A hybrid gene was constructed containing a fusion between the DNA sequences encoding the secretory precursor of the yeast mating pheromone α-factor and a synthetic sequence encoding a biologically active 24-amino acid carboxyl-terminal portion of the human atrial natriuretic peptide (hANP) precursor. Transformation of Saccharomyces cerevisiae with the hybrid gene resulted in the yeast cells secreting biologically active hANP into the extracellular medium. The secreted hANP was purified and found to be accurately processed at the junction in the chimeric α-factor/hANP protein, producing the desired mature hANP amino terminus. The secreted product was also folded correctly with respect to the single disulfide bond. However, the carboxyl terminus of the secreted hANP material was heterogeneous such that the major form lacked the last two amino acids of the peptide while the minor form was the full length material. The observed processing at the carboxyl terminus of the secreted hANP may reflect a normal processing event involved in α-factor peptide maturation.

The mating pheromone α-factor is one of the best characterized of the proteins traversing the secretory pathway of the yeast Saccharomyces cerevisiae. This 18-amino acid peptide is initially synthesized from the structural gene MFb1 as part of a 155-residue precursor protein (Fig. 1) which contains (i) a hydrophobic signal sequence at the amino terminus, (ii) an additional leader region of about 60 residues containing three glycosylation sites, (iii) four copies of the mature α-factor sequence, and (iv) four spacer peptides, one preceding each of the four α-factor repeats (1, 2). Two of the enzymes involved in the proteolytic processing of this precursor have been characterized in some detail: an endopeptidase that cleaves on the carboxyl-terminal side of -Lys-Arg- amino acid pairs in the spacer peptides (3, 4) and a dipeptidyl aminopeptidase that removes the -Glu-Ala- and -Asp-Ala- residue pairs of the spacers to produce the mature α-factor amino terminus (5).

Genetic and biochemical studies have indicated that the -Glu-Ala--Glu-Ala-endopeptidase is encoded by the KEX2 gene (3), while the dipeptidyl aminopeptidase is encoded by STE13 (5). The prototype involved in the maturation of the α-factor carboxyl terminus (needed to remove the -Lys- and -Arg- residues proposed to be left on three of the four α-factor repeats after endopeptidase cleavage of the spacers (Fig. 1)) have not yet been definitively identified, although Achstetter and Wolf (4) have detected membrane-bound peptidase activities that cleave two synthetic substrates mimicking possible intermediates of the carboxyl-terminal maturation process (Cbz-Tyr-Lys-Arg-NH₂ and Cbz-Tyr-Lys-Arg-NA).

Several groups have utilized knowledge of the α-factor gene structure and maturation process to construct hybrid genes joining the coding sequence for the prepro-region of α-factor to the coding sequence for a heterologous protein. In some cases, the fusion proteins expressed from these hybrid genes were found to be transported into the secretory pathway and efficiently processed, such that the heterologous product was cleaved out of the fusion protein and secreted from the yeast cell (6-8). These results indicated that the α-factor prepro-region is itself sufficient to direct both secretion and the amino-terminal processing events that occur in the first spacer region and might, therefore, be used to direct the export of a wide variety of protein products. Two of the earliest reported fusions placed the mature amino terminus of human epidermal growth factor (6) or human leukocyte interferon D (IFN-α1) (8) directly after the -Lys-Arg- cleavage site at position 85 of prepro-α-factor. Both the epidermal growth factor and IFN-α1 secreted into the medium in these two cases had the correct amino terminus with no α-factor sequences remaining. These data indicated that the -Glu-Ala--Glu-Ala-residues in the first spacer region are not required for KEX2-dependent cleavage at -Lys-Arg-.

