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Expression of human α_1-antitrypsin cDNA in the yeast *Saccharomyces cerevisiae*

(emphysema/plasma protein/molecular cloning/electrophoretic transfer blotting)

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ABSTRACT **Nucleotide sequences coding either for the precursor or the mature form of human α_1-antitrypsin have been placed under the control of the yeast *ARG3* expression signals. Recombinant plasmids pRIT10782 and pRIT10787 express the precursor or the mature α_1-antitrypsin species, respectively, in two different yeast strains, with yields ranging between 0.3 and 1% of total soluble proteins. The α_1-antitrypsin synthesized in yeasts was specifically recognized by polyclonal and monoclonal antibodies raised against human α_1-antitrypsin. In addition, it was shown to be biologically active in its mature form only, with optimal activity in a peptidase-deficient yeast strain.**

α_1-Antitrypsin, one of the main components of blood proteins, is synthesized in the liver and secreted into the plasma (1, 2). The enzyme acts as an inhibitor of various proteases, such as thrombin, plasmin, and trypsin, but its main target is elastase, a protein involved in the degradation of lung tissues (3). The association of α_1-antitrypsin hereditary deficiency with disease is well documented (4–6); deficient variants of α_1-antitrypsin are relatively frequent and are mainly confined to people of European origin. When associated with environmental factors, hereditary deficiencies lead to cumulative pulmonary damage known as emphysema (6, 7). It has been reported recently (8) that human emphysematous conditions can be relieved by intravenous injection of partially purified α_1-antitrypsin. It thus appears worthwhile to produce large quantities of human α_1-antitrypsin through recombinant DNA technology to assess its therapeutic value in animal models and possibly in human medicine. In this context, we reported previously (9, 10) the cloning of full-length cDNA coding for human α_1-antitrypsin and its expression in *Escherichia coli*.

We describe here the construction of multipurpose yeast vectors, pRIT10774 and pRIT10779, which contain the yeast *ARG3* expression signals and are appropriate for the expression of human α_1-antitrypsin. We show that the derived recombinant plasmids, pRIT10782 and pRIT10787, carrying the genetic information coding either for the precursor or for the mature α_1-antitrypsin, efficiently express the protein in two different yeast strains and that the mature product displays biological activity. We also used a set of monoclonal antibodies against α_1-antitrypsin to characterize the yeast-produced molecule in terms of antigenic specificity.

MATERIAL AND METHODS

General Methods. Restriction endonucleases, T4 DNA polymerase, and T4 DNA ligase were purchased from Boehringer-Mannheim and used as recommended by the manufacturer. *BAL*-31 and mung bean nucleases were obtained from P-L Biochemicals and used as described in Manniatis *et al.* (11).

Plasmids used in this work, pULB1523, YEp13, pMC200, and pRIT10749, have been described (9, 12–14). Yeast strains Ic1697d (*argJ*$^{\pm}$, *leu2*) and 10S44c (*leu2-3*, *leu2-112*, *pep4-3*) were grown in yeast nitrogen base medium (Difco) supplemented, when needed, with 4 μg of arginine per ml. Transformations of yeast strains and of *E. coli* strain MM294 (*endoA*, *thi*$^-$, *hsd*R, *sup*E) were done as described (15, 11). Colony hybridization experiments follow the procedure of Grunstein and Hogness (16).

Plasmid DNA purification was achieved on cesium chloride gradients and small-scale DNA preparations for rapid analysis were performed as described (11). DNA fragments were analyzed and purified either on agarose or polyacrylamide gels (11).

Preparation of Yeast Extracts. Yeast extracts for the immunoassay and for the activity assay of α_1-antitrypsin were prepared as follows: 200-ml cultures of appropriate strains were cultivated at 30°C up to OD_{620} of about 0.4. Cells were collected by centrifugation, washed in distilled water, resuspended in 10 ml of extraction buffer (50 mM Tris·HCl, pH 8/1 mM EDTA/0.1 mM cysteine), and disrupted by two passages through a French pressure cell at 12,000 psi (1 psi = 6895 Pa). Extracts were then centrifuged for 30 min at 15,000 rpm (Sorvall SS.34) to eliminate cell debris, and supernatants were assayed for protein concentration by using the procedure of Lowry *et al.* (17). Supernatants were immediately assayed or frozen at −20°C until used.

