Korean J Biol Sci 5: 199-203, 2001

Peptide Sequence Analysis of the CNBr-Digested 34-36 kd Sperminogen

Hyunkyung Yu and Lee S. H. Yi*

Department of Biological Science and the Institute for Basic Science, Sungkyunkwan University, Suwon 440-746, Korea

Key Words:
Sperminogen
Proacrosin
Acrosin
Spermatozoa

Sperminogen was purified from the acid extracts of boar spermatozoa and partial peptide sequence of the 34-36 kd sperminogen was determined. Acid extracts of boar spermatozoa was gel-filtered through Sephadex G-75, and the 34-36 kd sperminogen was purified by preparative SDS-PAGE. The sperminogen bands were sliced out, and 34-36 kd sperminogen were eluted from the gel fragments and was subjected to peptide sequencing. Since the amino termini were blocked for Edman degradation method, internal amino acid sequences of the eluted 34-36 kd sperminogen were obtained from CNBr-digested peptides of sperminogen. Among several bands resolved on tricine SDS-PAGE, 14, 22 and 26 kd peptides were subjected to peptide sequencing. The analyzed amino acid sequences of the 26 and 22 kd peptides showed high homologies with that of the zona pellucida binding protein, Sp38, and the analyzed amino acid sequence of the 14 kd peptide showed neither sequence homology nor similarity with any known proteins.

Capacitation and acrosomal reaction are the two major preparatory events for successful penetration of sperm into oocyte, which eventually leads to successful fertilization. Physiologically occurring in the female reproductive track, capacitation is a complex process accompanying modification of membrane characteristics, enzyme activity and motility property of spermatozoa (Baldi et al., 1996). Only the capacitated spermatozoa are capable of binding the zona-intact egg and undergoing the acrosomal reaction (Tulsiani, 1998). The acrosomal reaction is a unique exocytotic event in the capacitated spermatozoa, which occurs with the fusion of the outer portion of the acrosomal membrane with the plasma membrane, which then vesiculates and disappears. Acrosomal enzymes such as proacrosin/acrosin system and speminogen are exposed at this time. These acrosomal proteases have long been believed to be involved in various aspects of fertilization, including the recognition and binding between the sperm and oocyte, and the sperm penetration of zona pellucida; therefore, researches on sperm proteases have attracted major attention. Sperminogen, which has trypsin-like specificity as does, the most representative sperm protease, proacrosin/acrosin system, was originally detected in human spermatozoa showing characteristics of low-molecular mass (32-36 kd) non-proacrosin zymogen (Siegel et al., 1987). As a newly discovered enzyme, speminogen has not drawn much attention since most of the research has been focused on the earlier discovered and the most abundant proacrosin/acrosin system. Composed of three proteolytic bands in SDS-PAGE analysis, sperminogen was distinguished from the proacrosin/acrosin system with marked differences in enzyme activation kinetics (Siegel et al., 1987; Yi, 2000). However, Cechova (1990) reported that sperminogen is an activation by-product of proacrosin through N-terminus peptide sequencing. Later, Yu and Yi also reported that 32 kd sperminogen is most likely a part of proacrosin/acrosin system from partial peptide sequence analysis (in press). Until now, however, the identity of the higher molecular mass (34-36 kd) sperminogen, has not been reported. Therefore, we report the partial peptide sequence analysis of the 34-36 kd sperminogen from boar spermatozoa.

* To whom correspondence should be addressed.
Tel: 82-31-290-7005, Fax: 82-31-290-7015
E-mail: shlee@yurim.skku.ac.kr

Materials and Methods

Materials

Boar epididymides were kindly donated by Shinwon Meat Co. (Suwon, Korea). Ham's buffer was purchased from GIBCO Laboratories (USA). Benzamidine and Sephadex G-75 resin were supplied from Sigma Chemical Co. (USA). Electrophoresis reagents and electro-elution reagents were obtained from Bio-Rad (USA). Cyanogen bromide and Problott membrane were purchased from Janssen Chemica (Belgium) and Applied Biosystems (USA), respectively. All other reagents were obtained in molecular biology or extra-pure grade from Sigma Chemical Co. (USA) and Fisher Scientific (USA).