In this report, we have used the α-factor promoter and prepro-region to successfully direct the synthesis and secretion of a human atrial natriuretic peptide (hANP) in yeast cells. The 24-residue hANP(128-151) is one of a group of hANPs, all derived from the same 151-residue precursor (9), that exhibit potent natriuretic, diuretic, and vasorelaxant activities and have been implicated in the regulation of cardiovascular homeostasis (10, 11). When a chimeric protein was produced in yeast cells that contains the hANP(128-151) sequence joined to the α-factor prepro-region at the -Lys-Arg-processing site, we found that biologically active hANP peptide with a mature amino terminus and properly formed disulfide bond was secreted from the host yeast cells. Unexpectedly, however, we found that the carboxyl terminus of the peptide(s) involved in the maturation of the α-factor carboxyl terminus (needed to remove the -Lys- and -Arg- residues proposed to be left on three of the four α-factor repeats after endopeptidase cleavage of the spacers (Fig. 1)) have not yet been definitively identified, although Achstetter and Wolf (4) have detected membrane-bound peptidase activities that cleave two synthetic substrates mimicking possible intermediates of the carboxyl-terminal maturation process (Cbz-Tyr-Lys-Arg-NH₂ and Cbz-Tyr-Lys-Arg-NA).

1 The abbreviations used are: Cbz, benzoyloxycarbonyl; NA, 4-nitroanilide; hANP, human atrial natriuretic peptide; RIA, radioimmunoassay; RPLC, reversed-phase liquid chromatography; bp, base pair(s); SDS, sodium dodecyl sulfate.

2 The numbering refers to the position of amino acids in the human propro-ANP sequence (9).
secreted hANP was heterogeneous, possibly reflecting a normal processing event involved in α-factor peptide maturation.

MATERIALS AND METHODS

Yeast and Bacterial Strains—S. cerevisiae strain W301-1A8 (ade2-1 leu2-3, 112 trpl-1 can1-100 uros-1 his1-11, 15) (12) was used as the host for yeast plasmid transformations (13). Escherichia coli strain DH1 (14) was used for all bacterial plasmid transformations (15). E. coli strain JM101 (16) was used for bacteriophage M13 transformations (14, 16).

Culture Media—E. coli were grown in LB medium supplemented with ampicillin (100 μg/ml). Yeast complete medium and synthetic complete medium lacking leucine were prepared as described (13).

Preparation of Synthetic Oligodeoxynucleotides—Synthetic oligodeoxynucleotides were prepared using an automated synthesizer (Applied Biosystems). Following deprotection, the oligodeoxynucleotides were purified by preparative 20% polyacrylamide gel electrophoresis in the presence of 8 M urea.

Vector Construction—The S. cerevisiae-E. coli shuttle vector used for the expression of hANP (pJC1-5 (Fig. 2)) was derived from the plasmid pCV7 (17), which contains a 2.2-kilobase XhoI-Sall fragment spanning the S. cerevisiae α72 gene (18, 19) cloned into the SacI site of pBR322 and the 2.24-kilobase EcoRI fragment of the B form gene (18) cloned into the Sall site of pBR322 and then phosphorylated at the EcoRI restriction site. The mutagenized gene fragment (M2) was ligated to pJC1-5 DNA, which had been partially cleaved with PstI in the presence of ethidium bromide and then completely digested with HindIII. A plasmid with the basic structure shown in Fig. 2 was isolated from this ligation mixture designated pJC1-5. DNA sequencing of the 494-bp PstI-Sall fragment and sequencing of the unique HindIII site (see Fig. 2).

Assembly of Synthetic ANP Gene Sequences—500 pmol of the number digest oligodeoxynucleotides shown in Fig. 3 (except 1 and 9) were phosphorylated individually in 60 mM Tris-Cl, pH 8.0, 15 mM dithiothreitol, 10 mM MgCl₂, 20 μCi of [32P]ATP (Amersham Corp.), and 20 units of T4-polyolodeoxyribonucleotide kinase (P-L Biochemicals) for 30 min at 37 °C. ATP was then added to a final concentration of 1.5 mM along with 20 units of polynucleotide kinase, and the mixtures were incubated for an additional 30 min. The reactions were terminated by incubating at 100 °C for 5 min. 500 pmol of the unphosphorylated terminal oligodeoxynucleotides 1 and 8 were diluted in the above buffer without ATP. 16.7 pmol of each oligodeoxynucleotide constituting a double-stranded pair (e.g. oligodeoxynucleotide 2 and 3 and 4 and 5) were annealed for 2 h at 90 °C for 2 min followed by slow cooling to room temperature. All of the oligodeoxynucleotide pairs in the construction were then combined, extracted with phenol/chloroform, and precipitated with ethanol. The combined oligodeoxynucleotide pairs were resuspended in 50 mM Tris-Cl, pH 8, 10 mM MgCl₂, and 20 mM dithiothreitol, heated to 50 °C for 10 min, and then allowed to cool to room temperature. The addition of ATP to a final concentration of 0.5 mM was followed by the addition of 800 units of T4-DNA ligase (New England Biolabs) and incubation at 12.5 °C for 12–16 h.

