A Small N-Terminal 60-kD Fragment of gp600 (Megalin), the Major Autoantigen of Active Heymann Nephritis, Can Induce a Full-Blown Disease

ANDREW V. OLEINIKOV, BRADY J. FELIZ, and SUDESH P. MAKKER

Department of Pediatrics, Division of Nephrology, School of Medicine, University of California, Davis, California.

Abstract. Active Heymann nephritis of rat, an autoimmune glomerular disease, is an immunohistological, ultrastructural, and clinical model of human membranous glomerulonephritis. Both diseases in their full-blown form are characterized by (1) the formation of large, subepithelial glomerular immune deposits, which stain for IgG, C3, and membrane attack (C5b-9) components of complement and (2) the excretion of large amounts of protein in the urine (proteinuria). The target autoantigen of active Heymann nephritis is a large transmembrane renal glycoprotein with a molecular weight of approximately 600 kD, variously named gp600, gp330, LRP-2, or “megalin.” This study was performed to identify the region in this enormously large glycoprotein that would produce full-blown active Heymann nephritis. A stable, small (60-kD) proteolytic fragment of gp600 was isolated and localized to the N-terminal end of the molecule using Western blot, sequencing, and amino acid analyses. Based on its primary structure, this fragment contains approximately 60 cysteine residues, the cross-linking of which to each other probably explains its stability. Immunization of rats with this fragment induced a full-blown disease that was comparable to the disease induced by a preparation containing the whole protein. These results indicate that this small fragment, retaining the natural disulfide bonds and probably its overall structure, contains those B and T cell epitopes that are sufficient to produce this organ-specific autoimmune disease.

Idiopathic membranous glomerulonephritis (MGN) (1), a common glomerular disease in humans, is responsible for much morbidity and many cases of end-stage kidney failure (1,2). Active Heymann nephritis (AHN) of rats, an autoimmune glomerular disease, is an immunohistologic, ultrastructural, and clinical model of human idiopathic MGN (3,4), and is the subject of intense investigation. The target autoantigen of AHN is a transmembrane renal glycoprotein with a molecular weight of approximately 600 kD, variously named gp600, gp330, LRP-2, or “megalin” (5–10). Recently, the primary structure of this protein has been determined. The protein contains a C-terminal cytoplasmic domain (213 amino acid residues), a single transmembrane domain (22 amino acid residues), and an extremely large ectodomain (4400 amino acid residues) (9). The protein belongs to the LDL-receptor family (9,11) but differs in the number and arrangements of the repeated sequences and in its distribution on various cell types. It is most abundant in proximal renal tubular cells, but is also present in small amounts in the glomerular epithelial cells, where in AHN it becomes the target for the binding of autoantibodies during the development of the disease.

AHN in rats can be produced by an intradermal injection of a crude fraction of rat kidney cortex termed Fx1A (3,12), or alternatively by an injection of the purified gp600 (13) mixed in complete Freund’s adjuvant (CFA). AHN, like human idiopathic MGN, in its full-blown form is characterized by (1) the formation of large, subepithelial glomerular immune deposits, which stain for IgG, C3, and membrane attack (C5b-9) components of complement and (2) the excretion of large amounts of protein in urine (proteinuria). It is unknown whether immunization with the whole protein is essential or whether only a part of the protein is sufficient to produce the full-blown AHN.

In this study we have purified an approximately 60-kD proteolytic fragment of rat gp600. This fragment is stable to further proteolysis, is located at the very N-terminal end of gp600, and includes the first ligand-binding domain and part of or the entire first YWTD spacer region. We found that this small fragment, retaining the natural disulfide bonds and probably its overall structure, was able to induce full-blown AHN.

