

# Reduced expression of multiple forms of the $\alpha$ subunit of the stimulatory GTP-binding protein in pseudohypoparathyroidism type Ia

(guanine nucleotide-binding proteins/hormone resistance/adenylate cyclase)

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**ABSTRACT** We examined the expression of the  $\alpha$  subunit of the stimulatory GTP-binding protein ( $G_s$ ) in fibroblasts of subjects with pseudohypoparathyroidism (PHP) type Ia by transfer blot hybridization and S1 nuclease analyses. Six subjects with PHP type Ia showed decreased steady-state content of  $G_s$   $\alpha$  mRNA. S1 nuclease analysis indicates that both long and short forms of  $G_s$   $\alpha$  mRNA are decreased, with no apparent change in the ratio of long to short forms in PHP compared with normal individuals. It appears likely that in some cases of PHP type Ia the genetic lesion affects the maintenance of mRNA levels for all forms of the  $G_s$   $\alpha$  subunit.

Pseudohypoparathyroidism (PHP) is a genetic disorder characterized by target organ resistance to parathyroid hormone (PTH) (1). Most subjects with PHP excrete significantly less cyclic AMP in the urine in response to PTH infusion than do normal subjects or subjects with other forms of hypoparathyroidism (2). This form of the disease is termed PHP type I to distinguish it from a rarer form, type II (3), in which urinary cyclic AMP excretion in response to PTH is normal. PHP type I has also been divided into two forms. In PHP type Ib physiognomy is normal and there is resistance only to PTH, whereas in PHP type Ia unusual physical features, collectively termed Albright's hereditary osteodystrophy, are displayed and there is resistance to multiple hormones that activate adenylate cyclase and thereby increase cyclic AMP production (4, 5).

Adenylate cyclase is under dual regulation by stimulatory ( $G_s$ ) and inhibitory ( $G_i$ ) guanine nucleotide-binding proteins (G proteins) (6, 7). With rare exceptions, subjects with PHP Ia show an approximately 50% reduction in  $G_s$  activity in plasma membranes from multiple cell types (4, 5, 8–11). Since  $G_s$  is believed to couple receptors for agonists that stimulate adenylate cyclase in all cell types (6, 7), reduction in functional  $G_s$  could play a role in resistance to multiple hormones in PHP Ia.

G proteins are composed of three distinct subunits. The  $\alpha$  subunit binds and hydrolyzes GTP, is a substrate for ADP-ribosylation by bacterial toxins, and confers specificity in receptor and effector interactions (6, 7). Two different forms of the  $G_s$   $\alpha$  protein, 45 and 52 kDa, have been characterized (12). These different forms of  $G_s$   $\alpha$  protein arise from several species of mRNA (12–14). In turn, the multiple species of mRNA appear to be products of alternative splicing of a common precursor (12, 13).

$G_s$  deficiency has been detected in PHP Ia both by functional assay (activation of adenylate cyclase activity in  $G_s$ -deficient mutant mouse S49 lymphoma cells,  $cyc^-$ ) and by

cholera toxin-catalyzed labeling of plasma membrane  $G_s$   $\alpha$  subunit with [ $^{32}$ P]ADP-ribose (8–11, 15). The molecular basis for reduced  $G_s$  activity in PHP Ia, however, has not been clearly defined. Since not only the adenylate cyclase assay but also cholera toxin-catalyzed ADP-ribosylation may require a functional protein,  $G_s$  deficiency could be due to synthesis of a defective protein or reduced synthesis of normal protein. Moreover, since four species of  $G_s$   $\alpha$  mRNA have been observed in humans (13) but only two species of protein had previously been characterized, it is unknown how the lesion in PHP affects each of the forms of  $G_s$   $\alpha$ . To gain a greater understanding of this disorder at the molecular level, we have used recently cloned bovine (16) and human (13) cDNA probes to examine the expression of  $G_s$   $\alpha$  at the RNA level in six subjects with PHP type Ia.

