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Influenza viral (A/WSN/33) hemagglutinin is expressed and glycosylated in the yeast *Saccharomyces cerevisiae*

(recombinant DNA/shuttle vector/alcohol dehydrogenase I promoter/glycosylation)

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ABSTRACT **Recombinant plasmids were constructed in which genes coding for either the entire or the signal-minus (amino acid residues 2–17 deleted) hemagglutinin (HA) of WSN influenza virus were placed under the control of the alcohol dehydrogenase I gene promoter of *Saccharomyces cerevisiae*. Both recombinant plasmids were shown to direct the synthesis of HA-specific polypeptides that were detected by immunoprecipitation with antiviral antibodies. The complete HA produced in yeast had an approximate M_r of 70,000 and was glycosylated, as determined by the endoglycosidase H sensitivity, and was bound to membrane. Therefore, the complete HA polypeptide possessing the signal sequence probably traversed the yeast secretory pathways. Signal-minus HA, on the other hand, had a lower molecular weight and was nonglycosylated. The specific binding of yeast HA with antiviral antibodies could be competitively inhibited by influenza viral HA, demonstrating that the HA produced in yeast contained antigenic determinants of the native viral HA.**

Influenza virus, a well-known human and animal pathogen, still causes pandemics and major epidemics in humans and animals. It is a segmented negative-strand enveloped RNA virus that codes for two membrane glycoproteins: hemagglutinin (HA) and neuraminidase. HA is quantitatively the major surface glycoprotein of influenza virus and the antigen against which neutralizing antibodies are elicited (see ref. 1). The advent of recombinant DNA techniques has aided greatly in understanding the structural features that determine the biological and antigenic properties of HA of influenza virus (see ref. 2). Moreover, the expression of HA of influenza virus in *Escherichia coli* has generated a great deal of interest in developing the recombinant HA as subunit vaccine against influenza (2, 3). However, lack of glycosylation by the bacterial host appears to affect the stability, secondary and tertiary structure, as well as the antigenicity of the HA (3). Therefore, an alternative host, such as yeast, would be desirable for expressing this important viral glycoprotein. Furthermore, since the yeast (*Saccharomyces cerevisiae*) is known to cause glycosylation and to possess complex protein secretory pathways (4) similar to those present in eukaryotic cells, it may be possible to overcome many of the difficulties encountered in expressing integral membrane glycoproteins in *E. coli*.

Many heterologous genes, such as those coding for interferons-α (IFN-α) (5–7), IFN-γ (8), the hepatitis B surface antigen (9, 10), and calf prochymosin (11, 12), have recently been expressed in *S. cerevisiae*. Proteins, like IFNs and chymosin, are typical examples of secretory proteins in mammalian cells. Recent studies have also shown that foreign eukaryotic proteins containing signal sequences are processed in yeast (13, 14). For example, when human pre-IFNs were expressed in yeast, a large fraction of the IFN-α-1 and IFN-α-2 polypeptides had the same amino termini as that of the mature IFN in human cells, suggesting that yeast was able to remove the same signal sequence processed by human cells (13). Moreover, a plant protein, thaumatin, has recently been expressed in yeast and the signal sequence of preprothaumatin is cleaved at the site used in plant cells (14). However, none of these proteins is an integral membrane protein requiring anchorage to the plasma membrane.

In the present study, the HA gene of influenza virus has been placed under the control of alcohol dehydrogenase I (ADHI) promoter of *S. cerevisiae*. A recombinant plasmid that retains the DNA encoding the hydrophobic signal sequence is shown to direct the synthesis of HA as glycosylated protein, whereas another recombinant plasmid that lacks the DNA for signal sequence produces nonglycosylated HA polypeptide.

MATERIALS AND METHODS

Organisms and Growth Conditions. *E. coli* 294 (*end-A, thi, hsdR*) was used for plasmid transformation and isolation. *S. cerevisiae* 20B12 (*α, trp-1, gal-7, SUC, pep 3-2*) (13) was used as recipient for transformation by recombinant yeast plasmids. The compositions of minimal medium (SD medium) and rich (YPD medium) medium have been described (15). Influenza virus A/WSN/33 (HIN1) was used and grown in Madin–Darby (MDBK) bovine kidney cells (16).

