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Mutations in the Gene Encoding the α-Subunit of the G_s Protein in Molar Pregnancy

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Molar pregnancy is a gestational trophoblastic disease associated with a trophoblastic proliferation and a protein synthesis alteration. It is characterized by the presence of hydatiform moles, which are fluid-filled cysts derived from the chorionic villi of the placenta. Recent studies have reported a reduced expression of several types of G proteins including $G_s\alpha$ in molar pregnancies suggesting alterations in G protein structure in hydatiform moles. To identify mutations that lead to $G_s\alpha$ deficiency, we isolated genomic DNA from hydatiform moles and used polymerase chain reaction to amplify all exons of the $G_s\alpha$ gene. Amplified $G_s\alpha$ gene fragments were analyzed by sequencing using the dideoxy chain termination method. Tissues obtained from three complete hydatiform moles and one partial hydatiform mole were examined. We have identified a heterozygous 8-bp deletion in exon 10 of the $G_s\alpha$ gene, in two complete hydatiform moles, that had evidence for a dysfunctional $G_s\alpha$ protein. This deletion produced a truncated protein. We have also identified a heterozygous polymorphism in exon 5 in two complete hydatiform moles, and a homozygous substitution (A → G) in intron 5 of the $G_s\alpha$ gene in the other complete hydatiform mole; these two last types of mutations should not have any effects on protein activity.

Key Words: G proteins; hydatiform moles; mutation.

Introduction

Regulatory guanine nucleotide (guanosine 5'-triphosphatase [GTP]-binding proteins (G proteins) that mediate signal transduction across cell membrane share a heterotrimeric structure composed of α-, β-, and γ-subunits. The α-subunit contains the guanine nucleotide-binding site, has intrinsic GTP activity, and is thought to confer functional specificity on each G protein, allowing it to discriminate among multiple receptors and effectors. At least two classes of G proteins regulate the activity of adenylyl cyclase: the α-subunit of G_s is responsible for stimulation of catalytic activity whereas the α-subunit of G_i, represented by at least three forms, mediates inhibition of the enzyme. The binding of hormones and neurotransmitters to their specific receptors stimulates adenylyl cyclase activity, and thus intracellular cyclic adenosine monophosphate (cAMP) formation, through activation of G_s *(1)*. G proteins have been shown to play a key role in the stimulus-response coupling of many important biological systems including peptide production by the placenta. Reduced expression of several types of G proteins including $G_s\alpha$ has been demonstrated in molar pregnancies *(2)*.

Molar pregnancy is a gestational trophoblastic disease associated with a trophoblastic proliferation and a protein synthesis alteration. The incidence of molar pregnancy in Japan (2 per 1000 pregnancies) is reported to be about three times higher than that in Europe or North America (about 0.6–1.1 per 1000 pregnancies) *(3)*. Molar pregnancies are characterized by the presence of hydatiform moles, which are fluid-filled cysts derived from chorionic villi of the placenta in which the fetal blood vessels are diminished or absent from the villi. The terms *complete mole* and *partial mole* have been used to describe the variations of molar pregnancies. With a complete mole, all placenta villi are swollen and the fetus, cord, and amniotic membrane are absent. In partial molar pregnancy, only some chorionic villi are swollen, whereas others appear normal and fetal tissues are present such as the amniotic membrane, cord, or even occasionally a full-term triploid fetus. With the partial mole, the trophoblastic hyperplasia is limited to the syncytiotrophoblast *(4)*. Genetically, complete moles are diploid and only paternal chromosomes are believed to be present. The development of a complete mole appears to result from the fertilization of an "empty egg" with an absent or inactive nucleus, by a haploid paternal set of chromosomes.

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These paternal chromosomes then duplicate to give a diploid number, a process known as androgenesis. Two haploid spermatozoa can also fertilize the "empty egg." The partial moles usually have 69 chromosomes (triploid) of both maternal and paternal origin. The most common mechanism for the origin of partial mole is a haploid egg being fertilized by two spermatozoa, resulting in three sets of chromosome *(5)*.