## MATERIALS AND METHODS

The bovine cDNA clone of  $G_s$   $\alpha$  (16) in pcD-X was a gift from J. D. Robishaw and A. G. Gilman (University of Texas Health Science Center, Dallas). Human cDNA clones  $\alpha_s$ -1 and  $\alpha_s$ -2 have been described by Bray *et al.* (13). A chicken  $\beta$ -actin clone (17) was obtained from Bruce Paterson (National Cancer Institute).

Skin biopsies were performed under a protocol approved by the National Institutes of Health Clinical Research Committee. Informed consent for biopsy was obtained in all cases. Six subjects with PHP Ia (all female; three sisters and three other unrelated subjects) were studied. Cultured skin fibroblasts from neonatal foreskin, as well as from skin biopsies performed on normal adults and subjects with PHP type Ib, were also studied. Fibroblasts were cultured as previously described (10). Subjects with PHP Ia showed the characteristic features of Albright's hereditary osteodystrophy, resistance to multiple hormones—including PTH, glucagon, and thyroid-stimulating hormone—and deficient  $G_s$  activity measured in erythrocyte and fibroblast membranes (4, 10). Normal subjects and those with PHP type Ib showed normal levels of  $G_s$   $\alpha$  protein by functional assay and by cholera toxin-catalyzed ADP-ribosylation (4, 15).

Total RNA was prepared from cultured skin fibroblasts of subjects with PHP and normal controls (18). Ten micrograms of total RNA from each sample was fractionated by formaldehyde/agarose gel electrophoresis and transferred to nitrocellulose (19).  $^{32}$ P-labeled probe was prepared to a specific activity of  $4\text{--}5 \times 10^7$  Cherenkov cpm/pmol by nick-translation of gel-purified, approximately 1367-base-

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Abbreviations: G protein, guanine nucleotide-binding protein;  $G_s$  and  $G_i$ , the stimulatory and inhibitory G proteins, respectively, associated with adenylate cyclase; PHP, pseudohypoparathyroidism; PTH, parathyroid hormone.

pair (bp), *Nco* I–*Hind*III fragment of bovine cDNA. RNA was hybridized with probe ( $3 \times 10^5$  cpm/ml) in 50% (vol/vol) formamide/1.2 M NaCl/120 mM sodium citrate/40 mM sodium phosphate, pH 7.0/1× Denhardt's solution (0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone) containing denatured salmon sperm DNA at 250  $\mu$ g/ml. Blots were treated with four 5-min washes of 0.6 M NaCl/60 mM sodium citrate/0.1% NaDodSO<sub>4</sub> at room temperature and two 30-min washes of 30 mM NaCl/3 mM sodium citrate/0.1% NaDodSO<sub>4</sub> at 50°C.

Single-stranded DNA probes for S1 nuclease mapping were obtained by primer extension of a phage M13 subclone containing an *Eco*RI fragment of either  $\alpha_s$ -1 or  $\alpha_s$ -2 cDNA. Total RNA (7.5  $\mu$ g) and <sup>32</sup>P-labeled probe (3000–5000 Cherenkov cpm) were hybridized and digested with S1 nuclease as previously described (13).

Genomic DNA was prepared from human skin fibroblasts (20). Twenty-five micrograms of DNA per sample was digested with *Hind*III, *Xba* I, *Eco*RI, *Bam*HI, *Msp* I, or *Pst* I, using conditions recommended by the supplier. Digests were fractionated through 1% agarose gels and blotted onto nitrocellulose (21). <sup>32</sup>P-labeled probes were prepared by nick-translation of gel-purified 413-bp (5') and 869-bp (3') *Eco*RI fragments of  $\alpha_s$ -2 (13). Hybridization was carried out for 16 hr at 65°C in 1.8 M NaCl/180 mM sodium citrate/5× Denhardt's solution/0.5% NaDodSO<sub>4</sub> containing denatured salmon sperm DNA at 250  $\mu$ g/ml, 10% dextran sulfate, and  $2 \times 10^6$  cpm of [<sup>32</sup>P]DNA per ml. Blots were washed with four 5-min washes of 0.6 M NaCl/60 mM sodium citrate/0.1% NaDodSO<sub>4</sub> at 50°C.