Expression Assay for α_1-Antitrypsin. Immunological detection and quantification of α_1-antitrypsin were performed by micro-ELISA using either polyclonal or monoclonal antibodies against α_1-antitrypsin as described (9, 10).

Assay for α_1-Antitrypsin Activity. The assay measures the inhibitory capacity of α_1-antitrypsin toward trypsin. It consists of a microtest using the chromogenic substrate S2444 (L-pyroglutamylglycyl-L-arginine, *p*-nitroanilide hydrochloride, Kabi Diagnostica, Stockholm, Sweden). The polystyrene microtest plates were incubated for 1 hr with 1% bovine serum albumin and washed extensively with distilled water. α_1-Antitrypsin at various concentrations was incubated with a fixed amount of trypsin for 20 min at 37°C in a 200-μl final reaction volume of (0.1 M Tris·HCl, pH 8.2/0.15 M NaCl/0.01 M EDTA/0.5% polyethylene glycol, M_r 6000). Samples were cooled slowly at room temperature and then exposed to the chromogenic substrate (20 μl of aqueous solution of 0.003 M S2444). After 5 min at room temperature, the reaction was stopped with 50 μl of 50% acetic acid.

Absorbance was read at 400 nm in a micro-ELISA automatic reader (Dynatech AM120). Calibration curves were determined for both trypsin and α_1-antitrypsin. Yeast extracts to be assayed were diluted serially in reaction buffer.

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Abbreviation: bp, base pair(s)

Electrophoretic Transfer Blotting and Immunodetection. $NaDodSO_4$/polyacrylamide gels slabs (18) were washed briefly with distilled water and washed three times for 20 min in 50 mM sodium phosphate buffer at pH 7.5. Transfer was performed on nitrocellulose sheets for 2 hr at 36 V and 1.5 A in the same buffer (transblot, Bio-Rad).

The nitrocellulose sheet was saturated with phosphate-buffered saline (0.15 M NaCl/10 mM sodium phosphate, pH 7.5) containing 0.1% Tween 80, 10% horse serum, and 1% bovine serum albumin (4 hr at room temperature). The sheet then was washed three times for 20 min each with phosphate-buffered saline/0.1% Tween 80 and incubated overnight at room temperature in saturation buffer supplemented with mouse anti-human α_1-antitrypsin serum (dilution 1:1000).

The sheet then was washed five times with 40 ml of phosphate-buffered saline/0.1% Tween 80 for about 2 hr and incubated for 4 hr at room temperature in 40 ml of phosphate-buffered saline/Tween 80 containing 10 μg of peroxidase-labeled goat anti-mouse serum. The sheet then was washed for about 2 hr in phosphate-buffered saline/Tween 80 (five times, 40 ml). Immunodetection was achieved by treating the nitrocellulose sheet for 25 min in the chromogenic substrate [10 mg of diaminobenzidine and 100 μl of urea peroxide (10%) in 50 ml of 0.1 M Tris·HCl at pH 7.6]. The sheet was finally washed with distilled water.

RESULTS

Yeast Expression Vectors. Yeast expression vectors were constructed to fulfill the following criteria: (*i*) to be able to replicate and be selected for in both *E. coli* and yeast; (*ii*) to contain an expression "cassette" in which sequences necessary for initiation and termination of transcription in yeast are separated by a unique restriction site; (*iii*) to allow expression of sequences whether or not they contain their initiation codon; (*iv*) to have a modular design allowing exchange of the regulatory sequences used for expression.

The *E. coli*/yeast shuttle vector YEp13 (12) was chosen as a backbone for the expression vectors. In the present study, we used plasmids in which the "expression cassette" is derived from the *ARG3* gene coding for ornithine carbamoyl transferase (EC 2.1.3.3). The vectors were constructed from pMC200 (14) and pRIT10779 (12) described previously. Their structure is shown in Fig. 1; their construction will be described in detail elsewhere. Briefly, by a combination of BAL-31 digestion and ligation with filled-in restriction sites, *Bam*HI sites were introduced either upstream (pRIT10774) or immediately downstream (pRIT10779) of the *ARG3* initiation codon. The *ARG3* coding sequences have been largely removed and the *Bam*HI site, which is unique on the plasmids, separates the promoter from the region where transcription terminates (M. Crabeel, personal communication). In plasmid pRIT10779 the promoter sequence has been left intact up to and including the *ARG3* initiation codon. Heterologous coding sequences containing an initiation codon can be expressed as authentic proteins after insertion in the *Bam*HI site of pRIT10774 or in the *Nco* I site of pRIT10779 after treatment with mung bean exonuclease. Sequences devoid of an initiation codon can be fused to the *ARG3* ATG at the *Bam*HI site of pRIT10779. The unique *Xho* I and *Sac* II sites can be used to replace the promoter or transcription termination elements (or both).