The observation that hydatiform moles have either reduced levels of $G_s\alpha$ protein and $G_s\alpha$ mRNA suggests that $G_s\alpha$ deficiency might originate from mutations in the gene *(2)*. Indeed, in other pathologies, all defects in the $G_s\alpha$ gene described to date are owing to mutations that result in abnormal RNA processing, production of a nonfunctional mRNA, or synthesis of a truncated protein *(6)*.

In the current study, our laboratory sought to identify mutations that may lead to synthesis of $G_s\alpha$ protein with altered biological activity in hydatiform moles. We report the identification of a heterozygous polymorphism and two mutations in the $G_s\alpha$ gene. One of these mutations may play a role in the reduced expression of $G_s\alpha$ protein. These are the first identified G protein mutations in molar pregnancies.

Results

G_sa Exon Analysis of Four Hydatiform Moles

The human $G_s\alpha$ gene is composed of 13 exons and 12 introns that span ~20 kb of genomic DNA *(7)* mapped to chromosome 20q13.2 → q13.3 in males *(8)*. In our study, each polymerase chain reaction (PCR) amplified exon of the $G_s\alpha$ gene was sequenced at least four times for the three complete hydatiform moles (diploid), and at least six times for the partial hydatiform mole (triploid). We found two mutations and a polymorphism in the $G_s\alpha$ gene in hydatiform moles. As shown in Table 1, one of the mutations (8-bp deletion) was found in two complete hydatiform moles (#1 and #2). The polymorphism was found in exon 5 in the same two complete hydatiform moles (#1 and #2), and the other mutation (A → G) was found in one complete hydatiform mole (#3). No mutations were found in the partial hydatiform mole (#4).

Analysis of Exon 10

An 8-pb deletion was identified in exon 10 of the $G_s\alpha$ gene in two complete hydatiform moles (#1 and #2) (Fig. 1). This deletion (ATG GTC AT), located from codon 255 in exon 10, is heterozygous since it was found in <50% of the clones sequenced. This mutation produces a frameshift of the coding region and the apparition of a STOP (TGA) codon 129 pb farther in exon 11, interrupting the generation of a normal $G_s\alpha$ protein (Fig. 2). This deletion also creates in *Sma*I restriction site that allows for rapid detection of the mutation.

Table 1
Mutations Identified in $G_s\alpha$ gene in Hydatiform Moles

Hydatiform mole	Mutation
Complete hydatiform mole	
#1	Polymorphism (ATT → ATC) in exon 5 8-bp deletion in exon 10
#2	Polymorphism (ATT → ATC) in exon 5 8-bp deletion in exon 10
#3	A → G substitution in intron 5
Partial hydatiform mole	
#4	None

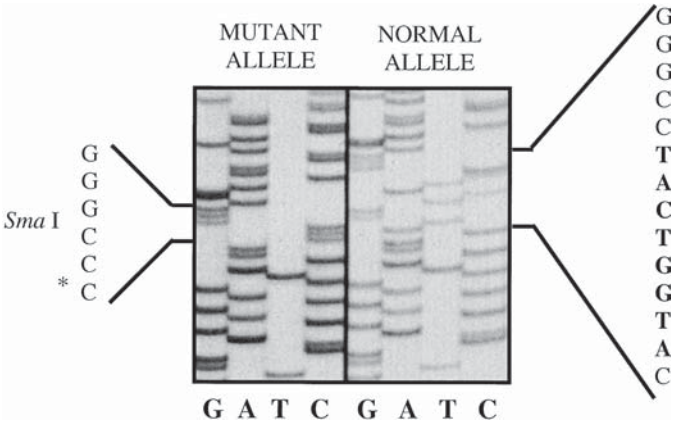

Fig. 1. An 8-bp deletion identified in exon 10 of the $G_s\alpha$ gene. **(Right)** An autoradiograph of the nucleotide sequence of the normal exon 10 of the $G_s\alpha$ gene in the complete hydatiform mole #1; **(left)** an autoradiograph with the corresponding nucleotide sequence of the mutant $G_s\alpha$ exon 10 from the same hydatiform mole. This mutation has also been found in the complete hydatiform mole #2. The asterisk indicates the site of the 8-bp deletion. As can be seen, the deletion created an *Sma*I restriction site. Bold letters on the right indicate the bases that have been deleted.

