

## **Expression of full length and deletion homologues of *Carcinoscorpius rotundicauda* Factor C in *Saccharomyces cerevisiae*: immunoreactivity and endotoxin binding**

J.L. Ding, C. Chai, A.W.M. Pui and B. Ho  
*Journal of Endotoxin Research* 1997 4: 33  
DOI: 10.1177/096805199700400105

The online version of this article can be found at:  
<http://ini.sagepub.com/content/4/1/33>

---

Published by:



<http://www.sagepublications.com>

On behalf of:

[International Endotoxin & Innate Immunity Society](#)

**Additional services and information for *Journal of Endotoxin Research* can be found at:**

**Email Alerts:** <http://ini.sagepub.com/cgi/alerts>

**Subscriptions:** <http://ini.sagepub.com/subscriptions>

**Reprints:** <http://www.sagepub.com/journalsReprints.nav>

**Permissions:** <http://www.sagepub.com/journalsPermissions.nav>

**Citations:** <http://ini.sagepub.com/content/4/1/33.refs.html>

>> [Version of Record](#) - Feb 1, 1997

[What is This?](#)

# Expression of full length and deletion homologues of *Carcinoscorpius rotundicauda* Factor C in *Saccharomyces cerevisiae*: immunoreactivity and endotoxin binding

J. L. Ding,<sup>1</sup> C. Chai,<sup>2</sup> A. W. M. Pui,<sup>1</sup> B. Ho<sup>2</sup>

<sup>1</sup>Marine Biotechnology Laboratory and BioScience Centre, and <sup>2</sup>Department of Microbiology, School of Biological Sciences, National University of Singapore, Singapore

**Summary** Deletion homologues of the cloned Factor C cDNAs from the horseshoe crab *Carcinoscorpius rotundicauda* were engineered to express in *Saccharomyces cerevisiae* under the regulation of a galactose-inducible promoter. Expression cassettes were constructed in the vectors: pEMBLyex4 and YEpsc1 to direct, respectively, the intracellular expression, and the secretion of the protein into the culture medium using a heterologous signal sequence. The effect of insert size on the efficiency of expression and the functionality of the resulting recombinant Factor C (rFC) were studied by creating expression constructs bearing various deletion and/or hybrid fragments of Factor C. Removal of the long 5' UTR from the Factor C cDNA improved expression of the rFC. 3' Deletions of up to 84%, or internal deletions of 65% of the Factor C cDNA resulted in either the lack of detectable amounts of Factor C or loss of immunoreactivity. Depending on the construct, full length or partial rFC-related proteins were correspondingly expressed intracellularly, regardless of the vector. The rFC partitioned with the insoluble cell fraction, was solubilised with either SDS or Triton X-100, and found to be immunoreactive. The rFCs were functionally active, being able to bind Gram-negative bacterial endotoxin, provided critical regions of the endotoxin-binding domain were preserved.

## INTRODUCTION

In the horseshoe crab, one of the main lines of defense against Gram-negative bacterial infection is a coagulation reaction in the hemolymph that immobilises invading bacteria.<sup>1</sup> The coagulation reaction is effected by the sequential activation of a series of serine proteases which

leads to the polymerisation of a soluble coagulogen to form a coagulin clot.<sup>2,3</sup> The mechanism of the coagulation cascade has been largely elucidated and its enzyme components identified.<sup>4</sup> The key enzyme which initiates the coagulation cascade is Factor C. It is activated in the presence of bacterial endotoxins.<sup>5–7</sup> The zymogen consists of single- and double-chain glycoproteins.<sup>7,8</sup> During endotoxin-induced activation of *C. rotundicauda* Factor C, the single chain form of the enzyme remains intact whereas the double-chain form is cleaved to give intermediates comprising an 80 kDa heavy chain and a 52 kDa light chain.<sup>7</sup> The light chain is subsequently cleaved at a unique internal Phe–Ile bond.<sup>9</sup>

Two distinct, full-length cDNAs encoding Factor C have been isolated from a recombinant phage library prepared from amoebocyte mRNA of the Singapore horseshoe crab, *C. rotundicauda*.<sup>10</sup> Designated CrFC21 and

Received 7 August 1996

Revised 10 December 1996

Accepted 13 December 1996

Correspondence to: Dr J. L. Ding, Marine Biotechnology Laboratory and BioScience Centre, School of Biological Sciences, National University of Singapore, Lower Kent Ridge Road, Singapore 119260  
Tel. +65 772 2776; Fax. +65 779 2486  
E-mail sbsdjl@leonis.nus.sg

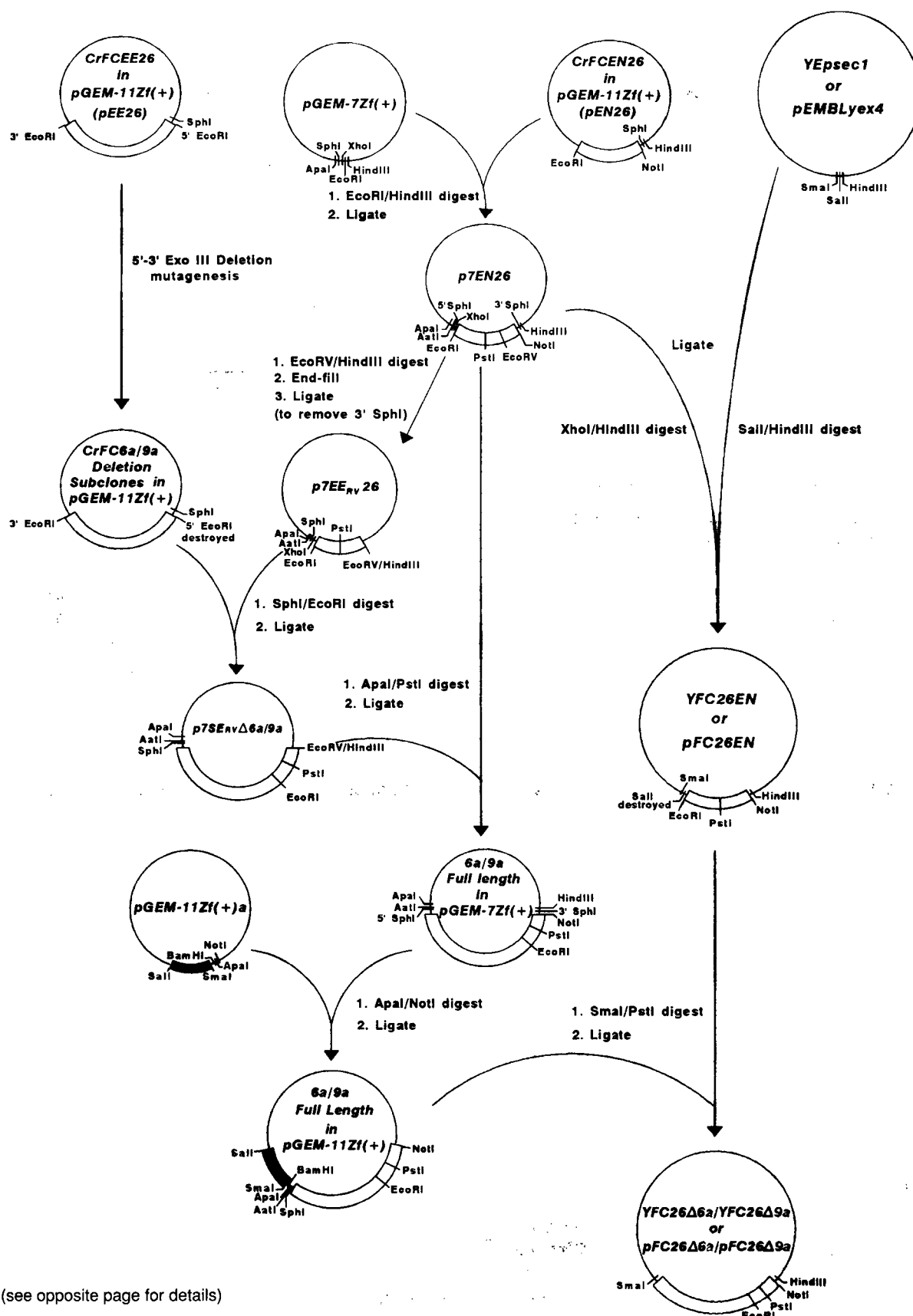


Fig. 1 (see opposite page for details)