The ligation mixture was extracted with phenol/chloroform and the DNA (from the Chao's precipitation) was then resuspended in 1 mM EDTA, 0.1% gelatin. The ligation mixtures were incubated for another 1.5 h and the DNA was precipitated with ethanol, and then ethanol precipitated and resuspended in 10 mM NH₄OAc, 50 mM Tris-Cl, pH 7.5 and subjected to electrophoresis on an 8% polyacrylamide gel and the appropriately sized bands excised and eluted. Following two ethanol precipitations, the synthetic gene fragments were ligated to HindIII-digested M13mp18 and then phosphorylated with HindIII, and then phosphorylated at the 5′ termini with polynucleotide kinase. Double-stranded DNA fragments were separated on an 8% polyacrylamide gel and the appropriately sized bands excised and eluted. Following two ethanol precipitations, the vector containing α-factor sequences, the cohesive ends of a 2.75-kilobase EcoRI fragment spanning the α-factor gene Mα1 (1) were first filled in with Klenow fragment of DNA polymerase I, and BamHI linkers (5′-CGGATCCG-3′) were ligated to the blunt ends of the unique HindIII site (see Fig. 2). The resulting plasmid, HinΔ2, has lost one EcoRI site and both HindIII sites found in CV7.

The resulting plasmid, pJC1-5, was used as the host for yeast plasmid transformations (14, 16).

Radioimmunooassay of hANP—Samples for radioimmunooassay (RIA) were typically suspended in 100 μl of solution containing phosphate-buffered saline, pH 7.4, 5 mM EDTA, and 0.1% gelatin (Sigma). ANP-specific antisera (kindly provided by Dr. Steven Atlas) was diluted 1:20,000 in the above buffer and added to the samples in a volume of 500 μl. 20,000 cpm of [3H]hANP (127–151) (prepared according to Schenk et al. (25)) was then added. The mixture was incubated overnight at 4 °C, and antibody-bound and free [3H]hANP (127–151) were separated by the addition of 0.5% charcoal and centrifugation. The supernatant was then filtered through 0.45-mm GF/C filters, and aliquots were applied to a 17.5% SDS/polyacrylamide gel (24).

Purification of Secreted ANP—Yeast cells containing plasmid pJC1-5 were grown in synthetic complete medium minus leucine to a cell density corresponding to an A600 of 5–6, and the cells were removed by centrifugation. The resulting supernatant (1 liter) was

Fig. 1. Schematic representation of events involved in the processing of prepro-α-factor. α-Factor peptides within the precursor protein are indicated by shaded boxes (αF1–αF4); spaces peptide regions are labeled S1–S4. Potential N-glycosylation sites within the leader peptide region are represented by asterisks. The proteolytic activities shown processing the carboxyl-terminal portion of the αF1 peptide are speculative. See text for further details.
adjusted from pH 2.5 to 8.0 with ammonium hydroxide and then subjected to another high speed centrifugation to remove any insoluble material. The supernatant from this centrifugation was applied to a DEAE-Sepacel column (2.5 x 20 cm) equilibrated with 10 mM ammonium acetate, pH 5.0, and the resulting eluate was collected and lyophilized. The dried post-DEAE material was resuspended in 0.5 M acetic acid and applied to a Sephacel G-10 column (2.6 x 100 cm) equilibrated in the same buffer. The loaded material was eluted with approximately 300 ml of 0.5 M acetic acid, and fractions were assayed by ANP-specific RIA (see above). Peak immunoreactive fractions were pooled and lyophilized. The dried post-Sephacel G-10 material was resuspended in a minimal volume of 3.5 M acetic acid and further fractionated by gradient elution reversed phase liquid chromatography (RPLC) employing a Series 4 solvent delivery system (Perkin-Elmer) in series with a Kratos model 757 UV detector (220 nm). A Perkin-Elmer model LCI-100 computing integrator provided integration and retention data. Fractionation was accomplished using a 1.0 x 25-cm C8 column (5u, Vydac 218TP510) eluted initially at 0.5 ml/min with 15% CH3CN in H2O containing 0.1% C8-COOH and then with a gradient from 2-22 min going to 35% CH3CN (1%/min) to elute the immunoreactive zone. Aliquots from 1-min fractions were collected and portions dried and assayed for immunoreactivity using the ANP-specific RIA. The peaks of immunoreactivity were collected and portions dried and assayed for immunoreactivity using the ANP-specific RIA. The supernatant from this centrifugation was applied to another high speed centrifugation to remove any insoluble material. The material was resuspended in water and dried by lyophilization.

