag) and a polyadenylation site (dotted), was cloned into the BamHI site of the antisense plasmid. This resulted in the p(as)GHneo plasmid used for transfections. The control plasmid pLTneo was made by excision of the HindIII rGH fragment from p(as)GHneo.



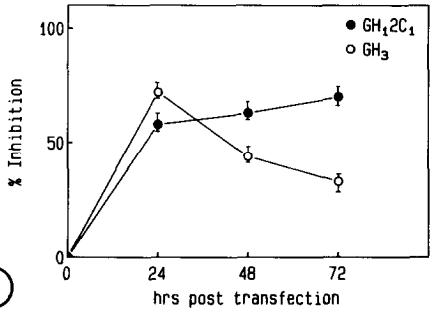


Figure 2. Northern blot analysis of $GH_1 2C_1$ and GH_3 cells transfected with the antisense rGH plasmid.
Total cell RNA from $GH_1 2C_1$ (10 µg/lane) and GH_3 cells (5 µg/lane) transfected with p(as)GHneo (+) or control plasmid (-) were separated and transferred to nylon membranes as described in Materials and Methods. The filters were hybridized to an antisense rGH RNA probe (A), an antisense $G_s\alpha$ RNA probe (B) and to an ß-actin probe (C). Molecular size DNA standards (in kb) are indicated. Times after transfections (in hours) are indicated at top. Optical scanning of rGH mRNA levels gave relative values (treated/control) 24, 48 and 72 hours post transfection: 0.77, 0.04 and 0.07 for $GH_1 2C_1$ and 0.28, 0.32 and 0.76 for GH_3 cells.

Figure 3. Inhibition of GH production in transfected $GH_1 2C_1$ and GH_3 cells.
The diagram shows the reduction in GH production (percent relative to controls) in $GH_1 2C_1$ and GH_3 cells after transfection with p(as)GHneo. Accumulation of GH into the medium was estimated by RIA in 24 hour collections. The variation of GH inhibition between duplicate determinations was less than 11 percent. The experiments are the same as those described in Fig.2 and were repeated twice with similar results. The concentration in control cultures after 24, 48 and 72 hours were (µg/mg protein x 24 hours): 0.62, 0.4 and 0.61 for $GH_1 2C_1$ and 0.09, 0.13 and 0.22 for GH_3 cells, respectively.

son to cells transfected with the control plasmid pLTneo (Fig.1). A time dependent reduction of rGH levels were observed for $GH_1 2C_1$ cells with maximal effect after 48 hours; in GH_3 cells the maximum decrease occurred 24 hours after transfection (Fig. 2A). The filters were rehybridized to a $G_s\alpha$ probe (Fig. 2B) and to a ß-actin probe (Fig. 2C), which show no significant alterations. A sense rGH RNA probe failed to detect any antisense RNA (data not shown).

Inhibition of GH production in transfected $GH_1 2C_1$ and GH_3 cells.
The hormone production as measured in the culture medium was estimated 24, 48 and 72 hours post electroporation (Fig.3). In

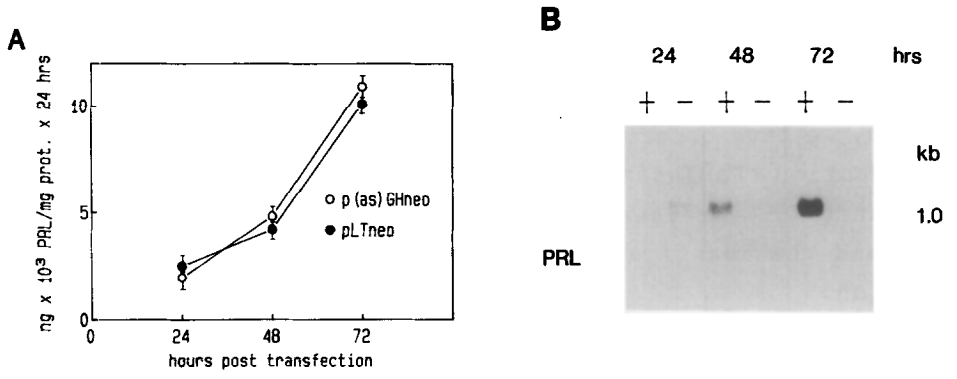


Figure 4. **PRL production and mRNA levels in GH_3 cells transfected with p(as)GHneo.**
A: Time course of PRL production in GH_3 cells transfected with p(as)GHneo or control plasmid pLTneo.
B: Northern Blot analysis of total cell RNA (5 µg per lane) 24, 48 and 72 hours after transfection of GH_3 cells with p(as)GHneo (+) or the control plasmid (-) hybridized to a PRL cDNA probe. Time points after transfections (in hours) are indicated.