**Fig. 1** (see page opposite) Construction of expression plasmids of CrFC26 cDNA deletion derivatives lacking the 5' UTR and portions of the leader peptide. The Eco RI–Eco RI (EE) fragment and the Eco RI–Not I (EN) fragments of CrFC26 cDNA were isolated from recombinant phage clones and inserted individually into the vector pGEM11Zf(+), giving pEE26 and pEN26, respectively. The entire 5' UTR and various lengths of the sequences coding for the leader peptide of CrFC26 were deleted by performing 5'–3' Exo III deletion mutagenesis on pEE26. From the deletion library, two mutants: CrFC6a/9a, also designated FC26Δ6a and FC26Δ9a (see Fig. 2) were selected for expression studies. The EN fragment of CrFC26 was excised from pEN26 with Eco RI and Hind III and inserted into the vector pGEM7Zf(+) to give p7EN26. From p7EN26, the same fragment was isolated using the flanking Xho I and Hind III sites and inserted into Sal I/Hind III digested YEpsc1 or pEMBLyex4, giving YFC26EN or pFC26EN, respectively. The Sph I–Eco RI fragments from FC26Δ6a and FC26Δ9a, viz., CrFC6a/9a deletion subclones in pGEM11Zf(+) were isolated and inserted through an intermediate step, into the Sph I and Eco RI sites of p7EE<sub>RV</sub>26. The resultant full-length deletion mutants in p7SE<sub>RV</sub>Δ6a/9a were subcloned into pGEM11Zf(+)-a, a derivative of pGEM11Zf(+) (see Fig. 3B). The Sma I–Pst I fragments were subsequently isolated from these subclones and inserted into Sma I/Pst I digested pFC26EN to give the plasmids pFC26Δ6a and pFC26Δ9a. The same fragments were inserted into YFC26EN, creating in-frame protein fusions to the *K. lactis* killer toxin signal sequence, to yield YFC26Δ6a and YFC26Δ9a.

CrFC26 (GenBank Database Accession numbers: S77063 and S77064, respectively), the complete nucleotide sequences of the cDNAs were determined and their lengths shown to be 3448 and 4182 bp, respectively. CrFC21 and 26 differ markedly in the 5' region. CrFC26 encodes an additional 64 amino acid residues at the N-terminus. Furthermore, an unusual feature of CrFC26 is a long 5' untranslated region (UTR) of 568 bp which contains nine ATG start codons preceding the putative true initiation codon. Translation initiation at these upstream ATGs would, in theory, produce peptides of 4–18 amino acids. Except for differences at the N-terminus, the predicted proteins encoded by the two cDNAs are essentially identical, with a homology of 99.1%.

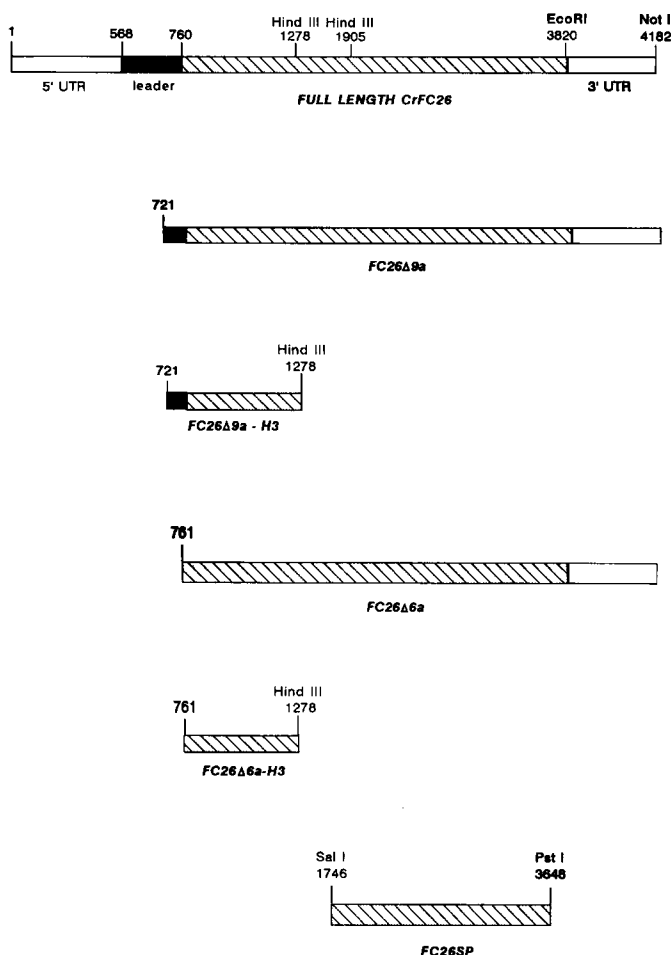
In view of its high affinity for LPS, it is envisaged that Factor C may be utilised as a biosensor for the detection of bacterial endotoxins. This paper reports the use of *S. cerevisiae* as a host organism to produce recombinant Factor C (rFC). *S. cerevisiae* has become a favoured host for the expression of heterologous proteins.<sup>11</sup> A number of attributes has made *S. cerevisiae* suitable as a host. It is endotoxin-free, easily manipulated and amenable to scale-up. Of greater importance is that, being an eukaryote, it allows post-translational processing of heterologous proteins in ways similar to those found in the natural producer organisms. A variety of recombinant proteins have been expressed in this yeast. Notable examples are viral antigens for vaccines, hormones, growth factors, blood proteins and multimeric protein complexes such as antibodies and transmembrane receptors.<sup>11</sup> We report here, the cloning and expression of full

length and deletion homologues of the CrFC cDNAs in *S. cerevisiae*, and investigation of the immunoreactivity and LPS-binding capability of the rFCs.

## MATERIALS AND METHODS

### Plasmids and construction of CrFC recombinant vectors

YEpsc1 and pEMBLyex4 are high copy number, 2 μm plasmid based vectors which direct the expression of



**Fig. 2** Schematic diagram of the CrFC26 cDNA fragments cloned into the yeast expression vector YEpsc1. The complete CrFC26 cDNA is shown at the top for reference: open box, untranslated region; shaded box, sequence coding for the leader peptide of CrFC26; hatched box, coding region. Exo III nuclease deletion mutagenesis was carried out on CrFC26 cDNA to yield the deletion mutants FC26Δ9a and FC26Δ6a which contain 5' deletions up to nucleotide positions 721 and 761, respectively. Further deletions were carried out on FC26Δ9a and FC26Δ6a by removing all sequence downstream of an internal Hind III site at nucleotide position 1278 to give FC26Δ9a-H3 and FC26Δ6a-H3, respectively. A 1902 bp internal Sal I/Pst I fragment of CrFC26 cDNA was inserted directly into YEpsc1 in-frame with the *K. lactis* killer toxin signal sequence.