Materials and Methods

Proteins and Protein Fragments

A gp600-rich preparation was isolated from frozen rat kidneys (Pel Freeze, Rogers, AR) by Lens culinaris agglutinin (LCA) affinity chromatography as described earlier (6,14), and called LCA gp600 preparation. It was digested under nonreducing conditions in 5% sodium dodecyl sulfate (SDS), 100 mM Tris-HCl, pH 8.3, 10% glycerol, and 5% (wt/wt) Staphylococcus aureus V8 protease (Boehringer Mannheim, Mannheim, Germany) at 37°C for 24 h (15). Proteolytic fragments were separated under nonreducing conditions by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE). A strongly
reactive 60-kD fragment, identified by immunoblotting with AHN autoantibodies eluted from the kidneys of rats with AHN, was electroeluted in the Elutrap apparatus (Schleicher & Schuell, Keene, NH). The fragment was dialyzed extensively against deionized water and further purified by reversed-phase HPLC on a Hitachi C4 column (4.6 mm × 250 mm; Bio-Rad Laboratories, Hercules, CA), using acetic acid gradient (0.1% trifluoroacetic acid) from 0 to 60% in 60 min at a flow rate of 1 ml/min. Its amino acid analysis was performed at the University of California, Davis Protein Structure Laboratory. A reduced and carboxamidomethylated (CAM) fragment was prepared by sequentially incubating the HPLC-purified nonreduced 60-kD fragment with 140 mM β-mercaptoethanol and 250 mM iodoacetamide followed by HPLC purification as described above. The N terminus sequence analysis of this 60-kD fragment was performed for both the nonreduced and reduced CAM forms using the Edman degradation cycle.

The fusion protein 7f-glutathione S-transferase (GST) was overexpressed in Escherichia coli. The expressed protein was insoluble and present in the inclusion bodies. These inclusion bodies were purified by sequential urea extractions followed by reversed-phase HPLC. This fusion protein contains a part (amino acid residues 180 to 453) of the rat gp600 in frame with the GST protein (16). The clone, 7f, containing the DNA coding for the amino acid residues 180 to 453 of gp600 was obtained as described earlier (17).

**Antibodies**

AHN autoantibodies were eluted from the glomeruli of AHN rats as described earlier (18) and used as primary antibodies in immunoblotting. Peroxidase-conjugated rabbit anti-rat IgG antibodies (Sigma Chemical Co., St. Louis, MO) were used as secondary antibodies for immunoblotting and enzyme-linked immunosorbent assay (ELISA). FITC-labeled goat anti-rat IgG and FITC-labeled goat anti-rat C3 complement component antibodies (Cappel Laboratories, Cochranville, PA) were used for direct immunofluorescence microscopy (IFM). Rabbit anti-rat C3b-9 membrane attack complement component antibodies were prepared as described earlier (18) and used for indirect IFM. Rabbit anti-rat gp600 antibody was prepared and characterized as described earlier (19). Rat autoantibody to the fusion protein 7f-GST was prepared by immunizing rats with the 7f-GST protein emulsified in CFA. Anti-7f sera reacted with the rat LCA gp600 preparation in both ELISA and immunoblot studies (16).

**Induction and Assessment of Active HN**

Female Lewis rats (Charles River, Wilmington, MA) weighing 150 to 200 g were used. Three groups of rats with three rats in each group were immunized. The first group was immunized with the LCA gp600 preparation (100 µg) emulsified in CFA, the second group with the 60-kD fragment (10 µg) emulsified in CFA, and the third group with phosphate-buffered saline (PBS) emulsified in CFA. Each rat was injected intradermally on the back and in one footpad. Booster immunizations with one-half of the dose of protein used in the first immunization were given to all rats at 4 and 8 wk after the first immunization. Twenty-four-hour urine samples were collected at 0, 7, 9, and 11 wk in metabolic cages with the animals having free access to water. No food was given during the urine collections. The protein content of the urine samples was determined by the sulfosalicylic acid method as described earlier (6). Fasting serum samples were collected at 0, 4, 8, and 12 wk and tested for autoantibodies against LCA gp600 by ELISA as described earlier (18). The rats were sacrificed at 12 wk and their kidneys were studied by IFM using unfixed, frozen sections (6 µm) as described earlier (19).

### Immunoblotting

Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) by the semidy blotting technique. The membrane was blocked with 5% nonfat dry milk in PBS, 0.1% Tween 20, and incubated with the primary antibodies for 2 h at room temperature. After washing, the membrane was incubated with secondary antibodies for 1 h, then extensively washed and developed with 4-chloro-1-naphthol.

### Statistical Analyses

Statistical analyses were performed using GraphPad Prism statistical software (GraphPad Software, Inc., San Diego, CA). Differences in the amounts of 24-h proteinuria between the two groups were analyzed for significance by the two-tailed t test.