Recombinant Plasmids for Expression of Human α_1-Antitrypsin in Yeast. α_1-Antitrypsin is synthesized as a 418 amino acid precursor containing a 24 amino acid long signal sequence (9, 19). To express the precursor and the mature form of α_1-antitrypsin, we constructed the following recombinant plasmids (Fig. 2 *Upper*). Plasmid pULB1523 (9) contains, as a *Pst* I insert, the DNA coding for human α_1-antitrypsin, including 14 base pairs (bp) of 5′ and 102 bp of 3′

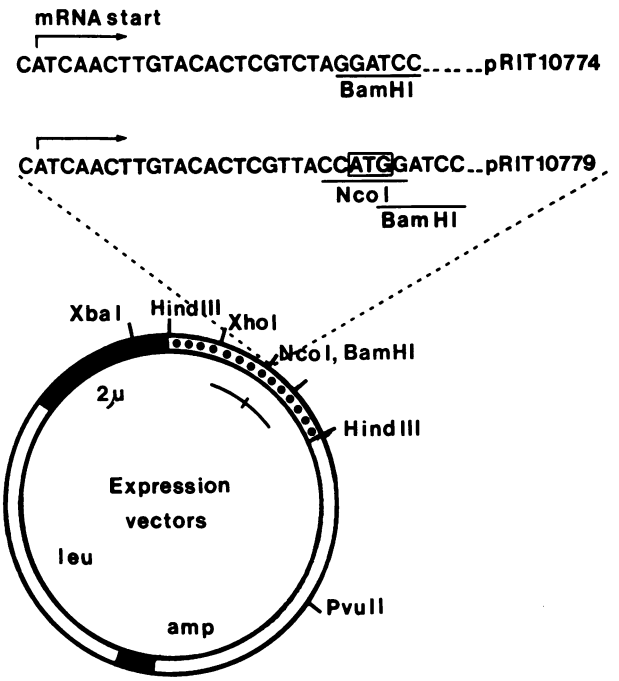

FIG. 1. Modular yeast expression vectors pRIT10774 and pRIT10779. The large *Hin*dIII fragment is plasmid YEp13 (12). The small *Hin*dIII piece contains the *ARG3* promoter and the transcription termination region separated by unique *Nco* I or *Bam*HI sites (or both) available for insertion of heterologous coding sequence (see text). The *Xho* I and *Sac* II sites can be used to replace the regulatory sequences used for expression.

noncoding sequence. This insert was isolated and treated with BAL-31 to remove unnecessary nucleotides upstream and downstream of the coding sequence. After treatment with T4 DNA polymerase it was ligated to the expression vector pRIT10774 previously treated with *Bam*HI and T4 DNA polymerase. The mixture was used to transform *E. coli* MM294. Several plasmids carrying the insert in the correct orientation for expression were recovered and used to transform yeast strain Ic1697d. Transformants were screened for expression of α_1-antitrypsin as described below. Plasmid pRIT10782 gave the highest expression level as measured by ELISA and was selected for further analysis. Its sequence around the fusion between *ARG3* and α_1-antitrypsin cDNA sequence is shown in Fig. 2 *Lower*.

To express mature α_1-antitrypsin, we took advantage of a *Bam*HI site overlapping the codon for the second amino acid of the mature protein (9, 19). The reading frame across this site is the same as in the expression plasmid pRIT10779. Recombining these *Bam*HI sites results in a recombinant molecule coding for authentic human α_1-antitrypsin, except that the NH_2-terminal glutamic acid is replaced by a methionine. The 13,700-bp *Xho* I–*Bam*HI fragment from pRIT10782 was purified and ligated to a 750-bp *Xho* I–*Bam*HI fragment derived from plasmid pRIT10779, which contains the *ARG3* promoter. Chimeric molecules were used to transform *E. coli* strain MM294 to ampicillin resistance and one of the recombinant plasmids, pRIT10787, was shown to have the correct structure to express human α_1-antitrypsin (Fig. 2 *Lower*).