Plasmid Vectors and Recombinant DNA Technology. Two-micron-based yeast shuttle plasmid vector containing ADHI promoter and terminator was obtained from Wyeth. Restriction endonuclease digestion and ligation with T4 DNA ligase were conducted as recommended by the suppliers. DNA-mediated transformation of yeast (17), plasmid purification, agarose gel electrophoresis, and other manipulations of nucleic acids were performed by standard methods (18).

Analysis of HA-Specific mRNA by RNA Transfer Blot and Dot-Blot Hybridization. Total and poly(A)$^+$ mRNAs were isolated, glyoxal-denatured, electrophoresed, and transferred to GeneScreen paper (New England Nuclear) as described (19). For dot-blot hybridization, denatured RNA samples were spotted onto GeneScreen. Blots were hybridized to ^{32}P-labeled HA cDNA-specific probes (2 ng/ml, 1.0 $\times$ 10^5 cpm/ng) obtained by nick-translation and were autoradiographed. For quantification, specific spots on the blot were excised and counted for radioactivity in a liquid scintillation counter.

Immunoprecipitation and Endoglycosidase H (endo H) Treatment of the Hemagglutinin Antigens. Trp$^+$ yeast trans-

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Abbreviations: HA, hemagglutinin; S$^-$ HA, signal-minus HA; ADH, alcohol dehydrogenase; IFN, interferon; MDBK, Madin–Darby bovine kidney; endo H, endoglycosidase H.

formants were grown at 30°C in SD selective medium [SD medium supplemented with uracil (20 μg/ml), adenine (20 μg/ml), tyrosine and phenylalanine (50 μg/ml)] to mid-exponential phase (A_{600} = 1.0–1.5). The cells were centrifuged, resuspended in fresh SD selective medium to an A_{600} of 2.0, and labeled with either [^{35}S]methionine or [^{35}S]cysteine (100 μCi/ml; 1 Ci = 37 GBq; 37°C for 2 hr). At the end of the labeling period, the cells were pelleted and washed once with 5 ml of 10 mM NaN_3 solution. Yeast cell lysates were prepared and immunoprecipitated as described (20) except that protein A-Sepharose was used to collect the immune complexes. Either the rabbit polyclonal antibodies made against the whole virus particles or the murine monoclonal antibodies made against PR/8 HA were used (21). The immune complexes were analyzed by $NaDodSO_4$/10% polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE). endo H treatment was done as described (20).

Isolation of Spheroplast and Preparation of Extracts. Trp$^+$ yeast transformants were grown in SD selective medium to an A_{600} of 1.5. Ten milliliters of the labeled cells was centrifuged, suspended in 4 ml of spheroplast buffer [1.2 M sorbitol/50 mM phosphate, pH 7.2/15 mM 2-mercaptoethanol/100 μg of zymolyase (60,000 units/g; Seikagaku Koygo, Tokyo, Japan) per ml], and incubated for 1 hr at 30°C with gentle shaking (10). The spheroplasts were pelleted (1000 × *g* for 10 min), washed three times with 6 ml of spheroplast buffer without zymolyase, and lysed by adding 1 ml of phosphate-buffered saline (P_i/NaCl) in the presence of 1 mM phenylmethylsulfonyl fluoride on ice for 30 min. The pellet fraction containing membranes was collected by centrifugation at 5000 × *g* for 20 min (22). The membrane fractions were further washed once in 2 ml of P_i/NaCl, solubilized in $NaDodSO_4$ (1%), and analyzed by $NaDodSO_4$/PAGE (20).

RESULTS

Construction of the Recombinant Plasmids for the Direct Expression of the HA Gene. The strategy for the construction of the expression plasmids containing both complete and signal-minus HA (S$^-$ HA) cDNA is outlined in Fig. 1*A*. The junction sequence of the promoter and the HA genes is shown in Fig. 1*B*. The HA gene of WSN virus has been cloned (24) and its complete nucleotide sequence has been determined (25). The WSN HA gene codes for a polypeptide of 565 amino acids from which the signal peptide (17 amino acids) at the amino terminus is cleaved to produce the mature HA. The complete HA insert was treated with BAL-31 to remove the first 14 nucleotide residues, including 4 guanosine residues (positions 11–14) in the noncoding region of the HA (25), as they may interfere with efficient translation initiation in yeast (26). Subsequently, the HA cDNA fragment was treated with *Eco*RI methylase to protect the internal *Eco*RI site. *Eco*RI linkers were added and the insert was cloned into the *Eco*RI site of pBR322. The full-length HA cDNA was obtained by partial *Eco*RI digestion and cloned into the *Eco*RI site of pWY4, a shuttle vector that can replicate both in *E. coli* and in *S. cerevisiae*. *E. coli* 294 was transformed with the recombinant plasmids containing the HA cDNA and ampicillin-resistant colonies were identified. The clones were further screened for the orientation of the HA inserts towards the ADHI promoter. Plasmids containing the correct (pWYHAC51) and the opposite (pWYCAH50) orientations were identified. A similar procedure was used for constructing recombinant plasmids containing the cDNA insert of the S$^-$ HA under the control of ADHI promoter. For the initial construction of S$^-$ HA, a synthetic DNA primer was used to remove the DNA sequences encoding the signal peptide and to add a synthetic ATG initiation