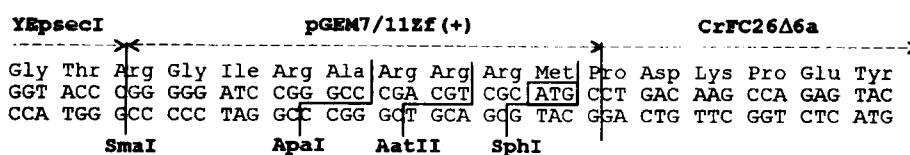
heterologous proteins under the control of a galactose inducible UAS<sub>Gal</sub>-CYC1 hybrid promoter.<sup>12-14</sup> YEpsc1 directs the secretion of heterologous proteins as fusion products of a *Kluyveromyces lactis* killer toxin signal sequence.<sup>12</sup> pEMBLyex4 is a non-secretory vector and analyses of heterologous protein expression would require lysis of the host cells.<sup>13,14</sup>

Figure 1 outlines the construction of expression plasmids carrying deletions of CrFC26 which lack the 5' UTR. The long 5' UTR of CrFC26 suggests that it may play a role in regulating the expression of the gene at the trans-

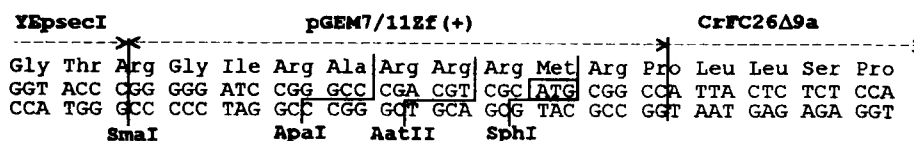
lational level. Studies using a coupled in vitro transcription and translation system showed no expression of CrFC26 whereas CrFC21, which lacks the long 5' non-coding sequence, expressed to high levels<sup>15</sup> suggesting that the 5' non-coding sequence may be involved in down-regulation of the translation of CrFC26. From a deletion library made by 5'-3' ExoIII deletion mutagenesis, two subclones designated FC26Δ6a and FC26Δ9a lacking the 5' UTR (Fig. 2) were cloned into YEpsc1 and pEMBLyex4. Further deletions of these subclones at the Hind III site (position 1278) removed sequences down-