the GH_12C_1 cells, there was a decreased rGH expression with a maximal inhibition of 75 percent after 72 hrs. The GH_3 cells showed a maximal inhibition of 75 percent after 24 hrs, which but decreased to 30 percent at 72 hours. Over all protein synthesis was unchanged in electroporated cells (data not shown).

Alteration in PRL production in GH_3 cells transfected with an antisense rGH plasmid. The rGH probe did not crosshybridize to rPRL mRNA, nor did the rPRL probe detect rGH mRNA (Data not shown). In GH_3 cells that transiently expressed antisense rGH mRNA, we found no change in PRL production as measured in the culture medium (Fig. 4A) compared to controls, while mRNA levels in the two situations varied significantly (Fig. 4B). The rPRL mRNA levels showed a increase at 48 and 72 hours after the transfection with p(as)GHneo and did not correlate with the lack of difference in PRL production.

DISCUSSION

In order to study the regulation of hormone expression in differentiated rat pituitary tumour cells, we used an approach employing expression of antisense RNA to inhibit rat GH production. The rGH cDNA used in our experiments specified the entire coding sequence for the rat GH precursor and contained part of the 5′ and all of the 3′ untranslated region of rGH mRNA.

Our results indicate that transfection of differentiated rat pituitary tumour cells with an antisense rGH plasmid construction results in specific decrease of levels of this mRNA. However, we have observed that electroporation itself may affect GH production (Paulssen et al., unpublished), which stresses the importance of a relevant control vector, the same amounts of cells and the identical experimental conditions in the control situation.

In GH_3 cells we found a correlation between reductions in GH production and mRNA levels. These findings complied with our investigations of an indicator plasmid carrying the gene for chloramphenicol acetyltransferase (CAT) (Paulssen et al., unpublished). For this plasmid, strong correlations between transcriptional and translational levels after transient expression were reported (32,33). mRNA analysis of transfected GH_3 cells show a maximum inhibition of GH production 24 hrs after transfection and a linear increase of GH production within the following 48 hrs. In the case of GH_12C_1 cells, transient expression of antisense rGH mRNA lasted longer. We observed at least a 75 percent inhibition of GH production 72 hrs after transfection (Fig.3) and these results were in agreement with the semiquantitative mRNA data obtained by optical scanning (Fig.2). To investigate whether the inhibition was specific for rGH, we hybridized the same northern blots with a ß-actin probe (30) and a $G_s\alpha$ probe (29). No significant changes in these mRNA levels occurred between cells transfected with the antisense plasmid or the control plasmid. Investigations to detect antisense rGH mRNA by Northern blot analysis of total cell RNA proved unsuccessful. This phenomenon was described earlier by Kim et al. (3) and it was suggested that the formation of RNA:RNA hybrids in the nucleus resulted in decreased transport of the double stranded hybrids into the cytoplasm and rapid degradation by double strand specific ribonucleases followed by extensive reduction of antisense and sense RNAs.

GH_3 cells also produce and secrete PRL in addition to GH. These hormones have partly homologous mRNA sequences and belong to the same gene family (34). We therefore studied PRL production in GH_3 cells transfected with the antisense rGH plasmid. We found that inhibition of GH production by antisense RNA was followed by increased PRL mRNA levels 48 hrs after transfection, but that the

hormone levels were not significantly different from the controls. This apparent inverse regulation of mRNA levels should be subject of further investigations.

We conclude that the expression of antisense rGH RNA specifically inhibits GH mRNA levels and hormone production. It should therefore be possible to use this technique to inhibit growth hormone production in transgenic animals and in a more general way to study the biological implications with regard to grossly reduced production of important regulatory proteins.

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