Isolation and nucleotide sequence of the *Aspergillus restrictus* gene coding for the ribonucleolytic toxin restrictocin and its expression in *Aspergillus nidulans*: the leader sequence protects producing strains from suicide

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ABSTRACT

We describe the cloning and characterization of the gene coding for the ribotoxin restrictocin, from *Aspergillus restrictus* (gene *res*, EMBL accession Number X56176). This toxin is a potent inhibitor of protein synthesis in eucaryotes and is of potential interest as a component of immunotoxins. To analyze the mechanism of self-protection in the producing organism, the *res* gene was cloned into the vector pFB39 and introduced into *Aspergillus nidulans*. The secretion of active restrictocin from transformants suggests that the pro-toxin is not an active nuclease but is activated during the process of secretion.

INTRODUCTION

The ribosome-inactivating proteins (RIPs), or ribotoxins, are potent inhibitors of protein synthesis produced by plants, fungi, and bacteria; they are among the most potent inhibitors of translation known. Their mode of action has been shown to require a highly specific interaction with the eucaryotic ribosome which results in enzymatic modification of the 28S rRNA within a universally conserved region. The ribotoxins can be divided into two classes: those such as ricin or pokeweed toxin, which cleave an N-glycosidic linkage between base and ribose, and those which cleave a single phosphodiester bond in the same target domain (1, 2). The latter mechanism is characteristic of the fungal toxins restrictocin, mitogillin and α-sarcin, produced by filamentous fungi of the *Aspergillus* genus (3, 4). These three ribotoxins are known to have ribonucleolytic activity and have sequence similarity with a number of ribonucleases (5, 6, 7, 8); α-sarcin and restrictocin show 86% amino acid sequence identity (9, 10). The fungal toxins have proved to be of great practical value in structural studies of ribosome function (11, 12). In recent studies cytotoxins related to restrictocin have been implicated in the pathogenicity of human aspergillosis (13, 14).

The structure and organization of the gene for the *Aspergillus restrictus* toxin, restrictocin, is described; it contains a single intron of 52 bp that interrupts the coding sequence immediately after the N-terminal amino acid of the mature nuclease. The restrictocin gene (gene *res*) was cloned and expressed in *Aspergillus nidulans* with no apparent toxic effects on this new host.

MATERIALS AND METHODS

Fungal and bacterial strains and vectors

A. restrictus ATCC 34475 and *A. giganteus* MDH 18894 were obtained from culture collections.

A. nidulans CS2008 (*wA4, pyroA4, argB2, hxA1)* and plasmid pFB39, a pUC8 derivative containing a *Sal*I fragment carrying the *arg*B gene of *A. nidulans* encoding ornithine carbamoyl transferase (15), were kindly provided by C. Scazzocchio (University Paris-Sud).

Gene isolation and analysis

Cloning techniques were performed by standard procedures (16). Preparation of DNA from *Aspergillus* is described elsewhere (17). Preliminary Southern analysis was done using a labelled degenerate oligonucleotide probe R3 (fig. 1), under stringent conditions (5× SSC, 0.1% SDS at 45°C).

Two independent libraries were constructed from *A. restrictus* genomic DNA partially digested with *Mbo*I (average insert size 19 kb or 7 kb) in λEMBL3 phage (Stratagene, Inc, San Diego, CA). Two inserts were isolated using the probe R3 and the same conditions as for the Southerns on screening the libraries. From the first, a 5-kb *Eco*RI fragment was cloned in pUC18 and further subcloned to a 800-bp *Eco*RI-*Hin*dIII fragment which was inserted and sequenced in M13mp18 and M13mp19 (Biolabs). From the second insert, a 7-kb *Sal*I fragment was cloned in the pBluescript SK- vector (Stratagene). The resulting plasmid, pBS7k, was used to determine the sequence of flanking regions. DNA sequencing was carried out by the dideoxy chain termination method using Sequenase (United States Biochemicals), according to the manufacturers protocols, with universal primers or appropriate oligonucleotides synthesized with a Cyclone DNA synthesizer (Biosearch, San Rafael, CA). All