Methods

Preparation and acid extraction of boar spermatozoa

Boar spermatozoa were flushed and acid-extracted according to Yi (1997). Briefly, boar epididymides were dissected from freshly excised tissue and the spermatozoa were flushed with Ham's buffer (pH 7.4) containing 50 mM benzamidine as an activation inhibitor of trypsin-like enzymes. The flushed spermatozoa were washed via centrifugation at 10,000 x g through 11% ficoll containing 50 mM benzamidine for 30 min. The sperm pellet was then resuspended in 50 mM benzamidine and adjusted to pH 4.0 with concentrated HCl. After overnight incubation at 4°C, the suspensions were centrifuged at 26,000 x g for 30 min. The supenatant was saved and gel-filtered through Sephadex G-75 resin on a 1.6 x 100 cm column (Pharmacia, UK) with 1 mM HCl. Fractions of 1.8 ml were collected in each tube, and protein concentration of each fraction was determined by measurement of the absorbance at 280 nm.

Purification of boar 34-36 kd sperminogen

The fractions that were shown to contain sperminogen from gelatin SDS-PAGE were pooled and lyophilized. The concentrated sample by lyophilization was then separated by preparative SDS-PAGE according to Laemmli (1970). The sample was resuspended in SDS buffer (0.125 M Tris-HCl, 2% SDS, 10% glycerol, 0.001% bromophenol blue, pH 3.0), and preparative SDS-PAGE was performed at 4℃ on a 3 mm thick slab gel with a 12.5% separating gel and a 5% stacking gel at 60 mA constant current. Following electrophoresis, one strip of the gel was excised vertically from one side of the gel, and stained with Coomassie brilliant blue R and destained with 50% methanol. After rehydration with distilled water, the gel strip was used as a marker to locate the 34-36 kd sperminogen bands in the unstained part of the gel. The bands were excised from the unstained part of the gel with a scalpel, and the protein was eluted from the sliced gel fragment using the Model 422 Electro-eluter (Bio-Rad, USA). Electro-elution was performed at 8 mA constant current for 12 h. The eluted sperminogen was then concentrated by ultrafiltration (Centricon, USA).

CNBr-digestion of 34-36 kd sperminogen

CNBr-digestion was performed according to Huh and Yi (1999) with minor modifications. Briefly, the concentrated sperminogen was dissolved in 70% formic acid. Ten µl (70 mg/ml) of cyanogen bromide was added per 10 µg protein. After incubation in dark at room temperature for 24 h, the reaction mixture was dried using Speed-Vac (Heto, Denmark). The digested peptide pellet was dissolved in distilled water according to Yu and Yi (in press).

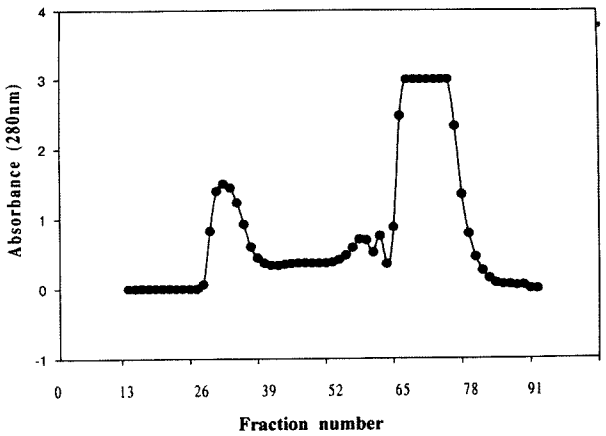

Fig. 1. Gel filtration of acid extracts from boar spermatozoa through a Sephadex G-75 column at pH 3.0. Sixty drops of eluent were collected for each fraction and the absorbance of odd-numbered fractions were measured at 280 nm for protein concentration.

Amino acid sequencing of the CNBr-digested peptides

The CNBr-digested peptides were separated according to their molecular mass using tricine SDS-PAGE, since tricine SDS-PAGE is more efficient in separating smaller peptides (Shagger and VonJagow, 1987). Conditions for electrophoresis were the same as SDS-PAGE. The separated peptides were electroblotted onto a Problott membrane using CAPS buffer (10 mM CAPS in 10% methanol) at constant voltage of 24 V for 2 h. The membrane was stained with Amido Black and destained with 50% methanol. The membrane stained for the peptide band was excised with a razor blade. The excised membrane was analyzed by automated Edman degradation method for its amino acid sequence using the AAA Amino Acid Analyzer (Water/HPLC, USA).