Expression of Human α_1-Antitrypsin. Two different yeast strains were used as host for the recombinant plasmids pRIT10782 and pRIT10787. The first one, Ic1697d, is bradytrophic for arginine (20) and was grown at low arginine concentration (4 μg/ml) to obtain maximum expression levels of human α_1-antitrypsin. Its drawbacks, however, are slow generation time and normal protease content. The second yeast strain, 10S44c, does not allow a regulation via the

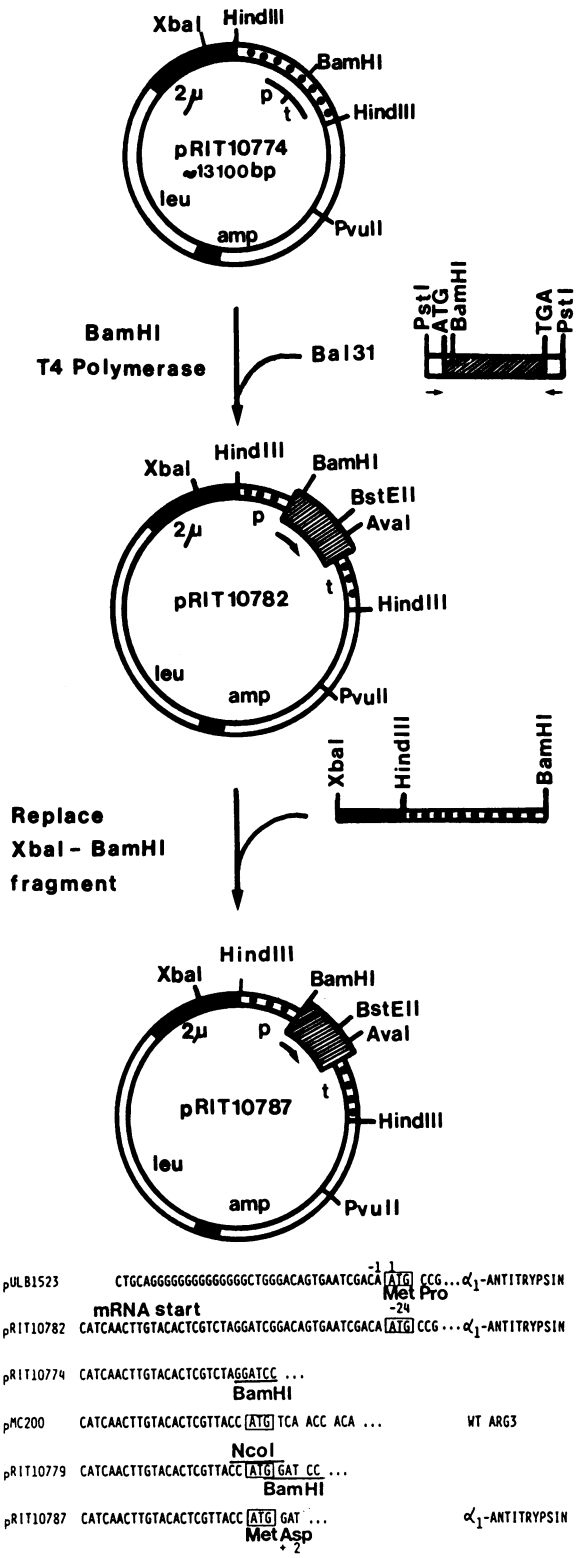

FIG. 2. Construction of recombinant plasmids expressing human α_1-antitrypsin in yeast. (*Upper*) Outline of cloning steps. The pRIT10774 vector was used to fuse a BAL-31-treated *Pst* I fragment encoding α_1-antitrypsin at the *Bam*HI site located in the 5′ nontranslated leader sequence of the *ARG3* gene. pRIT10782 contains the α_1-antitrypsin coding sequence in the correct orientation for expression. This plasmid was then used as a backbone to replace the promotor region with an *Xba* I–*Bam*HI fragment from pRIT10779 and derive pRIT10787. In pRIT10779 the *Bam*HI site is located at the *ARG3* ATG codon and has the same reading frame as the natural *Bam*HI site located in the second codon of the α_1-antitrypsin coding sequence. (*Lower*) Nucleotide sequences at the promoter–coding sequence junction in the different constructs.

