Leptin Receptor Isoforms Expressed in Human Adipose Tissue

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Leptin and its structural gene, Ob, are exclusively expressed in adipose tissue. Leptin is secreted into the blood and is responsible for fat mass regulation via leptin receptors in the hypothalamus. This has been considered the major role of leptin, but leptin receptor isoforms are expressed not only in the brain but also in most other tissues in humans and rodents: heart, placenta, lung, liver, muscle, kidney, pancreas, spleen, thymus, prostate, testes, ovary, small intestine, and colon. This implicates leptin regulation in other systems apart from fat mass regulation, and leptin action has been demonstrated in human fetal development and reproductive development, liver metabolism, hematopoiesis, and insulin secretion. Four splice variants of the leptin receptor have been identified in humans: the long isoform huOb-R and the shorter isoforms B219.1 to B219.3. It is known that the long isoform has full intracellular signaling capacity, and is responsible for anorectic action in the hypothalamus. The roles of the other isoforms are yet to be elucidated. Here, we report the identification by reverse transcriptase–polymerase chain reaction (RT-PCR) of three leptin receptor isoforms coexpressed in human visceral adipose tissue: the long isoform huOb-R and the short isoforms huB219.1 and huB219.3. The possible roles of these isoforms are discussed.

ADIPOSE TISSUE has recently been discovered to have an endocrine character, and specifically expresses the hormone leptin. A well-established role of leptin is in fat mass regulation via hypothalamic control of food intake and thermogenesis.[1-3] However, leptin can affect fat mass regulation directly, without involvement of the brain. Recently published data indicate that leptin can directly affect adipocyte metabolism: leptin inhibits adipocyte acetyl coenzyme A carboxylase gene expression and activity,[4] suppresses in vitro lipid synthesis,[4,5] and counteracts induction of lipid synthesis by glucocorticoids and insulin.[4] Identification of the possible leptin receptor isoforms involved in adipose tissue is the main purpose of the present study.

A second direct effect of leptin has been demonstrated on human hepatoma cells (hepG2) expressing the Ob-R receptor.[6] Exposure of these cells to leptin attenuated several insulin-induced activities, including insulin receptor signaling, and downregulated gluconeogenesis. The investigators suggested a possible role of hyperleptinemia in obesity-associated insulin resistance. There is a third direct effect of leptin on pancreatic β cells, as ^{125}I-leptin has been found to bind to β insulinoma cells,[7] and Ob-Rb (long isoform of leptin receptor) is expressed in pancreatic islets of *ob/ob* and wild-type mice.[8] Moreover, it has been shown that leptin reduces basal and glucose-stimulated insulin secretion in a perfused pancreas preparation from *ob/ob* mice but not from Zucker *fa/fa* rats or *db/db* mice,[8] which have mutated Ob-Rb receptors. Presumably, the latter two direct effects both result in hyperinsulinemia, hyperglycemia, and diabetes in vivo in the case of leptin resistance.

In addition to fat mass regulation, leptin has several important roles that have already been clarified, and probably others awaiting discovery. Leptin receptors have a wide distribution in humans and rodents, and therefore many peripheral actions, including regulation of hematopoiesis,[9] stimulation of cytokine production and macrophage phagocytosis,[10] and control of the development of reproductive systems.[11-13]

Taken together, these findings point to the importance of identifying the presence of the different leptin receptor isoforms in different tissues. Elucidation of their function and intracellular signaling, plus mutations or disruptions in these, may enable the prevention of the effects of leptin resistance and its consequences.

MATERIALS AND METHODS

Preparation of RNA

Biopsies of visceral adipose tissue were obtained from female patients in the fasted state undergoing laparoscopic cholecystectomy, with cholelithiasis but no other serious illness, after approval of the protocol by the Pomeranian Medical Academy Ethical Committee. All participants provided written consent after being informed of the nature, purpose, and possible risks of the study.

Columns from a Total RNA Isolation kit from A and A Biotechnology (Gdansk, Poland) were used to isolate RNA from this tissue. About 0.5 g frozen tissue was first pulverized in Fenozol solution (1 mL, from the kit) and homogenized using a glass cylinder homogenizer for 3 minutes, and then the manufacturer's instructions for the kit were followed (modified from Chomczynski and Sacchi[14]). Solutions of RNA were quantified spectrophotometrically at 260 nm, and the integrity of the RNA was verified using the absorption ratio at 260/280 nm and agarose gels. Solutions of RNA were suspended in water and stored at −80°C.