### RESULTS

We have examined the expression of G<sub>s</sub>  $\alpha$  mRNA in six different subjects with PHP type Ia. Total RNA was extracted from cultured skin fibroblasts from subjects with PHP Ia and normal controls. Ten micrograms of each sample was subjected to transfer blot hybridization. A representative autoradiogram is displayed in Fig. 1 and the results from multiple blots are summarized in Fig. 2. All six subjects with PHP Ia display lower than normal steady-state levels of the approximately 1900-base G<sub>s</sub>  $\alpha$  mRNA. Mean values for the six vary from 9% to 30% of control values. No RNAs of altered sizes have been detected in any subject with PHP type Ia.

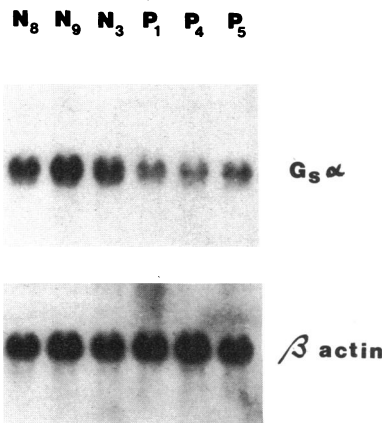


FIG. 1. Blot hybridization analysis. Ten micrograms of total RNA from each subject was fractionated through a formaldehyde/agarose gel and blotted onto nitrocellulose. Blots were probed with *Nco* I–*Hind*III fragment from bovine G<sub>s</sub>  $\alpha$  cDNA (Upper). Several exposures were made. The G<sub>s</sub>  $\alpha$  probe was removed, and the blot was reprobed with chicken  $\beta$ -actin (Lower). N<sub>8</sub>, N<sub>9</sub>, and N<sub>3</sub> are normal subjects; P<sub>1</sub>, P<sub>4</sub>, and P<sub>5</sub> are unrelated subjects with PHP type Ia.

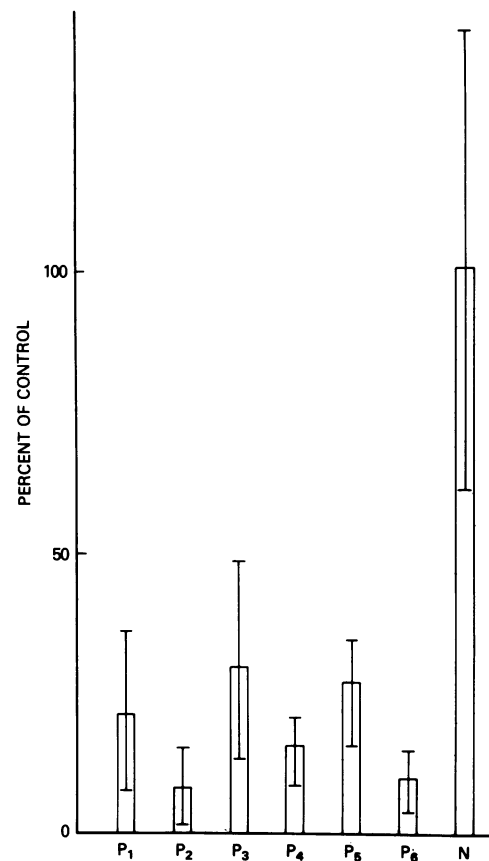


FIG. 2. Summary of blot hybridization experiments. Blots were prepared from several different preparations of RNA from each subject as described for Fig. 1. Densitometric scans were made. The ratio of G<sub>s</sub>  $\alpha$  to  $\beta$ -actin RNA was determined for each sample. For each blot, the average of the ratio of G<sub>s</sub>  $\alpha$  to  $\beta$ -actin for normal subjects is designated 100. Results (percent of control) are expressed as mean and range for PHP subjects (P<sub>1</sub>–P<sub>6</sub>) and seven normal controls (N). Data for P<sub>1</sub>–P<sub>5</sub> were from a minimum of three separate experiments. Results for P<sub>6</sub> were obtained in two separate experiments.