ARG3 promoter but carries a *pep4-3* mutation, which results in a deficiency of several proteases (21).

These two strains were transformed with the recombinant plasmids pRIT10782 and pRIT10787, and transformants were analyzed for α_1-antitrypsin production as follows: cell extracts were prepared (see *Material and Methods*) and then assayed in the amplified ELISA described previously (9, 22). This assay, which allows detection of low levels of α_1-antitrypsin (down to 2 ng/ml), was calibrated with a standard human α_1-antitrypsin preparation and applied to serial dilutions of the yeast extracts. As seen in Table 1, both plasmids pRIT10782 and pRIT10787 direct the expression of human α_1-antitrypsin in the two yeast strains used as host. Levels of expression, as determined by this assay, varied from 0.3 to 1% of soluble proteins and were reproducibly higher in the yeast strains carrying plasmid pRIT10787, which codes for the mature α_1-antitrypsin molecule.

In the case of pRIT10782, detection of human α_1-antitrypsin in yeast extracts was also performed by using, as a second antibody in the immunoassay, a set of 26 monoclonal antibodies (22) raised against human α_1-antitrypsin instead of the usual polyclonal mouse antiserum. We measured the binding efficiency of these monoclonal antibodies on α_1-antitrypsin produced in yeast and compared it to their affinity for the natural molecule. These experiments revealed four classes of monoclonal antibodies. Sixteen of them recognized the protein produced in yeast as efficiently as the natural species, 6 others showed decreased recognition (40–65% of control), 2 antibodies displayed weak recognition (20–30% of control), and the last 3 showed no recognition at all. Since α_1-antitrypsin is naturally glycosylated (23), it is not surprising that some antibodies exhibit weak or no affinity for the molecule synthesized in yeasts that could be either nonglycosylated or only partially glycosylated (see below). Nevertheless, on the whole, these data suggest a close structural relationship between plasma-derived α_1-antitrypsin and the protein obtained by DNA recombinant technology.

Biological Activity of Human α_1-Antitrypsin Produced in Yeasts. The inhibitory capacity of human α_1-antitrypsin toward trypsin was taken as a measure of its activity in yeasts extracts. The microassay described in *Material and Methods* involves the cleavage of a chromogenic substrate by trypsin in the presence of either a standard α_1-antitrypsin preparation or extracts derived from clones carrying pRIT10774, pRIT10782, and pRIT20787. As seen in Fig. 3 and Table 1, no activity could be detected in cells producing the preprotein in either the *PEP4* or the *pep4-3* strain. In contrast, the mature α_1-antitrypsin produced in the yeast clone containing plasmid pRIT10787 was shown to be active, with an optimal activity in the peptidase-deficient yeast strain. On the basis of the amount of α_1-antitrypsin measured by ELISA and comparison with the standard activity curve, the mature α_1-antitrypsin produced in yeast exhibits at least 30% of the ac-

Table 1. Expression and activity of human α_1-antitrypsin in yeast

Plasmid	Coding sequence	Strain	Expression	Activity*
pRIT10774	None	Ic1697d	0	−
		10S44c	0	−
pRIT10782	Preprotein	Ic1697d	0.34	−
		10S44c	0.3	−
pRIT10787	Mature protein	Ic1697d	1.2	+
		10S44c	1.0	+ + +

Expression level is given as percentage of total soluble protein.
*Activity: −, no activity; +, moderate activity (at least 10% of the standard activity); + + +, higher activity (at least 30% of the standard). Activity measurements have only a qualitative value since the assay is performed on crude yeast extracts.

