Expression of the Extracellular Domain of the Human Immunodeficiency Virus Type 1 Envelope Protein and Its Fusion with \( \beta \)-Galactosidase in \textit{Saccharomyces cerevisiae}  

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Two envelope glycoprotein gene fragments were cloned from the proviral genome of the HXB2 isolate of human immunodeficiency virus (HIV). For the production of the two domains of the envelope gene product these cloned gene fragments were inserted into an \textit{Escherichia coli}-yeast inducible shuttle vector fused to the galactokinase (GAL1) promoter. Cell extracts from strains of \textit{Saccharomyces cerevisiae} harboring these two vectors (pYENV1 and pYENV2) were found to contain a specific protein with a size of 50 kDa when induced by galactose, while the protein could not be detected in extracts from control cells containing only the \textit{E. coli}-yeast vector in the presence of galactose. Furthermore, another expression plasmid coding for fusion proteins from the majority of the external envelope glycoprotein (gp120) moiety and a large part of the \( \beta \)-galactosidase was constructed. Antibodies from HIV type 1-positive sera could react with recombinant fusion polypeptides. Transfectants could produce this fusion protein to a level of about 1.6% of the total protein content, as deduced from \( \beta \)-galactosidase activity.

Human immunodeficiency virus (HIV), the causative agent of AIDS, is a retrovirus belonging to the lentivirus subfamily. The envelope (\textit{env}) gene of HIV type 1 (HIV-1) is predicted from the DNA sequence to encode a precursor polypeptide of 856 amino acids (16). The 160-kDa glycoprotein (gp160), observed in infected cells, is the glycosylated precursor which undergoes endoproteolytic cleavage to yield the mature gp120-gp41 complex (6, 16). The envelope glycoprotein complex is anchored to the virion in infected cells and infected cell membranes through gp41, an integral membrane protein, while gp120, an external membrane protein, is attached to gp41 through non-convalent interactions (6, 9). Studies have shown that gp120 and gp41 play, as in the case of other enveloped viruses, a critical role in the interaction with the virus receptor (14) in the fusion process with the host cell membrane (13) and in syncytium formation (12). It is now becoming increasingly clear that most envelope protein functions require the interaction between nonadjacent sequences within gp120 or between gp120 and gp41 (8). Since these proteins are the primary targets for antibody-mediated neutralization and cytotoxic immunity (3), a full understanding of the role that HIV envelope proteins have in eliciting and mediating protective immunological responses is crucial in projects aiming at vaccine development.

Because the amount of HIV-1 envelope glycoprotein produced by virus-infected cells is comparatively small, and also because gp120 is readily shed from the surfaces of purified virions (9), it is not practical to purify quantities of gp120 or the gp120-gp41 complex sufficient for immunogenicity and binding studies of virus-infected cells. Fragments of gp160 have been produced in a wide variety of systems, including \textit{Escherichia coli} (13), recombinant baculovirus (20), adenovirus (5), and Chinese hamster ovary cells (2). However, either expression levels were too low or large-scale preparation was too difficult. The yeast \textit{Saccharomyces cerevisiae} has been widely used as a host organism, especially for the production of eucaryotic heterologous proteins. Unlike bacteria, \textit{S. cerevisiae} does not produce endotoxins, and products of yeast cells are considered safe for use in pharmaceutical and food products. We describe here the production of polypeptides representing the majority of the protein moiety associated with the HIV-1 isolate HXB2 envelope gene in genetically engineered yeast. The expression of a fused polypeptide located between the extracellular domain of the envelope protein and the \( \beta \)-galactosidase (\( \beta \)-Gal) has also been studied.

DNA-encoding regions of the \textit{env} gene, defined as \textit{env}A and \textit{env}B, were excised from plasmid pHXB2, which contains the HIV-1 HXB2 proviral genome (7), with appropriate restriction enzymes. For expression of \textit{env}A, plasmid pHXB2 was digested with \textit{Hind}III and a 2.11-kb DNA fragment was purified with the GeneCleanII kit (Bio 101, Inc., La Jolla, Calif.). This DNA fragment was then cloned into plasmid pGEM-3zf(+) (Promega Corp.), which was also digested with \textit{Hind}III and dephosphorylated with calf intestinal alkaline phosphatase. The resulting plasmid, pH211-ENV, was digested with \textit{Kpn}I and \textit{Hind}III. The resulting 1.8-kb DNA fragment was purified and inserted into plasmid pBV221 (21) to acquire plasmid pBV18-ENV, into which an in-frame initiation codon was introduced for the cloned fragment. Finally, this cloned 1.8-kb DNA fragment was inserted into plasmid pYES2 (Invitrogen Corp.), a galactose-inducible \textit{E. coli}-yeast shuttle vector. For \textit{env}B, plasmid pBV18-ENV was completely digested with EcoRI and then partially digested with \textit{Bgl}II. A 1.3-kb DNA fragment was purified with the GeneCleanII kit and subcloned into pBV221 digested with \textit{Bam}HI and EcoRI to produce pBV13-ENV before it was ligated to prepared pYES2 with T4 DNA ligase. Verified recombinant plasmids were used for the transformation of yeast strain BJ2168 (11) by the method of Ito and Murate (10). Yeast transformant colonies were picked and