Gelatin SDS-PAGE

Gelatin SDS-PAGE was performed according to Siegel et al. (1987). Briefly, conditions for gelatin SDS-PAGE were the same as SDS-PAGE except that the gel contained 0.1% gelatin. After gel electrophoresis, the gelatin polyacrylamide gel was soaked in 2.5% Triton X-100 for 30 min, washed extensively with distilled water to remove SDS from the protein and then incubated in 0.1 M Tris buffer (pH 8.0) for enzyme activation at 37℃ overnight. The gel was then stained with 0.1% Amido Black and destained with 50% methanol.

Results

Analysis of the acid extracts of boar spermatozoa

An aliquot of sperm extract at pH 4.0 was fractionated on a Sephadex G-75 column. Fractions of 60 drops (1.8 ml) were collected in each tube and a total of 150 tubes were obtained. The protein concentrations in odd-numbered fractions were determined by measuring the absorbance at 280 nm. Fraction numbers from 29

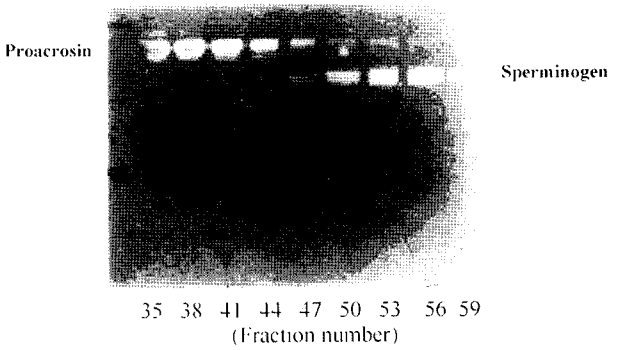

Fig. 2. Gelatin SDS-PAGE analysis of the Sephadex G-75 fractionated acid extracts of boar spermatozoa. Ten μl aliquots of the fractions in each tube were loaded and were electrophoresed in a 12.5% separating gel containing 0.1% gelatin at 20 mA constant current. Following electrophoresis, the gel was incubated in Triton X-100 for 1 h and then further incubated in 0.1 M Tris buffer (pH 8.0) at 37℃. Both proacrosin and sperminogen protein bands appear as clear zones on the gel.

to 113 were shown to contain proteins (Fig. 1). When these proteins were analyzed for protease activity using gelatin SDS-PAGE, both proacrosin and sperminogen appeared to have a proteolytic activity (Fig. 2). The fractions that contained proacrosin and sperminogen were from 35 to 47 and from 50 to 59, respectively. Sperminogen was shown to be composed of proteins with three different molecular masses ranging from 32 to 36 kd.

Purification and CNBr-digestion of the 34-36 kd sperminogen

For peptide sequence analysis of the 34-36 kd sperminogen, fractions which were shown to contain sperminogen from gelatin SDS-PAGE were pooled and concentrated by lyophilization. Following preparative SDS-PAGE, 34-36 kd sperminogen bands were excised (Fig. 3), and the proteins were eluted from the gel fragments. The eluent was concentrated to 500 pmol by ultrafiltration and the enzymatic activity was reconfirmed by SDS-PAGE (data not shown). After CNBr-digestion, the digested peptides were separated by

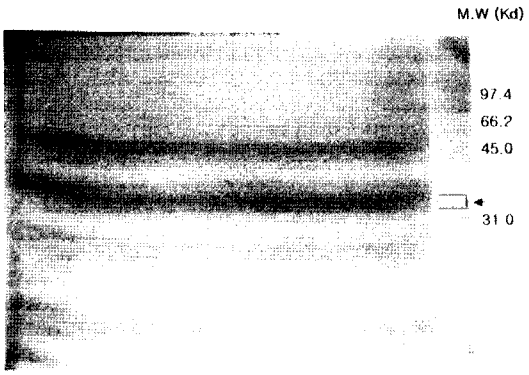

Fig. 3. Preparative SDS-PAGE of the lyophilized samples of fractions from 50 to 59. Arrow denotes excised bands for elution.