The proposal that multiple forms of G<sub>s</sub>  $\alpha$  result from alternative splicing (13) raises the question whether the decrease in G<sub>s</sub>  $\alpha$ -specific message observed in the PHP subjects represents a general decrease of all forms of G<sub>s</sub>  $\alpha$  or a decrease of a particular form. We performed S1 nuclease analysis of RNA from several subjects to test for possible changes in the relative amounts of the different species of G<sub>s</sub>  $\alpha$  RNA.

Probes used for this analysis were made from 412- and 413-bp fragments of human  $\alpha_s$ -1 and  $\alpha_s$ -2 cDNA, respectively, by primer extension. The entire cDNA portion of each probe is protected by both  $\alpha_s$ -1 and  $\alpha_s$ -2 mRNA. Under the conditions used, there is no discrimination between  $\alpha_s$ -1 and  $\alpha_s$ -2 mRNA. Two shorter species of G<sub>s</sub>  $\alpha$  mRNA,  $\alpha_s$ -3 and  $\alpha_s$ -4 (13), can be detected with these probes. Protected fragments corresponding to those predicted for  $\alpha_s$ -3 mRNA are observed with the use of the  $\alpha_s$ -1 probe. Similarly, the  $\alpha_s$ -2 probe allows detection of smaller protected fragments corresponding to  $\alpha_s$ -4 mRNA. By determining the ratio of short to long protected fragments for each probe, we can gain some insight into the relative amounts of  $\alpha_s$ -3 or  $\alpha_s$ -4 mRNA to the longer mRNA species.

A representative S1 nuclease analysis is shown in Fig. 3. Both normal and PHP individuals express both long and short forms of G<sub>s</sub>  $\alpha$  mRNA. Moreover, regardless of which probe is used, in the PHP samples, both long and short forms are reduced. Densitometric scans of the autoradiograms indicate

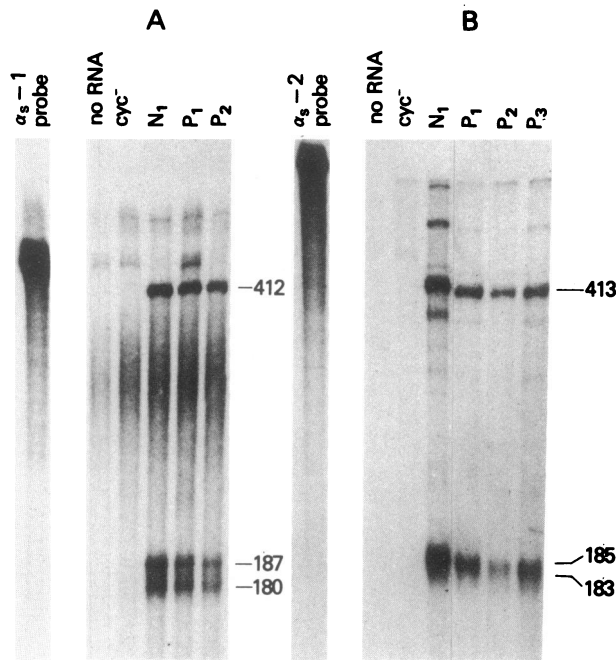


FIG. 3. S1 nuclease analysis of RNA from subjects with PHP type Ia. Total RNA (7.5  $\mu$ g) was allowed to anneal to a primer extended probe containing either  $\alpha_s$ -1 (A) or  $\alpha_s$ -2 (B) cDNA sequences. RNA-DNA hybrids were treated with S1 nuclease as described by Bray *et al.* (13). The single-stranded  $\alpha_s$ -1 probe is 412 bases of  $\alpha_s$ -1 cDNA flanked by 47 bases of vector sequence. The single-stranded  $\alpha_s$ -2 probe is an 873-base fragment containing two 413-base copies of  $\alpha_s$ -2 cDNA flanked by 47 bases of vector sequence. Probes were incubated with no RNA, RNA from S49 *cyc*<sup>-</sup> mouse lymphoma cells, RNA from a normal subject (N<sub>1</sub>), and RNA from subjects with PHP type Ia (P<sub>1</sub>-P<sub>3</sub>). Sizes of protected fragments were established by comparison to *Hpa* II fragments of pBR322 DNA. Expected sizes of fragments after hybridization of [<sup>32</sup>P]DNA probes with  $\alpha_s$  RNA and digestion with S1 nuclease are as follows: 412 or 413 bases after hybridization with  $\alpha_s$ -1 and  $\alpha_s$ -2 long-form RNAs; 187 and 180 after hybridization with  $\alpha_s$ -3 short-form RNA; 185 and 183 after hybridization with  $\alpha_s$ -4 short-form RNA.