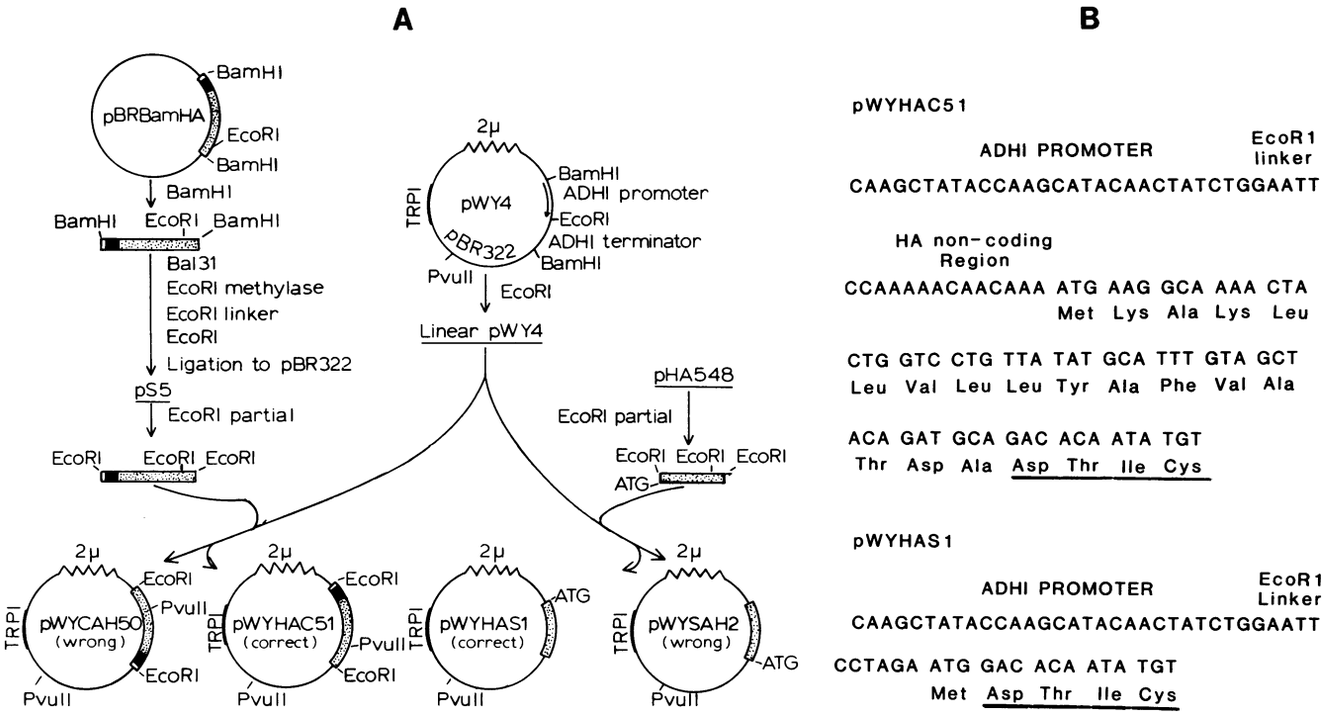

FIG. 1. (*A*) Construction of yeast plasmid vector containing complete and S$^-$ HA cDNA insert of A/WSN/33 virus. The complete HA cDNA was cloned into the *Eco*RI site of the shuttle vector (pWY4) containing the ADHI promoter and terminator. The colonies were screened for correct (pWYHAC51) and wrong (pWYCAH50) orientations. Similarly, S$^-$ HA cDNA was obtained from pHA548 and ligated into pWY4 to obtain pWYHAS1 (correct orientation) and pWYSAH2 (wrong orientation). DNA represents the 5′-noncoding region (▭), signal region (■), and mature HA (▨). (*B*) Nucleotide sequence of the junction of the yeast ADHI promoter, *Eco*RI linker, and either the complete or S$^-$ HA cDNA. S$^-$ cDNA lacks 48 nucleotides coding for the 16 amino acids (residues 2–17) of the signal sequence of complete HA (23). Amino-terminal amino acid sequences of complete and S$^-$ HA polypeptide are also shown. The first 4 amino acids of the mature HA polypeptide are underlined.