### Results

**Identification and Purification of the 60-kD Fragment**

The 24-h V8 digest of the LCA gp600 preparation separated under nonreducing conditions by SDS-PAGE revealed several polypeptides (Figure 1, lane 1, Coomassie blot). Immunoblot analysis with eluted AHN autoantibodies identified a 60-kD polypeptide that was found to be both the smallest and the most reactive of all the polypeptides (Figure 1, lane 1, immunoblot, and Figure 2, lane 2). This 60-kD fragment was isolated by electroelution (Figure 1, lane 1, Coomassie blot) and shown to react with the same AHN autoantibodies described above by immunoblotting (Figure 1, lane 2, immunoblot). When the

![Figure 1](image-url)
electroeluted 60-kD fragment was run under reducing conditions, no reaction with AHN autoantibodies was seen by immunoblotting. The nonreduced electroeluted 60-kD fragment was further purified by reversed-phase HPLC and eluted as a single sharp peak (Figure 3, nonreduced 60-kD fragment peak and Figure 4, lane 2). The material in this single peak was reduced, carboxamidomethylated, and again separated by reversed-phase HPLC under the same conditions. The reduced and carboxamidomethylated material also eluted as a sharp peak (Figure 3, major peak of the reduced CAM 60-kD fragment indicated by an arrow, and Figure 4, lane 1) with two other small peaks. When the purified nonreduced 60-kD fragment was rerun in SDS-PAGE under reducing conditions, or when this fragment was first reduced and carboxamidomethylated and rerun under nonreducing conditions, a significant decrease in its mobility in the gel was noted (Figure 4, lane 1 versus lane 2). This shift from the 60-kD to the 70-kD position is most likely due to the reduction of the disulfide bonds present in this fragment. In an effort to obtain a smaller fragment, we digested the nonreduced 60-kD fragment with V8 protease under the same conditions for 96 h. This digestion produced neither a smaller fragment nor a change in the mobility of the 60-kD fragment in SDS-PAGE under the nonreducing conditions. Digestion with trypsin (5% wt/wt) in the presence of 4 M urea (20) also gave the same results (data not shown).

Figure 2. Mapping of the 60-kD fragment on the gp600 sequence by immunoblot analysis. V8 protease digest of LCA gp600 was separated by SDS-PAGE in nonreducing conditions, transferred to membrane, and probed with rat autoantibody to 7f-glutathione S-transferase (GST-7f) fusion protein (lane 1) and with autoantibodies eluted from AHN glomeruli (lane 2). The same 60-kD band (arrow) reacts with both antibodies.

Figure 3. Reversed-phase HPLC of 60-kD gp600 fragment in nonreduced and reduced carboxamidomethylated (CAM) states. The material in the single HPLC peak of nonreduced 60-kD fragment was reduced, carboxamidomethylated, and separated by reversed-phase HPLC in the same conditions. To prevent confusion, the main peak of this chromatogram is designated as “Reduced CAM 60-kD fragment,” although it moves as a 70-kD band on SDS-PAGE (Figure 4).

Figure 4. SDS-PAGE analysis of HPLC-purified 60-kD reduced and carboxamidomethylated (lane 1) and nonreduced (lane 2) fragments. The material in lane 1 was taken from the major peak (reduced CAM) shown in Figure 3. Lane M shows prestained molecular weight markers (Bio-Rad).

Location of the 60-kD Fragment in the Structure of gp600

Immunoblot analysis of the purified nonreduced 60-kD fragment with the eluted AHN autoantibodies and the rat antiserum
to the 7f-GST gp600 fusion protein showed that both antibodies react with the 60-kD protein (Figure 2). Because the 7f-GST gp600 rat antiserum is directed against the amino acid residues Thr180 through Asn453 of gp600, this indicated that the 60-kD fragment was located near the N terminus of the gp600 structure.