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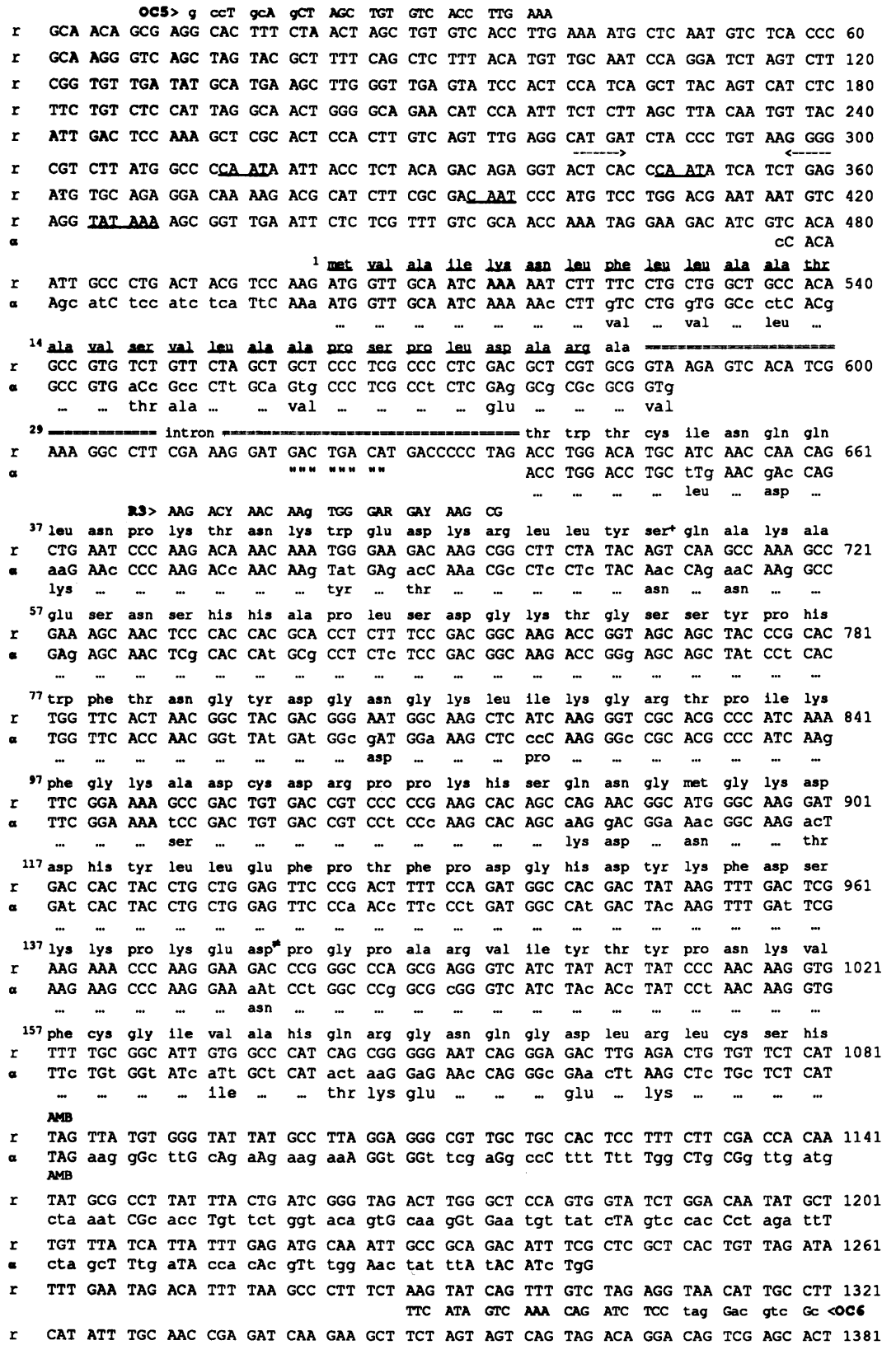

Figure 1. Alignment of the nucleotide sequences of the *res* gene (r) (EMBL accession Number X56176) and cDNA for α-sarcin (α). Sequences of the oligonucleotides R3, OC5, and OC6, used in this work are indicated (Y= C or T, R= A or G). Lower case letters denote bases not homologous with the restrictocin sequence. The restrictocin intron is underlined: (=). The lariat signal is indicated: (' '). The proposed TATA box and the three CAAT boxes are underlined, one CAAT box is flanked by inverted repeats (arrows). The deduced aminoacid sequence of restrictocin is shown over, and the aminoacid differences in α-sarcin under the nucleotide sequences (Dots: ··· indicate identical aminoacids). The predicted signal peptide is underlined. The serine residue (position 52) which differentiates restrictocin and mitogillin is indicated: (+). The aspartic acid residue (position 142) of restrictocin identified as asparagine in peptide sequence determination is indicated: (≠).

sequences were determined on both strands and confirmed by repetition. Other DNA manipulations were as previously described (16, 18).