Leukocytes were isolated from fresh blood samples (10 mL each) from two overweight female patients, using an osmotic gradient method with Gradisol G (Polfa, Kutno, Poland) and the manufacturer's protocol. RNA was isolated exactly following the method of Chomczynski and Sacchi.[14]

Primers

The original terminology for the leptin receptor isoforms[9] is used here, but it should be noted that huOb-R is homologous to muOb-Rb and huB219.3 is homologous to muOb-Ra. (The human isoforms are sometimes referred to as huOb-Rb and huOb-Ra, respectively[10]).

A set of primers (Table 1) for the Ob receptor isoforms were designed using the software PrimerSelect (DNASTAR, Madison, USA). Four reverse primers, B1R, B2R, B3R, and Ob-RR, were designed—one for each of the receptor isoforms already identified[9] plus another reverse primer, B0R, designed to produce a product with any B219/Ob-R transcript. All reverse primers were theoretically compatible with the forward primer, B3F.

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Table 1. Primers Used to Detect the Different Splice Variants of the Ob Receptor Gene

Primer	Length (mer)	Melting Temperature (°C)	Product With B3F (bp)
Ob-R forward			
B3F TTGTGCCAGTAATTATTTCCTCTT	24	51.3	
Ob-R reverse			
B0R—detects all isoforms ATAGCTTTTTCATTCTTTGGTGTG	24	51.6	78
B1R—detects huB219.1 CTGTGGCCTTCCGCAGTG	18	54.7	227
B2R—detects huB219.2 ACCTCCACCCAGTAGTTCCTT	21	50.9	176
B3R—detects huB219.3 AGTTGGCACATTGGGTTCAT	20	51.6	200
Ob-RR—detects huOb-R CTGATCAGCGTGGCGTATTT	20	52.8	439

The Ob gene was used as a marker for adipose tissue RNA. Ob primers were kindly provided by Dr H. Vidal (INSERM, Lyon, France[15]).

DNase Preincubation

For some experiments, DNase I (Boehringer, Mannheim, Germany) was used in a preincubation before reverse transcription, according to the protocol of Huang et al.[16] In this case, 1 μL (1 U) DNase I (replacing 1 μL dNTPs) was added to the reverse transcriptase (RT) reaction mixture (see below) minus the RT enzyme. The tubes were incubated at 37°C for 30 minutes and 75°C for 5 minutes. The RT enzyme was then added and the procedure continued as described later.

Reverse Transcription

First-strand cDNA synthesis was performed in a total volume of 20 μL using 20 U RT (M-MuLV) plus 4 μL 5X M-MuLV–specific buffer (Boehringer), 40 U RNAsin (N211B; Promega, Madison, USA), 8 μL dNTPs (2.5 mmol/L each; Promega), and 2 μg/μL RNA, plus either reverse primer (10 pmol, designed as before; synthesized by TIB MOLBIOL, Poznan, Poland) or random hexamers (100 pmol, pol $(N)_6$ cDNA synthesis; Promega). The following program was used: 20°C for 10 minutes, 37°C for 60 minutes, and 94°C for 5 minutes. Products were stored at −20°C. (All reactions in this study were performed in an Eppendorf Mastercycler 5330, Warsaw, Poland).

Polymerase Chain Reaction

Amplification of cDNA was performed in a total volume of 100 μL using 2.5 U *Taq* polymerase plus 8 μL 10X *Taq*-specific buffer (Boehringer), 10 μL dNTPs (2.5 mmol/L each; Promega), 20 μL reverse transcription product, water (Water for Injection; Polfa, Starogard, Poland), and 10 pmol forward primer only (or if random hexamers were used for the RT step, 10 pmol of both forward and reverse primers). The following program was used: 94°C for 3 minutes and then 34 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 90 seconds, and 72°C for 10 minutes. Products were stored at 4°C.