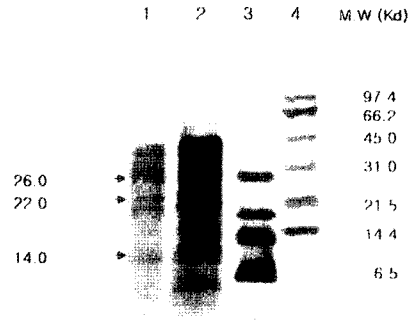

Fig. 4. Electroblotting of the CNBr-digested peptides onto Problott membrane. Lanes 1 and 2: CNBr-digested peptides of 34-36 kd sperminogen. Lanes 3 and 4: molecular weight markers. Arrows denote the peptides which were subjected to peptide sequencing.

tricine SDS-PAGE and electroblotted onto a Problott membrane (Fig. 4). As shown in lanes 1 and 2 of Fig. 4, many peptides bands were produced after CNBr-digestion. Among these, only the well-separated and prominent bands, such as 26, 22 and 14 kd bands, were subjected to peptide sequence analysis.

Amino acid sequence analysis

Twenty six, 22 and 14 kd peptides from the CNBr-digested 34-36 kd sperminogen were analyzed for their amino acid up to 28, 22 and 22 amino acids, respectively. The amino acids that were not clearly identified were marked X and the amino acids that were resolved but shown to have more than one possibility were marked as both amino acids with one amino acid has higher chance than the other one. Amino acid sequence of 26 kd peptide showed a 55% homology with that of porcine zona binding protein Sp38 when the homology search was made against NIH data base, and the homology was even higher (67%) when the first 18 amino acids were compared (Fig. 5). When the amino acid sequence of 22 kd peptide was analyzed, it showed a 83% homology with the same protein Sp38 (Fig. 6). However, the amino acid sequence of the 14 kd peptide did not show any homology with any other proteins whose sequences are deposited in the NIH database (Fig. 7).

Discussion

Acrosomal proteases have attracted major attention in the areas of reproductive biology and developmental biology since they might be responsible for the penetration and/or fertilization of mammalian spermatozoa into an oocyte by dissolving one of the egg's vestments, zona pellucida. Proacrosin/acrosin system is the most studied protease among the acrosomal proteases

(A)

F-Q-L-T-Q-N-S-A-V, K-I-V-T> G-Y-S-P-
Phe Gln Leu Thr Gln Asn Ser Ala Val, Lys Ile Val Thr> Gly Tyr Ser Pro

N-F-X-V-V, K-L-K-A(?)-I-L-D(?)-N, Q-K(?)-I
Asn Phe Val Val, Lys Leu Lys Ala(?) Ile leu Asp(?) Asn, Gln Lys(?) Ile

Blast search :

34 ~ 36 Kd sperminogen vs zona-pellucida-binding protein (Sp38)

Sperminogen : **FQLTQNSAVIVTSPNFXVVLKA I LDNK**
Sp38 : **FHLTQESAKIVGSPNFPVKVYVMLHQK 79**

(B)

M-E-A-S-A-P-D-R-A-R-R-G-W-R-R-A-R-A-A-A-S-P-L
-S-R-A-A-V-V-L-L-L-S-A-L-V-L-R-A-P-P-S-V-G-Y-L-
D-R-L-P-R-S-**F-H-L-T-Q-Q-S-A-K-I-V-G-S-P-N-F-P**
-V-K-V-Y-V-M-L-H-Q-K-S-P-H-V-L-C0.-V-T-Q-R-L-
R-N-F-Q-L-V-D-P-S-F-Q-W-H-G-P-K-G-K-I-V-S-E-N-
S-T-A-Q-V-T-S-T-G-S-L-V-F-Q-N-F-Q-Q-S-M-S-G-V-
Y-T-C-F-L-E-Y-K-P-T-V-E-E-V-V-K-N-L-Q-L-K-
Y-I-I-Y-A-Y-R-E-P-R-Y-Y-Y-E-F-T-A-R-Y-H-A-A-P-C
-N-S-I-Y-N-I-S-F-E-K-K-L-L-E-I-L-S-K-L-V-L-N-L-S-
C-E-V-S-L-L-K-S-E-K-H-R-V-K-M-Q-R-A-G-L-Q-N-E-
L-P-P-T-P-S-V-S-S-L-D-T-E-K-G-P-K-P-C-A-G-H-S-C
-E-S-S-K-R-L-S-K-A-K-N-L-I-E-R-P-P-N-Q-Q-V-E-V-
L-G-R-R-A-E-P-L-P-E-I-Y-Y-I-E-E-T-L-Q-M-V-W-I-N-
R-C-F-P-G-Y-G-M-N-I-L-K-H-P-K-C-P-E-C-C-V-I-C-S-
P-G-T-Y-N-S-R-D-G-I-H-C-L-Q-C-N-S-S-L-V-F-G-A-K
-A-C-L