A

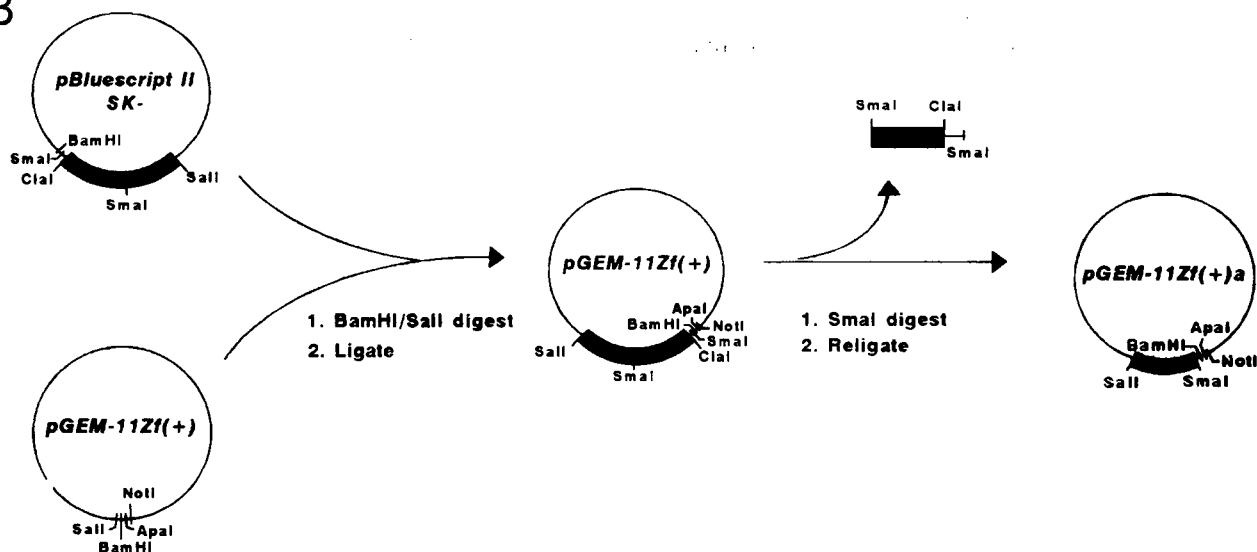
## CrFC26 DELETION SUBCLONE 6a



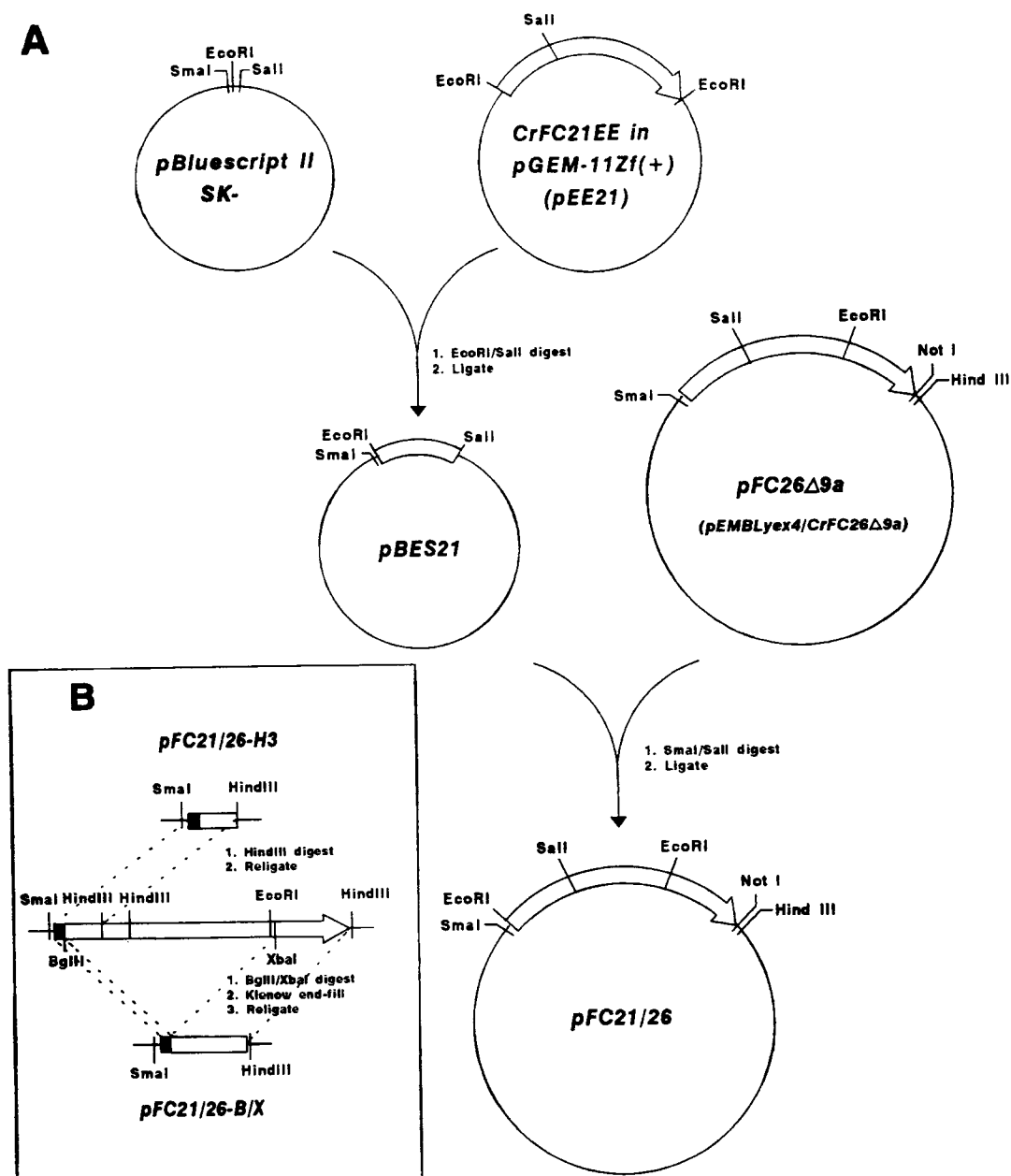
## CrFC26 DELETION SUBCLONE 9a



B



**Fig. 3** (A) Sequences at the junction between *K. lactis* killer toxin signal peptide (in YEpsc1) and CrFC26 deletion mutants 6a and 9a. The *K. lactis* killer toxin signal peptide is linked in-frame to CrFC26 deletion mutants 6a and 9a (p7SE<sub>RV</sub>Δ6a/9a, see Fig. 1) by a short stretch of polylinker sequence derived from pGEM7Zf(+) and pGEM11Zf(+). This polylinker sequence contains an ATG codon (boxed) which would serve as a translation initiation codon in pFC26Δ6a and pFC26Δ9a. (B) Construction of pGEM11Zf(+).a, a derivative of pGEM11Zf(+). A Cla I/Sal I stuffer DNA fragment (shaded) was inserted into pBluescript II SK-. This fragment was then isolated by Bam HI/Sal I digestion and inserted into pGEM11Zf(+), effectively introducing a Sma I site into the multiple cloning site of pGEM11Zf(+). The resultant plasmid was digested with Sma I and religated, removing a 300 bp Sma I fragment to give pGEM11Zf(+).a.



**Fig. 4** Construction of a hybrid cDNA of CrFC21 and CrFC26 and its deletion derivatives in the yeast expression vector pEMBLyex4. (A) The 5' Eco RI/Sal I fragment of CrFC21, which contains the ribosomal binding site and initiation codon of CrFC21, was isolated from pEE21 and inserted into the Eco RI and Sal I sites of pBluescript II SK- to give pBES21. This fragment was excised from pBES21 using Sma I and Sal I digestion and introduced into Sma I/Sal I digested pFC26 $\Delta$ 9a (pEMBLyex4/CrFC26 $\Delta$ 9a) to give pFC21/26 (pEMBLyex4/CrFC21/26). (B) The deletion mutant pFC21/26-H3 was created by digesting pFC21/26 with Hind III, thus removing 2286 bp of 3' sequences, followed by religation of the plasmid. Internal deletion of 2257 bp fragment by double digestion of pFC21/26 with Bgl II and Xba I, followed by subsequent ligation of their filled ends produced the deletion construct, pFC21/26-BX.

stream, giving rise to FC26 $\Delta$ 6a-H3 and FC26 $\Delta$ 9a-H3, respectively. These inserts were similarly cloned into the two vectors and transformed into *S. cerevisiae* for expression analyses. Selection of these subclones was based on the creation of an open reading frame upon religation of the deleted ends to the vector (Fig. 3A,B). The DNA sequences at the junctions between the vectors and

FC26 $\Delta$ 6a and 9a were verified by sequencing (Fig. 3A). These constructs utilise the ATG codon within the Sph I site of the vector for translation initiation.

Figure 4A shows a hybrid cDNA consisting of the 5' portion of CrFC21 and the 3' portion of CrFC26, inserted into pEMBLyex4. This was done by substituting the SmaI-SalI fragment of pFC26 $\Delta$ 9a (pEMBLyex4/CrFC26 $\Delta$ 9a) with the



SmaI–SalI fragment isolated from pBES21 to give pFC21/26 (pEMBLyex4/CrFC21/26) hybrid. At the amino acid level, the fusion gene product would be expected to be identical to the product encoded by CrFC21 except for a single Arg to Ser substitution at residue 427 of CrFC21. Translation initiation in pFC21/26 would rely on the cognate ATG codon of CrFC21. Deletion constructs of this hybrid cDNA are shown in Figure 4B, where pFC21/26-H3 has 84% of the 3' end of its insert deleted, and pFC21/26-BX has 65% of its internal fragment removed.

### Transformation and culture of microbial strains

Recombinant CrFC constructs were introduced into *Escherichia coli*, strain DH5 $\alpha$  by CaCl<sub>2</sub> transformation.<sup>16</sup> Transformants were cultured in LB medium supplemented with 50  $\mu$ g/ml ampicillin.

*S. cerevisiae* strain S150-2B (leu2 his3 ura3 trp1), a generous gift from Dr M. Broecker (Behringwerke, Marburg, Germany), was transformed by a modified lithium acetate procedure.<sup>17</sup> Transformed yeast cells were recovered by selection on uracil deficient synthetic complete (SC-ura) medium containing 2% glucose and 0.67% yeast nitrogen base (YNB) without amino acids (Difco) and supplemented with the required amino acids.

### Induction of Factor C synthesis

Yeast cells harbouring secretory plasmids were cultured at 30°C in complex YPD medium (2% glucose, 1% yeast extract, 2% peptone) to late log phase when the cells were induced by supplementing the medium with 2% galactose. For the induction of intracellular Factor C expression, recombinant yeasts were first cultured in SC-

ura medium supplemented with 40  $\mu$ g/ml leucine. At late log phase, cells were harvested and resuspended in fresh SC-ura medium containing 60  $\mu$ g/ml leucine and 2% galactose. After 24 h induction at 25°C, the cells were harvested for protein analyses.

### Yeast nucleic acid isolation and analysis

Transformed yeast cells were cultured overnight at 30°C in 5 ml SC-ura medium. Total DNA was isolated from the yeast cultures according to the procedure of Treco.<sup>18</sup> Following digestion with restriction endonucleases, DNA samples were fractionated on 0.8% agarose gel and transferred to nylon filter. The filter was hybridised to [<sup>32</sup>P]-labelled CrFC26 Eco RI–Not I (CrFCEN26) DNA fragment. Total yeast RNA was prepared from induced yeast cells as described by Treco.<sup>18</sup> RNA samples were denatured with glyoxal and dimethyl sulfoxide, and fractionated on a 1.2% agarose gel by the method of Selden.<sup>19</sup> Following transfer to nylon filters, the RNA was hybridised against pooled [<sup>32</sup>P]-labelled CrFCEE21 and CrFCEN21 fragments. Hybridised filters were autoradiographed.

### Protein extraction, partial purification and Western analysis

Yeast cells were collected from 10 ml induced cultures and resuspended in 0.2 ml disruption buffer, containing 25 mM Tris-Cl, pH 8.0 and 0.1 M NaCl with or without SDS. For solubilisation of insoluble proteins, SDS was added to the samples to a final concentration of 0.5, 1, 2, 3, 4, and 5%. Cell suspensions were mixed with an equal volume of chilled, acid washed glass beads (450–500  $\mu$ m, Sigma) and disrupted by vortexing 5 times for 1 min

