Spermine deficiency in Gy mice caused by deletion of the spermine synthase gene

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Two mouse mutations gyro (Gy) and hypophosphatemia (Hyp) are mouse models for X-linked hypophosphatemic rickets and have been shown to be deleted for the 5′ and 3′ end of the mouse homolog of PHEX (phosphate regulating gene with homologies to endopeptidases on the X chromosome; formerly called PEX), respectively. In addition to the metabolic disorder observed in Hyp mice, male Gy mice are sterile and show circling behavior and reduced viability. The human SMS (spermine synthase) gene maps ∼39 kb upstream of PHEX and is transcribed in the same direction. To elucidate the complex phenotype of Gy mice, we characterized the genomic region upstream of PheX by establishing the genomic structure of mouse Sms, a 160–190 kb deletion was shown in Gy mice, which includes both Phex and Sms. There are several pseudogenes of SMS/Sms in man and mouse. Northern analysis revealed three different Sms transcripts which are absent in Gy mice. Measurement of polyamine levels revealed a marked decrease in spermine in liver and pancreas of affected male Gy mice. Analysis of brain tissue revealed no gross or histological abnormalities. Gy provides a mouse model for a defect in the polyamine pathway, which is known to play a key role in cell proliferation.

INTRODUCTION

Gy (1) and Hyp (2) mice constitute mouse models for X-linked dominant hypophosphatemic rickets and are caused by mutations in the Phex gene (formerly called Pex) (3–5). In accordance with the phenotype observed in humans, both mouse mutants have renal phosphate wasting, impaired mineralization and growth retardation (1). Affected Gy males and some affected female animals also show inner ear abnormalities, deafness, hyperactivity and circling behavior (1,6,7). Male Gy mice are also sterile (8), are smaller than their normal sibs from birth onwards and weigh less than Hyp males (1). The Gy mouse is bred on the B6C3H background and the Hyp mouse is bred on the C57BL/6J background. Interestingly, male Gy mice do not survive on the C57BL/6J background (9) and have a reduced viability from birth, with sudden death occurring in adults on the B6C3H background (1).

The Gy deletion extends upstream of the 5′-untranslated region (5′-UTR) of Phex (5). It seemed likely that the additional symptoms in Gy mice are caused by a contiguous gene deletion syndrome which includes at least one additional gene to Phex. We have mapped the human SMS gene distal to PHEX (formerly called PEX), and have shown that both genes have the same orientation (5). The genomic organization of the region containing SMS and PHEX has been reported (10) (GenBank accession nos: U53331, U72789, U72790, U73024 and Y10196). Human SMS is therefore known to be 39 kb upstream of PHEX.

Sms constitutes an attractive candidate gene for the additional symptoms in Gy mice because polyamines are required for normal growth and differentiation. The polyamines spermidine and putrescine are found ubiquitously in prokaryotic and eukaryotic cells whereas spermine is rarely found in prokaryotes but is widespread in eukaryotes [for review, see refs (11–14)]. Synthesis and degradation of polyamines are tightly regulated (15). Putrescine is synthesised from l-ornithine by ornithine decarboxylase, the rate-limiting enzyme in polyamine synthesis. Putrescine and decarboxylated S-adenosylmethionine are substrates for the synthesis of spermidine, which is converted to spermine by spermine synthase (16) (Fig. 1). Polyamines are organic polycations which interact with a variety of intracellular targets including nucleic acids, phospholipids and proteins. Putrescine and spermidine are known to play specific roles in the maturation of the eukaryotic translation initiation factor eIF-5A (17). Furthermore, specific interactions of spermine and...
of clone, 308A23, by PCR screening using primers from the 5′ end.

We then isolated a mouse bacterial artificial chromosome (BAC) and sequenced the entire length of the BAC clone. Restriction enzyme digestion of 308A23 gave a size of 1400 bp, suggesting the presence of exons 2–11 as well as exons 3 and 4 kb. PCR amplification with primers from the 5′-UTR revealed a deletion of the entire Sms gene. This deletion was confirmed by sequencing the PCR products.