**Amino- and Carboxy-terminal Amino Acid Sequence Analysis**—Amino-terminal sequence was determined on approximately 100 pmol of RPLC-purified material using an Applied Biosystems 470A gas phase sequenator. Twenty-two cycles were run on two independent samples of recombinant peptide. Phenylthiohydantoin amino acids were identified with a Beckman 3355T high pressure liquid chromatograph using a 0.46 x 25-cm IBN CM column and a gradient slightly modified from that of Hunkapiller and Hood (26). Repetitive yields were in excess of 95%.

Carboxy-terminal sequence was determined on approximately 200 pmol of RPLC-purified material using carboxypeptidase Y digestion and analysis. Typically, samples were dissolved in 50 mM sodium acetate, pH 5.5, containing norleucine as a standard. 0.2 mg of carboxypeptidase Y (Cooper Biomedical Inc.) was added amino acids were identified by precol- larization and analysis. Typically, samples were dissolved in 50 mM sodium acetate, pH 5.5, containing norleucine as a standard. 0.2 mg of carboxypeptidase Y (Cooper Biomedical Inc.) was added amino acids were identified by precol- larization and analysis. Typically, samples were dissolved in 50 mM sodium acetate, pH 5.5, containing norleucine as a standard. 0.2 mg of carboxypeptidase Y (Cooper Biomedical Inc.) was added amino acids were identified by precol- larization and analysis. 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identified by precol- larization and analysis. 0.2 mg of carboxypeptidase Y (Cooper Biomedical Inc.) was added amino acids were identified by precol-
Expression of the α-Factor/hANP Gene Fusion, pJCNF-1—
*S. cerevisiae* strain W301-18A was transformed with plasmid pJCNF-1 or the control plasmid pJC1-5, and leucine prototrophs were selected. These transformants were then grown in synthetic complete medium lacking leucine at 30 °C to a cell density corresponding to an *A*<sub>600</sub> of 5. To determine whether the transformants were secreting ANP-like peptides, intact cells were removed from the cultures by centrifugation, and the extracellular medium was assayed using an ANP-specific RIA. The results of this assay indicated that cells containing pJCNF-1 consistently produced 530–570 pg of immunoreactive hANP/liter of culture medium whereas no detectable amount of immunoreactivity was seen in culture media derived from cells containing the control plasmid pJC1-5.

We also investigated the variety of new peptide sequences being secreted by the yeast transformants by labeling the cells with [35S]methionine, fractionating the resulting culture media on SEP-PAK C<sub>18</sub> cartridges, and then analyzing the eluted products using SDS-polyacrylamide gel electrophoresis (see "Materials and Methods"). As shown in Fig. 4, cells containing plasmid pJCNF-1 secrete two [35S]methionine-labeled polypeptides not found in the medium from the control cell culture. The major new labeled peptide migrated with a mobility similar to that seen with synthetic hANP(128–151) (indicated by the arrow in Fig. 4), while the minor peptide had a mobility which was slightly lower. Culture media from [35S]methionine-labeled W301-18A[pJCNF-1] and W301-18A[pJC1-5] cultures were also treated with CCl<sub>4</sub>COOH (to precipitate a more representative class of total secreted polypeptides) and subjected to SDS-polyacrylamide gel electrophoresis analysis. As was the case for the SEP-PAK-absorbed polypeptides, the only difference detected between the two samples using this analysis was the presence of the small molecular weight peptides shown in Fig. 4 (data not shown).

