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PRODUCTION OF A SOLUBLE AND SECRETED ANTIGENIC FRAGMENT OF HBsAg IN YEAST

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Summary: We have produced a fragment of hepatitis B surface antigen (HBsAg) corresponding to amino acids 1-60 as a fusion protein with the alpha mating factor of yeast. The product is secreted from yeast as a soluble monomer that expresses HBsAg antigenicity. Unlike other heterologous fusion proteins, it is not processed by the Lys-Arg endoprotease, possibly due to a proline in the linker between the two coding sequences. The resulting soluble fragment will enable us to map the immunodominant sites of HBsAg recognized by T cells and to identify additional factors contributing to vaccine potency.

Immunization with the surface antigen of hepatitis B virus, HBsAg, elicits protective immunity against subsequent infection (1,2). The vaccine is immunogenic over the broad range of human HLA types, and certain epitopes may predominate in the helper T cell response to HBsAg and contribute to vaccine potency (3-5). Since T cells respond to antigen only after antigen processing (6, 7), the immunodominant epitopes ordinarily could be mapped using proteolytic fragments (8, 9) or synthetic peptides corresponding to the known sequence (10). But due to its resistance to enzymatic degradation (11, 12) and its hydrophobic amino acid sequence, most fragments and synthetic peptides of HBsAg are insoluble and difficult to introduce into cell culture without toxicity.

Abbreviations used: yeast alpha mating factor, MFalpha; hepatitis B virus surface antigen, HBsAg; human major histocompatibility complex, HLA; fragment 1-60 of HBsAg, F1A.

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The alpha mating factor of yeast (MF alpha) is a well characterized protein that is transported out of the cell by a specialized secretory pathway (13- 15). When heterologous protein sequences are linked to MF alpha, they are also secreted (16, 17). Since native HBsAg is efficiently synthesized in yeast but not secreted (18), we reasoned that a hybrid between HBsAg fragment 1-60 (termed fragment F1A) and the mating factor would be synthesized and secreted by yeast. In fact, the MF-F1A fusion protein was secreted as a soluble hybrid protein, which has been fairly easy to purify while retaining HBsAg antigenicity as measured in a Western blot.

Materials and Methods

Bacteria and Yeast: E. coli strain DH5alpha was obtained as transformation competent cells from Bethesda Research Labs, Bethesda, MD, and was selectable in LB medium with ampicillin. S. cerevisiae haploid strain 2602 (MAT alpha ura3-52 leu 2,3-112 his 6 K_1+), a gift of Dr. Scott Emr, Cal. Tech., Pasadena, CA, was selectable in synthetic complete medium lacking uracil.
HBsAg gene fragment F1A: Synthetic oligonucleotide primers were produced on an automated DNA synthesizer (Applied Biosystems) and purified on a C14 HPLC column. The 5′ primer (Table IA) contains a Bam H1 restriction site followed by an additional base to maintain the reading frame and a 21 base overlap with the amino terminus of HBsAg, and the 3′ primer has a termination codon and Hind III site following a 21 base overlap corresponding to amino acids 54 to 60 of HBsAg. They were used to amplify the F1A fragment of HBsAg DNA in 35 rounds of polymerase chain reaction in a Cetus-Perkin Elmer thermal cycler. The DNA product was phenol extracted, cut with Bam H1 and Hind III, and electrophoresed on a 1.7% agarose gel, yielding a 180 bp band corresponding to the F1A fragment.
pAlpha-F1A expression plasmid: The plasmid palpha F-X (a gift of Dr. Scott Emr) (16) was digested with BamH1 and Hind III and phenol extracted. Plasmid DNA (0.375 ug) was mixed with fragment F1A DNA (0.19 ug) and annealed by heating to 70^{o} for 5 min, followed by slow cooling to room temperature. They were ligated with 2 units of T4 DNA ligase for 14 hours at 14^{o}, which was stopped by heating to 70^{o}.
Bacterial transformation: Competent DH5alpha were transformed with palpha-F1A by the heat shock method (19). Transformants were selected on LB agar in 100 ug/ml Ampicillin and screened by colony hybridization with a ^{32}P-labeled probe coding for HBsAg (20).
Yeast Transformation: S. cerevisiae haploid strain 2602 was transformed with p alpha-F1A by the lithium acetate method (21). Briefly, yeast were grown to OD_{600} of 1.5, approximately 2 X 10^{6} cells/ml, washed three times with Tris EDTA, resuspended in 1 ml of 100 mM lithium acetate in Tris EDTA. and incubated 1 hr at