Cloning in *A. nidulans*

Polymerase chain reaction (PCR) methodology was applied as described (19) to amplify a 1,3-kb DNA fragment (from 480 bp 5′ to 225 bp downstream of the coding sequence of the gene; fig. 1). Plasmid pBS7k was used as template and oligonucleotide primers, OC5 and OC6 (fig. 1) were employed to generate *Pst*I sites . The amplification was carried out for 25 cycles (94°C/2 min, 47°C/2 min, 70°C/1 min) using Taq polymerase (Cetus). The resulting fragment (PrT1300) was inserted at the *Pst*I site of plasmid pFB39. A 333-bp probe (NX333: from the *Nsi*I site (position 646) to the *Xma*I site (position 979); fig. 1) purified from pBS7k, was used to identify the two plasmids pFB39c1 and pFB39c2, containing the insert in either orientation (fig. 2A).

These plasmids were used to transform *A. nidulans* CS2008 as described by Tilburn (17). Transformants were selected on minimal medium (20) and DNA analyzed by Southern blotting using the NX333-fragment as probe.

To examine expression of the toxin, *A. nidulans* transformants were grown in medium MOA4, modified from that described: beef extract (Oxoid) 2% w/v, Bacto-peptone (Difco) 1% w/v, corn starch (Prolabo) 2% w/w, sodium chloride 0.5% w/v, pH adjusted to 6,8 (21).

Protein isolation and analysis

The culture medium of *Aspergillus* strains was filtered on a Blutex nylon filter (pore size 70μm) and centrifuged for 1 hour at 40,000×g. Samples of the crude filtrate were diluted 1/1 in SDS-PAGE loading buffer or precipitated by the addition of an equal volume of acetone at 4°C for concentration before resuspension in the minimum volume of loading buffer. SDS-PAGE and immunoelectrophoresis (Western) were carried out following conventional protocols (22). Rabbit antiserum to α-sarcin was obtained from Drs Y.Endo and G.Stoeffler. These antibodies cross react strongly with restrictocin revealed with alkaline phosphatase-conjugated anti-rabbit IgG (Promega). The serum was used at a hundred fold dilution to test for the presence of toxin in supernatants of *A. nidulans*, *A. restrictus* or *A. giganteus*. For use in analyzing transformants of *A. nidulans*, antiserum was presaturated with a crude filtrate of a culture of *A. nidulans* CS2008.

Purification of restrictocin from culture medium was carried out on MonoS HR5/5 (Pharmacia LKB) with a NaCl gradient, after an initial batch extraction with SP-Sephadex C-25 (Pharmacia LKB).

Measurements of protein synthesis inhibition, and the production of the 'α-fragment' were carried out with rabbit reticulocyte lysates (Promega) (23). The ribosomes were incubated 5 minutes at 37°C in the presence of 100 ng of toxin, and the extracted RNA analyzed on 2.4% agarose gels.

RESULTS

Isolation and sequencing of the *res* gene

The identification of restrictocin-encoding fragments of *A. restrictus* DNA employed labeled oligonucleotide probes derived from the aminoacid sequence of restrictocin (10). The probes were chosen using the codon usage determined for *Aspergillus* species (24). Oligonucleotide probe R3 (fig. 1) hybridized to genomic Southern blots of *A. restrictus* and *A. giganteus* (the α-sarcin producer) but not to *A. nidulans* (data not shown). Subsequently, probe R3 was used to screen λEMBL3 libraries of *Mbo*I-digested *A. restrictus* DNA. From two independent libraries, the hybridization-positive plaques were picked, grown and DNA isolated. One contained an 800-bp *Eco*RI-*Hin*dIII fragment which was subsequently found to contain the entire coding sequence for restrictocin but contained only about 20 bp upstream of the translation start. From the second library, a 7-kb *Sal*I fragment was isolated which permitted the sequencing of 500 nucleotides upstream, and up to 880 nucleotides downstream of the open reading frame.

Analysis of the *res* gene sequence

Based on the amino acid sequence of restrictocin, it was possible to identify the coding sequence for the toxin; this was interrupted by an intron of 52 nucleotides (fig. 1) having the characteristics typical of introns identified in aspergilli. There is a short, contiguous open reading frame 5′ of the known N-terminal amino acid of the mature protein, with an ATG at position 501; we propose that the sequence 501 to 585 represents a signal peptide of 27 residues required for secretion of the toxin. There is a GAC codon (aspartic acid) at position 977, although the published amino acid sequence assigns asparagine (10).