**Table 1** *C. rotundicauda* Factor C gene constructs in the *S. cerevisiae* expression plasmid vectors, YEpsc1 (secretory) and pEMBLyex4 (non-secretory)

| Vector    | Construct name       | cDNA insert                                 | Insert size<br>(bp) | Expression status |     |  |
|-----------|----------------------|---|---------------------|-------------------|-----|--|
|           |                      |   |                     | mRNA (kNt)        | rFC |  |
| YEpsc1    | YEpsc1               | nil   |                     | nil               |     |  |
|           | YFC26 $\Delta$ 6a    | Deletion clone CrFC26 6a                    | 3447                | 3.5 1.3           | +   |  |
|           | YFC26 $\Delta$ 9a    | Deletion clone CrFC26 9a                    | 3492                | 3.5 1.3           | +   |  |
|           | YFC26 $\Delta$ 6a-H3 | Deletion clone CrFC26 6a – HindIII fragment | 543                 | 0.9               | –   |  |
|           | YFC26 $\Delta$ 9a-H3 | Deletion clone CrFC26 9a – HindIII fragment | 588                 | 0.9               | –   |  |
|           | YFC26SP              | Sal I–Pst I fragment of CrFC26              | 1902                | 2.1 1.3           | +   |  |
| pEMBLyex4 | pEMBLyex4            | nil   |                     | nil               |     |  |
|           | pFC26 $\Delta$ 6a    | Deletion clone CrFC26/6a                    | 3447                | 3.5 1.3           | +   |  |
|           | pFC26 $\Delta$ 9a    | Deletion clone CrFC26/9a                    | 3492                | 3.5 1.3           | +   |  |
|           | pFC21/26             | CrFC21–CrFC26 hybrid                        | 3448                | 3.5 1.3           | +   |  |
|           | pFC21/26-BX          | CrFC21–CrFC26 hybrid – BglIII/XbaI fragment | 1191                | 1.2               | –   |  |
|           | pFC21/26-H3          | CrFC21–CrFC26 hybrid – HindIII fragment     | 535                 | 0.9               | –   |  |

These constructs were transformed in *S. cerevisiae* 150-2B.

kNt: kilonucleotide. Expression status for rFC refers to transcription of Factor C mRNA, and translation of rFC protein that was detected by Western blot (see Fig. 7).

each, with 5 min chilling between. Cells were examined for complete lysis by microscopy. Lysates were clarified by centrifugation at 17 600 *g* for 1 h. Partial purification

of recombinant Factor C was carried out using  $(\text{NH}_4)_2\text{SO}_4$  precipitation at 20% saturation cut. The precipitated protein samples were reconstituted in pyrogen-free water and desalted through a Sephadex G-25 column (Pharmacia). Desalted protein samples were electrophoresed on denaturing SDS/10% polyacrylamide gel according to the methods of Laemmli<sup>20</sup> and blotted onto Immobilon PVDF membrane (Millipore) by electrotransfer. Factor C-related proteins were detected with rabbit anti-Factor C antibody and visualised with horseradish peroxidase-conjugated goat anti-rabbit antibody using 4-chloro-1-naphthol (Sigma) and  $\text{H}_2\text{O}_2$  as substrates.

### Lipopolysaccharide- and lipid A-binding assay of rFC

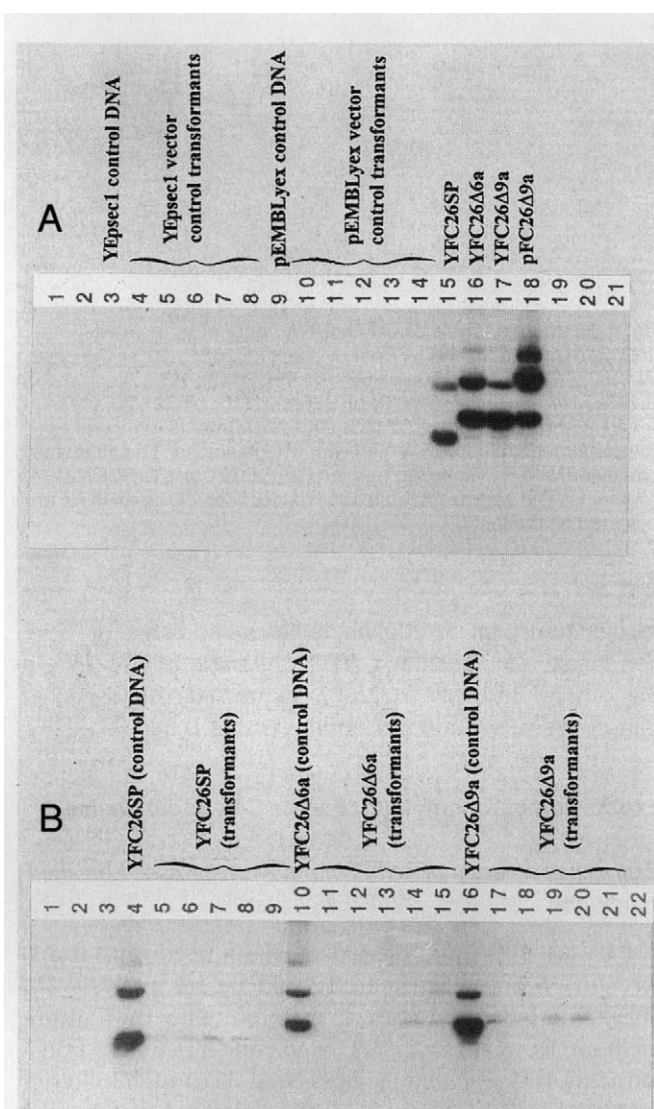
Aliquots (10  $\mu\text{g}$ ) of reconstituted lipopolysaccharide, LPS (*E. coli* O55:B5, Sigma) or diphosphoryl lipid A (*E. coli* K12, D31M4, List Biologicals, Inc., USA) were electrophoresed in each well of a 15% SDS-polyacrylamide gel. After the gel run, the LPS/lipid A was electroblotted onto Immobilon PVDF membrane. The electroblotted membrane was blocked in PBS containing 30 mg/ml BSA for 30 min at 37°C. The membrane was subsequently cut into strips corresponding to each lane of the electrophoretically resolved LPS/lipid A. Each LPS/lipid A strip was incubated separately with 300  $\mu\text{g}$  of the respective rFC sample that was partially purified by 20%  $(\text{NH}_4)_2\text{SO}_4$  cut. PBS was used as the reaction buffer and the reaction volume was 1 ml/cm<sup>2</sup> membrane area. Incubation was carried out overnight at 37°C with slight agitation. The LPS/lipid A strips were washed 3 times with PBS containing 0.2% Tween-20. This was followed by incubation for 3 h at 37°C with anti-Factor C antibody diluted 1:500 in PBS containing 0.2% Tween-20 and 1 mg/ml BSA. After 3 washings with 0.2% Tween-20 in PBS, the strips were incubated at 37°C for 1 h with the secondary alkaline phosphatase-conjugated goat anti-rabbit antibody (Dako). The strips were subsequently rinsed in 0.2% Tween-20 in PBS, and stained with the substrate, 5-bromo-4-chloro-3-indolyl-phosphate p-nitro blue tetrazolium chloride (BCIP/NBT, Moss, Inc., USA).

## RESULTS AND DISCUSSION

Table 1 summarises CrFC cDNA constructs in pEMBLyex4 and YEpsc1 vectors, and their expression status in *S. cerevisiae* host, 150-2B.

### Presence of recombinant plasmids in transformants

Total DNA was prepared from yeast cells transformed with FC26SP, FC26Δ6a and FC26Δ9a (see Fig. 2). DNA from YEpsc1 and pEMBLyex4 vector transformants and untransformed yeast cells were included as controls.



**Fig. 5** Southern hybridisation of total DNA preparations from yeast transformants and purified plasmid constructs. DNA samples from transformants were digested with KpnI restriction endonuclease and fractionated on 0.8% agarose gel before transfer to nylon filters. The nylon filters were hybridised to [<sup>32</sup>P]-labelled CrFCEN26 fragment. (A) (1, 21)  $\lambda$ Hind III marker, (2) pCrFC26EE, (3) YEpsc1, (4–8) total DNA from independent YEpsc1 transformants, (9) pEMBLyex4, (10–14) total DNA from independent pEMBLyex4 transformants, (15) YFC26SP, (16) YFC26Δ6a, (17) YFC26Δ9a, (18) pFC26Δ9a, (19, 20) control DNA from untransformed *S. cerevisiae*, 150-2B. (B) (1, 22)  $\lambda$ Hind III marker, (2) pCrFC26EE, (3) YEpsc1, (4) YFC26SP, (5–9) total DNA from independent YFC26SP transformants, (10) YFC26Δ6a, (11–15) total DNA from independent YFC26Δ6a transformants, (16) YFC26Δ9a, (17–21) total DNA from independent YFC26Δ9a transformants.