Analysis of Exon 5

A nucleotide variation was identified in exon 5 of the $G_s\alpha$ gene in two complete hydatiform moles (#1 and #2). The variation, a nucleotide transition (ATT → ATC), is located on the codon corresponding to amino acid 131 of the mature $G_s\alpha$ protein (Fig. 3). This nucleotide transition has been identified on the two strands of the molecule in 50% of the sequenced clones of exon 5 and is therefore considered a heterozygous. No alteration in gene function is expected as a result of the T → C nucleotide substitution in codon 131 because it conserves the isoleucine residue normally present at this location in the $G_s\alpha$ protein.

Analysis of Intron 5

A mutation (A → G) was found in intron 5, 18 bp before the beginning of exon 6 in the $G_s\alpha$ gene (Fig. 4). This

Normal Gs ATG GGC TGC CTC GGG AAC AGT AAG ACC GAG GAC CAG CGC AAC GAG GAG AAG GCG CAG CGT 20
Mutant Gs ATG GGC TGC CTC GGG AAC AGT AAG ACC GAG GAC CAG CGC AAC GAG GAG AAG GCG CAG CGT

Normal Gs GAG GCC AAC AAA AAG ATC GAG AAG CAG CTG CAG AAG GAC AAG CAG GTC TAC CGG GCC ACG 40
Mutant Gs GAG GCC AAC AAA AAG ATC GAG AAG CAG CTG CAG AAG GAC AAG CAG GTC TAC CGG GCC ACG

Normal Gs CAC CGC CTG CTG CTG CTG GGT GCT GGA GAA TCT GGT AAA AGC ACC ATT GTG AAG CAG ATG 60
Mutant Gs CAC CGC CTG CTG CTG CTG GGT GCT GGA GAA TCT GGT AAA AGC ACC ATT GTG AAG CAG ATG

Normal Gs AGG ATC CTG CAT GTT AAT GGG TTT AAT GGA GAG GGC GGC GAA GAG GAC CCG CAG GCT GCA 80
Mutant Gs AGG ATC CTG CAT GTT AAT GGG TTT AAT GGA GAG GGC GGC GAA GAG GAC CCG CAG GCT GCA

Normal Gs AGG AGC AAC AGC GAT GGT GAG AAG GCA ACC AAA GTG CAG GAC ATC AAA AAC AAC CTG AAA 100
Mutant Gs AGG AGC AAC AGC GAT GGT GAG AAG GCA ACC AAA GTG CAG GAC ATC AAA AAC AAC CTG AAA

Normal Gs GAG GCG ATT GAA ACC ATT GTG GCC GCC ATG AGC AAC CTG GTG CCC CCC GTG GAG CTG GCC 120
Mutant Gs GAG GCG ATT GAA ACC ATT GTG GCC GCC ATG AGC AAC CTG GTG CCC CCC GTG GAG CTG GCC

Normal Gs AAC CCC GAG AAC CAG TTC AGA GTG GAC TAC ATT CTG AGT GTG ATG AAC GTG CCT GAC TTT 140
Mutant Gs AAC CCC GAG AAC CAG TTC AGA GTG GAC TAC ATT CTG AGT GTG ATG AAC GTG CCT GAC TTT

Normal Gs GAC TTC CCT CCC GAA TTC TAT GAG CAT GCC AAG GCT CTG TGG GAG GAT GAA GGA GTG CGT 160
Mutant Gs GAC TTC CCT CCC GAA TTC TAT GAG CAT GCC AAG GCT CTG TGG GAG GAT GAA GGA GTG CGT

Normal Gs GCC TGC TAC GAA CGC TCC AAC GAG TAC CAG CTG ATT GAC TGT GCC CAG TAC TTC CTG GAC 180
Mutant Gs GCC TGC TAC GAA CGC TCC AAC GAG TAC CAG CTG ATT GAC TGT GCC CAG TAC TTC CTG GAC