**Purification and Characterization of the Secreted hANP Produced by W301-18A[pJCNF-1]—**The above results suggested that cells containing plasmid pJCNF-1 were secreting significant quantities of immunoreactive hANP into the extracellular medium. To definitively demonstrate the nature of the secreted material, the ANP-like peptides were purified from the culture medium. As described under "Materials and Methods," the purification protocol involved chromatography on three successive columns: DEAE-Sephacel, Sephadex G-50, and finally C<sub>18</sub>RPLC. Each separation step was monitored through the use of an ANP-specific RIA.

In a preliminary experiment, total secreted material from cells containing pJCNF-1 was separated by C<sub>18</sub>RPLC without prior chromatography on DEAE-Sephacel or Sephadex G-50. RIA of the resulting fractions indicated that only a single peak of immunoreactivity was present which eluted at a CH<sub>3</sub>CN concentration of approximately 29%. No such peak of immunoreactivity was observed in material secreted from the control W301-18A[pJC1-5] cells (data not shown). The hANP in the single peak of immunoreactive material from the pJCNF-1-containing cultures was purified using the three-column protocol described under "Materials and Methods." As shown in Fig. 5A, the purified material eluted as a single peak from a C<sub>18</sub>RPLC column. In addition, as shown in Fig. 5B, this material also co-eluted with synthetic hANP(128–151) run under identical RPLC conditions.

Amino-terminal sequence analysis of the immunoreactive peak as described under "Materials and Methods" resulted in the sequence, NH<sub>2</sub>-Ser-Ser-Gla-Ser-Cys-Arg-Ser-Gla-Ser-Asp-Asp-Arg-Ile-Glu-Glu-Leu-Cys-Asp-Ser-Phe. . . As expected, the amino terminus of this material begins with
The prepro-hANP sequence (see Fig. 3B). The amino-terminal sequence analysis also indicated that the immunoreactive material within this peak was >95% pure over the range shown above.

Because the sequence shown above is only a partial sequence of the purified material, we utilized carboxypeptidase Y digestion of the immunoreactive material to determine its composition at the carboxyl terminus. As a control in the carboxypeptidase Y digestions, synthetic hANP(128-151) was used. As shown in Fig. 6A, digestion of synthetic hANP(128-151) with carboxypeptidase Y resulted in the sequential liberation of tyrosine, arginine, and phenylalanine residues, respectively. However, the results obtained following carboxypeptidase Y digestion of the immunoreactive material isolated from yeast were more complicated. As shown in Fig. 6B, the major carboxypeptidase Y digestion product detected was phenylalanine, with tyrosine and arginine appearing as minor residues. Identical results were obtained on several different occasions using different peptide preparations. These results indicated that the purified material shown in Fig. 5A actually contains two peptide species, a major form corresponding to hANP(128-149) which ends with phenylalanine and a minor form corresponding to the expected hANP(128-151) sequence terminating in tyrosine. The carboxypeptidase Y digestion results were verified by amino acid composition analysis of the immunoreactive material, which indicated the ratio of hANP(128-149) to hANP(128-151) in the purified material was approximately 70:30 (data not shown).