Fig. 5. Amino acid sequence of the CNBr-digested 26 kd peptide of the 34-36 kd sperminogen and the comparative locations of the sequenced peptides in the porcine zona binding protein Sp38 amino acid sequence. (A) Analyzed sequence was displayed from the N-terminus. When the amino acid was analyzed for more than one possibility, both of the amino acids were written in that slot. And when the amino acid in that slot is not analyzable, the slot was marked as X. The amino acid which is questionable is marked with ? in the bracket. Blast search against NIH database was made and the highest homologous protein is presented together in comparison to the sequenced peptide. (B) Specific locations of analyzed 26 kd and 22 kd peptide sequences in the Sp38 amino acid sequence reported by Baba et al. (1995). Analyzed 26 kd and 22 kd peptide sequences are denoted in their respective locations of the Sp38 as bold characters and italicized characters, respectively.

since proacrosin is the most abundant protease in the acrosome of the mammalian spermatozoa. It is naturally believed that this proacrosin/acrosin system might be the one which is responsible for the sperm

F-T-X-F-L- E-Y-G-F-T- V-E-E- L- V-R-V- L-Q
Phe Thr Phe Leu Glu Tyr Gly Phe Thr Val Glu Glu Leu Val Arg Val Leu Gln

Blast search :

34 ~ 36 Kd sperminogen vs zona-pellucida-binding protein (Sp38)

Sperminogen : FTX**FLEYGPTVEELVKVLQ**
Sp38 : **YTCFLEYKPTVEELVKRLQ 156**

Fig. 6. Amino acid sequence of the CNBr-digested 22 kd peptide of the 34-36 kd sperminogen.

A-V- R- F - T- T(?) > K - K- S- Y / I- V- G -K-
Ala Val Arg Phe Thr Thr(?)> Lys Lys Ser Tyr/ Ile Val Gly Lys

Y - N- D - L- L(?) - F- R - V- L- K
Tyr Asn Asp Leu Leu(?) Phe Arg Val Leu Lys

Fig. 7. Amino acid sequence of the CNBr-digested 14 kd peptide of the 34-36 kd sperminogen.

penetration of the zona pellucida. However, Baba et al. (1994) reported that the sperm produced from the mouse whose proacrosin gene has been knocked out could fertilize an egg, which strongly suggested that the proacrosin/acrosin system might not be the sole protease which is responsible for the sperm penetration. In this regard, mammalian spermatozoa are known to contain more than one trypsin-like enzyme (Meizel, 1972; McRorie et al., 1976; Siegel et al., 1987; Yi, 1997). Actually, it has been already reported that the sperm contained several proteolytic enzymes different from acrosin (Meizel and Cotham, 1972). However, none of these enzymes has yet been characterized well.

Among these non-acrosin proteases in the spermatozoa, sperminogen has been the most controversial. Sperminogen was originally reported from the acid extracts of human sperm by Siegel et al. (1987) with marked differences in enzyme activation kinetics from that of the proacrosin/acrosin system. As an acrosomal protease, sperminogen is composed of three proteolytic bands ranging from 32 kd to 36 kd which have trypsin-like specificity upon activation. When the peptide sequence of the 32 kd sperminogen was analyzed, however, Cechova et al. (1990) and Yu and Yi (in press) concluded that the 32 kd sperminogen might be a part of the proacrosin/acrosin family, which is modified posttranslationally. The identity of the two higher molecular mass sperminogen (34-36 kd) has yet to be reported. The two higher molecular mass sperminogen were, therefore, purified and analyzed for their peptide sequences in the present study. For peptide sequence analysis, the 34-36 kd sperminogen was purified altogether since the two protein bands of sperminogen were so closely spaced in the preparative SDS-PAGE that it was almost impossible to separate them without cross-contamination during purification process. Therefore, these two sperminogen bands were used together for the current experiment. When the CNBr-digested pattern of the 34-36 kd sperminogen was compared with that of the 32 kd sperminogen, two patterns were shown quite different, signifying that the 34-36 kd sperminogen might be structurally unrelated to the 32 kd sperminogen. When three most prominent bands from the CNBr-digested 34-36 kd sperminogen peptides were sequenced, two peptides (26 and 22 kd) showed high homologies with that of the zona binding protein, Sp38, which was reported by Baba et al. (1994). Sp38 has been reported to bind to one of the egg's vestment, zona pellucida, without any mention of its