Normal Gs AAG ATC GAC GTG ATC AAG CAG GCT GAC TAT GTG CCG AGC GAT CAG GAC CTG CTT CGC TGC 200
Mutant Gs AAG ATC GAC GTG ATC AAG CAG GCT GAC TAT GTG CCG AGC GAT CAG GAC CTG CTT CGC TGC

Normal Gs CGT GTC CTG ACT TCT GGA ATC TTT GAG ACC AAG TTC CAG GTG GAC AAA GTC AAC TTC CAC 220
Mutant Gs CGT GTC CTG ACT TCT GGA ATC TTT GAG ACC AAG TTC CAG GTG GAC AAA GTC AAC TTC CAC

Normal Gs ATG TTT GAC GTG GGT GGC CAG CGC GAT GAA CGC CGC AAG TGG ATC CAG TGC TTC AAC GAT 240
Mutant Gs ATG TTT GAC GTG GGT GGC CAG CGC GAT GAA CGC CGC AAG TGG ATC CAG TGC TTC AAC GAT

Normal Gs GTG ACT GCC ATC ATC TTC GTG GTG GCC AGC AGC AGC TAC AAC ATG GTC ATC CGG GAG GAC 260
Mutant Gs GTG ACT GCC ATC ATC TTC GTG GTG GCC AGC AGC AGC TAC AAC CCG GGA GGA CAA CCA GAC

Normal Gs AAC CAG ACC AAC CGC CTG CAG GAG GCT CTG AAC CTC TTC AAG AGC ATC TGG AAC AAC AGA 280
Mutant Gs CAA CCG CCT GCA GGA GGC TCT GAA CCT CTT CAA GAG CAT CTG GAA CAA CAG ATG GCT GCG

Normal Gs TGG CTG CGC ACC ATC TCT GTG ATC CTG TTC CTC AAC AAG CAA GAT CTG CTC GCT GAG AAA 300
Mutant Gs CAC CAT CTC TGT GAT CCT GTT CCT CAA CAA GCA AGA TCT GCT CGC TGA Stop

Normal Gs GTC CTT GCT GGG AAA TCG AAG ATT GAG GAC TAC TTT CCA GAA TTT GCT CGC TAC ACT ACT 320

Normal Gs CCT GAG GAT GCT ACT CCC GAG CCC GGA GAG GAC CCA CGC GTG ACC CGG GCC AAG TAC TTC 340

Normal Gs ATT CGA GAT GAG TTT CTG AGG ATC AGC ACT GCC AGT GGA GAT GGG CGT CAC TAC TGC TAC 360

Normal Gs CCT CAT TTC ACC TGC GCT GTG GAC ACT GAG AAC ATC CGC CGT GTG TTC AAC GAC TGC CGT 380

Normal Gs GAC ATC ATT CAG CGC ATG CAC CTT CGT CAG TAC GAG CTG CTC TAA -Stop 395

Fig. 2. Nucleotide sequence of normal and mutant (8-bp deletion) $G_s\alpha$ gene. Underscores indicate the change of amino acid in the sequence bearing the 8-bp deletion (ATG GTC AT).

mutation in intron 5 was identified owing to its proximal location to exon 6. Indeed, the upper primer of exon 6 allowed the sequencing of a small part of intron 5 in front of exon 6. This A → G substitution was identified on the two DNA strands in all the clones sequenced of one complete hydatiform mole (#3) and is therefore homozygous. This mutation should not alter the gene function because it should be eliminated during splicing. Table 1 presents a summary of the mutations identified in the four hydatiform moles.

