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Biochemistry

The *Saccharomyces cerevisiae BAR1* gene encodes an exported protein with homology to pepsin

(yeast/mating factors/protease/secretion)

VIVIAN L. MACKAY*†, S. K. WELCH*, MARGARET Y. INSLEY*, THOMAS R. MANNEY‡, JULIE HOLLY*, GENA C. SAARI*§, AND MICHAEL L. PARKER*¶

*ZymoGenetics, Inc., 2121 N. 35th Street, Seattle, WA 98103; and ‡Department of Physics, Kansas State University, Manhattan, KS 66506

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ABSTRACT *Saccharomyces cerevisiae* a cells secrete an extracellular protein, called "barrier" activity, that acts as an antagonist of α factor, the peptide mating pheromone produced by mating-type α cells. We report here the DNA sequence of *BAR1*, the structural gene for barrier activity. The deduced primary translation product of 587 amino acids has a putative signal peptide, nine potential asparagine-linked glycosylation sites, and marked sequence similarity of the first two-thirds of the protein with pepsin-like proteases. Barrier activity was abolished by *in vitro* mutation of an aspartic acid predicted from this sequence homology to be in the active site. Therefore, barrier protein is probably a protease that cleaves α factor. The sequence similarity suggests that the first two-thirds of the barrier protein is organized into two distinct structural domains like those of the pepsin-like proteases. However, the *BAR1* gene product has a third carboxyl-terminal domain of unknown function; deletion of at least 166 of the 191 amino acids of this region has no significant effect on barrier activity.

Saccharomyces cerevisiae a and α cells each secrete peptide mating pheromones (a factor and α factor) that act on cells of the opposite mating type to promote several physiological changes that precede cell fusion—e.g., the induction of cell-surface agglutination factors and the arrest of cells in the G_1 phase of the cell cycle (1). Mating-type a cells produce an additional extracellular activity (called "barrier" activity) that is an antagonist of α factor (2, 3). The recovery of a cells from α-factor arrest is facilitated in part by barrier activity, since a strains with mutations in the *BAR1* gene lack the activity, are super-sensitive to α factor, and are slower to recover from G_1 arrest (4, 5). As a *bar1* mutants also mate less efficiently with α cells in mass mating mixtures, barrier activity apparently functions to establish optimal pheromone concentrations for conjugation.

It is unclear whether the extracellular barrier activity also exists in a cell-associated form (3) or if it is identical to a membrane-bound endopeptidase that cleaves α factor between leucine and lysine, the sixth and seventh amino acids (6). However, unlike barrier activity (2, 3), this endopeptidase was not detected in the culture medium. Some endopeptidase activity could be found in preparations from α and a/α cells, although barrier activity is not detected in these cell types (2, 3). Moreover, *BAR1*, shown in the present paper to be the structural gene for the barrier protein, is transcribed only in a cells (ref. 7; V.L.M., unpublished).

S. cerevisiae naturally exports very few proteins to the culture medium and only four of these have been identified: a factor (8, 9), α factor (10, 11), killer toxin (12), and barrier activity (2, 3). The first three are relatively small peptides, but barrier protein has not yet been purified and characterized. Therefore, we determined and analyzed the sequence of the *BAR1* gene‖ to learn more about the mature protein as well as features of its processing and export.

MATERIALS AND METHODS

Plasmids. YEp13 bears the *S. cerevisiae LEU2* gene for selection of transformants in either *S. cerevisiae* or *Schizosaccharomyces pombe* (13, 14). The promoter from the *S. cerevisiae ADH1* gene (15) encoding alcohol dehydrogenase I was obtained from plasmid AM5 (kindly donated by G. Ammerer, ZymoGenetics) and the transcription terminator from the *TPI1* gene (triose phosphate isomerase) (16) was isolated from plasmid M210 (also provided by G. Ammerer). Expression plasmids were constructed and transformed into *Escherichia coli* strains RR1 (17) and JM83 (18) by standard techniques.

Yeast Strains. *S. cerevisiae* strain XP635-10C (a *bar1 leu2-3,112 gal2*) was used for expression experiments. An isogenic Bar^+ revertant XP635-10CR was constructed by cotransformation with YEp13 and a linear fragment containing the *BAR1* gene (19); Leu^+ transformants were screened for those that exhibited the Bar^+ phenotype. The *BAR1* gene replacement was confirmed by Southern blot analysis (20). *S. cerevisiae* transformations were done by the method of Beggs (21). *Schizo. pombe* strain PR118-14 (h^- *leu1*) was transformed as described by Russell (14).