One hundred picomoles of the nonreduced 60-kD fragment that eluted as a single sharp peak on reversed-phase HPLC (Figure 3 and Figure 4, lane 2) was sequenced by the Edman degradation method and revealed the following sequence, G375 YILERGGH of gp600. The amount of amino acid residues identified during the Edman degradation sequencing cycles was approximately 20 pmol. On our attempt to sequence 100 pmol of the material present in the sharp reduced CAM peak (Figure 3), which moved as a 70-kD fragment in electrophoresis (Figure 4, lane 1), we found that it was blocked at its N terminus. Therefore, amino acid analysis of this fragment was performed and those results are shown in Table 1. The most remarkable feature of this analysis was the large number of cysteine residues present in this fragment.

### Induction of AHN with the 60-kD Fragment

Because the purified 60-kD fragment in its reduced form did not react with the AHN autoantibodies, the nonreduced 60-kD fragment was used to study the induction of AHN. LCA gp600 preparation containing the full-length gp600 was used as a positive control. Figure 5 shows the results of the autoantibody response to LCA gp600 in sera measured by ELISA. It is clear that both the LCA gp600 preparation and the 60-kD fragment produce comparable levels of autoantibody response against rat gp600, even though it should be noted that the amount of 60-kD fragment injected in each animal was only one-eighth the amount of LCA gp600 preparation. The control group immunized with PBS and CFA did not produce autoantibodies to gp600.

Assessment of the severity of the disease measured by the levels of 24-h proteinuria also revealed that the disease in the rats immunized with the 60-kD fragment was as severe as the disease in the rats immunized with the LCA gp600 preparation (Figure 6). No significant differences in the amounts of 24-h proteinuria were seen between the groups of rats immunized with the 60-kD fragment and the LCA gp600 at week 7 ($P = 0.38$), week 9 ($P = 0.52$), or week 11 ($P = 0.47$). The rats in the control group failed to develop abnormal amounts of 24-h proteinuria. Also, the fasting sera collected at 12 wk from the rats immunized with the 60-kD fragment and the rats immunized with the LCA gp600 preparation were lipemic, confirming the severity of the disease in both groups of diseased rats. The sera from the control group immunized with PBS were not lipemic.

Immunofluorescence microscopy performed on kidneys of rats immunized with the 60-kD fragment and the rats immunized with the LCA gp600 preparation showed similar staining in the glomerular immune deposits for rat IgG (Figure 7) and rat C5b-9 (Figure 8). The staining for rat C3 was similar to that of C5b-9 in all of the rats immunized with the 60-kD fragment or the LCA gp600 preparation (data not shown). Both groups

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<th>Amino Acid</th>
<th>Theoretical % for Two Different Fragments$^a$</th>
<th>Experimental (%)</th>
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<td></td>
<td>$Q_{375} \text{E}_{1013}$</td>
<td>$Q_{26} \text{E}_{691}$</td>
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$^a$ Both fragments are of approximately the same size as the unfolded 60-kD fragment (about 70 kD). Fragment $Q_{375} \text{E}_{1013}$ starts from N-terminal residue obtained by sequencing analysis of nonreduced 60-kD fragment. Fragment $Q_{26} \text{E}_{691}$ starts from N terminus of mature gp600. Amino acid composition was predicted using DNASIS computer program (Hitachi Software) and amino acid sequence of gp600 (9).
developed a severe disease. No deposits were found in the rats immunized with PBS and CFA. Also, we studied the kidney sections for the presence of rat gp600 in the glomerular immune deposits by indirect IFM using rabbit anti-rat gp600 antibody (19). The staining for rat gp600 in the glomerular immune deposits in the rats immunized with LCA gp600 preparation or with the 60-kD fragment was similar (data not shown) and duplicated the results described previously for the rats immunized with gp600 (19). All of these data show that the 60-kD fragment is as potent in inducing AHN as the LCA gp600 preparation.

Discussion

This study clearly shows that a small N-terminal 60-kD proteolytic fragment of gp600 that is approximately one-eighth of the length of the whole protein can produce as severe an AHN as that produced by a preparation containing the whole protein. The data also show that the fragment is located at the very N terminus of gp600. We base this conclusion on the results of Western blot analysis, amino acid sequencing, and amino acid composition of the isolated fragment. Western blot results showed that it contained part or all of amino acid residues 180 to 453 of gp600, and the amino acid sequencing and amino acid composition results indicated that it was located at the very N terminus of gp600.