Discussion

In the present study, we identified two new mutations and a neutral polymorphism in the $G_s\alpha$ gene in the same complete hydatiform moles in which previous studies have demonstrated a reduced $G_s\alpha$ protein and mRNA expression *(2)*. The most interesting mutation found is an 8-bp deletion identified in exon 10 of the $G_s\alpha$ gene in two of the three complete hydatiform moles studied. This deletion produced a $G_s\alpha$ mRNA which the reading frame was shifted after codon Asn^{254}, resulting in a change in all other downstream amino acid residues of the protein sequence. Moreover, this deletion also resulted in the introduction of a premature stop codon 129-bp downstream in exon 11 and to a truncated $G_s\alpha$ protein. Thus, it was deduced from the nucleotide sequence that only the first 254 amino acid residues of the truncated protein are identical to that of the wild-type $G_s\alpha$ protein. This 8-bp deletion should therefore lead to

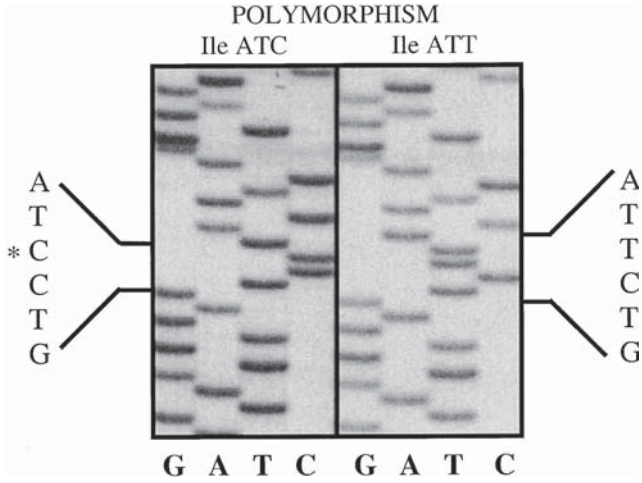

Fig. 3. A polymorphism (ATT → ATC) identified in exon 5 of the $G_s\alpha$ gene. **(Right)** An autoradiograph of the nucleotide sequence of the polymorphism ATC in exon 5 in the complete hydatiform mole #1; **(left)** an autoradiograph with the corresponding nucleotide sequence of the polymorphism ATT in exon 5 of the same hydatiform mole. This polymorphism has also been found in the hydatiform mole #2. The asterisk indicates the site of the T → C substitution within exon 5 corresponding to codon 131 of the $G_s\alpha$ gene. This substitution does not change the amino acid residue.

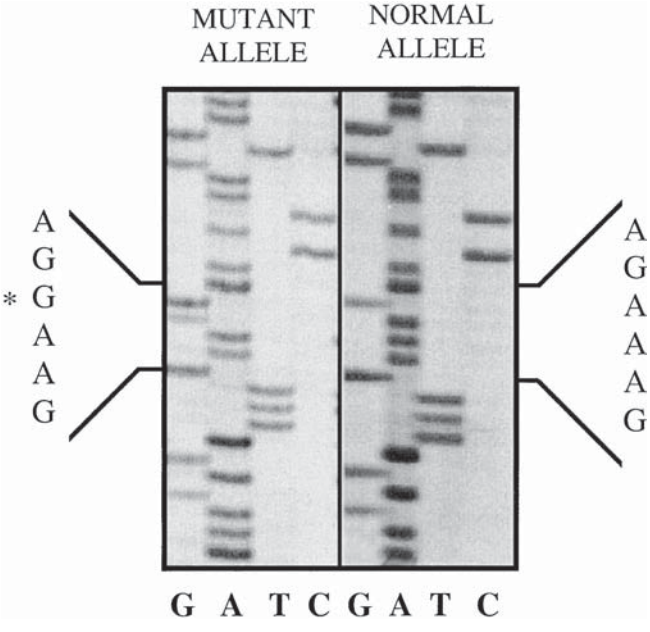

Fig. 4. A missense mutation (A → G) identified in intron 5 of the $G_s\alpha$ gene. **(Right)** An autoradiograph of the nucleotide sequence of the normal intron 5 of the $G_s\alpha$ gene in complete hydatiform mole #3; **(left)** an autoradiograph with the corresponding nucleotide sequence of the mutant $G_s\alpha$ intron 5 from the same hydatiform mole. The asterisk indicates the site of A → G substitution within intron 5.

dramatic changes in protein function since it is known that the $G_s\alpha$ protein amino acids 349–395 are important for stimulation of adenylyl cyclase and for $G_s\alpha$ coupling of certain receptors *(9,10)*. Indeed, amino acid residues 349–356 are part of the effector domain of the protein implicated in adenylyl cyclase stimulation whereas residues 367 to 376 are proposed to play a role in membrane attachment of $G_s\alpha$ protein.