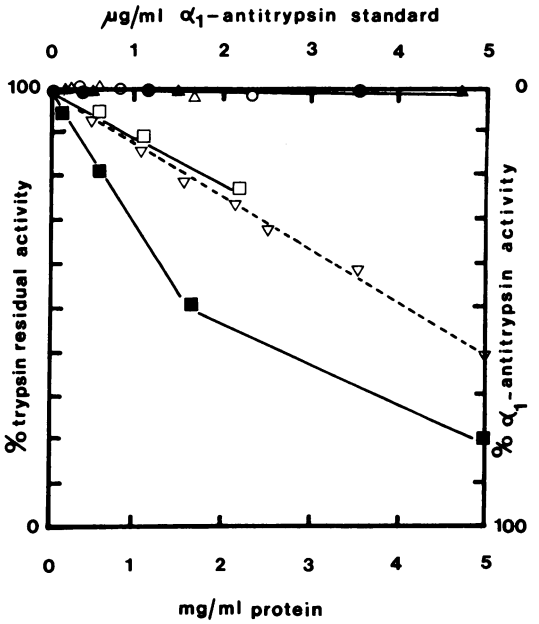

FIG. 3. Biological activity of yeast-produced human α_1-antitrypsin. The inhibitory capacity of α_1-antitrypsin toward trypsin was measured in a microtest using the chromogenic substrate S2444 as described (9). The results are expressed as % residual trypsin activity as a function of protein concentration in the assay. The 100% value corresponds to systems without α_1-antitrypsin (OD_{405} = 1.04 corresponds to the absorbance measured in a system in which trypsin activity is monitored by its action on the chromogenic substrate). The standard α_1-antitrypsin curve (▽) ranges from 0 to 5 μg/ml in the assay. Control plasmid pRIT10774 in strains Ic1697d (○) and 10S44c (●); plasmid pRIT10782 in strains Ic1697d (△) and 10S44c (▲); plasmid pRIT10787 in strains Ic1697d (□) and 10S44c (■).

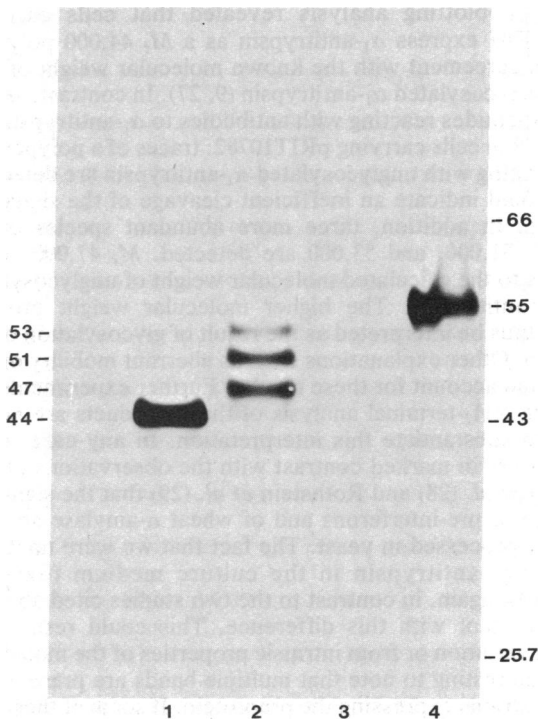

FIG. 4. Immunological characterization of human α_1-antitrypsin produced in yeast. Yeast extracts were analyzed on $NaDodSO_4$/polyacrylamide gels (18). Proteins were then electrically transferred onto nitrocellulose sheets and reacted with mouse antihuman α_1-antitrypsin serum followed by treatment with peroxidase-labeled goat anti-mouse serum and the appropriate chromogenic substrate (9). Lane 1, plasmid pRIT10787 in strain 10S44c; lane 2, plasmid pRIT10782 in the same yeast strain; lane 3, control plasmid pRIT10774 in the same yeast strain; lane 4, native human α_1-antitrypsin (50 ng). Molecular weights (shown as $M_r \times 10^{-3}$) of standards (right) and of yeast-produced α_1-antitrypsin molecules (left) are indicated.

tivity of authentic glycosylated human α_1-antitrypsin. Preliminary data obtained on purified material indicate that the protein made in yeast exhibits equivalent activity in the trypsin inhibition test as authentic human α_1-antitrypsin (data not shown).

Molecular Characterization of Human α_1-Antitrypsin Synthesized in Yeasts. To identify the α_1-antitrypsin molecules produced in yeasts, we performed $NaDodSO_4$/polyacrylamide gel electrophoresis on yeast extracts derived from clones carrying pRIT10774, pRIT10782, and pRIT10787. The separated proteins were then electrically blotted onto nitrocellulose sheets, which, in turn, were treated with mouse antihuman α_1-antitrypsin serum, peroxidase-labeled goat antimouse serum, and the appropriate chromogenic substrate (see *Material and Methods*).