codon before the codon of aspartic acid, the first amino acid of the mature HA polypeptide (23). The correct- and opposite-orientation plasmids for S^- HA were named pWYHAS1 and pWYSAH2, respectively. Yeast strain 20B12 was transformed by these recombinant plasmids and the transformants harboring these plasmids will be referred to as 20B12/pWYHAC51, 20B12/pWYCAH50, 20B12/pWYHAS1, and 20B12/pWYSAH2.

HA-Specific mRNAs Are Transcribed from the Chimeric Plasmids. Since all of the recombinant plasmids carry the wild-type *trp* allele, tryptophan-independent transformants were obtained with all four plasmids carrying either the entire or the S^- HA inserts in both orientations. To determine if the insert HA cDNA in either orientation was transcribed, poly(A)$^+$ RNA was isolated and analyzed by RNA transfer blot and by dot-blot hybridization (19) with ^{32}P-labeled nick-translated HA cDNA probe. The RNA analysis by both procedures showed that mRNA containing HA sequences were transcribed from all four plasmids. Similar results were obtained whether the total RNA or the poly(A)$^+$ RNA was used in analysis. The size of the HA-specific mRNA (≈2.0 kilobases) indicated that both the yeast promoter and the yeast terminator flanking the HA insert were used and that the HA insert was transcribed in both orientations as expected. Furthermore, RNA estimation by dot-blot analysis showed that approximately 0.11%, 0.24%, 0.29%, and 0.20% of the total RNA was HA-specific in 20B12/pWYHAC51, 20B12/pWYHAC50, 20B12/pWYHAS1, and 20B12/pWYSAH2, respectively. Yeast transformants containing the complete HA insert in correct orientation consistently yielded a lower percentage of HA-specific mRNA when compared to the other three transformants.

HA Polypeptides Are Expressed from the Chimeric Plasmids. To determine if HA-specific polypeptides were made in 20B12/pWYHAC51 and 20B12/pWYCAH50, they were labeled with [^{35}S]methionine or [^{35}S]cysteine. The cell lysates were immunoprecipitated with rabbit polyclonal anti-WSN antibodies that have been preadsorbed with the cell extract made from a yeast strain without the HA insert and were analyzed by NaDodSO$_4$/PAGE (Fig. 2). A specific broad heterodisperse polypeptide (HAC51 polypeptide) was present in the lysate of 20B12/pWYHAC51 (Fig. 2, lanes B and F) but was absent in the lysate of 20B12/pWYCAH50 (Fig. 2, lanes C and G), indicating that plasmid pWYHAC51 directs the synthesis of a polypeptide that should be HA. Furthermore, the HAC51 polypeptide was not precipitated by normal rabbit serum (data not shown) and migrated at the same position as the HA made in MDBK cells infected with WSN virus (Fig. 1, lanes A and H). Also, a number of independently isolated yeast transformants carrying the HA insert in correct orientation exhibited the same polypeptide after immunoprecipitation, but none of the yeast transformants carrying the HA insert in wrong orientation demonstrated the presence of this specific polypeptide (data not shown). Furthermore, to test the association of HA polypeptides with pWYHAC51 plasmids, a number of segregants that are cured of the plasmid pWYHAC51 were isolated and five of them were analyzed. None of these tryptophan-requiring segregants produced the HAC51 polypeptide. This would also strongly indicate that the HAC51 polypeptide was pWYHAC51 plasmid-borne. Similarly, the S^- HA polypeptide appears to be synthesized in yeast harboring pWYHAS1 (correct orientation) (Fig. 2, lane D) but not in yeast containing pWYSAH2 (wrong orientation) (Fig. 2, lane E). The complete and S^- HA polypeptides were also immunoprecipitated by monoclonal antibodies. However, the common yeast polypeptides were also present in these lysates.