RESULTS

Sms/Sms pseudogenes

Screening a mouse P1 library with Sms cDNA revealed five different clones (703K15175, 703K23169, 703D07320, 703E04130 and 703C14122). PCR with primers from exon 2 (SpSyF3) and the 3′-UTR (SpSyR1) using P1 DNA as template revealed discrete products of ∼1400 bp, suggesting the presence of pseudogenes which have arisen by integration of reverse-transcribed RNA. The PCR products were sequenced and all of them showed deletions and insertions causing frameshifts.

Southern blot analysis of EcoRI digests of genomic mouse DNA showed various bands between 1 and 14 kb. The absence of dosage differences between male and female genomic DNA on Southern blots suggests the existence of several autosomal pseudogenes in humans (data not shown).

Sms is deleted in Gy mice

In order to characterize the distal deletion breakpoint in Gy mice, we then isolated a mouse bacterial artificial chromosome (BAC) clone, 308A23, by PCR screening using primers from the 5′-UTR of Phex. Restriction enzyme digestion of 308A23 gave a size of ∼150 kb. Hybridization of various Phex and Sms RT-PCR products to the BAC DNA showed the presence of the entire Sms gene and of exon 1 of the Phex gene. Intron–exon boundaries of Sms exons were determined by preparing a shotgun library of 308A23 and sequencing clones which hybridized with the mouse Sms cDNA (5). The BAC ends were determined by direct sequencing and by sequencing of products generated by vector-Alu PCR. In addition, a 1.5 kb HindIII SP6 end fragment was subcloned and sequenced. The T7 end lies on the same 4.3 kb BamHI end fragment as the exon 1 of Phex. Partial restriction mapping indicated that the BAC clone extends from intron 1 of the Phex gene to ∼56 kb upstream of the first exon of the Sms gene (Fig. 2).

Sms/Sms is highly conserved between man and mouse. The open reading frame (ORF) comprises 1101 nucleotides. The mouse gene shows 91% identity at the DNA level and 96% identity on the protein level to the human gene. The mouse gene comprises 11 exons showing the same exon–intron structure as the human homolog (GenBank accession no. U53331).

PCR amplification from Gy genomic DNA with intronic primers of the 5′-UTR, exons 2, 4, 7 and 10 revealed a deletion of the entire Sms gene. The T7 end of the BAC clone was shown to be deleted while the 1.5 kb HindIII SP6 end fragment was present, which was confirmed by sequencing the PCR products and hybridization to HindIII digest of genomic Gy DNA. Hybridization of the 1.5 kb HindIII fragment to genomic mouse EcoRI Southern blots revealed an ∼6.5 kb junction fragment in Gy mice, instead of an 11 kb genomic EcoRI fragment in control mice (Fig. 3). Furthermore, a 2.5 kb EcoRI fragment, which lies ∼34 kb distal to exon 1 of the Sms gene, was subcloned and sequenced. PCR amplification with primers from this sequence showed that the deletion in Gy extends up to this EcoRI fragment. Thus, the distal deletion breakpoint of the Gy deletion lies ∼50 kb distal to exon 1 of the Sms gene.

Sms expression

Sequencing of cDNA clones isolated from a mouse embryonic cDNA library revealed two variant transcripts, with exons 3 and 4 absent or present. Poly(A)^+ RNA from normal mouse embryos and from adult Gy mice and controls was subjected to northern analysis using Sms cDNAs as probes. A northern blot (Clontech) containing RNA from total mouse embryos of day 7, 11, 15 and 17 was hybridized with a full-length mouse cDNA probe and separately with a probe of exons 3 and 4. In both cases, three transcripts of 2, 3 and 4 kb were detected (Fig. 4A). The 3 kb transcript produced only a weak signal. The highest expression could be detected on embryonic day 11.

RNA was extracted from male B6C3H Gy tissue. RNA from male unaffected littersmates was used as control. Spleen expressed the 2 and 3 kb transcripts, brain expressed the 2 and 4 kb transcripts. Even exposure of the blot to autoradiograph film for 2 weeks gave no evidence of a 4 kb transcript in spleen tissue and a 3 kb transcript in brain tissue. In Gy mice, no Ssms expression could be detected in brain or spleen tissues (Fig. 4B). The same data were obtained using probes comprising exons 2–11 as well as exons 9–11. Thus, none of the transcripts detected on northern blots are caused by processed pseudogenes or Sms homologs because all three are absent in Gy mice.