The reduced level of $G_s\alpha$ mRNA and protein observed with these four hydatiform moles by Petit and al. *(2)* can be caused, at least in two complete moles, by the 8-bp deletion in exon 10, which affects 50% of the sequenced $G_s\alpha$ gene alleles. The use of an antibody directed against the C-terminal region of the protein may have failed to recognize the truncated $G_s\alpha$ protein lacking its C-terminal region. Moreover, mutations such as base substitutions at a splice junction or coding frameshift mutations are consistent with abnormal RNA processing, and thus with the expression of an abnormal and presumably deficient form of the protein *(10)*. Inactivating mutations may create generalized hormone resistance as seen in patients with Albright Hereditary Osteodystrophy (AHO) and Pseudohypoparathyroidism type Ia, a variant of AHO *(11)*. These hormones include parathyroid hormone, thyroid-stimulating hormone, gonadotropins, and glucagon, all coupled to the stimulation of adenylyl cyclase through G_s *(10)*. Indeed, multiple distinct heterozygous mutations (base substitution at a splicing junction, coding frameshift mutations, missense mutations) of the Gsa gene were identified to be the cause of abnormal RNA processing, production of nonfunctional mRNA, or truncated protein, which lead to an approx 50% reduction in functional $G_s\alpha$ protein; this is thought to impair the cAMP response to hormone stimulation *(12)*. The 8-bp deletion cannot explain the reduced level of expression in the third complete mole and the partial hydatiform mole that were not mutated at that position. It is possible that other regulatory elements such as G-coupled receptors, effectors, and kinase proteins are altered. Moreover, we cannot rule out that other mutations may be present in the promoter region and splicing regions that could lead, respectively, to a diminution of the transcription of the $G_s\alpha$ gene and modification of the transcript itself.

Many disorders have been reported to be caused by defects in G proteins. In fact, endocrine tumors such as pituitary adenoma *(13)*, thyroid neoplasm *(14)*, and adrenocortical adenomas *(15)* have been reported to be caused by activating mutations of $G_s\alpha$. These mutations converted the $G_s\alpha$ gene to an oncogene termed *gsp (16)* that enabled the $G_s\alpha$ subunit to remain in the active GTP-bound state, resulting in accumulation of cAMP and subsequently leading to cell proliferation and malignant transformation. The most frequently reported mutations in the Gsa gene are Arg^{201} and Gln^{227}; the latter amino acid residue corresponds to Gln^{61} in $p21^{ras}$, which confers its oncogene activity. Arg^{201} and Gln^{227} are part of the domains required for guanosine 5'-diphosphate/GTP binding and intrinsic GTPase activation, respectively *(16,17)*. These residues are highly

conserved in all G protein α-subunits, and naturally occurring mutations involving these amino acids have been found in the $G_{i2}\alpha$ gene in adrenocortical as well as ovarian neoplasms *(18)*.

Furthermore, the detection of heterozygous mutations in complete hydatiform moles suggests that they result from the fertilization of an empty egg by two spermatozoa rather than from the duplication of the DNA from one spermatozoid. In the latter case, the mutation should be duplicated and the mutation would be homozygous.

The polymorphism, a T → C nucleotide substitution in codon 131, was found in two complete hydatiform moles and is heterozygous. The isoleucine normally present at this location of the $G_s\alpha$ protein is conserved. This polymorphism has already been reported in patients with AHO. The relative frequency of polymorphism in exon 5 of the $G_s\alpha$ gene has been evaluated in 20 normal unrelated Caucasian individuals who had no history of $G_s\alpha$ deficiency, and results showed that 38% of the chromosomes contained a thymidine and 62% contained a cytosine at this position *(11)*. Therefore, it is unlikely that this polymorphism had any effect on Gsa protein expression.