Complete HA Expressed in Yeast Is Glycosylated and Membrane-Bound. The heterodisperse nature of the HAC51 polypeptide present in cells transformed with plasmid carrying the full-length HA cDNA could be due to the glycosylation of HA since secretory and other glycoproteins are known to be glycosylated as they pass through the secretory pathways in *S. cerevisiae* (4). *S. cerevisiae* has been shown to produce N-linked glycosyl chains similar to the mannose-rich core oligosaccharides attached to the asparagine residue in mammalian glycoproteins (27). However, unlike mammalian systems, which add complex sugars at a later step during protein transport (28), the yeast system synthesizes only high mannose-type sugars (29). To determine if the HA produced in yeast is also glycosylated, the immunoprecipitate from 20B12/pWYHAC51 cells was exhaustively digested with endo H to remove N-linked carbohydrate chains from the peptide backbone (30). Fig. 3 shows that the endo H treatment produces a discrete band (lane C) that migrates faster than the untreated HA (lane D) but slightly slower than the S^- HA (lane A). Slower mobility of the endo H-treated yeast HA compared to S^- HA polypeptide is probably due to two or three sugar residues that still remain attached to the polypeptide after the endo H treatment. These results demonstrate that the influenza viral HA produced in yeast is glycosylated in the same way as are the resident secretory and other glycoproteins. In mammalian cells, we and others have often observed two species of HA polypeptides (31, 32) (Fig.

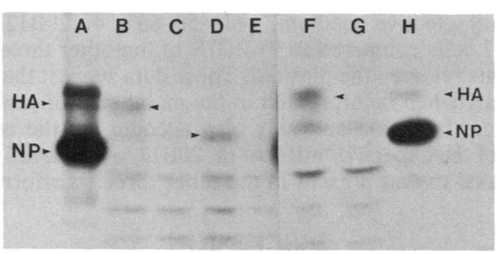

FIG. 2. Expression of influenza virus WSN HA polypeptide in yeast. The transformants were labeled with either [^{35}S]cysteine (lanes B–E) or [^{35}S]methionine (lanes F and G), lysed, immunoprecipitated, and analyzed by NaDodSO$_4$/PAGE. Lanes A and H, [^{35}S]methionine-labeled lysate of WSN-infected MDBK cells was used as a positive control. Lanes B and F, 20B12/pWYHAC51, complete HA in correct orientation; lanes C and G, 20B12/pWYCAH50, complete HA in wrong orientation; lane D, 20B12/pWYHAS1, S^- HA in correct orientation; lane E, 20B12/pWYSAH2, S^- HA in wrong orientation. Lanes A–E and F–H represent two separate runs. Arrowheads indicate the positions of the complete and S^- HA polypeptides. NP denotes the nucleoprotein of WSN virus.

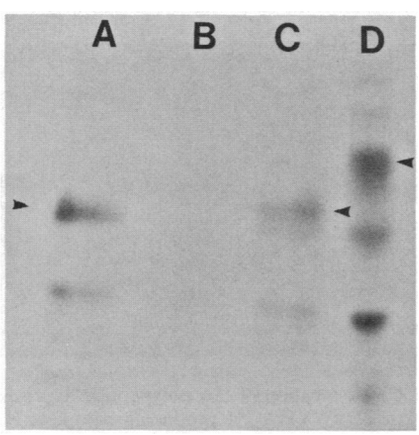

FIG. 3. endo H treatment of HA polypeptide expressed in yeast. [^{35}S]Methionine-labeled yeast lysates were immunoprecipitated and treated with endo H. Arrowheads indicate the positions of glycosylated HA and endo H-treated HA. 20B12/pWYHAS1 extract with (lane B) and without (lane A) endo H treatment. 20B12/pWYHAC51 extract with (lane C) and without (lane D) endo H treatment.

2, lanes A and H) that possibly represent partially and completely glycosylated forms of HA (32). The HA synthesized in yeast comigrates with the partially glycosylated form of HA.