Restriction Analysis

Confirmation that a RT-polymerase chain reaction (PCR) product was from the B219.3 leptin receptor was obtained by restriction with the enzyme *Alu*I (Peterfarm, Sierdz, Poland) as follows: 8.5 μL RT-PCR product, 1 μL (5 U) *Alu*I, plus 1 μL *Alu*-specific buffer (Peterfarm) at 37°C for 90 minutes.

Gels

Products were viewed using 1% to 3% agarose gels, and photographs were taken with a Polaroid DS34 camera (UK) and Print Film type 667 (Sigma-Aldrich, Deisenhofen, Germany). Product lengths were estimated by comparison with two size markers (VI and VIII; Boehringer).

RESULTS

Samples from 12 female patients were used in this study: six obese (body mass index [mean ± SE], 32.8 ± 4.26 kg/m^2; waist to hip ratio, 0.84 ± 0.027; age, 56 ± 8.9 years) and six lean (body mass index, 22.95 ± 1.64 kg/m^2; waist to hip ratio, 0.78 ± 0.046; age, 43 ± 8.8 years). RNA was isolated from adipose tissue (mean, 38.5 μg from ~0.5 g tissue) and from blood (4.7 μg from 10 mL). The blood volume in adipose tissue was not measured, but we estimate it to be 5% or less. This yields an estimated blood RNA to adipose RNA ratio in adipose tissue of less than 1:1,000.

After RT-PCR, huB219.3 product bands were detected using random hexamers in the reverse transcription step, but the strongest and most consistent bands were obtained using the reverse primer (B3R) in this step (Fig 1). huB219.3 product bands obtained with adipose tissue RNA were consistently strong in these experiments, plus and minus DNase treatment. No differences were noted in the product strength from six lean and six obese patients. The huB219.3 product was estimated on gels to be of the correct length (200 bp), and the identity of the product was confirmed using restriction analysis with *Alu*I—yielding two products estimated to be of the correct lengths, 74 and 126 bp (Fig 2).

Additionally, huB219.3 was also detected in leukocyte samples from two patients, but the band strength (with DNase treatment) was less than obtained with adipose tissue (Fig 3). The RT-PCR techniques used here are semiquantitative, and the finding of submaximal band strength from blood samples shows that blood perfusion of adipose tissue was not responsible for the huB219.3 products detected in adipose tissue. (However, it should be noted that without DNase treatment, one leukocyte sample did yield a band of similar strength as those from adipose tissue (lane 4, Fig 3)). As a marker for nondegraded

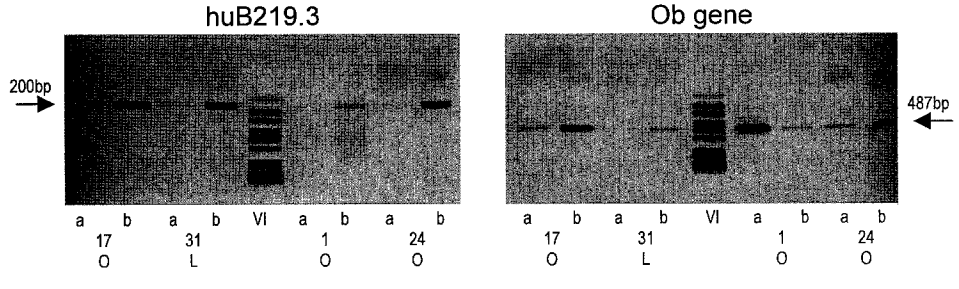

Fig 1. Expression of huB219.3 and Ob gene in 4 patients, measured by 2 different RT-PCR methods. In the RT step, (a) random hexamers and (b) reverse primers were used. Size markers are No. VI (Boehringer). Patient number is shown; L, lean; O, obese.