proteolytic activity. If one of the 34-36 kd sperminogen is proven to be identical with Sp38, this is the first report that there is another protease that has a zona binding activity in the acrosome of mammalian spermatozoa other than proacosin. Furthermore, it is quite interesting to note that both sperminogen and proacrosin, which are known for their proteolytic activities, also have a zona binding capacity. The other sequenced peptide (14 kd) did not show any homology upon search against NIH database. This suggested that one of the 34-36 kd spermingoen might be a novel protease which has not been reported until now; however, since partial peptide sequence is the only information to claim for the novel protease, further characterization, such as cDNA sequence analysis encoding these proteins, is necessary to conclude that there is another novel protease in the acid extract of spermatozoa, and this work is under way in our laboratory.

In conclusion, the 34-36 kd sperminogen are different from the 32 kd sperminogen which is presumed to be a part of the proacrosin/acrosin family. Based on peptide sequence analysis, it is highly likely that one of the two sperminogen bands of 34-36 kd might be identical with the zona binding protein Sp38. The other sperminogen band has a chance to be a novel protease from the peptide sequence analysis.

Acknowledgement

This work is partly supported by Sungkyunkwan Research Grant.

References

Baba T, Azuma S, Kashiwabara S, and Toyoda Y (1994) Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization. *J Biol Chem* 269: 31845-31849.

Baldi E, Luconi M, Bonaccorsi L, Krausz C, and Forti G (1996) Human sperm activation during capacitation and acrosome reaction: role of calcium, protein phosphorylation and lipid remodeling pathways. *Front Biosci* 1: 189-205.

Cechova D, Topfer-Peterson E, Zucner A, and Jonakova V (1990) Isolation, purification, and partial characterization of low molecular mass boar proacrosin. *Biol Chem Hoppe-Seyler* 371: 317-323.

Huh K and Yi L (1999) Purification and partial peptide sequence analysis of the boar proacrosin binding protein. *Mol Reprod Dev* 54: 76-80.

Laemmli U (1970) Cleavage of structural protein during the assembly of the head bacteriophage T4. *Nature* 227: 680-685.

McRorie R, Turner R, Bradford M, and Williams W (1976) Acrolysin, the aminoproteinase catalyzing the initial conversion of proacrosin to acrosin in mammalian fertilization. *Biochem Biophys Res Commun* 71: 492-498.

Meizel S (1972) Biochemical detection and activation of an inactive form of a trypsin-like enzyme in rabbit testes. *J Reprod Fertil* 31: 459-462.

Meizel S and Cotham J (1972) Partial characterization of a new bull sperm arylamidase. *J Reprod Fertil* 28: 303-307.

Schagger H and VonJagow G (1987) Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of protein in the rage 1 to 100 kda. *Ann Biochem* 166: 368-379.

Siegel M, Bechtold D, Willand J, and Polakoski K (1987) Partial purification and characterization of human sperminogen. *Biol Reprod* 36: 1063-1068.

Tulsiani D, Abou-Haila A, Loeser C, and Pereira B (1998) The biological and functional significance of the sperm acrosome and acrosomal enzymes in mammalian fertilization. *Exp Cell Res* 240: 151-164.

Yi L (2000) Amino acid composition analysis of the 32 kda sperminogen. *J Biochem Mol Biol* 33: 510-513.

Yi L (1997) Purification and partial immuno-characterization of boar sperm proteinase sperminogen. *J Biochem Mol Biol* 30: 448-452.

Yu H and Yi L Purification and partial peptide sequence analysis of the boar 32 kd sperminogen. *Mol Cells:* in press.

[Received June 28, 2001; accepted July 21, 2001]