that the ratio of either short form,  $\alpha_s$ -3 or  $\alpha_s$ -4, to long forms is essentially the same for normal individuals and PHP subjects. Whatever the genetic lesion is in PHP type Ia, it appears to decrease all species of  $G_s \alpha$  mRNA without causing a major change in the amount of either short form relative to the long forms.

We have attempted to find physical changes in the  $G_s \alpha$  gene itself by restriction analysis of genomic DNA taken from PHP subjects and normal volunteers. We have used six enzymes and primarily two probes: a 413-bp fragment from near the 5' end of human  $G_s \alpha$  cDNA (5' probe) and an 869-bp fragment of cDNA that includes the 3' coding portion of the gene (3' probe) (13). Fig. 4 illustrates the rather simple restriction patterns obtained with several enzymes tested. Each probe hybridizes to a small number of fragments in each digest. Furthermore, both probes hybridize to few fragments of the same size. So far, we have been unable to detect any differences in restriction pattern among normal individuals and subjects with PHP type Ia.

## DISCUSSION

The observation that the steady-state content of  $G_s \alpha$  mRNA in fibroblasts from six subjects with PHP Ia is lower than normal is consistent with reports of reduced  $G_s \alpha$  protein detected by ADP-ribosylation (9, 11, 15) and with our preliminary observations of decreased  $G_s \alpha$  on immunoblots of erythrocyte membranes from affected subjects (S. Mumby and A.S., unpublished observations). It is conceivable that deficient  $G_s$  activity in this disease could be due to reduced synthesis of normal functional  $G_s \alpha$  protein as a direct consequence of the lower level of  $G_s \alpha$ -specific RNA. Our results, therefore, suggest that in the subjects we tested the genetic lesion in PHP Ia exerts a significant effect prior to translation of the  $G_s \alpha$  protein.

S1 nuclease analysis shows that all forms of  $G_s \alpha$  mRNA are proportionately decreased in PHP Ia. The genetic lesion in the subjects tested has a similar effect on all forms of  $G_s \alpha$  mRNA. Processing steps involved in generating long and short forms thus appear to be unaffected by the disease.