Barrier Assay. Yeast strains were assayed for barrier activity as described by Manney *et al.* (22). Transformants to be assayed were applied to a selective (minus leucine) agar plate that had been overlaid with 0.75% agar containing RC629 cells (a *sst1-2 ade2 his6 met1 ura1 can1 cyh2 gal2*; *bar1* and *sst1* mutations are in the same complementation group) (4) and enough α factor (Sigma) to arrest their growth (≈200 ng/ml). Barrier activity exported from the transformant colonies inactivates α factor surrounding the colony and thereby allows the a *bar1* assay cells to grow up as a fringe of cells around the transformant colony.

DNA Sequencing. A 2750-base-pair fragment from plasmid pBAR2 was sequenced on both strands by a combination of the chemical cleavage procedure (23) and the phage M13 dideoxy procedure (24).

Computer Analysis. The deduced amino acid sequence of the *BAR1* primary translation product was compared with

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†To whom reprint requests should be addressed.
§Present address: Zoologisches Institut der Universitat, Zurich, Winterthurstrasse 190, CH-8057 Zurich, Switzerland.
¶Present address: The Pennsylvania State University, 525 Biotechnology Institute Building, University Park, PA 16802.

‖The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03573).

sequences in the Protein Identification Resource.**

***In Vitro* Mutagenesis.** Site-specific mutagenesis of Asp-287 was achieved by the methods of Zoller and Smith (25) using the following sense-strand mutagenic oligonucleotides: 5′ CCAGTTTTATTAGCTTCAGGAACCT 3′ (aspartic acid to glutamic acid) and 5′ CCAGTTTTATTAGAATCAGGAACCT 3′ (aspartic acid to alanine); the antisense-strand template was the 1.3-kilobase *Sal* I–*Xho* I fragment (nucleotides 752–2070 in Fig. 2) from pBAR2 subcloned in M13mp18. Oligonucleotides were synthesized with an Applied Biosystems DNA synthesizer. Following mutagenesis, the entire template was sequenced to confirm the presence of only the desired mutation.

Tagging Barrier Protein with a Peptide Antigen. A DNA sequence encoding 14 amino acids including the 5-amino acid substance P antigen (26) and a translation stop signal was obtained from H. R. B. Pelham (Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.). This sequence was ligated to the *BAR1* coding sequence at the *Eco*RI site at nucleotide 1573 (Fig. 2), corresponding to amino acid 524 of the primary translation product. The *BAR1* *Eco*RI protruding end had been filled in with *E. coli* polymerase (Klenow fragment) and ligated with *Bam*HI linkers treated with polynucleotide kinase (27); the newly generated *Bam*HI site was ligated in frame with the substance P oligonucleotide. A *Sma* I site 3′ to the substance P translation stop signal was ligated to an *Xba* I–*Bam*HI fragment containing the transcription terminator from the *S. cerevisiae* *TPI1* gene (16); the *Xba* I end of the terminator fragment had been filled in with Klenow polymerase. A *Pvu* II–*Bgl* II fragment including nucleotides 1268–1576 of *BAR1*, substance P sequence, and the *TPI1* terminator was ligated with an *Sph* I–*Pvu* II fragment containing the *S. cerevisiae* *ADH1* promoter (15) and nucleotides 1–1267 of the *BAR1* coding sequence and inserted into YEp13 digested with *Sph* I and *Bam*HI. The wild-type *BAR1* coding sequence and the two Asp-287 mutant DNAs were used in the constructions. Yeast cells transformed with the hybrid genes were grown in selective medium and the supernatants were analyzed on electrophoretic transfer blots with anti-substance P antibody (NC1/34 HL, Accurate Chemical & Scientific, Westbury, NY) as described by Munro and Pelham (26).

Enzymes. Restriction endonucleases, DNA ligase from bacteriophage T4, polynucleotide kinase, and DNA polymerases were obtained from Bethesda Research Laboratories, New England Biolabs, and Boehringer Mannheim and were used according to suppliers' recommendations.