Figure 1. Polyamine biosynthesis pathway. Key to enzymes: 1, ornithine decarboxylase; 2, S-adenosylmethionine decarboxylase; 3, spermidine synthase; 4, spermine synthase.
Figure 2. Genomic organization of the SMS/Sms and of PHEX/Phex genes. In the top line, the genomic organization of the human genes is drawn to scale. The genomic distances are derived from the sequence of this region. In the second line, the genomic organization of the syntenic region in mice is shown. The regions of the mouse genes, which are covered by genomic clones, are indicated by black boxes. Exon 1 of the Phex gene lies on the 4.3 kb BamHI end fragment of BAC 308A23. Exon 1 of the Sms gene lies ∼56 kb downstream of the SP6 end of BAC 308A23. Below, the deletion in Gy mice is shown, comprising 160–190 kb. The dotted line indicates the deleted region, black boxes indicate the region present in Gy and the hatched boxes indicate the regions which bear the deletion breakpoints. Hybridization of the 1.5 kb HindIII SP6 end fragment to genomic mouse EcoRI Southern blots revealed an ∼6.5 kb junction fragment in Gy mice, instead of an 11 kb genomic EcoRI fragment in control mice. The proximal deletion breakpoint lies within intron 3 of the Phex gene. The BAC clone 308A23 was partially restriction mapped. The bottom line shows the restriction map from the SP6 end up to exon 1 of the Sms gene (X, XhoI; N, NruI; K, KspI; S, SalI).

Table 1. Tissue concentrations of polyamines in 12-day-old male Gy mice (1,2), control littermates (3–5), male Hyp mice (6,7) and control littermates (8,9)

<table>
<thead>
<tr>
<th>No.</th>
<th>Mouse</th>
<th>Putrescine</th>
<th>Spermidine</th>
<th>Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>Pancreas</td>
<td>Liver</td>
</tr>
<tr>
<td>1</td>
<td>Gy/Y</td>
<td>0.063</td>
<td>0.253</td>
<td>1.77</td>
</tr>
<tr>
<td>2</td>
<td>Gy/Y</td>
<td>0.194</td>
<td>0.083</td>
<td>2.55</td>
</tr>
<tr>
<td>3</td>
<td>+/Y</td>
<td>0.111</td>
<td>0.193</td>
<td>1.87</td>
</tr>
<tr>
<td>4</td>
<td>+/Y</td>
<td>0.098</td>
<td>0.237</td>
<td>2.53</td>
</tr>
<tr>
<td>5</td>
<td>+/Y</td>
<td>0.094</td>
<td>0.273</td>
<td>1.44</td>
</tr>
<tr>
<td>6</td>
<td>Hyp/Y</td>
<td>0.054</td>
<td>0.098</td>
<td>1.54</td>
</tr>
<tr>
<td>7</td>
<td>Hyp/Y</td>
<td>0.141</td>
<td>0.272</td>
<td>1.81</td>
</tr>
<tr>
<td>8</td>
<td>+/Y</td>
<td>0.039</td>
<td>0.122</td>
<td>1.76</td>
</tr>
<tr>
<td>9</td>
<td>+/Y</td>
<td>0.104</td>
<td>0.299</td>
<td>1.72</td>
</tr>
</tbody>
</table>

n.d. = not detectable.
The concentrations of putrescine, spermidine and spermine are given in µmol/g fresh weight.

**Polyamine levels**

Polyamine levels were measured by reversed phase HPLC after pre-column derivatization with benzoyl chloride, in liver and pancreas of 12-day-old male affected Gy mice (n = 2), male control littermates (n = 3), male affected Hyp mice (n = 2) and control littermates (n = 2). In male affected Gy mice, the spermine levels were clearly decreased in pancreas and below the detection limit in liver. The putrescine and spermidine levels were not different between Gy, control and Hyp mice. Male affected Hyp mice showed normal spermine levels when compared with control male littermates (Table 1).