The results described above indicate that hANP(128-151) and hANP(128-149) peptides isolated from yeast co-elute under the C18 RPLC conditions used here. To further verify that the two hANP sequences are not separated by the gradient conditions employed, a mixture of synthetic versions of hANP(128-151) and hANP(128-149) was analyzed. As shown in Fig. 5C, these peptides also co-elute from the C18 column when run under the same conditions used for the analysis of recombinant hANP. To separate the two recombinant hANP peptides, we examined the use of a phenyl RPLC column instead of C18. The rationale for using the phenyl support was based on the fact that the hANP(128-149) sequence lacks the aromatic tyrosine residue found at the carboxyl terminus of hANP(128-151) making it likely that the retention time of hANP(128-149) on the phenyl column would be sufficiently different from that of the hANP(128-151) sequence to allow their separation. As shown in Fig. 5D, mixtures of the synthetic versions of hANP(128-151) and hANP(128-149) were clearly resolved using the phenyl column. The latter sequence has a slightly shorter retention time on this column, probably reflecting the predicted difference in aromaticity between the two peptides and their interaction with the phenyl support. A similar result was obtained when the immunoreactive hANP peptides isolated from yeast were submitted to phenyl RPLC (Fig. 5E). The addition of synthetic hANP(128-149) to this material enhanced the peak with the shorter retention time (Fig. 5F), verifying the identity of the earlier-eluting material, while the later-eluting material was found to co-elute with synthetic hANP(128-151) (data not shown). From the areas of the two peaks resolved on the phenyl column (Fig. 5E), the ratio of hANP(128-149) to hANP(128-151) in the material isolated from yeast was calculated to be approximately 65:35, which is in close agreement with the 70:30 ratio obtained from the amino acid composition data.

The purified recombinant hANP material appeared to have formed the single disulfide bond between the two cysteine residues, as judged by the retention time of the peptides on C18 RPLC. Reduced forms of synthetic hANP(128-151) or hANP(128-149) were found to be retained on C18 columns longer (as indicated by the arrow in Fig. 5C) when compared to their respective disulfide bridged forms.

Biological Activity of Recombinant hANP—To assess the biological activity of the recombinant hANP material purified from yeast, we utilized the functioning isolated perfused rat kidney model (29,30). This system has been used extensively to analyze the renal hemodynamic effects of a variety of ANPs and has been shown to be a relevant model for evaluating the renal effects of these peptides in vivo (11, 31). As shown in Table 1, 1 μg of C18 RPLC-purified material caused significant increases over control levels in glomerular filtration rate, urine flow rate, sodium and potassium excretion rates as well as the urinary sodium and filtration fraction. Renal plasma flow remained relatively constant during the experiment. The natriuretic effects of the recombinant material were very similar to those observed previously with other ANPs (29, 31), indicating similar specific activities.
Fig. 5. Analytical reverse-phase liquid chromatography of recombinant hANP purified from yeast and hANP peptides made synthetically. Chromatographic conditions were the same as described under "Materials and Methods" except that a 0.46-cm inner diameter x 15-cm C18 column (5µ, Vydac 218TP5415) or 0.46-cm inner diameter x 15-cm phenyl column (5µ, Vydac 219TP5415) (panels A, B, C) or 0.46-cm inner diameter x 15-cm CIS column (5µ, Vydac 218TP5415) (panels D, E, F) was used at a flow rate of 1.0 ml/min. Panels: A, recombinant hANP (2 µg); B, mixture of recombinant hANP (2 µg) and synthetic hANP(128-151) (2 µg); C, mixture of synthetic hANP(128-151) (4 µg) and hANP(128-149) (4 µg); D, mixture of synthetic hANP(128-151) (8 µg) and hANP(128-149) (4 µg); E, recombinant hANP (4 µg); and F, mixture of recombinant hANP (4 µg) and synthetic hANP(128-149) (8 µg). See text for details.

Fig. 6. Carboxyl-terminal amino acid composition determination using carboxypeptidase Y digestion of synthetic hANP(128-151) (panel A) and recombinant hANP purified from yeast (panel B). Designations are: X, tyrosine; □, arginine; ○, phenylalanine. See text for details.