As seen in Fig. 4, a single polypeptide of M_r 44,000 is detected in extracts of yeast carrying pRIT10787. Its molecular weight is consistent with that calculated from the DNA sequence (44,679). On the other hand, several polypeptides were detected in extracts of cells carrying pRIT10782, which codes for the preprotein: a minor M_r 44,000 species and more intense bands at M_r 47,000, 50,000, and 53,000. The M_r 44,000 comigrates with the polypeptide encoded by pRIT10787 and could thus result from processing of the signal sequence. As judged from its molecular weight, the M_r 47,000 species corresponds to the unglycosylated preprotein (calculated M_r = 47,404). The two heavier species could be the result of partial or incorrect glycosylation.

DISCUSSION

We have constructed yeast expression vectors using expression signals from the *ARG3* gene. The design of the vectors is such that the coding sequence of a heterologous gene can be inserted in a unique restriction site located between 5′ and 3′ sequences of the *ARG3* gene. In the vector pRIT10779, the *ARG3* 5′ sequence is left intact up to and including the initiation codon, which is overlapped by unique *Nco* I and *Bam*HI sites. In pRIT10774, a unique *Bam*HI site is located within the 5′ untranslated sequence. Since, in yeast the first ATG present on the mRNA is used to initiate translation (24), heterologous coding sequences can be expressed as authentic proteins when translated from their own ATG after insertion into the *Bam*HI site of pRIT10774 or in the *Nco* I site of pRIT10779. Alternatively, foreign genes can be fused in the *ARG3* ATG at the *Bam*HI site of pRIT10779. The vector also contains *ARG3* 3′ sequences. Indeed, efficient transcription termination has been shown to be important for high-level expression of proteins in yeast (25, 26). These vectors are able to direct the synthesis of heterologous protein up to levels of a few percent of yeast proteins (this study and unpublished results from our laboratory). This may be seen as a surprising result in view of the fact that the *ARG3* gene does not code for an abundant protein in yeast.

The cDNA coding for human α_1-antitrypsin was introduced into the expression vectors to obtain expression of either the preprotein (pRIT10782) or the mature protein (pRIT10787). The expected product coded by pRIT10787 is authentic α_1-antitrypsin, except that the NH_2-terminal glutamic acid is replaced by methionine (methionine α_1-antitrypsin). In fact, preliminary data (data not shown) obtained with the protein purified from yeast suggest that the methionine is cleaved *in vivo* and that the product starts with an aspartic acid residue (second amino acid of the mature protein).

Immunoblotting analysis revealed that cells carrying pRIT10787 express α_1-antitrypsin as a M_r 44,000 polypeptide, in agreement with the known molecular weight of mature unglycosylated α_1-antitrypsin (9, 27). In contrast, several polypeptides reacting with antibodies to α_1-antitrypsin are detected in cells carrying pRIT10782: traces of a polypeptide comigrating with unglycosylated α_1-antitrypsin are detected. This could indicate an inefficient cleavage of the signal sequence. In addition, three more abundant species of M_r 47,000, 51,000, and 53,000 are detected. M_r 47,000 corresponds to the calculated molecular weight of unglycosylated pre-α_1-antitrypsin. The higher molecular weight proteins could thus be interpreted as the result of glycosylation of the protein. Other explanations such as aberrant mobility on the gel could account for these results. Further experiments, including NH_2-terminal analysis of these products are necessary to substantiate this interpretation. In any case, these results are in marked contrast with the observations of Hitzeman *et al.* (28) and Rothstein *et al.* (29) that the signal sequence of pre-interferons and of wheat α-amylase are efficiently processed in yeast. The fact that we were unable to detect α_1-antitrypsin in the culture medium (data not shown)—again, in contrast to the two studies cited above—is consistent with this difference. This could result from strain variation or from intrinsic properties of the molecules. It is interesting to note that multiple bands are present only in the strains expressing the preprotein. If some of these species are glycosylated, it would indicate that the presence of the signal sequence is necessary for this process to occur.

Based on the ELISA, expression levels appear higher in cells expressing the mature protein as compared to cells expressing the preprotein (1% versus 0.3% of soluble proteins). However, the membrane fraction was not examined for the presence of α_1-antitrypsin.

Biological activity of α_1-antitrypsin was dependent on the strain used, the mature protein synthesized in yeast being more active in the *pep4-3* strain than in the *PEP4* host. Also, biological activity was only associated with the product coded for by plasmid pRIT10787—i.e., where the coding sequence for α_1-antitrypsin lacks the signal sequence. It should be pointed out that the lack of a glutamic acid residue, the first amino acid of authentic mature α_1-antitrypsin, did not impede biological activity.

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