WSN HA polypeptide contains a hydrophobic signal peptide at the amino terminus and another hydrophobic region of 26 amino acids at the carboxyl terminus, which functions for anchoring the polypeptide to the lipid bilayer of the membrane (25). Since the HA produced in yeast is also glycosylated, we wanted to determine the cellular localization of the glycosylated HA. Accordingly, 20B12/pWYHAC51 cells were subjected to both mechanical and enzymatic fractionation procedures and cellular fractions were immunoprecipitated. HA appears to be predominantly localized in the membrane fraction of the yeast cells (Fig. 4, lanes A and D). However, mechanical breaking of the cells does not clearly separate HA into membrane or cytoplasmic fractions, even though the membrane fraction (lane A), when compared with the cytoplasmic fraction (lanes B and C), appears to be enriched in HA. The enzymatic separation, on the other hand, clearly identifies the HA in the membrane fraction (Fig. 4, lane D) since little or no HA is detected in the cytoplasmic or periplasmic fractions (lanes E and F). Preliminary experiments to demonstrate the presence of HAC51 polypeptide on the surface of the yeast spheroplast by hemagglutination and hemadsorption have been negative. Thus, although membrane-bound and glycosylated, the subcellular location of HA is yet to be determined. Further experiments are needed to analyze in detail the steps in the yeast secretory pathways traversed by influenza HA.

Yeast HA Retains the Antigenic Determinants of the Viral HA. To confirm that the HA made in yeast is antigenically related to viral HA, immunocompetition of the yeast HA by the viral HA was performed. Unlabeled lysate of MDBK cells infected with WSN virus was used as the competing antigen against the labeled HAC51 polypeptide. Fig. 5 shows that the immunoprecipitation of the HAC51 polypeptide was competitively inhibited by the unlabeled viral HA, demonstrating that the HA produced in yeast contains the antigenic determinants of the native viral HA.

Growth Characteristics of Yeast Transformants. When grown in tryptophan-free SD selective medium, three transformants—namely, 20B12/pWYCAH50, 20B12/pWYHAS1, and 20B12/pWYSAH2—had almost an identical growth pattern, with a doubling time of 2.5 hr at 30°C. However, 20B12/pWYHAC51 (complete HA in correct orientation) grew slowly with a generation time of ≈3.5 hr.

Yeast transformants carrying the 2-μm origin plasmid are known to lose the plasmid frequently when grown nonselectively. To determine the segregational loss of the plasmid from transformants carrying the HA insert, transformants were grown nonselectively in rich medium (YPD medium) overnight and cells were plated on the tryptophan-containing SD medium to obtain individual colonies. These colonies were then tested for their ability to grow on tryptophan-free SD selective medium to determine retention of the recombinant plasmids. Ninety-five percent of the 20B12/pWYHAC51 and 76% of the 20B12/PWYCAH50 cells lost the plasmid. This indicates that cells expressing the complete HA polypeptide tend to lose the plasmid more frequently than cells that do not produce HA. Stability of plasmids in selective medium was also determined for all four yeast transformants. Accordingly, each transformant was grown to a logarithmic phase in tryptophan-free SD selective medium and cells were plated on both nonselective medium (total number of colonies) and selective medium (cells containing 2-μm origin plasmid). Our results show that when grown even in selective medium, only 50–60% of 20B12/pWYHAC51 cells compared to 95–100% of the other three transformants retained the plasmid. These data suggest that influenza HA when incorporated in the membrane of yeast may be somewhat toxic and may also account for the reduced level of HA-specific mRNA in 20B12/pWYHAC51 cells compared to that present in the other three transformants.

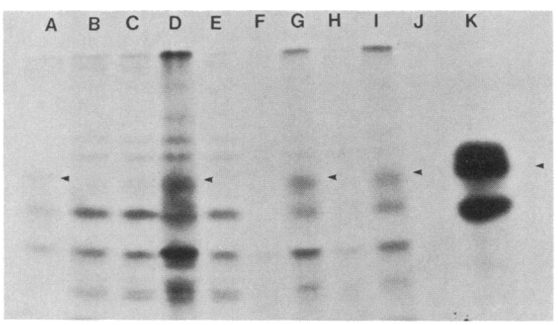

FIG. 4. Cellular location of HA polypeptide expressed in yeast. Yeast cells 20B12/pWYHAC51 producing complete HA polypeptide were fractionated either by mechanical breaking (lanes A–C) or by enzymatic treatment (lanes D–F). Arrowheads indicate the positions of HA polypeptides. Lanes: A, membrane fraction; B and C, cytoplasmic fraction; D, membrane fraction; E, cytoplasmic fraction; F, periplasmic fraction; G and I, total extract of 20B12/pWYHAC51; H and J, total extract of 20B12/pWYCAH50; K, MDBK cells infected with WSN virus.