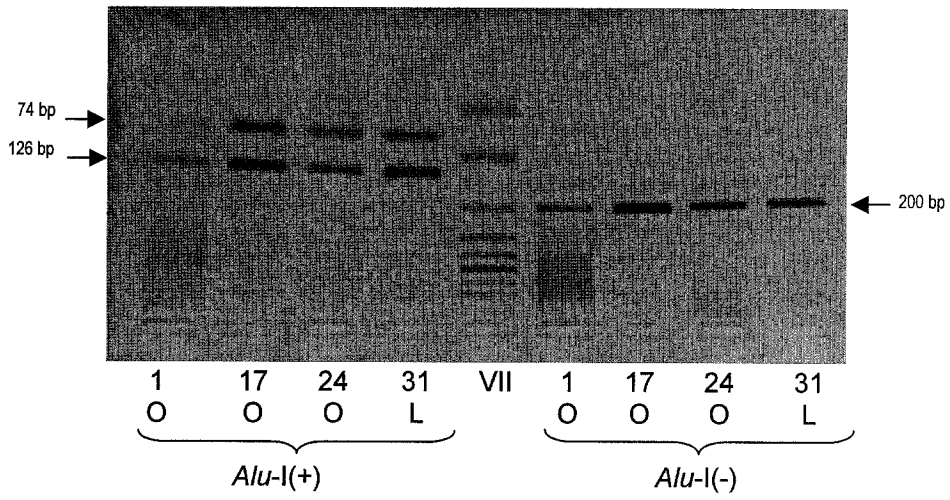

Fig 2. Restriction of huB219.3 RT-PCR products from Fig 1 (reverse primer in RT step) by *AluI*. Size markers are No. VIII (Boehringer). Patient number is shown; L, lean; O, obese.

adipose tissue RNA, the *ob* gene was detected in samples from four patients (Fig 1).

Three experiments showed consistent (but weak) huB219.1 product bands (Figs 3 and 4) from adipose tissue, both plus and minus DNase treatment. These were also detected in leukocyte samples, but the bands were even weaker (Fig 3)—on one gel with less product applied (not shown), four bands could be seen from adipose tissue and none from blood samples.

Results using the Ob-RR reverse primer have not been so consistent, perhaps because of reduced or variable expression. Bands varying from very weak to strong were seen in samples from six patients (two strong bands are shown in Fig 4). The differences noted do not correlate with obesity: in one experiment, a sample from a lean patient yielded a slightly weaker band than from the obese patients (not shown), but in another experiment, the sample from the obese patient yielded a slightly weaker band than from the lean patient (Fig 5). The Ob-R product was also detected in one leukocyte sample (Fig 5).

Products using the B0R reverse primer (not shown) were consistently produced by all samples tested, but the bands were weak. No products were detected using the B2R reverse primer (Fig 4).

DNase I treatment effectively destroyed all DNA (Fig 5). With either the huB219.1 or huB219.3 reverse primer, the band of the correct length was unaffected (Fig 3), and with the huB219.1 primer, some weak nonspecific bands were eliminated by the treatment. However, using the protocol of Huang et al[16] is not recommended without first checking the extent to which RNA is degraded by the DNase I, especially as a higher pH is used than recommended by the manufacturer, and RNA degradation has been detected in this laboratory (results not shown).

DISCUSSION

The extent of distribution of the leptin receptor in mouse tissues[9,10,17,18] has been shown using primers that detect but do not distinguish between all mouse isoforms, or that detect only the short-form Ob-Ra or the long-form Ob-Rb (which are homologous to the human isoforms B219.3 and Ob-R, respectively[9]). Ghilardi et al[19] found that both the short-form Ob-Ra and long-form Ob-Rb are apparently coexpressed in most tissues. Ob-Ra was found in all 21 tissues tested, and the high level of expression indicates the importance of this isoform (and its huB219.3 homolog). Ob-Rb was detected in all but three tissues to a variable extent (and always less than Ob-Ra).

One study has clarified the distribution of all five mouse isoforms, Ob-R a to e, in several tissues.[17] This study indicated an Ob-Ra distribution different from that detected by Ghilardi et al, and detected expression of all five isoforms in adipose tissue.

Less study has been performed on human tissues. Cioffi et al[9] detected the leptin receptor (isoforms not distinguished) in 15 human tissues, and Gainsford et al[10] detected coexpression of long and short isoforms (huB219.3 and Ob-R) in four human tissues: brain, bone marrow, fetal liver, and spleen. Surprisingly, none of these studies tested human adipose tissue, and there has been no attempt to show the distribution of huB219.1 or huB219.2 isoforms in the human since their discovery in fetal liver[9] (or indeed, whether these isoforms can be detected in mouse tissues). Additionally, no studies have attempted to show the significance of blood (or indeed, fibroblasts) in the tissue samples.