**Histology**

On day 12, autopsy was performed in two affected male Gy mice. At this time, the body weight of male Gy mice (4.5 and 4.8 g) was less than in control littermates (6.0, 6.0 and 7.3 g). Examination of brain tissues in male Gy mice showed no gross or histological abnormalities. Analysis of 12 serial sections of the mes- and metencephalon revealed a normal architectural organization of the cerebellar cortex and cochlear nucleus and gave no evidence for a decreased number of granule cells, which have been reported in rats and hamsters treated postnatally with the inhibitor of
DISCUSSION

Gy and Hyp are mouse models for X-linked dominant hypophosphatemic rickets bearing deletions of the Phex gene. The disease shows a similar phenotype in man and mouse. In both species, low serum phosphate levels are caused by a defect of the proximal tubular phosphate reabsorption, causing rickets and osteomalacia. In spite of low phosphate levels, serum vitamin D levels are not elevated but are in the normal range. The phenotype in females is as severe as in males. Male Gy mice, however, show additional symptoms (1): they have a reduced viability whereas affected men and male Hyp mice have a normal viability. Male Gy mice are smaller from birth onwards, whereas in man and in Hyp mice birth weight is normal and short stature is acquired during postnatal life. Male Gy mice are sterile whereas male Hyp mice are fertile and have only been reported to possibly have a reduced fertility (1). Apart from these clearly distinguishable symptoms, there may be some overlap concerning the circling behavior. Gy males and some affected females show circling behavior, and this has also been observed occasionally in Hyp mice on the B6C3H background (9). Gy mice are bred on the B6C3H, Hyp mice on the C57BL/6J background. Nevertheless, the differences in viability, birth weight and sterility of Hyp and Gy mice do not seem to be related to modifier gene effects: when Gy mice are transferred to the C57BL/6J background, affected males do not seem to be related to modifier gene effects: when Gy mice are transferred to the C57BL/6J background, affected males do not survive (9). The Gy phenotype suggests that, apart from hypophosphatemia, an X-linked recessive trait is inherited in this mouse model.

We show that, in addition to Phex, the Sms gene is deleted in Gy mice. The BAC clone 308A23 covers ~150 kb and contains the entire Sms gene. It extends from intron 1 of the Phex gene to ~56 kb upstream of the first exon of the Sms gene. We have determined a partial genomic sequence of the mouse Sms gene, showing that the genomic organization of the mouse gene is comparable with the human gene. Comparison with the human genomic sequence gave no evidence of further genes in between the differences between predicted and observed sizes are still obvious abnormalities. Testes have not yet been examined in this study.

Northern blot analysis of Sms shows three transcripts of ~2, 3 and 4 kb. All three transcripts are absent in Gy mice, which excludes the possibility that they are due to transcribed pseudogenes or homologs of Sms. During embryogenesis, Sms expression seems to be highest at day 11, suggesting a role for this enzyme in development. In adult mice, tissue-dependent expression was observed: the 4 and 3 kb transcripts are not expressed in spleen and brain, respectively. So far, we do not know whether the different transcripts are caused by alternative splicing or the use of alternative polyadenylation signals. A large number of Sms expressed sequence tags (ESTs) found in dBEST all seem to suggest the use of an unusual polyadenylation signal, ATTAA, situated 410 bp downstream of the stop codon. This polyadenylation signal is conserved in human and mouse gene sequences, and predicts a 1.6 kb transcript size. However, a 2 kb transcript has also been reported in human northern blot experiments (16), and the differences between predicted and observed sizes are still

Figure 3. Southern blot of HindIII and EcoRI digests of mouse genomic DNA. Hybridization with the 1.5 kb HindIII SP6 end fragment of BAC 308A23 revealed an ~6.5 kb junction fragment in Gy mice, instead of an 11 kb genomic EcoRI fragment in control mice.

Figure 4. (A) A mouse embryo northern blot (Clontech) was hybridized with a full-length Sms cDNA probe (exposure: 4 days) and rehybridized with β-actin cDNA as control (exposure 1 day). (B) Sms RNA expression in male B6C3H Gy mice and unaffected male littersmates. 2 µg of poly(A)+ RNA from adult spleen, brain and 12.5 day total embryo was electrophoresed in a formaldehyde–agarose gel, transferred to a nylon membrane and hybridized with an Sms cDNA probe comprising exons 2–11 (exposure: 2 days). The same filter was rehybridized with a GAPDH cDNA probe to control for RNA loading (exposure: 12 h).