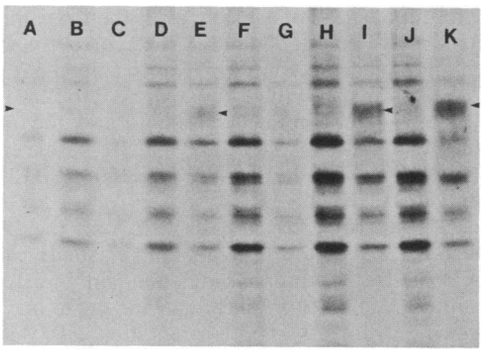

FIG. 5. Immunocompetition of HA expressed in yeast with the HA from WSN-infected MDBK cells. Polyclonal anti-WSN antibodies were preincubated with unlabeled lysate of WSN virus-infected or uninfected MDBK cells in P_i/NaCl containing 0.1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride at 0°C for 30 min. [^{35}S]Methionine-labeled extract of 20B12/pWYHAC51 was added and incubation at 0°C was continued for 2 hr. MDBK cell lysate (infected or uninfected) containing ≈250 μg of protein was used in each competition experiment. Immunoprecipitates were collected and analyzed by NaDodSO$_4$/PAGE. Uninfected MDBK cell extract was added in lanes A, C, E, G, I, and K. WSN virus-infected MDBK cell extract was added in lanes B, D, F, H, and J. Arrowheads indicate the positions of glycosylated HA expressed in yeast 20B12/pWYHAC51. Varying amounts of anti-WSN antibodies were used: 0.1 μl, lanes A and B; 0.5 μl, lanes C and D; 1.0 μl, lanes E and F; 2.0 μl, lanes G and H; 5.0 μl, lanes I and J; 10 μl, lane K.

DISCUSSION

In this report we have shown that HA, a viral integral membrane glycoprotein, is expressed in *S. cerevisiae* and that the expression is under the control of a promoter signal of the yeast ADHI gene to which the cloned HA cDNA was linked. In *E. coli*, the HA polypeptide has been expressed as a fusion protein from plasmids in which HA cDNA was fused in phase with either the *trpLE'* or *lacZ* gene of *E. coli* (23, 33). Even though in bacteria, the fusion HA is made in abundance, antibody response to this polypeptide appears to be qualitatively different from the antibodies made against the native viral HA (3, 33). The difference in the antibody re-

sponse could probably be due to an altered tertiary structure because of the presence of extra bacterial peptides, *trpLE'* or *lacZ* at the amino termini of the fusion protein (33). In *E. coli*, influenza HA could not be expressed directly, even when a strong promoter (i.e., *trpP*) was used (23). Furthermore, lack of glycosylation in *E. coli* might affect the stability, tertiary structure, and immunogenicity of the HA fusion proteins (33).

In yeast, unlike in *E. coli*, both the complete HA and the S^- HA polypeptides are expressed directly. Furthermore, the complete HA in yeast is glycosylated, whereas S^- HA is not. The carbohydrate moiety in complete HA is of high mannose type as expected for yeast. Therefore, this would suggest that the signal sequence of HA is recognized by yeast for translocation across the membrane and that the HA was traversing the yeast secretory pathways. The signal sequence of HA is possibly cleaved as in higher eukaryotic systems, although we have not directly demonstrated the cleavage of HA signal in yeast. Therefore, HA produced in yeast is likely to possess a more native conformation similar to that observed in influenza virus. The ability of the yeast HA to be immunoprecipitated by antiviral antibodies and the fact that viral HA competes with yeast HA suggest a similarity in their tertiary structure and, possibly, antigenic epitopes. Experiments are necessary to test the immunogenicity of the HA polypeptide produced in yeast as well as to determine if the yeast HA is present in the trimeric form as is found in both higher eukaryotic cells and influenza viruses.

Results reported here show that the yeast system can be used to express the glycosylated forms of complex integral membrane proteins such as influenza viral HA, and therefore, may be potentially useful for the develoment of a pure subunit vaccine. An added advantage of the yeast system is that the pathways for processing and transporting HA could be systematically explored in detail since conditionally defective mutants have been identified and the order of the events in the yeast secretory pathways have been worked out (4). Thus, it would be possible to compare the similarity and the difference in the steps involved in transport and processing of HA in lower and higher eukaryotic cells.

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