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grown in SD-ura medium (17a) to an optical density at 600 nm of about 1.2 and induced for the expression of the envelope gene fragments by the addition of galactose. After galactose induction for different time intervals, cells were harvested and lysates were prepared from yeast cultures transformed with expression vectors and from cells transformed with control plasmids. The recombinant proteins produced by yeast were found to be almost all in the insoluble fraction, as has been found for many secreted proteins when produced intracellularly. The insoluble fractions from these lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (Fig. 1). New protein bands were seen at approximately 50 kDa in the preparations from yeasts transformed with vectors carrying either envA or envB, a mass which corresponds to the molecular mass expected from the inserted envB DNA. Unexpectedly, a new protein species with a molecular mass of 68 kDa, expected to result from the inserted envA DNA, could hardly be discerned in the Coomassie-stained gel. The new protein bands seen by Coomassie staining were also shown to be reactive with HIV-1 antibody-positive sera (data not shown).

It has already been established that specific antibodies against a protein can be induced with a β-Gal fusion molecule containing amino acid regions from that protein (18). In addition synthesis of a β-Gal fusion protein might increase the number of copies of the N-terminal antigenic determinant because β-Gal can form an enzymatically active complex of four monomeric subunits and these uniform complexes might potentially improve the immunogenic properties of the antigens. To easily detect the expression of the cloned envelope gene fragment and to lay a basis for facilitating further purification of the expressed antigen protein, we further constructed expression plasmid pYENVG12 for the expression of a fusion protein between the gp120 fragment and E. coli β-Gal. Plasmid pBV13-ENV was digested with EcoRI and SauI. The terminus produced by SauI was made blunt with a DNA polymerase I Klenow fragment, and the resulting 1.3-kb DNA fragment was purified with a GeneCleanII kit. Meanwhile, plasmid pSV-β-galactosidase (Promega Corp.) was digested with HindIII and then digested with exonuclease Bal 31 under conditions in which approximately 200 to 400 bp at each DNA terminus was removed. The plasmid was then digested with restriction endonuclease XbaI, and DNA fragments located between bp 3530 and 3330 were isolated by preparative agarose gel electrophoresis. The above fragments, including the purified 1.3-kb DNA fragment, were ligated to prepared pYES2 digested with EcoRI and XbaI to give rise to pYENVG12. Plasmid pYESGal was constructed as a control plasmid by directly fusing the β-Gal gene to the GAL1 promoter of pYES2. BJ2168 cells were transformed by selecting directly for Ura⁺ and Lac⁺ by the Li-acetate procedure. β-Gal from transformants was assayed in the presence of different concentrations of galactose. The result showed that β-Gal levels increased to reach a maximal value from which it was estimated that the produced fusion protein might represent 1.6% of total cell proteins. The maximal value depended on the initial concentration of galactose added to the culture for induction. The immunodot blot method was employed to further establish that the fused expression plasmid encoded the expected fusion protein, which was specifically recognized by HIV-1-positive serum antibodies, whereas plasmid pYESGal, with only the β-Gal gene (lacZ) under the control of the GAL1 promoter, did not (Fig. 2). The enzyme-linked immunosorbent assay data obtained by employing HIV-1 antibody-positive sera also gave the same result (data not shown).

Our results here show the cloning and subsequent expression of two env gene fragments from the proviral genome of the HXB2 isolate of HIV-1 in S. cerevisiae. Furthermore, we show for the first time that fusion proteins from the majority of the external env gp120 moiety and a large part of the β-Gal were also produced. A key feature of such a β-Gal fusion protein may be the ease with which pure fusion proteins can be prepared for use as reagents or to raise antisera (19), thus providing a rapid means of preparing and evaluating HIV antigens for a variety of immunological purposes. Although there have been reports on the secretion of HIV-1 envelope protein from S. cerevisiae and Pichia pastoris, the products were shown to be either hyperglycosylated or substantially proteolytically degradable and hardly reacted with antibodies to mammalian cell-derived material (1, 17). On the other hand, taking into consideration the potential immunosuppressive effects that may result from gp120 binding to CD4 and inhibiting T-cell function in humans, the use of immunogens containing neutralization determinants but deficient in conformation-dependent CD4 binding may be advantageous (4, 15). In addition, while Barr et al. (1) have further shown that the nonglycosylated gp120 polypeptide could elicit neutralizing antibodies in mice, results also implied that in order to elicit protective humoral immunity effective against all HIV isolates, env-encoded polypeptides from multiple HIV strains including isolate HXB2 might be required to evaluate the ability of the polypep-
tides to induce neutralizing antibodies that are effective against divergent HIV variants.

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