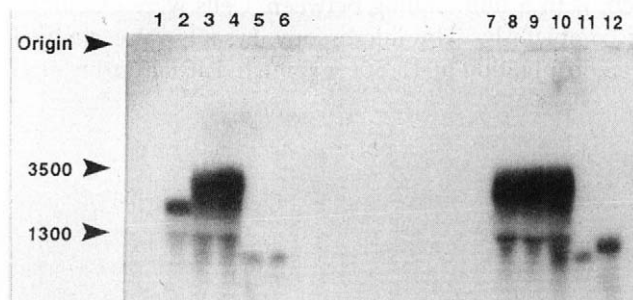


Transformant DNAs were digested with KpnI. Following fractionation on agarose gel and transfer to nylon membranes, Southern blot hybridisation was carried out with [ $^{32}$ P]-labelled CrFCEN26. Figure 5 shows that the recombinant plasmids were present in the yeast host.

### Transcription of Factor C sequences in *S. cerevisiae*

The transcription of Factor C sequences under the regulation of the inducible UAS<sub>gal</sub>-CYC1 promoter was analysed by Northern hybridisation. Figure 6 shows the transcription of some representative clones. Table 1 summarises the transcripts produced by each clone. It is apparent from the sizes of the mRNAs that transcription has terminated efficiently in some of the plasmid constructs. The transcripts from constructs which lack the 3' UTR of Factor C cDNA were observed to be approximately 200–300 bases larger than would be predicted from the sizes of the cDNA inserts (Fig. 6, lanes 2, 5, 6, and 11). This would be consistent with transcription termination at the FLP gene terminator within the vectors. This terminator sequence is located 205 bp downstream from the multiple cloning sites of pEMBLyex4 and YEpsec1.<sup>12,13</sup>

A smaller transcript of ~1300 nucleotides was observed for YFC26SP, YFC26Δ6a, YFC26Δ9a, pFC26Δ6a, pFC26Δ9a and pFC21/26 transformants (Fig. 6, lanes 2, 3, 4, 8, 9 and 10, respectively). This low level transcript could have arisen as a result of a fortuitous transcription initiation from an internal site or a premature transcription termination event. The latter possibility may be ruled out, since the transcript is observed to be uniform in size for different constructs despite the difference in lengths of the cDNA inserts. Premature termination would be expected to yield correspondingly smaller transcripts, assuming that termination occurs at the same point. Transcription initiation from a cryptic internal initiation site would most likely account for the smaller transcript observed. It is known that, in yeasts, the transcriptional machinery recognises a functional TATA box and initiates transcription 40–120 bp downstream from the site.<sup>21</sup> The cDNA coding for CrFC26 contains five TATA boxes of which three are also present at corresponding positions in the cDNA sequences coding for CrFC21.<sup>10</sup> At least two of these elements, located at regions corresponding to nucleotide positions 1823–1827 and 3105–3110 of CrFC21 are present in the plasmid constructs which could have yielded the ~1300 nucleotides transcript when induced in yeast cells. Transcription initiation directed from the first TATA box would produce transcripts in the size range of about 1500 nucleotides. Therefore, it seems likely that the smaller transcript is due to internal transcription initiation. However, its expression level is low, possibly due to the lack of transactivating *cis* element proximal to internal TATA box. A similar explanation may be offered for the



**Fig. 6** Northern analysis of CrFC cDNA sequences in yeast. Total RNA was purified from (1) YEsec1, (2) YFC26SP, (3) YFC26Δ6a, (4) YFC26Δ9a, (5) YFC26Δ6a-H3, (6) YFC26Δ9a-H3, (7) pEMBLyex4, (8) pFC26Δ6a (9) pFC26Δ9a, (10) pFC21/26, (11) pFC21/26-H3, and (12) pFC21/26-BX transformants induced in complete medium supplemented with 2% galactose. The filter was hybridised with [ $^{32}$ P]-labelled purified CrFCEE21 and CrFCEN21 fragments. The approximate sizes in nucleotides of the mRNAs are indicated on the left.

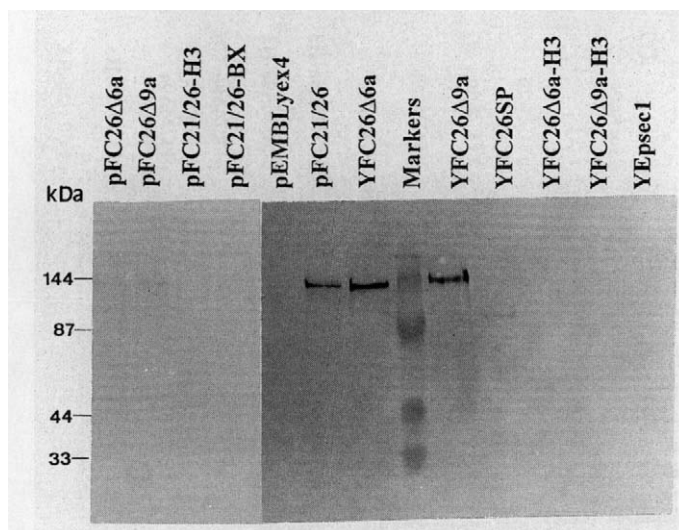
smaller transcript of 900 nucleotides expressed by deletion constructs YFC26Δ6a, YFC26Δ9a and pFC21/26. On the other hand, pFC21/26-BX expressed the expected transcript size of 1200 nucleotides (Table 1).

### Expression of recombinant Factor C in *S. cerevisiae*

#### *Expression of recombinant Factor C from YEsec1 plasmid constructs*

The culture medium from galactose-induced yeast transformants was concentrated 10-fold before analysis. No detectable level of Factor C was found in the culture medium. However, with YFC26Δ6a and YFC26Δ9a transformants, rFC prepared by glass bead disruption followed by 20% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation showed substantial amounts of immunoreactive recombinant protein in the insoluble intracellular fractions (Fig. 7). YFC26SP which contained only 1.9 kb of internal fragment of the CrFC cDNA insert expressed limited levels of truncated, albeit immunoreactive, rFC of ~90 kDa. Generally, the yield of rFC increased when SDS was included in the extraction buffer, indicating that a substantial amount of the expressed Factor C was associated with the insoluble cell fraction. Maximal solubilisation was achieved with either 1% Triton X-100 or SDS.

It is evident from the results that the *K. lactis* killer toxin signal sequence did not direct the secretion of the recombinant product. This could be due to improper processing of the signal peptide, although the hypothetical cleavage site for the signal endopeptidase was preserved



**Fig. 7** Western analysis of partially purified rFC from *S. cerevisiae* clones containing: (1) pFC26Δ6a, (2) pFC26Δ9a, (3) pFC21/26-H3, (4) pFC21/26-BX, (5) pEMBLyex4 control vector, (6) pFC21/26, (7) YFC26Δ6a, (8) molecular weight markers (9) YFC26Δ9a, (10) YFC26SP, (11) YFC26Δ6a-H3, (12) YFC26Δ9a-H3 and (13) YEpsec1 control vector. The molecular weight markers are indicated on the left in kDa.

in each case. An alternative cleavage site, which was recognised by the endopeptidase during the processing of a killer toxin-human interleukin  $\beta$  fusion product,<sup>12</sup> is also present in YFC26SP. It is also possible that the folding of the signal peptide with respect to the overall conformation of the recombinant protein has adversely affected the processing by the endopeptidase.