30°. After adjusting the yeast to 1 X 10^8 cells/ml, 200 µl were incubated with 10 ug of CsCl purified palpha-F1A DNA for 30 min at 30°, followed by resuspension in 1.5 ml of 35% PEG 4000 and incubation for 1 hr at 30°. The cells were heat shocked 5 min at 42°, then they were incubated 50 minutes at 30°, washed in distilled H_2O, and plated in SCM agar without uracil. Out of 160 colonies screened with an HBsAg-specific probe, 115 were positive for F1A insert DNA. Of these 10 were screened for expression of MF-F1A fusion protein by SDS PAGE of concentrated culture supernatants and one was found to secrete the fragment at high levels.

Electrophoretic detection of F1A protein: Following centrifugation of yeast from the culture, the supernatant (200 ml) was lyophilized and resuspended in 1 ml of 0.05 M Tris-HCl pH 7.5 at a protein concentration of 0.4 mg/ml. This material was run on a 15% SDS PAGE gel and either silver stained (22) or transferred to nitrocellulose and probed with guinea pig antibodies to denatured HBsAg (a gift of Dr. James Shih, Dept. of Transfusion Med., NIH). Bound antibodies were detected with affinity purified goat anti-guinea pig antibodies coupled to horseradish peroxidase (Kierkegaard and Perry Labs, Inc., Gaithersburg, MD).

Results

The yeast plasmid p alpha F-X is a shuttle vector capable of replication in both E. coli and yeast (Fig. 1). It contains pBr322 for growth in E. coli, with Ampicillin selection, and the 2-µm replication origin for yeast, with Ura-3 for selection. The F1A insert is located downstream from the alpha mating factor promoter, in the same reading frame, and is followed by the polyadenylation site of hepatitis B virus. This arrangement should yield a secreted form of fragment F1A, under control of the MF alpha promoter.

The size of the F1A DNA insert was determined by digestion with Bam H1 and Hind III, and its correct orientation was demonstrated by digestion with Pst I outside the insert and Ava II inside the insert (not shown). The DNA sequence at the hybrid junction was determined by dideoxy sequencing (23), starting from a primer 48 bp upstream and extending through the hybrid junction. As shown in Fig. 2, the sequence confirmed that the insert was in the correct orientation and reading frame. At the hybrid junction, the mating factor sequence 1-87 was linked through the tetrapeptide linker Pro-Gly-Asp-Pro to HBsAg sequence 1-60 (Table IB).

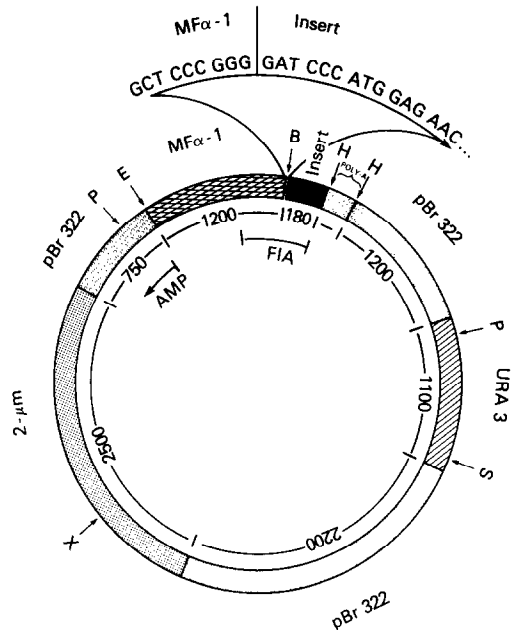

Fig. 1. Plasmid Construction. Yeast expression vector p alpha-F1A contains the yeast 2-μm origin of replication, the yeast URA3 gene as a selectable marker, the bacterial origin of replication and the ampicillin resistance gene. Fragment F1A (coding for amino acid residues 1-60 of HBsAg) and the polyadenylation site of hepatitis B virus were inserted downstream from the alpha mating factor, in the same reading frame, in order to form a fusion protein for secretion. The DNA sequence of the junction region is shown in detail. Restriction sites relevant to the construction are shown (B=Bam H1, H= Hind III).