RESULTS AND DISCUSSION

The *BAR1* gene was cloned by *in vivo* complementation of a *bar1* mutation in yeast strain XP635-10C, using a library of *S. cerevisiae* total DNA constructed in the yeast shuttle vector YEp13 (28). Bar^+ transformants were first selected for growth on 0.15–0.3 unit of α factor per ml, a concentration that is inhibitory to a *bar1* cells (22), and then screened for production of barrier activity. Plasmids pBAR2 and pBAR3 were isolated from two of the Bar^+ yeast transformants, transformed into *E. coli*, and shown by restriction endonuclease digestion to contain a common region. Chromosomal integration experiments using a 3.2-kilobase fragment that complements the *bar1* mutation established that the cloned gene was *BAR1* (data not shown; see also ref. 7).

We initially demonstrated that *BAR1* is the structural gene for the extracellular barrier protein by transforming the fission yeast *Schizo. pombe* with plasmid pBAR2 and with the vector YEp13 and assaying the transformants for barrier activity (Fig. 1*A*). Although *Schizo. pombe* cells transformed with YEp13 do not secrete any barrier-like activity (rows 4 and 5), the pBAR2 transformants produce an activity that permits the growth of some a *bar1* assay cells around the *Schizo. pombe* colonies (rows 1–3). The low level of activity exported by the *Schizo. pombe* pBAR2 transformants compared to *S. cerevisiae* pBAR2 transformants (compare Fig. 1 *A* and *B*) presumably reflects in part incorrect transcription starts with the *S. cerevisiae* promoter in the fission yeast (14). The identification of *BAR1* as the structural gene for barrier protein was confirmed in later experiments (see below).

Subsequent subcloning, complementation tests, and integration experiments demonstrated that the functional *BAR1* gene and adjacent sequences necessary for its expression and regulation were contained within 2750 base pairs extending from an *Xba* I site to an *Xho* I site (ref. 7; T.R.M. and V.L.M., unpublished). As shown in Fig. 2, DNA sequencing of this region revealed only one long open reading frame beginning at position 1 and extending for 1761 base pairs. This was demonstrated to be the *BAR1* coding sequence by expression experiments in which this open reading frame was fused at the second *Xba* I site (position −16) to the *S. cerevisiae* *ADH1* promoter (15). Yeast transformed with a plasmid bearing the fusion of the *ADH1* promoter and the *BAR1*

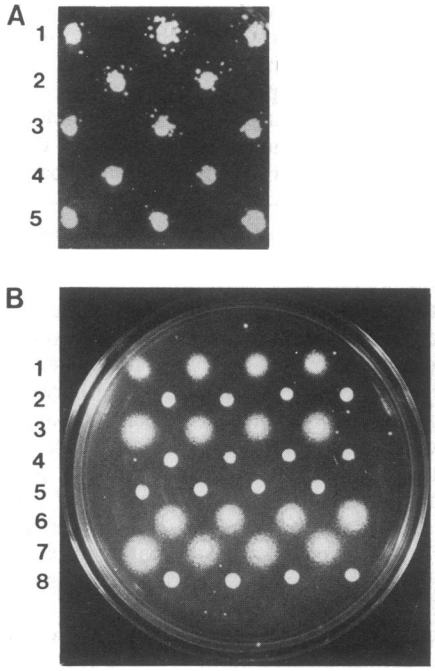

FIG. 1. Extracellular barrier activity produced by yeast transformed with the *BAR1* gene or mutant forms. (*A*) *Schizo. pombe* strain PR118-14 transformed with pBAR2 (rows 1–3) or with the vector YEp13 (rows 4 and 5). (*B*) Row 1, *S. cerevisiae* strain XP635-10CR (a *BAR1*) transformed with the vector YEp13. Rows 2–8, isogenic a *bar1* mutant strain XP635-10C transformed with YEp13 plasmids containing forms of the *BAR1* gene as indicated: row 2, YEp13 vector control; row 3, wild-type *BAR1* gene; row 4, Asp-287 mutated to glutamic acid; row 5, Asp-287 mutated to alanine; row 6, wild-type *BAR1* coding sequence with the *ADH1* promoter; row 7, deletion of 166 amino acids from the *BAR1* carboxyl terminus (*ADH1* promoter); row 8, deletion of 199 amino acids from the *BAR1* carboxyl terminus (*ADH1* promoter). Plasmids were constructed by standard methods and contain either the *BAR1* promoter or, where indicated, the *S. cerevisiae* *ADH1* promoter (15). The two carboxyl-terminal deletion genes utilize the transcription terminator from the *S. cerevisiae* triose phosphate isomerase (*TPI1*) gene (16) in place of the *BAR1* terminator. Yeast transformants were transformed with the expression plasmids and assayed for extracellular barrier activity (22).