YFC26Δ6a-H3 and YFC26Δ9a-H3 did not yield any detectable levels of rFC. It is possible that sequences downstream of the Hind III site at position 1278 contribute to major epitopes of Factor C, thus even if rFC was produced from these two clones, it probably lacked immunoreactivity.

#### Expression of recombinant Factor C from pEMBLyex4 plasmid constructs

A single immunoreactive band with an apparent size of ~130 kDa was observed for rFC expressed in pFC21/26 transformants (Fig. 7). This recombinant protein is approximately 11 kDa larger than the calculated size based on the cDNA insert length. It is tempting to speculate that the rFC was post-translationally modified by glycosylation since the cDNA encodes six potential glycosylation sites.<sup>10</sup> pFC26Δ6a and pFC26Δ9a also produced immunoreactive rFC (Fig. 7). On the other hand, deletion subclones such as pFC21/26-H3 (devoid of 84% of 3' end) and pFC21/26-B/X (lacking 65% of internal fragment) transformants (see Fig.

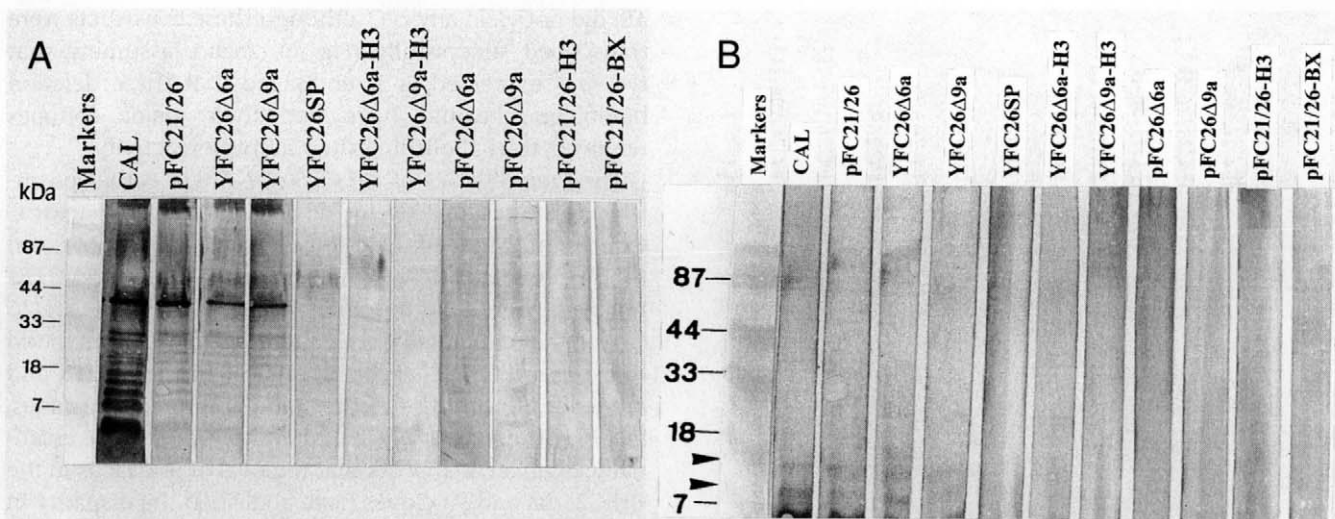
4B) did not yield any rFC although these constructs were transcribed successfully (Fig. 6). Again, assuming that rFC was expressed, it is envisaged that these deletion homologues would have had their major epitopes removed, thus abolishing their immunoreactivity.

The long 5' UTRs of CrFC26 cDNA was earlier postulated to be responsible for down-regulation of Factor C expression.<sup>15</sup> Indeed, deletion subclones pFC26Δ6a and 9a, YFC26Δ6a and 9a, which have their long 5' UTRs removed, were able to express rFC. Similarly, pFC21/26 hybrid that contained CrFC21 at the 5' region was found to express rFC. Except for differences in the 5' UTR and sequences encoding the first nine amino acid residues, the cDNA inserts of pFC21/26 and pFC26Δ6a are essentially identical. It appears that removal of 5' UTR as in the CrFC26/6a and 9a clones have abolished the disparity in expression levels of clones 21 and 26. The effects of alterations in the bases surrounding the AUG initiation codon on the translation levels of various yeast genes have been reported.<sup>22,23</sup> It was found that an A at the -3 position was the most effective in initiating translation. In the context of NUCAUG (in which N refers to any base), the efficiency of bases in initiating translation was found to be  $A > G > C = U$ . The relative levels of translation were 100%:90%:50% for A:G:C or U.<sup>24,25</sup> The other positions were found to have minimal effects on translation. The mRNA generated by pFC21/26 would contain an A at -3 while those of pFC26Δ6a and pFC26Δ9a would both contain a C at this position. Indeed, in the present recombinant constructs, this difference in sequence context has caused a drastic reduction in the level of translation of pFC26Δ6a and pFC26Δ9a as compared to pFC21/26 hybrid.

It is evident from the immunoblot (Fig. 7), that YFC26Δ6a and YFC26Δ9a have vastly improved levels of expression of rFC as compared to their expression in pEMBLyex vector. The presence of the *K. lactis* killer toxin signal sequence in the YEpsec1 vector may be responsible for directing the optimal expression of the recombinant proteins.<sup>12</sup>

#### Recombinant Factor C binds LPS

The functionality of the rFC was determined by its ability to bind to LPS, in particular, the lipid A moiety which is the main endotoxic component of LPS. Distinctive bands ranging from 7–20 kDa were observed on LPS strips that were incubated with rFC derived from pFC21/26, YFC26Δ6a and YFC26Δ9a (Fig. 8A). Similarly, with lipid A strips, specific bands were observed within this range of molecular sizes corresponding to lipid A moieties, in particular, 8.3 and 15.5 kDa (Fig. 8B), thereby, confirming the specific biological activity of rFC. The ability of these rFCs to bind LPS suggests strongly the presence of a



**Fig. 8** Endotoxin-binding assay of rFC to (A) LPS and (B) purified diphosphoryl lipid A. LPS/lipid A was resolved on SDS PAGE and electroblotted onto Immobilon PVDF membrane. The membrane strips were then incubated with (1) *Carcinoscorpius* amoebocyte lysate (CAL), and rFC from (2) pFC21/26, (3) YFC26Δ6a, (4) YFC26Δ9a, (5) YFC26SP, (6) YFC26Δ6a-H3, (7) YFC26Δ9a-H3, (8) pFC26Δ6a, (9) pFC26Δ9a, (10) pFC21/26-H3 and (11) pFC21/26-BX. After rFC has bound to LPS/lipid A, the membrane strips were incubated with rabbit anti-rFC followed by visualisation using alkaline phosphatase/BCIP-NBT detection system. The heavy band at approximately 40 kDa (A) is probably due to non-specific binding to the O-specific chain of LPS. Absence of this band in lipid A binding assay (B) further substantiates this possibility. (B) Shows that rFC was specifically bound to the lipid A moieties of 8.3 and 15.5 kDa (arrow).

functional endotoxin-binding domain in these recombinant proteins. On the other hand, although pFC26Δ6a and 9a produced immunoreactive rFCs, the expression of these deletion subclones in the pEMBLyex4 system might have resulted in differently folded proteins which were unable to bind LPS/lipid A. rFC from clones with major deletions such as YFC26Δ6a-H3 and YFC26Δ9a-H3, pFC21/26-H3 and YFC26SP (see Figs 2 & 4B) containing only a small portion of CrFC and lacking the postulated endotoxin-binding site were unable to bind LPS. In particular, rFC from clone YFC26SP was immunoreactive (see Fig. 7) but incapable of binding LPS since it lacked the endotoxin-binding region. This confirms the requisite presence of the 5' region of the CrFC cDNA to encode the endotoxin-binding domain.