The homozygous mutation in intron 5 was a nucleotide substitution A → G located 18 bp upstream of exon 6. This mutation has been found in only one complete hydatiform mole. Since this nucleotide is located in intron 5, 18 bp outside of the reading frame, it should not interfere and it should be normally eliminated during splicing. It is not excluded, however, that this mutation might affect or prevent the production of splice variants of the mRNA and therefore affecting the formation of a normal Gsa protein.

The results of the present study provide a molecular basis for $G_s\alpha$ deficiency in hydatiform moles identified by Petit et al. *(2)* in human placentae from molar pregnancies. These results, however, do not allow us to conclude whether the mutations are a direct or an indirect cause of the formation of hydatiform moles. Other mutations might be present in the other types of G protein ($G_o\alpha$, $G_{i2}\alpha$, and $G_{i3}\alpha$) in which dramatic decreases of expression have been observed in molar pregnancies *(2)*. Mutations may also be present in the promoter region and in sequences required for splicing. Hydatiform moles are also associated with an overexpression of oncogenes such as *c-myc*, *c-fms*, *c-ras*, *v-alb*, *v-erb A*, *v-fms*, *v-mos*, *v-myc*, *N-ras*, and *v-src*, which suggests that the activation of cellular oncogenes might be strongly associated with neoplasic changes in placenta, and thus play a role in hydatiform mole formation *(19,20)*. Also, as mentioned previously, oncogenes such as *ras* are effectively activated by single nucleotide substitutions and associated with a significant percentage of human tumors from diverse tissues. They are believed to contribute to both the initiation and progression of human neoplasia *(21)*. The effects of mutations in G protein genes on the expression of placental hormones and cAMP formation remains to be fully investigated.

Materials and Methods

Tissue Specimens

The Human Subject Research Committee of our institution and participating hospitals approved this project. Placentae from molar pregnancies between 11 and 16 wk were obtained from the obstetrical service of the Montreal Jewish General Hospital (three cases) and the Royal Victoria Hospital (one case). The villous layers of trophoblastic tissues were dissected, frozen on dry ice, and stored at –80°C until time of DNA preparation.

Isolation of Genomic DNA and PCR Amplification

Genomic DNA was isolated from three complete hydatiform moles and one partial hydatiform mole with the DNAzol™ Reagent according to the protocol provided by the manufacturer (Gibco-BRL, Burlington, Ontario). Genomic DNA served as template for in vitro amplification by PCR. The PCRs were performed using intron-specific oligonucleotide primers flanking exons of the $G_s\alpha$ gene, synthesized with an *Xba*I restriction site in 5' of each primer *(7)* (Table 2). The PCRs were performed with a DNA thermal cycler 480 (Perkins-Elmer, Mississauga, Ontario). Genomic DNA (0.5–1.0 μg) was amplified in a 100-μL volume containing 100 m*M* Tris-HCl (pH 8.85), 250 m*M* KCl, 50 m*M* $(NH_4)_2SO_4$, 2 m*M* $MgSO_4$, 0.15 *M* dNTP, 15 p*M* of each primer, and 2.5 U of *Pyroccocus woesei* (PWO) DNA polymerase (Boehringer Mannheim, Laval, Quebec). After an initial denaturation for 4 min at 94°C, the samples underwent a touchdown consisting of gradually reducing the annealing temperature of by 1 to 2 degrees each cycle until the desired annealing temperature was reached. The touchdown procedure allows a more specific annealing of the primers. The amplification cycles, consisting of denaturation for 1 min at 94°C, annealing for 1 min 30 s, and extension for 1 min and 30 s at 72°C immediately follow. The number of amplification cycles ranged from 30 to 40, and the annealing temperature ranged from 52 to 68°C depending on the specific primer pair used. The amplification cycles are followed by an extension for 15 min at 72°C. To assess the reaction products of PCR, 10–20% of each reaction mixture was analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide (1 μg/mL), and examined by ultraviolet (UV) transillumination.