The fragment is highly cross-linked by disulfide bonds. This is suggested by: (1) a considerable decrease in the mobility of reduced form versus the nonreduced form of the fragment in SDS-PAGE; (2) resistance of the nonreduced form to additional proteolytic degradation; and (3) the known primary structure of the N-terminal end of gp600 (9). In the N-terminal region, 60 cysteine residues are present in the first 700 amino acid residues. Interestingly, 54 of those 60 cysteine residues are present in the first 370 amino acid residues. All of these cysteine residues belong to complement-like and epidermal growth factor (EGF)-like repeats (9), where they are cross-linked to each other in a certain order (21,22).

Edman degradation sequencing of the nonreduced 60-kD fragment produced the gp600 sequence starting at Gly375 (9). However, sequencing of the reduced CAM preparation of the same fragment revealed that it was blocked at the N terminus. This apparent contradiction is resolved if the nonreduced preparation of the 60-kD fragment was the N-terminal part of the gp600, which was blocked at the end by chemical modification, and the preparation also contained approximately 20% of the same molecules that were internally cut by protease at Glu374. Disulfide bonds in proteins considerably reduce their susceptibility to proteolytic digestion (23), and when the digestion is performed under nonreducing conditions, the various smaller fragments produced by digestion between the cross-linked cysteine residues are retained together. Because the overall structure of these molecules would be similar to the intact fragment due to disulfide cross-linking, they would be purified together with the intact nonreduced fragment by HPLC. Naturally blocked N-terminal ends, precluding sequencing by Edman degradation, are common and occur in 50 to 80% of the eukaryotic proteins. The internal cut of the 60-kD fragment created one unblocked N terminus, which was sequenced on the nonreduced preparation. Therefore, we concluded that the original nonreduced 60-kD fragment, eluting on HPLC as a single peak, consisted of two components (Figure 9). The major component was the intact fragment with blocked the N terminus and the minor component was the same fragment digested between the amino acid residues Glu374 and Gly375. The two parts of this internally digested fragment were apparently held together in the nonreduced preparation through disulfide bonding (Figure 9). Indeed, the two cysteine residues, Cys384 and Cys372, surrounding the internal cut, belong to the second EGF-like repeat in the first cluster (or ligand-binding domain) of the gp600 cysteine repeats. These cysteine residues are likely to be involved in disulfide bonding typical for the EGF-like cysteine repeats (22), holding the two smaller fragments together. The two small peaks on the chromatogram of the reduced CAM fragment apparently are the reduced products of an internal cut of the nonreduced fragment, referred to above as the minor component.

The amino acid analysis of this fragment confirmed that the original nonreduced 60-kD fragment was located at the very N terminus of gp600. The amino acid composition is in good agreement with the theoretical prediction for the fragment with a molecular weight of approximately 70 kD (the approximate size of the unfolded fragment) starting from the N terminus (residues 26 to 691, molecular weight 73,869 Daltons) of the mature protein, unlike a fragment starting from the internal cut at residue 375 (residues 376 to 1012, molecular weight 72,007 Daltons). Both theoretical fragments end by the Glu residue at their C termini, the cleavage site for the V8 protease. All of these data suggest that the original nonreduced 60-kD fragment is the N-terminal fragment of the gp600, which is blocked at its N-terminal end.

The identified pathogenic 60-kD gp600 fragment includes the first ligand-binding domain and part or all of the first YWTD spacer region (24). We cannot exclude the possibility that other regions of the molecule may also play a role in the
induction or development of AHN. Two other protein fragments with higher molecular mass (90 and 110 kD) also reacted with autoantibodies (Figures 1 and 2), although not as strongly as the 60-kD fragment. Additionally, a fusion protein containing a fragment of gp600 located in the second ligand-binding domain (amino acid residues 1114 to 1250) has been shown to induce a mild AHN characterized by small glomerular immune deposits but no proteinuria (25). We obtained similar results with a recombinant protein, 7f-GST, containing a smaller fragment (273 amino acid residues) of the pathogenic 60-kD fragment (16). One of the possible explanations may be that neither of these short fragments contained all of the B and T cell epitopes involved in the expression of the full-blown disease. It is also possible that these short fragments did not contain the gp600 T cell epitopes, and that all of the T cell help for autoantibody production after immunization was provided.