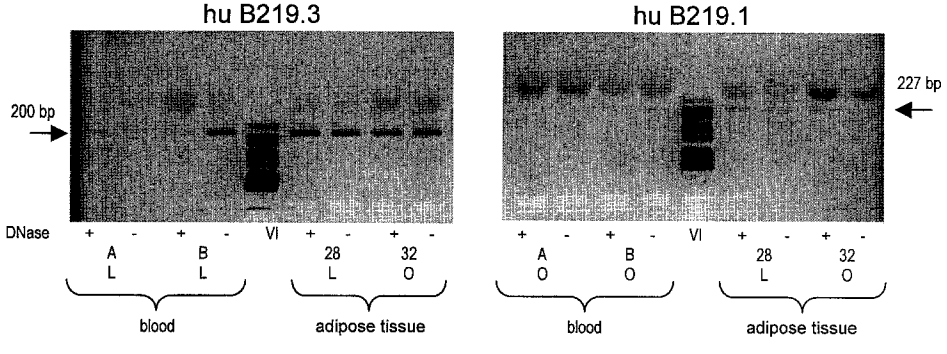

Fig 3. Expression of huB219.3 and huB219.1 in adipose tissue and blood leukocytes. RT-PCR (reverse primer in RT step) was used plus or minus DNase pretreatment. Size markers are No. VI (Boehringer). Patient number or letter is shown; L, lean; O, obese.

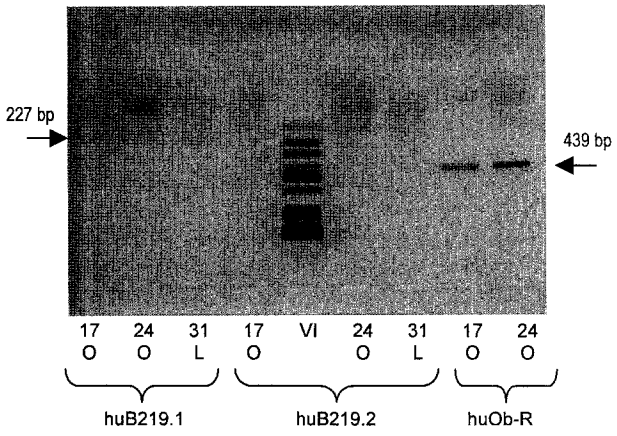

Fig 4. RT-PCR (reverse primer in RT step) using primers for huB219.1, huB219.2, and huOb-R. Size markers are No. VI (Boehringer). Patient number is shown; L, lean; O, obese.

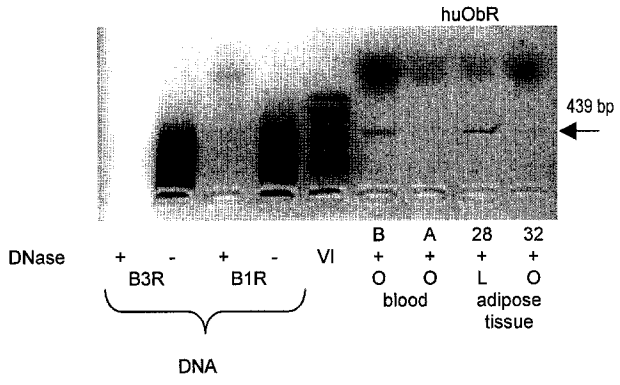

Fig 5. Expression of huOb-R in adipose tissue and blood leukocytes using RT-PCR with DNase pretreatment and reverse primer in RT step. Controls showing degradation of nonspecific products from DNA from patient no. 28 are also shown (using B3R and B1R primers). Size markers are No. VI (Boehringer). Patient number or letter is shown; L, lean; O, obese.