Comparison with the cDNA sequence of α-sarcin (25) confirms our assignments; there is a high degree of similarity between the coding sequences for the two toxins. These similarities are less evident outside of the open reading frames except for the five adjacent nucleotides at the transcription start for α-sarcin (position

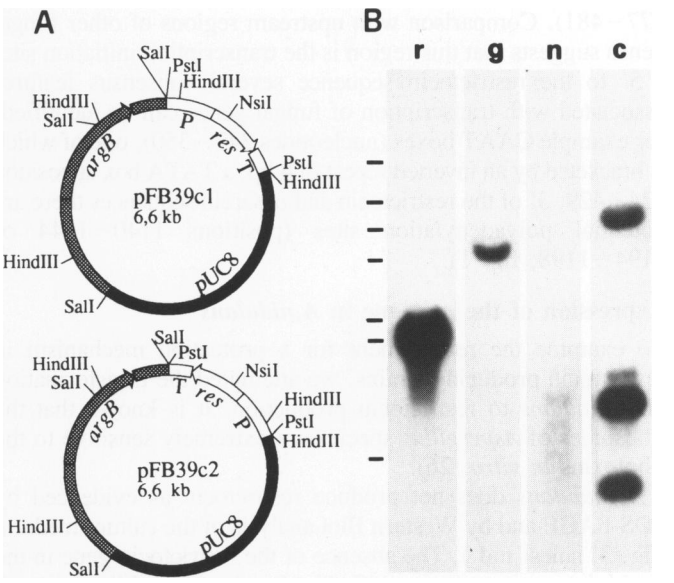

Figure 2. Vectors used for *A. nidulans* transformation, and Southern blot analysis of the *res* transformant compared to the host *A. nidulans* and parent *A. restrictus* strains. **A**: A 1.3 kb DNA fragment (PrT1300 in Materials and Methods) of *A. restrictus* (white box), containing the restrictocin gene (*res*), with 5′ and 3′ regions including the putative promoter (P), and the terminator(T) was introduced at the *Pst*I site in pFB39: (pUC8 : black box, including the *arg*B gene of *A. nidulans* : shaded box). The plasmids pFB39c1 and pFB39c2 have opposite insert orientations. **B** : DNAs were digested with *Pst*I, and analyzed by Southern hybridization using the 333-bp probe (NX333 fragment). Lanes r : *A. restrictus*, g : *A. giganteus*, n : *A. nidulans* CS2008, c : transformant *A. nidulans* CS2008-pFB39c1. *Hin*dIII fragments from λ phage DNA as size markers are shown on the left.

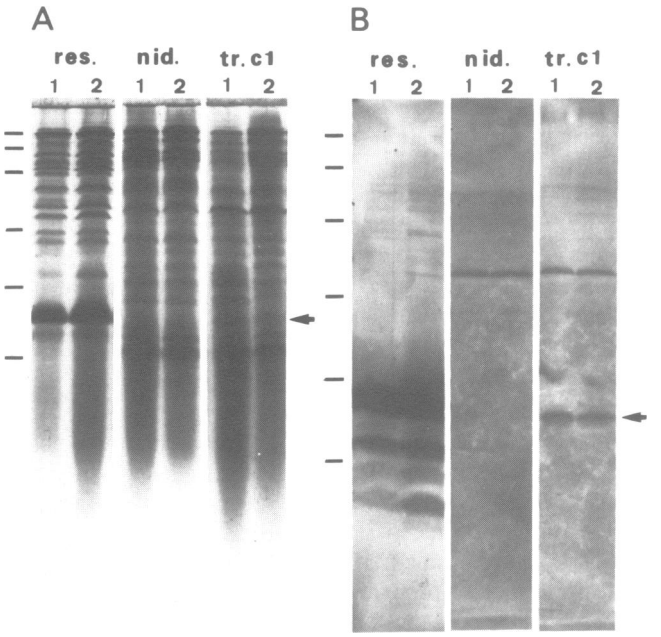

Figure 3. Detection of restrictocin in the culture medium of *A. nidulans* transformant CS2008-pFB39c1. A: SDS PAGE of concentrated culture media from strains (15 μg total proteins per lane) stained with Coomassie blue, and B : Western blot of culture medium (20 μl per lane) using rabbit anti α-sarcin serum. Mycelia were grown in MOA4 medium for either 50 h (lanes 1) or 72 h (lanes 2). Culture medium of *A. nidulans* transformant CS2008-pFB39c1 (tr. c1) compared to that of the host *A. nidulans* CS2008 (nid) and *A. restrictus* (res). The arrows on the right indicate restrictocin, and marks on the left indicate molecular weight standards: 94; 67; 43; 30; 20; 14 kD.