The first ~250 amino acid residues at the N-terminus have been reported to be involved in endotoxin-binding.<sup>26</sup> In this study, the integrity of the endotoxin-binding domain of the rFC homologue from YFC26Δ9a-H3 was adversely affected (Fig. 8) due to deletions of: (i) 51 amino acids from the 5' extremity; and (ii) 17 amino acids from the 3' extremity of the endotoxin-binding domain. This latter deletion was incurred by the removal of the HindIII fragment (downstream of 1278 bp position, see Fig. 2) which overlapped 51 bp of the 3' region of the postulated endotoxin-binding site. It appears that the remaining internal sequence encompassed within 721–1278 bp<sup>10</sup> encodes a truncated endotoxin-binding domain which has adversely affected LPS binding. By the same token, rFC

from YFC26Δ6a-H3 was also defective in binding LPS since a further 41 bp deletion was incurred at the 5' end of the CrFC cDNA insert. This indicates the critical importance of the integrity of the amino acids at the 5' and 3' extremities of the LPS-binding domain. Alternatively, the amino acids in close proximity appear to exert a strong influence on LPS binding. Either scenario has affected the proper folding conformation of the rFC molecule, giving rise to cryptic endotoxin-binding domain.

## CONCLUSION

Recombinant constructs of *C. rotundicauda* Factor C cDNA in *S. cerevisiae* shuttle vectors: pEMBLyex4 (intracellular expression) and YEpsec1 (secretion vector) were expressed intracellularly after galactose induction of the yeast transformants, yielding post-translationally modified and, possibly, glycosylated and immunoreactive rFC. The *K. lactis* secretory signal in YEpsec1 was, however, unable to direct secretion of the rFC into the medium. 3' Deletions of up to 84%, and internal deletion of 65% of the CrFC cDNA probably abolished immunoreactivity and LPS-binding ability of the rFC. Removal of the 5' UTR of CrFC26 resulted in improved expression of rFC. Where the endotoxin-binding domain was present, these rFCs were biologically functional, being capable of binding LPS. This study also indicates that the deletion constructs of rFC could be used to investigate and delineate the precise region of LPS-binding.



## ACKNOWLEDGEMENTS

We thank Dr J.A.H. Murray, Cambridge, UK for the plasmids: YEpsc1 and pEMBLyex4; Dr M Broker, Marburg, Germany for *S. cerevisiae* strain 150-2B, and Mr H.K. Yip for photography. This work was supported by National University of Singapore, Grant RP950303.

## REFERENCES

- Levin J, Bang F.B. The role of endotoxin in the extracellular coagulation of *Limulus* blood. *Bull Johns Hopkins Hosp* 1964; **115**: 265–274.
- Nakamura T, Morita T, Iwanaga S. Intracellular proclotting enzyme in *Limulus* (*Tachypleus tridentatus*) hemocytes: its purification and properties. *J Biochem (Tokyo)* 1985; **97**: 1561–1574.
- Ho B., Kim J.C., Ding J.L. Electrophoretic analysis of endotoxin-activated gelation reaction of *Carcinoscorpius rotundicauda* amoebocyte lysate. *Biochem Mol Biol Int* 1993; **29**: 687–694.
- Iwanaga S., Morita T., Miyata T., Nakamura T. Hemolymph coagulation system in *Limulus*. In: Lieve L. et al. (eds) *Microbiology*. Washington: American Society of Microbiology, 1985; 21–24.
- Nakamura T., Horiuchi T., Morita T., Iwanaga S. Purification and properties of intracellular clotting factor, Factor B from horseshoe crab (*Tachypleus tridentatus*) hemocytes. *J Biochem (Tokyo)* 1986a; **99**: 847–857.
- Tokunaga F., Miyata T., Nakamura T. et al. Lipopolysaccharide-sensitive serine protease zymogen (Factor C) in horseshoe crab hemocytes: identification and alignment of proteolytic fragments produced during the activation show that it is a novel type of serine protease. *Eur J Biochem* 1987; **167**: 405–416.
- Ding J.L., Navas III M.A.A., Ho B. Two forms of Factor C from the amoebocytes of *Carcinoscorpius rotundicauda*: purification and characterisation. *Biochim Biophys Acta* 1993; **1202**: 149–156.
- Nakamura T., Morita T., Iwanaga S. Lipopolysaccharide sensitive serine-protease zymogen (Factor C) found in *Limulus* hemocytes: isolation and characterization. *Eur J Biochem* 1986; **154**: 511–521.
- Muta T., Miyata T., Misumi Y. et al. *Limulus* Factor C: an endotoxin sensitive serine protease zymogen with a mosaic structure of complement-like, epidermal growth factor-like and lectin-like domains. *J Biol Chem* 1991; **266**: 6554–6561.
- Ding J.L., Navas M.A.A., Ho B. Molecular cloning and sequence analysis of Factor C cDNA from the Singapore horseshoe crab, *Carcinoscorpius rotundicauda*. *Mol Marine Biol Biotechnol* 1995; **4**: 90–103.
- Hadfield C., Raina K.K., Shashi-Menon K., Mount R.C. The expression and performance of cloned genes in yeast. *Mycol Res* 1993; **8**: 897–944.
- Baldari C., Murray J.A.H., Ghiara P., Cesareni G., Galeotti C.L. A novel leader peptide which allows efficient secretion of a fragment of human interleukin 1 $\beta$  in *Saccharomyces cerevisiae*. *EMBO J* 1987; **6**: 229–234.
- Cesareni G., Murray J.A.H. Plasmid vectors carrying the replication origin of filamentous ss phages. In: Setlow J.K., Hollaender A. (eds) *Genetic Engineering: Principles and Methods* Vol. 9. New York: Plenum, 1987; 135–154.
- Murray J.A.H., Scarpa M., Rossi N., Cesareni G. Antagonistic controls regulate copy number of the yeast 2 $\mu$  plasmid. *EMBO J* 1987; **6**: 4205–4212.
- Roopashree S.D., Chai C., Ho B., Ding J.L. Expression of *Carcinoscorpius rotundicauda* Factor C cDNA. *Biochem Mol Biol Int* 1995; **35**: 841–849.
- Sambrook J., Fritsch E.F., Maniatis T. *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
- Schiestl R.H., Gietz R.D. High-efficiency transformation of intact yeast cells using single-stranded nucleic-acids as a carrier. *Curr Genet* 1989; **16**: 339–346.
- Treco D.A. In: Ausubel M., Brent R., Kingston R.E. et al. (eds) *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley-Interscience 1989; **2**: Unit 13.
- Selden R.F. In: Ausubel M., Brent R., Kingston R.E. et al. (eds) *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley-Interscience 1987; **1**: Unit 4.
- Laemmli U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–684.
- Struhl K. Promoters, activator proteins, and the mechanism of transcriptional initiation in yeast. *Cell* 1987; **49**: 295–297.
- Baim S.B., Sherman F. mRNA structures influencing translation in the yeast *Saccharomyces cerevisiae*. *Mol Cell Biol* 1988; **8**: 1591–1601.
- Clements J.M., Laz T.M., Sherman F. Efficiency of translation initiation by non-AUG codons in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1988; **8**: 4533–4536.
- Kozak M. Bifunctional messenger RNAs in eukaryotes. *Cell* 1986; **44**: 283–292.
- Cigan A.M., Pabich E.K., Donahue T.F. Mutational analysis of the HIS4 translational initiator region in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1988; **8**: 2964–2975.
- Miura Y., Tokunaga F., Miyata T., Moriasu M., Yoshikawa K., Iwanaga S. Preparation and properties of monoclonal antibodies against lipopolysaccharide-sensitive serine protease zymogen, Factor C from the horseshoe crab (*Tachypleus tridentatus*) hemocytes. *J Biochem* 1992; **112**: 476–481.