Table I

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DISCUSSION

We have demonstrated that the α-factor promoter and leader coding region can be used to direct the expression and secretion of biologically active hANP material in S. cerevisiae. To achieve expression, a synthetic gene sequence encoding hANP(128-151) was joined to the α-factor gene sequence at a site immediately following the coding sequence for one of the natural processing sites (-Lys-Arg-) of the α-factor precursor (see Fig. 3). Endoproteolytic cleavage at this dibasic pair has been previously shown to occur independent of any other downstream α-factor precursor protein sequences (6, 8). Consistent with these reports, our data indicate that the purified immunoreactive hANP material secreted from yeast cells carrying the fusion gene has a homogeneous amino terminus beginning with the amino-terminal residue of mature hANP(128-151) and that the processing of the -Lys-Arg-endoproteolytic site in the chimeric α-factor/hANP protein therefore occurred accurately.
The purified secreted hANP material was found to elute as a single immunoreactive species on C18 RPLC, suggesting a homogeneous peptide sequence. However, in contrast to the amino-terminal sequence analysis, a combination of the results obtained from carboxy-terminal analysis, amino acid composition data, and phenyl column RPLC analysis demonstrated that this material actually consists of two peptides. The full length sequence, ending in -Ser-Phe-Arg-Tyr (hANP(128–151)) is the minor peptide, while the major peptide terminates in -Ser-Phe (hANP(128–149)). These results clearly demonstrate the need to fully characterize immunoreactive hANP peptides purified by RPLC by utilizing techniques which determine the integrity of both the amino and carboxyl terminus.

The cause of the observed carboxy-terminal processing of the secreted hANP is not known. However, since the primary translation product of the gene fusion should terminate with -Ser-Phe-Arg-Tyr (Fig. 3B), the loss of the -Arg- and -Tyr- residues could result from an endoproteolytic cleavage of the -Phe-Arg- bond or successive cleavage at this terminus by a carboxypeptidase activity. The observed processing of hANP(128–151) does not appear to have been the result of nonspecific proteolytic activity since only a single form of truncated hANP was detected. However, it should be emphasized that the secreted hANP material was purified with the aid of an ANP-specific radioimmunoassay, and only hANP sequences that reacted with the antibody would be detected during purification.

For the native α-factor precursor protein, it has been proposed that an endoproteolytic cleavage on the carboxy-terminal side of the -Lys-Arg- pair in the spacer regions results in this dibasic pair being linked to the carboxy-terminal -Tyr- residue of three of the four α-factor peptides initially processed out of the precursor (1, 4) see Fig. 1). Two peptidase activities have been proposed for the removal of these extra -Lys-Arg- residues from the putative intermediate forms of the α-factor peptides. The first is a chymotrypsin-like endoproteolytic activity which cleaves the -Tyr-Lys- bond (1). The second is a carboxypeptidase activity which sequentially removes the carboxy-terminal basic amino acid residues yielding the -Tyr carboxyl terminus of the mature α-factor peptide (1, 4). It is interesting to note that the properties of the amino acids surrounding the putative cleavage sites in the two models described above (aromatic-basic; -Tyr-Lys-) are similar to that of the bond processed to give the shortened form of hANP(128–151) (-Phe-Arg- (Fig. 2B)). This correlation supports the possibility that the observed processing of the hANP(128–151) sequence may have been catalyzed by an enzyme normally involved in the maturation of the α-factor peptide.

The biological activities of the purified recombinant hANP were determined using the isolated perfused rat kidney model which measures a variety of different effects brought about by the actions of ANP peptides. Two carboxy-terminal -Arg-Tyr residues have been shown not to be absolutely required for the natriuretic activity exhibited by ANP peptides (29–31). The carboxy-terminal -Arg-Tyr residues must have formed the single disulfide bond, since this bond has been shown to be absolutely required for the biological activities of ANP peptides (10, 11). A more quantitative measure of the percentage of recombinant material which was properly folded was obtained by observing the retention time of the purified material using C18 RPLC analysis (Fig. 5C). It is known that the reduced forms of synthetic hANP(128–151) or hANP(128–149) peptides elute later than their corresponding oxidized forms shown in Fig. 5C. None of the reduced form of either recombinant hANP(128–151) or hANP(128–149) was observed using this form of analysis at the detection limits used. Combining this result with the observed biological activity data we conclude that the recombinant hANP material was secreted from yeast cells with a properly formed single disulfide bond and, therefore, does not require in vitro oxidation following purification as is required for the synthetic versions of hANP peptides.

We are currently attempting to elucidate the mechanisms responsible for the observed carboxy-terminal processing of hANP(128–151). We feel that these investigations will not only help in improving the utility of S. cerevisiae in producing heterologous protein but will also provide greater insight into the mechanisms of peptide hormone processing in this organism.

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