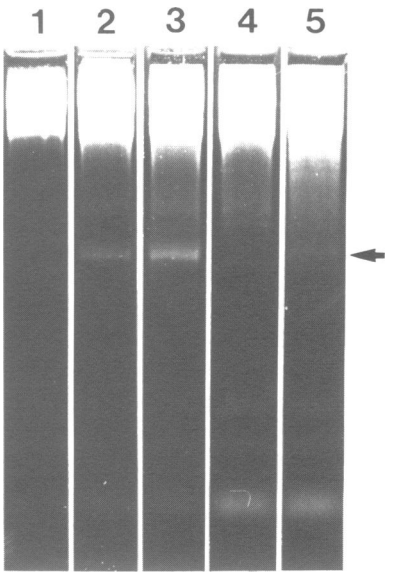

Figure 4. Specific ribonuclease activity of restrictocin shown by release of the 400 base 'α-fragment' from the 28S rRNA of eucaryote ribosomes on incubation with the ribotoxin. RNA from a rabbit reticulocyte lysate treated with ribotoxins was extracted and electrophoresed on an agarose gel as described in Materials and Methods. (1) ribosomes were incubated without toxin;(2) with 100 ng of α-sarcin or (3) restrictocin; or with crude culture medium (12 μl) of (4) the non-transformed strain : *A. nidulans* CS2008, and of (5) the transformant *A. nidulans* CS2008-c1. The arrow shows the position of the 'α-fragment' (*ca* 400 nucleotides) cleaved from 28S rRNA.

477−481). Comparison with upstream regions of other fungal genes suggests that this region is the transcription initiation site.

5′ to the restrictocin sequence several consensus features associated with transcription of fungal genes can be identified, for example CAAT boxes (nucleotides 347−350), one of which is bracketed by an inverted repeat of 4 bp; a TATA box at position 424−429. 3′ of the restrictocin and α-sarcin sequences there are potential polyadenylation sites (positions 1140−1144 or 1194−1198; fig. 1).

Expression of the *res* gene in *A.nidulans*

To examine the requirement for a protection mechanism in restrictocin producing strains, we attempted the transformation of *A.nidulans* to restrictocin production. It is known that the ribosomes of *Aspergillus* species are extremely sensitive to the ribotoxins *in vitro* (26).

A. nidulans does not produce restrictocin as evidenced by SDS-PAGE and by Western Blot analysis of the culture medium (fig. 3, lanes: nid.). The absence of the restrictocin gene in the *A.nidulans* genome was verified by Southern hybridization with the probe NX333 from *A.restrictus* (fig. 2, lane: n).

A PCR fragment of 1.3 kb was cloned into the vector pFB39 to give pFB39c1 (see Materials and Methods) which was transformed into *A. nidulans* CS2008 (fig. 2A). Twenty colonies were isolated and screened for the presence of the restrictocin gene by Southern hybridization with the NX333 probe. One pFB39c1 transformant which apparently contains several insertions of the *res* gene was chosen for further study (fig. 2B). This transformant was grown in liquid medium and tested for the presence of secreted restrictocin by SDS electrophoresis and cross-reaction with antibody specific for α-sarcin and restrictocin (fig. 3). The size of the crossreactive protein was found to be identical to that of mature restrictocin. This establishes that maturation of the preprotein occurs in *A. nidulans* as in *A. restrictus*. The level of expression of restrictocin in the culture supernatant of this *A. nidulans* CS2008-pFB39c1 transformant was lower than in the parent *A.restrictus*.

The culture supernatant of the *A. nidulans* transformant showed typical restrictocin activity on rabbit reticulocyte ribosomes indicating the same specific cleavage of 28S rRNA as the restrictocin control (fig. 4). The total protein secreted by transformants was subjected to chromatography on MonoS, and fractions assayed for ribonuclease activity against calf liver ribosomal RNA. The active fraction induced specific cleavage of 28S rRNA and levels of inhibition of protein synthesis *in vitro* using a rabbit reticulocyte system, by restrictocin produced by *A. restrictus* and by the *A. nidulans* transformant were equivalent (results not shown).