**Protein Identification Resource (1985) Protein Sequence Database (Natl. Biomed. Res. Found., Washington, DC), Release 6.

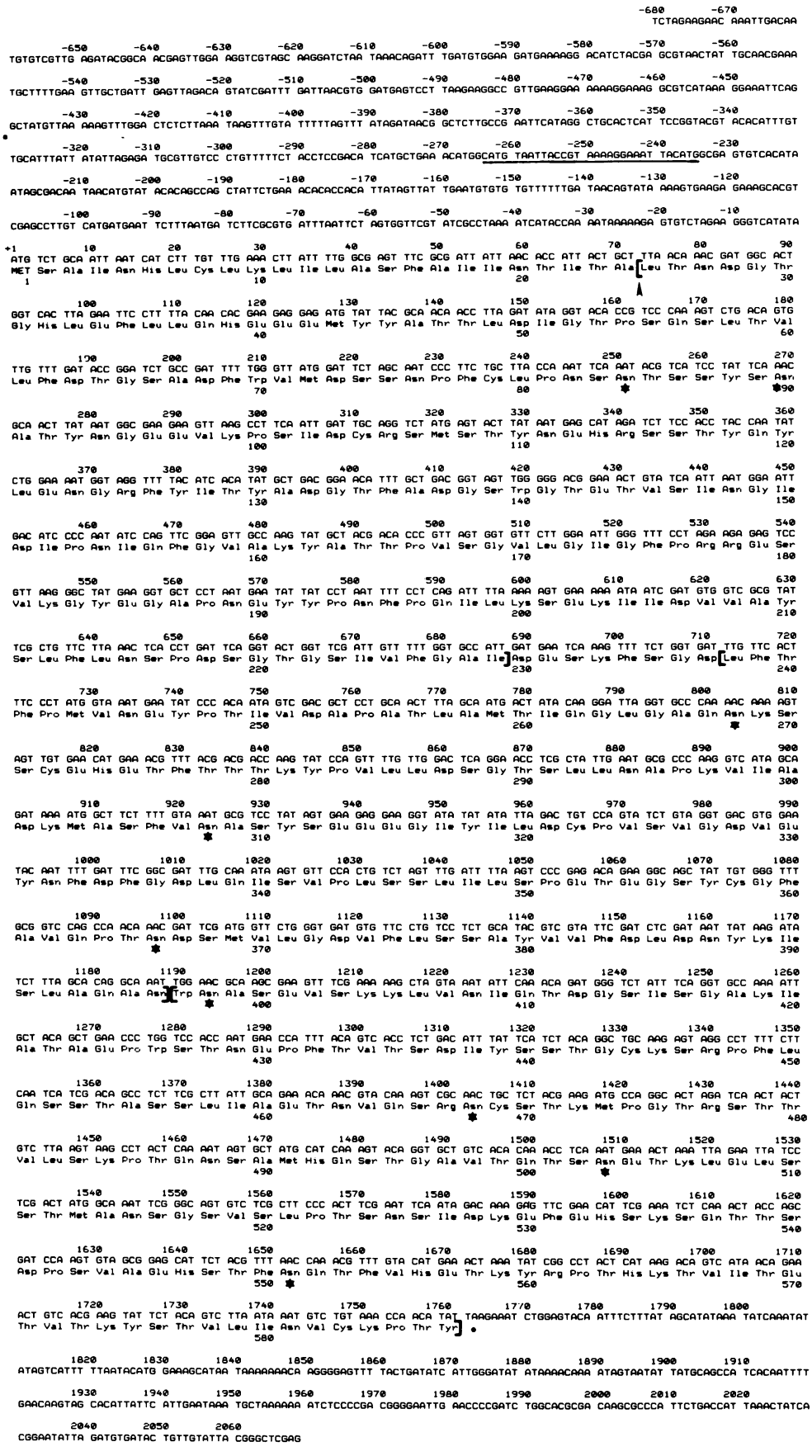

FIG. 2. Nucleotide sequence of 2750 base pairs encompassing the *BAR1* coding and regulatory sequences and the deduced amino acid sequence of the primary translation product. The *MATα2* binding site is underlined, the putative signal peptide cleavage site after amino acid 24 is indicated with an arrowhead, the potential asparagine-linked glycosylation sites are starred, and the three proposed domains (by analogy with pepsin-like proteases) are delimited with brackets.