1-ornithine decarboxylase, DL-α-difluoromethylornithine (DFMO) (Fig. 5). At autopsy, aside from rickets, no other gross or histological abnormalities were observed. In particular, a histological examin-
Figure 5. Photomicrographs of coronal sections of the hindbrain in 12-day-old mice: male Gy mouse (A), male control littermate (B). The top of the panel shows the dorsal cochlear nucleus and the bottom shows the ventral cochlear nucleus (magnification 160x, staining: HE). Both sections show comparable sizes of these nuclei and normal numbers of granule cells, giant cells and fusiform cells.

unclear. In humans, a second polyadenylation signal is predicted by GRAIL (23) to lie 809 bp downstream of the stop codon, but only a single truncated EST is present in dbEST which fits this site. It is known that spermine tissue levels differ markedly in different cells: they are high in prostate and seminal plasma, and low in brain and muscle (24). The presence of multiple transcripts and tissue-specific transcription patterns may contribute to the tissue-specific regulation of spermine levels.

Polyamine metabolism has been studied using inhibitors both in vivo and in vitro. Treatment with the highly selective, irreversible inhibitor of l-ornithine decarboxylase, DFMO, has revealed that the rate-limiting enzyme of polyamine synthesis has an essential role in mammalian embryogenesis. DFMO treatment arrests early stages of mammalian embryogenesis (25). When the dose or duration of treatment with DFMO is reduced, organ-specific growth deficits become apparent, affecting especially developing brain tissue (for review, see ref. 26). DFMO treatment causes near total depletion of putrescine and a marked decline in spermidine levels, but leaves spermine levels unaltered. This is a different pattern from that observed in Gy mice. There, putrescine and spermidine levels are not altered at day 12; only spermine levels are markedly decreased in pancreas and not detectable in liver. Since all mammalian cells are able to import polyamines (27), spermine levels in Gy mice may be less altered during embryogenesis. During postnatal life, further changes in polyamine levels may occur.

Despite the differences in polyamine levels, there are some similarities in the clinical symptoms between Gy mice and DMFO-treated animals. Hearing impairment (6,7) has been reported in Gy mice and this was also found in DFMO-treated guinea pigs (28). Male Gy mice are small at birth, have a reduced viability on the B6C3H background and do not survive on the C57BL/6J background. No gross or histological abnormalities of brain tissue were detected at day 12. Particularly, no alteration of the morphology of the cerebellum and the dorsal cochlear nucleus was detected, which has been reported in DFMO-treated rats (26) and hamsters (29), respectively. Spermine deficiency may cause more subtle alterations such as changes in channel activity of certain potassium and glutamate receptors in heart or brain (18–20) or changes of the regulation of mitochondrial Ca$^{2+}$ transport (30).

Recently, spermine synthase inhibitors have become available (31,32). So far, they have only been tested in vitro. Cell culture experiments have shown that the spermine content can be reduced profoundly and cell growth can be decreased in human breast cancer cells (33) and mouse leukemia cells (34). However, there have been no obvious effects on growth and viability in rat hepatoma cells (32). The Gy deletion provides a mouse model for
spermine synthase deficiency without the disadvantages of inhibitors such as unspecificity and toxicity. This model will allow the investigation of spermine deficiency and may help to unravel some of the mysteries of spermine function.

**MATERIALS AND METHODS**

**Genomic clones**

P1s were isolated by screening gridded mouse P1 library filters. This P1 library was prepared using MboI partially digested DNA obtained from C57BL/6 female mice spleen (F. Francis, unpublished). The cloning vector was pAd10sacBII and the host strain NS3145 (21). Primers SpSyF3 (GTG AGG CCA TTC TGA AAG GC) and SpSyR1 (CTA AGT CAA TTT GGG GGT GAG) were used to amplify the pseudogenes from P1 clones.

The Bac clone, 308A23, was isolated by PCR screening Bac DNA pools with primers from the 5′-UTR of *Phex* (P1MF1; GCT TGA GCA AAA AGC CTG CC; P1MR1: ACC AGG GTG CCA ACC ATA AAC (Research Genetics Inc.).

**cDNA libraries**

A human cDNA clone containing the 3′ cDNA libraries CCA A TA AAC) (Research Genetics Inc.). TGA GCA AAA AGC CTG CC; P1MR1: ACC AGG GTG CCA ACC ATA AAC) (Research Genetics Inc.).