Yeast transformed with the plasmid were grown to 4 OD_{600}, and the culture supernatants were concentrated and run on SDS-PAGE gels to determine the size of the secreted product (Fig. 3). The fusion protein gave a band at 16 Kd that was not detected in control cultures. This is consistent with the size of the alpha mating

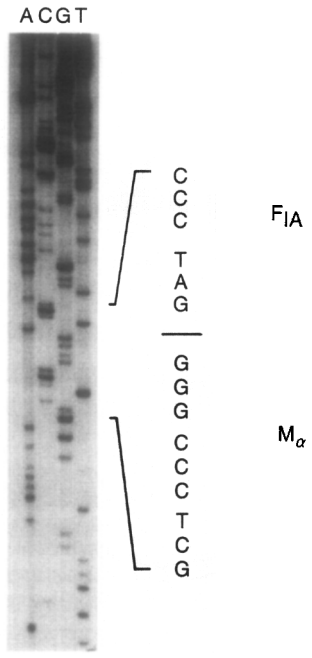

Fig. 2. DNA sequence of the F1A insert. Dideoxy sequencing of pa-F1A plasmid using a synthetic 21-nucleotide primer complementary to the mating factor sequence and reading through the hybrid junction indicates that the insert is in the correct orientation and reading frame to produce a fusion protein between mating factor and the F1A fragment 1-60 of HBsAg, with the linker shown in Table IB.

factor (10 Kd) and the F1A fragment (6 Kd), in the absence of normal processing, but is surprising in comparison with most other proteins produced this way (24). Based on the protein concentration of the 200-fold concentrated supernatant (0.4 mg/ml), we estimate the yield of F1A was 1-2 μg/ml, consistent with the levels reported for other heterologous proteins secreted from yeast (24). The MF alpha-F1A fusion protein was secreted into the culture medium and was readily soluble in aqueous solutions.

The F1A fusion protein was tested for antigenicity in an immunoblot (Fig. 3). Guinea pig antibodies to denatured HBsAg

TABLE I

A.	Oligonucleotide Primers	Restriction Site
	#12A 5′ GCG GGA TCC CAT GGA GAA CAT CAC ATC AGG A 3′	Bam HI
	#13A 5′ GCG AAG CTT TTA TGA GTG ATT GGA GGT TGG GGA 3′	Hind III
B.	Predicted amino acid sequence at the hybrid junction:	
	Lys-Arg-Glu-Ala-Pro-Gly-Asp-Pro-Met-Glu-Asn	
	—— MF_α ——→ linker ←—— F1A ——	

detected the 16 Kd band with intensity nearly equal to that for full length HBsAg, while there was no band with culture supernatants from control yeast expressing mating factor without the insert. This result demonstrates that the fusion protein expresses antigenic determinants of HBsAg.

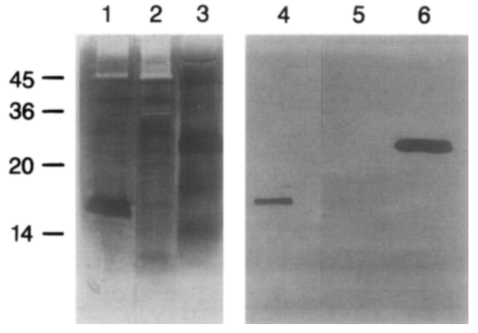

Fig. 3. Expression and secretion of the fusion protein. SDS-Page analysis of yeast culture supernatants containing the p alpha-F1A plasmid (lane 1 and 4) or the p alpha plasmid without F1A (lanes 2 and 5) or 0.5 ug HBsAg controls (lanes 3 and 6). Lanes 1 to 3 were stained with silver stain, while lanes 4 to 6 were transferred to nitrocellulose, and detected with guinea pig antisera prepared against denaturated HBsAg, followed by horseradish peroxidase labeled goat anti-guinea pig immunoglobulin. Molecular weight markers are indicated on the left.