DISCUSSION

We describe the cloning and characterization of the gene for the ribotoxin, restrictocin, from *A. restrictus*; this strain is closely related (or identical) to the ubiquitous *A. fumigatus*. The *res* gene possesses a short (52 bp) intron that interrupts the coding sequence immediately after the N-terminal amino acid; typical splice junction and internal lariat consensus sequences can be assigned. Potential transcription signals and a polyadenylation site can be identified in flanking regions of the open reading frame (fig. 1).

The sequence of the cDNA encoding the related ribotoxin α-sarcin, from *A. giganteus* has been reported recently (25); Southern hybridization studies of the two producing organisms

using a restrictocin probe indicate that the two genes are present in different genomic organizations (fig. 2B).

One of the objects of this work was to ascertain the mechanism by which the producing *A. restrictus* protects itself from suicide; restrictocin is an extremely potent inhibitor of protein synthesis and its catalytic activity as a ribosomal ribonuclease implies that one free molecule inside a cell would be lethal. It is known that the ribosomes of the α-sarcin producing organism are sensitive to inhibition by the toxin (26). One could imagine that the producing *A. restrictus* is protected from suicide a) either because pro-restrictocin is enzymatically inert and only becomes activated during processing in the Golgi system; restrictocin is known to be unable to enter fungal cells (26) or, b) an inhibitor of restrictocin is produced in *A. restrictus* which complexes the toxin and prevents enzymatic activity in the complexed form, as is known for the *Bacillus* ribonuclease barnase and its inhibitor barstar (27, 28).

Cloning of the restrictocin gene with its signal sequence into *A. nidulans* results in the secretion of active restrictocin into the culture medium. This result suggests that pro-restrictocin produced in the cell is enzymatically inactive and is converted to the active form during the secretion process. It is thus probable that *A. restrictus* requires no specific mechanism of self-protection against the ribotoxin although we cannot exclude the possibility that such exists.

Since restrictocin is efficiently secreted from the producing strain, the leader peptide identified for restrictocin might be useful in developing heterologous secretion systems for *Aspergillus* species and other filamentous fungi.

The ribotoxins are good indicators for investigation of the permeabilization of eucaryote cell membranes. Alpha-sarcin has been used to analyze the modification of membrane permeability during viral infections (29, 30, 31). In addition, targeting of toxins such as restrictocin has been proposed as an approach to obtain immunotoxins, because of their relatively low toxicity *in vivo* and *in vitro* (32); several immunoconjugates have been prepared and shown to be effective in animal models (33). With the availability of the cloned restrictocin gene it will be possible to construct fusions of toxin and target signal genes; such single chain immunotoxins are stable and should be readily available from bacterial expression systems.

Another application of the restrictocin gene will be its incorporation into suicide cassettes, under the control of a tightly regulated promoter. Such cassettes will be useful in studying the function of specific cells or tissues during development (34). Production of the toxin could be induced at specific times to block protein synthesis irreversibly by inactivation of all of the ribosomes in the cell containing the gene.

Restrictocin and the related fungal ribotoxins are among the most potent inhibitors of protein synthesis known and act by cleaving a single phosphodiester bond, between G3025 and A3026 in the large ribosomal subunit RNA of yeast (23, 35). How these agents pick out one single phosphodiester bond in 7000 is not known, but it implies that there is a very specific interaction between the ribosome and the toxin. Studies of the ribosomal ribonucleolytic activity of the restrictocin family of toxins by directed site mutagenesis is now possible, using the cloned gene. One of the most obvious domains for investigation is the region in which restrictocin and its related ribotoxin α-sarcin show sequence similarity to ribonuclease U2 from *Ustilago sphaerogena* (9). This domain probably represents the site of nucleolytic activity and the region(s) conferring specificity for ribosomal binding have yet to be identified.

Finally it should be noted that a cytotoxin of the restrictocin family has been isolated from the urine of human aspergillosis patients (13, 14). This disease is an increasingly important cause of mortality in severely immunocompromised patients. Until recently, no potential virulence factor had been identified in systemic fungal infections. The characterization of the restrictocin gene will permit analysis of the molecular pathogenicity of these diseases. Inactivation of the restrictocin related gene by insertion or deletion in *A. fumigatus* can be envisaged in studies of the role of ribotoxins as factors of virulence in fungal infections of man and animals.

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