The intracellular signaling following leptin binding to its receptor has only been elucidated in the case of the huOb-R isoform (and muOb-Rb), which has the correct structure (with homology to class 1 cytokine receptors) and has been shown to activate STAT (signal transducers and activators of transcription) proteins 1, 3, 5, and 6.[18,19] It has therefore been proposed that this isoform is the one responsible for anorectic action, and indeed, this isoform is strongly expressed in the hypothalamus.[2]

The results of the present study provide the first detection of leptin receptor isoforms in human adipose tissue and blood leukocytes and add to the list of tissues in which several isoforms are coexpressed in the same tissue. The detection of different isoforms in mouse (a, b, c, d, and e) and human adipose tissue (a = huB219.3, b = huOb-R and huB219.1) should be kept in mind when rodent models are used. Additionally, the variation in expression in blood leukocytes is especially interesting, and further study is needed to discover the full extent and importance of this variation. The intracellular signaling or interactions between the short isoforms huB219.1 to huB219.3 or muOb-R a, c, d, and e are completely unknown. The importance of the autoregulation of the fat mass demands that further study be initiated to clarify the roles of these isoforms.

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REFERENCES

1. Tartaglia LA, Dembski M, Weng X, et al: Identification and expression cloning of a leptin receptor, OB-R. Cell 83:1263-1271, 1995

2. Mercer JG, Hoggard N, Williams LM, et al: Localisation of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridisation. FEBS Lett 387:11-116, 1996

3. Caro JF, Kolaczynski JW, Nyce MR, et al: Decreased cerebrospinal-fluid/serum leptin ratio in obesity: A possible mechanism for leptin resistance. Lancet 348:159-161, 1996

4. Yinglin B, Zhang S, Kim KS, et al: Obese gene expression alters the ability of 30A5 preadipocytes to respond to lipogenic hormones. J Biol Chem 271:13939-13942, 1996

5. Shimabukuma M, Koyama K, Chen G, et al: Direct antidiabetic effect of leptin through triglyceride depletion of tissues. Proc Natl Acad Sci USA 94:4637-4641, 1997

6. Cohen B, Novick D, Rubinstein M: Modulation of insulin activities by leptin. Science 274:1185-1188, 1996

7. Kieffer TJ, Heller RS, Habener JF: Leptin receptor expressed on pancreatic β-cells. Biochem Biophys Res Commun 224:522-527, 1996

8. Emilson V, Liu YL, Cawthrone MA, et al: Expression of the functional leptin receptor mRNA in pancreatic islets and direct inhibitory action of leptin on insulin secretion. Diabetes 46:313-316, 1997

9. Cioffi JA, Shafer AW, Zupancic TJ, et al: Novel B219/OB receptor isoforms: Possible role of leptin in hematopoiesis and reproduction. Nat Med 2:585-589, 1996

10. Gainsford T, Willson TA, Metcalf D, et al: Leptin can induce proliferation, differentiation and functional activation of hemopoietic cells. Proc Natl Acad Sci USA 93:14564-14568, 1996

11. Hassink SG, Sheslow DV, de-Lancey E, et al: Serum leptin in children with obesity: Relationship to gender and development. Pediatrics 98:201-203, 1996

12. Barash IA, Cheung CC, Weigle DS, et al: Leptin is a metabolic signal to the reproductive system. Endocrinology 137:3144-3147, 1996

13. Chehab FF, Lim ME, Lu R: Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. Nat Med 12:318-320, 1996

14. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156-159, 1987

15. Auboeuf D, Vidal H: The use of the reverse transcription–competitive polymerase chain reaction to investigate the in vivo regulation of gene expression in small tissue samples. Anal Biochem 245:141-148, 1997

16. Huang Z, Fasco MJ, Kaminsky LS: Optimisation of Dnase I removal of contaminating DNA from RNA for use in quantitative RNA-PCR. Biotechniques 20:1012-1020, 1996

17. Lee GH, Proenca R, Montez JM, et al: Abnormal splicing of the leptin receptor in diabetic mice. Nature 379:632-635, 1996

18. Takahashi Y, Okimura Y, Mizuno I, et al: Leptin induces tyrosine phosphorylation of cellular proteins including STAT-1 in human renal adenocarcinoma cells, ACHN. Biochem Biophys Res Commun 228:859-864, 1996

19. Ghilardi N, Ziegler S, Wiestner A, et al: Defective STAT signalling by the leptin receptor in diabetic mice. Proc Natl Acad Sci USA 93:6231-6235, 1996