DNA Sequence Determination

Nucleotide sequence of amplified gene fragments was determined by sequencing DNA fragments that had been subcloned into plasmid vectors. The DNA was purified by centrifugation through Sephadex G-50 *(22)* and was sequenced by the dideoxy chain termination method *(23)* using the T7 Sequencing Kit (Pharmacia, Baie d'Urfé, Quebec). To subclone amplified fragments, DNA was ligated into a unique *Xba*I site of the plasmid pSV-SPORT1 (Gibco-BRL, Burlington, Ontario) and used to transform competent HB101 cells. The clones were verified by PCR,

Table 2
Oligonucleotide primers used to amplify exons of the $G_s\alpha$ gene

Exon	Size (bp)	Upper primer	Lower primer	Annealing temperature
2	234	5'-TGCTCTAGAAAATGCCTCCTTCATAACCTGAGA-3'	5'-TGCTCTAGATCTGTTCCTCTTACTTGGTC-3'	53
3	239	5'-TGCTCTAGATGATGGTTGAGGAATGTAGAGAGACTGTG-3	5'-TGCTCTAGACAGTATGATCTTCATGTTTGTTT-3'	53
4–5	461	5'- TGCTCTAGAATGAAAGCAGTACTCCTAACTGA-3'	5'-TGCTCTAGAGTGCCCATGTGCAGGGCTGTCACTCATGTT-3'	62
5	308	5'-TGCTCTAGATCTTGTAGCGCCCTCCCA-3'	5'-TGCTCTAGAGTGCCCATGTGCAGGGCTGTCACTCATGTT-3'	59
6	231	5'-TGCTCTAGATTAGTTCAAGCTCTTGCCTTTCTCTA-3'	5'-TGCTCTAGATTGTCTGTTTTATGTGCTGATGG-3'	68
7	235	5'-TGCTCTAGATGAGCCTGACCTTGTAGAGAGACACA-3'	5'-TGCTCTAGAGTAGTTTGGAAAGAGGGCTCAGAG-3'	67
7–8	466	5'-TGCTCTAGATGAGCCTGACCTTGTAGAGAGACACA-3'	5'-TGCTCTAGAGGTTATTCCAGAGGGACTGGGGTGAA-3'	67
8	166	5'-TGCTCTAGACTTCTGGAATCTTTGAGACCAAG-3'	5'-TGCTCTAGAGGTTATTCCAGAGGGACTGGGGTGAA-3'	62
9	189	5'-TGCTCTAGACATTCACCCCAGTCCCTCTGGAAT-3'	5'-TGCTCTAGAAGCGTTCTTTACGAACAGCCAAGC-3'	62
10	280	5'-TGCTCTAGATGTTGTTAGGGATCAGGGTCGCTG-3'	5'-TGCTCTAGAACAGTGCAGACCAGGGCCTCCTG-3'	64
9–11	698	5'-TGCTCTAGACATTCACCCCAGTCCCTCTGGAAT-3'	5'-TGCTCTAGAGAACCACCGCAATGAACAGCC-3'	58
12	214	5'-TGCTCTAGATGCTAGCACCCCAGCTCTGCTTGAA-3'	5'-TGCTCTAGAGGAGGAACAAGAGAGGAAACT-3'	55
13	284	5'-TGCTCTAGACATCAGGGATAGGGTGGTTCCTGGC-3'	5'-TGCTCTAGATTAAAGGCTTTAATTAAATTTGGGGGTTCC-3'	62

using the vector primers in a 10- μL volume, as described previously (without the touchdown procedure). The vector without insert was used as negative control. The colony was resuspended in part in the PCR solution, and the remainder was resuspended in 2 mL of Luria broth medium with ampicillin (100 μg/mL) followed by incubation for 16h at 37°C. The PCRs were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide (1 μg/mL) and examined by UV transillumination. The vectors containing the inserted fragment were isolated by alkali lysis *(24)* and sequenced by the dideoxy chain reaction method using the vector primers.

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