Protein	Precursor/ proenzyme	Active form	Sequence
BAR	45-71	21-47	YATTLDIGTPSQSLTVLFDTGSADFWV
PEHU	76-102	14-40	Y T* IGTP*Q *TV*FDTGS* *WV
PEPO	59-85	18-44	Y T* IGTP*Q *TV*FDTGS* *WV
PEPG	58-84	14-40	Y T* IGTP*Q *TV*FDTGS* *WV
PECH	59-85	17-43	Y T* IGTP Q **V*FDTGS* *WV
REMSS	83-109	20-46	Y * IGTP Q** V*FDTGSA *WV
KHPGD		15-41	Y * IGTP Q *TV*FDTGS* *WV
CMBO	74-100	16-42	Y * *GTP Q *TVLFDTGS*DFWV
CMUMF		20-46	YA * IGTP Q * *LFDTGS*D WV
PEPLBJ		17-41	Y T * IG * L FDTGSAD*WV
BAR	284-310	260-286	VLLDSGTSLLNAPKVIADKMASFVNAS
PEHU	274-300	212-238	**D*GTSLL P * S * AS
PEPG	256-282	212-238	**D*GTSLL P * S * AS
PECH	257-283	215-241	**D*GTSLL P ** * S
REMSS	283-309	220-246	V**D*G*S** AP * A
KHPGD		221-247	**D*GTSL* P * **
CMBO	271-297	213-239	*LD*GTS L P * * A*
CMUMF		226-252	* *D*GT ** P A K*
PEPLBJ		210-236	* D*GT*LL * SV *
BAR	370-393	346-369	VLGDVFLSSAYVVFDLDNYKISLA
PEHU	362-385	300-323	*LGDVF* * VFD N * LA
PEPG	344-367	300-323	*LGDVF* Y VFD N K* LA
PECH	341-364	299-322	*LGDVF* YV*FD N K* L*
REMSS	375-398	312-335	VLG F* Y FD N *I *A
KHPGD		313-336	*LGDVF* Y VFD D ** LA
CMBO	355-378	297-320	*LGDVF* Y VFD N * LA
CMUMF		320-342	* G *FL V*D* N *I *A
PEPLBJ		297-320	**GD*FL S YVVFD D * *A

FIG. 3. Amino acid homologies between barrier activity and pepsin-like proteases. Only identical amino acids are shown; conservative changes are denoted with an *. Proteins: BAR, barrier activity; PEHU, human pepsinogen; PEPO, bovine pepsinogen A; PEPG, porcine pepsinogen A; PECH, chicken pepsinogen; REMSS, mouse renin; KHPGD, porcine cathepsin D; CMBO, bovine prochymosin; CMUMF, mucor carboxyl protease; PEPLBJ, penicillopepsin. Amino acid numbering is shown for the enzymes in their proenzyme or precursor forms (primary translation product for BAR) and their active forms (BAR minus a 24-amino acid signal peptide). The three conserved regions are aligned with each other at the aspartic acid residues. Amino acids are shown in the one-letter code: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

coding sequence export as much or more barrier activity than yeast transformed with the wild-type *BAR1* gene (Fig. 1*B*, row 6 vs. row 3). The 5′ noncoding sequence contains the *MATα2* binding site (nucleotides −235 to −264) (29, 30), which is responsible for repression of *BAR1* transcription in α and **a**/α cells and other elements necessary for *BAR1* expression (7). The 587-amino acid primary translation product includes a putative signal peptide with a predicted cleavage site after amino acid 24 (31) and nine potential asparagine-linked glycosylation sites, features that are consistent with the secretion and export of the polypeptide.

The deduced amino acid sequence of the *BAR1* primary translation product was compared with sequences in the Protein Identification Resource.** Strong similarities were found between short regions of the *BAR1* product and conserved sequences of members of the "acid protease" family or pepsin-like proteases (Fig. 3). It has been reported that the yeast vacuolar protease A and a number of retroviral proteases (including the protease encoded by the acquired immunodeficiency syndrome human immunodeficiency virus) are also homologous to pepsin (32–34). Additional homology was demonstrated by computer-generated alignment between porcine pepsinogen (35–38) and the first 396 amino acids of the *BAR1* product (Fig. 4). Their similarity extends over the entire length of pepsinogen (PEPG) and results from a high percentage of identities and an accumulation of many conservative replacements. However, relative to pepsinogen, the *BAR1* product has three large insertions between PEPG amino acids 86 and 87, 182 and 183, and 253 and 254. Comparison of the *BAR1* sequence with the three-dimensional structure of pepsinogen (41) indicates that these may be surface loops and that the hydrophobic core of pepsin is conserved in the *BAR1* protein. The pronounced sequence similarity between the two proteins strongly suggests that the part of the *BAR1* product shown in Fig. 4 is homologous (i.e., coancestral) with pepsin and that it functions as a proteolytic enzyme.