Figure 7. Direct immunofluorescence microscopy of frozen kidney sections stained for rat IgG. (A) Rat immunized with the LCA gp600 preparation. (B) Rat immunized with the 60-kD gp600 fragment. Magnification, ×400.

Figure 8. Indirect immunofluorescence microscopy of frozen kidney sections stained for rat C5b-9 membrane attack complement component. (A) Rat immunized with the LCA gp600 preparation. (B) Rat immunized with the 60-kD gp600 fragment. Magnification, ×400.
by the GST part of the fusion protein. It has been shown earlier that T cells may play an important role in the development of AHN (26–28). Absence of recruitment of gp600-specific T cells during the immune response may explain the milder nature of AHN induced by these fusion proteins.

Recently, it was suggested that a rodent complement inhibitor, Crry, was present in Fx1A prepared from fresh kidneys but absent in frozen commercially available kidneys, and was critical for the development of full-blown AHN (29). Also, although no immunizations with megalin were performed or any literature cited, it was stated that “active immunization with megalin is insufficient to recapitulate the full disease of HN.” In our hands, Fx1A prepared from commercially available frozen kidneys (19), highly purified gp600 alone (13), and a small fragment of gp600 (present study) can all induce full-blown AHN. Our results also argue against an obligatory role of receptor-associated protein in the induction of AHN, as has been claimed by some investigators (30).

To prevent confusion between the pathogenic epitopes of AHN and passive Heymann nephritis (PHN), we would like to comment on the studies performed in PHN. PHN is produced by directly injecting heterologous antibodies against rat gp600 into normal rats and serves as a model for the study of the binding of these antibodies to gp600 and the formation of immune deposits in the glomerulus. PHN, unlike AHN, is not an autoimmune disease. In the two studies performed in PHN (31,32), the heterologous antibodies against all four ligand-binding domains could bind in glomeruli. The heterologous antibodies are more diverse in their epitope specificity than autoantibodies, and it would not be surprising if the heterologous antibodies against any surface-exposed part of gp600 would bind to gp600 in situ after an intravenous injection. Nevertheless, these regions were called pathogenic for PHN even though proteinuria did not develop in any animal and staining for complement was not performed on the immune deposits. Also, no experiments were performed to test whether immunizations with these regions would produce AHN. Hence, these findings in PHN may or may not be relevant to the B cell epitopes involved in AHN. Additionally, in AHN the pathogenic region(s) must contain regions that bind autoantibodies (B cell epitopes), as well as regions that contain self-reactive T cell epitopes. The 60-kD fragment of gp600, identified by us, contains those B and T cell epitopes that are sufficient to produce the full-blown AHN. Thus, this region of gp600 can be claimed to be autoimmunopathogenic in AHN.

Whether our new findings in AHN are relevant to the idiopathic MGN in humans is unknown. Our preliminary data suggest that autoantibodies to gp600 can be detected in the sera of some patients by immunoprecipitation (33), particularly in the early stages of the disease. Also, the sera from AHN rats, in which the disease had been induced by human gp600, react by immunofluorescence with rat and human glomerulus in a similar pattern (15). This pattern is similar to the pattern seen on the rat glomerulus with sera from AHN rats in which the disease had been induced with rat gp600 (15). These findings indicate the presence of gp600 in the human glomerulus. Also, in the kidney biopsies of some cases of idiopathic MGN using anti-human gp600 antibody and indirect immunoperoxidase technique, we have seen granular staining for the antibody in the glomerular capillary loops in the location of the IgG deposits (S. P. Makker, unpublished observations). Additional studies are needed to clearly establish the connection between AHN and the human disease.

Acknowledgments

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References

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Figure 9. Schematic representation of the material present in the HPLC-purified nonreduced 60-kD gp600 fragment preparation. mQ26 is the putative modified N-terminal amino acid residue. The modification precludes the Edman degradation sequencing. Only the disulfide bond holding two smaller fragments together is shown. The percentages indicate the approximate relative amount of each component.

MW ~ 70 kDa

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\begin{align*}
mQ_{26} & \quad S-S \\
E_{374} & \quad G_{375} \quad -20\% \\
S_{26} & \quad -80\%
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member of the low density lipoprotein receptor gene family. *Proc Natl Acad Sci USA* 91: 9725–9729, 1994