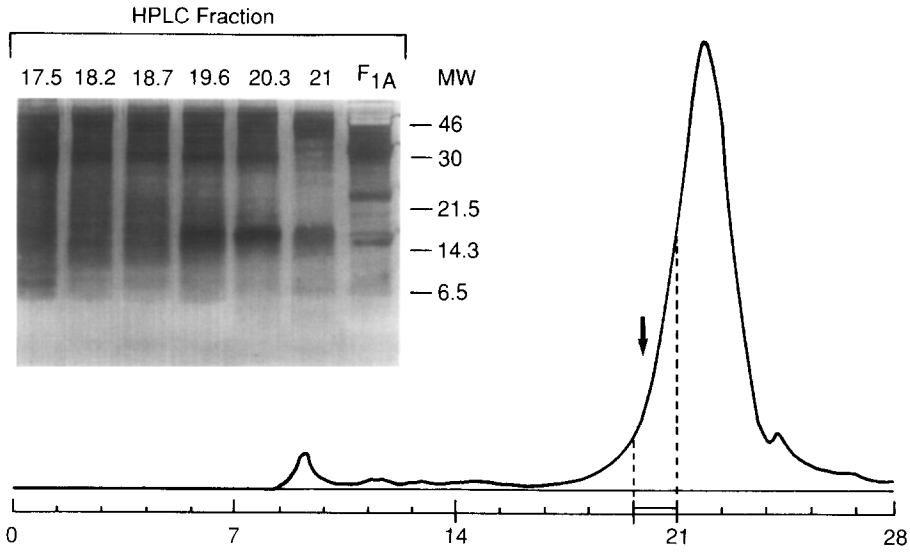

Fig 4. Molecular size of MF alpha-F1A hybrid protein. Concentrated culture supernatants (0.2 ml) were applied to a 28 ml size exclusion HPLC column of Spherogel TSK 3000 SW and eluted with phosphate buffered saline pH 6.5 in 1 M urea, and 0.7 ml fractions were collected. Each fraction was analyzed by SDS PAGE gel (insert), and the F1A peak was detected in fractions corresponding to 20.3- 21 ml elution volume. MW standards of 17 Kd, 44 Kd, and 158 Kd were run in parallel and eluted at 19.7, 16.6, and 13.7 ml, respectively.

F1A was partially purified and its native size determined by size exclusion chromatography on a Spherogel TSK 3000 SW HPLC column in phosphate buffered saline at pH 6.5 with 1M urea. Each fraction was analyzed by PAGE gel (Fig.4), and the MF alpha-F1A antigen was recovered in fractions 20-21 out of a total column volume of 28 ml. Based on MW standards, this corresponds to a MW of 15 Kd, indicating that MF alpha-F1A ran as a monomer under these conditions.

Discussion

The MF alpha-F1A product consists of yeast mating factor alpha precursor (87 amino acids) linked to amino acids 1 to 60 of HBsAg

via a 4 amino acid linker. The mating factor contributes secretory signals to the hybrid and then, because it is not processed, also contributes to the solubility of the product. The resulting hybrid expresses HBsAg antigenicity in a soluble form, facilitating purification from culture supernatants. It is monomeric in solution and retains antigenicity as measured by Western blot.

Our most unexpected finding was the failure of KR protease to process the fusion protein into separate MF alpha and F1A fragments. The sequence at the hybrid junction (Table IB) may explain the failure to cleave. Normally, the Lys-Arg processing site is followed by three repeats of Glu-Ala, but in this construct there is only one repeat, followed by a Pro. This result is similar to a previous report (25) in which two Glu-Ala repeats followed by Tyr resulted in a loss of KR processing, so the secreted product was the intact fusion protein, with MF alpha still attached to the heterologous protein. Both results suggest a role of Glu-Ala repeats in constituting the substrate site for KR protease.

HBsAg is an important example of a potent vaccine antigen in man that elicits a protective response over a broad range of HLA types. If we can identify the factors contributing to immunogenicity, we may be able to apply them to improving the potency of other vaccines. One goal of this work is to map the predominant epitopes of HBsAg that are recognized by T cells during immunization and drive the response. Celis has reported that an immunodominant epitope resides within F1A at residues 19-28 (3, 4) for HLA-DPw4 restricted T cell clones. We have also derived human T cell clones from vaccinees (5), restricted to HLA-DR6 and DR7, but preliminary experiments show that the DR7 clone fails to respond to this epitope while giving a strong response to native HBsAg (> 800 times background), indicating the presence of other

epitopes elsewhere. It is unlikely that the great diversity of human HLA types would all present the same epitope, but fragments like F1A will be useful in discovering the predominant epitopes, particularly if there are immunological hot spots.

Another factor in the potency of HBsAg vaccine may be efficient antigen processing. We have previously found that HBsAg can enter either of two processing pathways (5). Since T cells recognize antigen only after processing into antigenic fragments capable of binding the MHC groove (6, 26, 27), it is likely that fragments as large as F1A may contain one or more epitopes and that F1A may require further processing (28, 29). By modifying the p alpha-F1A plasmid DNA, we could identify the features of F1A structure, such as size, hydrophobicity, membrane permeability and sequence, which contribute to entry and exit from each processing pathway.

Acknowledgments

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