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PEPG:  LVKVPLVRKKSLQNLIKDGKLKDFLKTHKHNPASKYFPEAAALICDEPLENYLDTFYFGT (60)
 BAR:  ------------MSAINHLCLKLILASFVIINTITALTNDGTGHLEFLLQHEEEMYYATT (48)
                     : *::  **  * ::        : :       :  *::  :  * :

PEPG:  IGIGTPAQDFTVIFDTGSSNLWVPSV----------------------YCSSLACSDHN (97)
 BAR:  LDIGTPSQSLTVLFDTGSADFWVMDSSNPFCLPNSNTSSYSNATYNGEEVKPSIDCRSMS (108)
       : ****:* :**:*****:::**                                *: *:  :
                          •

PEPG:  PFNPDDSSTFEA-TSQELSITYGTGSM-TGILGYDTVQVCGISDTNQIFGLSETEPGSFL (155)
 BAR:  TYNEHRSSTYQYLENPSFYITYADGTFADGSWGTETVSINGIDIPNIQFAVAKYAT---- (164)
        :*:: ***::  :: :: *** *:: *  * :**:: **   *  * ::: :

PEPG:  YYAPFDGILGLAYPSISASGATPVFDN---------LWDQGLVSQDLFSVYLSSNDDSGS (206)
 BAR:  --TPVSGVLGIGFPRRESVKGYEGAPNEYYPNFPQILKTEKIIDVVAYSLFLNSPDSGTG (222)
         *  *:**: :*: ::    :  *         *  : ::    :*::*:* *

PEPG:  VVLLGGIDSSYYTGSLNWVPVSVEGYWQITLDSITMDGETIACSGGC------------- (253)
 BAR:  SIVFGAIDESKFSGDLFTFPMVNE------YPTIVDAPATLAMTIQGLGAQNKSSCEHET (276)
        :::* **:* ::* *   *:  *       :*    :*:* :

PEPG:  ------QAIVDTGTSLLTGPTSAIANIQSDIGASENSDGEMVISCSSIDSLPDIVFTING (307)
 BAR:  FTTTKYPVLLDSGTSLLNAPKVIADKMASFVNASYTEEEAIYILDSPV-SVGDVEYNFDF (335)
               ::*:***** *    :: * : ** :: :: *  * : *: *: :  :
                  •

PEPG:  VQPYLSPSAYILQDDDSCTSGFEGMDVPTSSGELWILGDVFIRQYYTVFDRANNKVGLAP (367)
 BAR:  GDLQISVPLSSLILSPETQGSYCGFAVQPTNDSM-VLGDVFLSSAYVVFDLDNYKISLAQ (394)
        :  :*    *   :   : * *  :: :: :*****::: * ***  * *: **

PEPG:  VA (369)
 BAR:  AN (396)
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FIG. 4. Alignment of the amino acid sequence of porcine pepsinogen and the first 396 residues of the *BAR1* primary translation product. Porcine pepsinogen is cleaved to pepsin after amino acid 43 (36). The nine-gap alignment was formed and evaluated statistically by the Sankoff algorithm (39). Nonparametric analysis of incremental scores demonstrated that nine was the maximum number of gaps that was allowed on a statistical basis. Comparison of the score of the alignment shown here with the scores of 100 pairs of randomized sequences of the same lengths and amino acid compositions (39) demonstrated that the similarity between the *BAR1* product and pepsinogen is statistically highly significant. The alignment of the authentic sequences generated a score that was 16 standard deviations greater than the mean of the scores of the randomized sequences. Under the assumption of a normal distribution, this deviation corresponds to a probability of 10^{-57}. Asterisks (*) indicate identities; colons (:) indicate conservative replacements—i.e., amino acid pairs having greater-than-average scores in the scheme of McLachlan (40). The two active site aspartic acid residues are indicated with closed circles (•). Amino acids are shown in the one-letter code: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

Two of the regions with strong homology between *BAR1* and pepsin-like proteases contain the two reactive site aspartic acid residues (corresponding to positions 63 and 287 in the *BAR1* precursor) that are characteristic of pepsin-like proteases (38, 42–46). We mutated one of these putative active site residues to test whether it is necessary for barrier activity. Asp-287 was mutated *in vitro* (25) to glutamic acid and to alanine and the mutant *BAR1* genes were transformed into yeast. As shown in Fig. 1*B* (rows 4 and 5), both mutations abolished detectable extracellular barrier activity.