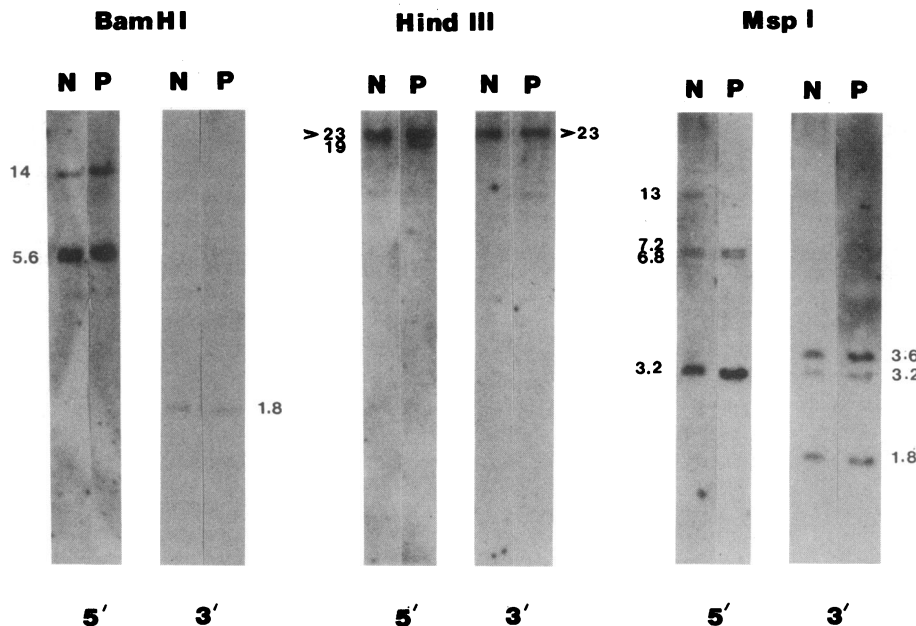


FIG. 4. Southern blot analysis of genomic DNA from subjects with PHP type Ia. Twenty-five micrograms of DNA was digested with the enzymes indicated, using conditions recommended by the suppliers. Nick-translated probes were 413-bp (5') or 869-bp (3') *Eco*RI fragments of human  $\alpha_s$ -2 cDNA (13). Approximate molecular sizes were estimated by comparison with *Hind*III fragments of  $\lambda$  phage DNA and are shown in kbp beside the bands. N, DNA from normal subjects; P, DNA from subjects with PHP type Ia.

Robishaw *et al.* showed that long and short forms of  $G_s \alpha$  mRNA (their study distinguished only two forms of mRNA) correspond to the 52- and 45-kDa forms of  $G_s \alpha$  protein, respectively (12). Their data suggest that multiple forms of  $G_s \alpha$  protein arise from translation of different  $G_s \alpha$  mRNAs rather than from post-translational changes. Decreases in both 45- and 52-kDa forms of  $G_s \alpha$  protein in fibroblast membranes from subjects with PHP Ia, as measured by cholera toxin-catalyzed ADP-ribosylation (10), are consistent with decreases in multiple forms of  $G_s \alpha$  mRNA.

We found no evidence for large deletions or rearrangements of the  $G_s \alpha$  gene(s) that could account for altered mRNA expression in subjects with PHP Ia. The simplicity of the genomic restriction patterns indicates a very low, if not single, copy number. Genetic and biochemical analysis of mouse S49 lymphoma mutants *cyc*<sup>-</sup> (no  $G_s \alpha$ ) and *unc* (altered isoelectric points of both long and short forms of  $G_s \alpha$  protein) indicate a single gene for  $G_s \alpha$  in mouse (22–25). A single gene has also been reported for the bovine species (26). Although the number of human  $G_s \alpha$  genes has not been definitively established, the restriction patterns in this study appear to lack sufficient complexity for four separate genes. Thus, the genomic blots lend additional support to the alternative splicing model.

While we found no differences in genomic blots among normal and affected subjects, subtle abnormalities, such as point mutations and small deletions, can have profound effects on gene regulation and still be undetectable by standard blot analysis. Decreased  $G_s \alpha$  mRNA in PHP Ia could be due to decreased transcription or decreased mRNA stability. A mutation within the promoter region for  $G_s \alpha$  could lead to lower efficiency of initiation of transcription. In view of the alternative splicing model of  $G_s \alpha$  expression, a single promoter mutation could cause reduced transcription of all forms of  $G_s \alpha$  mRNA. The same splicing model, however, allows the possibility that a single mutation capable of affecting RNA stability might affect all species of  $G_s \alpha$  mRNA equally, since such a mutation would likely be found in all forms (exceptions being mutations within the 42-bp long-form-specific region). Although alterations within the  $G_s \alpha$  gene or its transcriptional regulatory regions could account for reduced expression of  $G_s \alpha$  in PHP Ia, these are not the only possible explanations. An abnormality in a gene encoding a factor that affects  $G_s \alpha$  transcription in trans could likewise lower levels of  $G_s \alpha$  mRNA. Since DNA sequence comparisons are not yet available for the  $G_s \alpha$  genes found in normal individuals and subjects with PHP Ia, the existence and locations of abnormalities affecting the  $G_s \alpha$  gene itself, or potential trans-acting factors, await further investigation.

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