**Exon–intron structure of mouse Sms**

A shotgun library of Bac 308A23 was constructed by ligation of sonicated Bac DNA to blunt-ended linearized plasmid DNA (pUC18). A total of 800 clones were gridded on filters and hybridized with the mouse *Sms* cDNA. Inserts of positive clones were amplified by PCR and sequenced from both sides using ABI dye primer chemistry. The following primers were used to amplify the 5′-UTR and exons 2, 4, 7 and 10: 5′-UTR, SSM5UTRF (GGA GAG GCA CAG CAA TCC ATG CGC) and SSM5UTRR (GAC TAT GAT TCG GGC AGC AC) and SSM4R (CAT ATC TTC AGC AGA CAT TAA G); exon 7, SSM7F (CTT ACC TGC C); exon 4, SSM4F (GAC TA T GA T TTG GGC TCC TTT GTC); exon 2, SSM2F (TCT TTT GTG) and BACT7R (GGT TTT CAG GTC CCA CCT CAG). BACT7F (GGT TTT CAG GTC CCA CCT CAC).

**BAC ends**

BAC ends were generated by direct sequencing of the BAC DNA and by sequencing of PCR products amplified with vector (T7/SPl6) and Alu primers (CL1, TCC CAA AGT GCT GGG ATT ACA G; CL2, CTG CAC TCC AGC CTG GG). The following primers were used to amplify the BAC ends from genomic DNA: BACSP6F2 (CTG AAA AGT GCC ATT AAG GTG) and BACSP6R2 (GAA AGG GTC CTG CTA TGT GG); 1.5 HindIII SP6 end fragment, SP6F3 (GCT AGA CCT TGG TTG AAT GGA) and BACH15R (ACC TGA AAA GGT CTG TGC CTT); BACT7F (CCC TTT CTT TAC TGT CTG CCC) and BACT7R (GGT TTT CAG GTC CCA CCT CAG).

**Sequencing**

PCR-amplified products and genomic fragments cloned into plasmid vectors were purified by gel extraction and plasmid preparation kits (Qiagen). Cycle sequencing was performed using a Tgy DyeDeoxy Terminator Cycle sequencing Kit (ABI). The sequences were determined with an Applied Biosystems 377 automated sequencer.

**Northern blots**

The mouse embryo northern blot was obtained from Clontech and hybridized according to the manufacturer’s instructions. Total RNA from *Gy* mice was prepared using TRIzol reagent (GIBCO/BRL). Poly(A)+ RNA was then isolated from total RNA using an Oligotex mRNA mini kit (Qiagen). About 2 μg of poly(A)+ RNA from each sample was electrophoresed on a 1% formaldehyde–agarose gel and transferred to Hybond-N nylon membranes (Amersham). The filters were then hybridized with 32P-labeled probes.

**Polyamine measurement**

Polyamines were determined by reversed phase HPLC after pre-column derivatization with benzoyl chloride (22). Dissected organs were snap-frozen in liquid nitrogen and pulverized in a microdissmembranator. Diaminohexane (40 nmol) was added as internal standard and protein was precipitated with 0.2 M perchloric acid. After neutralization with 4 M KOH, polyamines were derivatized with benzoyl chloride, extracted with diethyl ether and dissolved in methanol:water (60:40 v/v). HPLC separation was performed on a Waters µBondapak C18 column (3.9×300 mm) with methanol:water (60:40 v/v) at a flow rate of 1 ml/min. The UV signal was monitored at 254 nm. Determination of polyamines displayed linearity over the whole range of tissue polyamine concentrations and was documented up to 5 nmol per 20 μl injection. Recovery of the internal standard ranged between 52 and 99%. Recovery of external standards after measurement of derivatized samples was 100–107%.

**Animals**

*Hyp* and *Gy* mice were obtained from the Jackson Laboratory. *Gy* mice are on the B6C3H background. Normal male mice (Y+/) were bred with heterozygous female mice (GY/+). *Hyp* mice are on the inbred C57BL/6j background. Normal male mice (Y+) were bred with heterozygous female mice (Hyp+). The genotype of the male littermates was determined by PCR amplification of *Phex* exons 3, 19 and 21.

**Histology**

To carry out histological studies, the samples were embedded in paraffin. Sections of 6 μm were prepared and stained with HE according to standard procedures.

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