To determine if the mutant proteins were still exported to the medium, we constructed hybrid genes in which DNA encoding the carboxyl-terminal 63 amino acids of the *BAR1* primary translation product was replaced with a synthetic DNA that codes for a 14-amino acid sequence containing a part of the neuropeptide substance P that can be detected with a monoclonal antibody (26). Yeast transformed with the

hybrid derived from the wild-type *BAR1* gene exported approximately as much active barrier protein as cells transformed with the full-length *BAR1* gene (data not shown), indicating that the carboxyl-terminal 63 amino acids are not required for barrier activity or export. As expected, yeast transformed with substance P hybrid genes derived from the Asp-287 mutant *BAR1* genes did not produce active barrier protein. Electrophoretic transfer blot analysis of culture medium from cells transformed with the wild-type or mutant *BAR1*-substance P hybrids indicated, however, that the mutant hybrid proteins were exported to the culture medium as efficiently as the wild-type hybrid protein (Fig. 5). Fig. 5 also demonstrates that the exported hybrid protein is heavily glycosylated, migrating as a heterogeneous band near the top of the gel with an apparent $M_r > 200,000$.

Pepsin-like proteases are organized into two distinct structural domains, each of which contains an active site aspartic acid. The two domains are related by a 2-fold axis of approximate rotational symmetry. Since the first two-thirds of the barrier sequence aligns well with the entire pepsinogen sequence, one can conclude tentatively that this region of barrier protein is also organized as two domains (indicated in Fig. 2). The role of the last third of the barrier protein is unclear, as a similar domain is not found in the other members of the pepsin family. This region should therefore not be required for the barrier protein to function as a protease. Accordingly, deletion of 166 amino acids from the carboxyl terminus of the *BAR1* product does not significantly affect barrier activity (Fig. 1*B*, row 7). However, a deletion of 199 amino acids, which extends into the second domain, abolishes activity (Fig. 1*B*, row 8).

Conservation of other amino acids near the conserved aspartic acid residues, not only in pepsinogen but also in other members of the family, argues that other positions are essential for protease activity and/or structure. The ease with which specific or localized random mutations can be generated in the *BAR1* gene and then reintroduced into yeast for assay suggests that this may be a useful system for analyzing the functions of active site and other conserved residues of pepsin-like proteases.

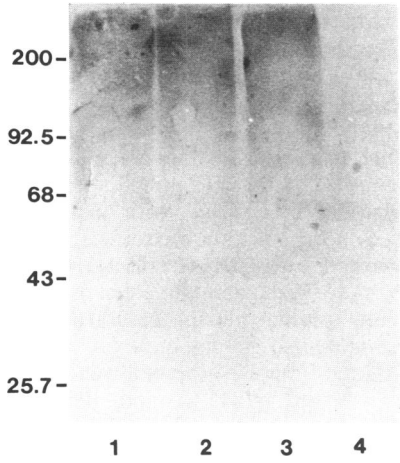

FIG. 5. Secretion of mutant barrier proteins. *S. cerevisiae* strain XP635-10C was transformed with plasmids bearing hybrid genes of the *ADH1* promoter-*BAR1*-substance P oligonucleotide-*TPI1* terminator. Transformants were grown in selective medium lacking leucine for 31 hr. Barrier protein was precipitated from 12.5 ml of the supernatant by the addition of an equal volume of cold 95% ethanol, and the dried pellet was resuspended in 200 μl of sample buffer for electrophoresis (after denaturation) on a 10% polyacrylamide gel containing sodium lauryl sulfate. Following electrophoresis, the proteins were transferred to nitrocellulose and immunoblotted as described by Munro and Pelham (26). Lane 1, Asp-287 mutated to alanine; lane 2, Asp-287 mutated to glutamic acid; lane 3, wild-type *BAR1*; lane 4, YEp13 vector. Molecular weights are shown as $M_r \times 10^{-3}$.

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