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# Purification of the Insulin-like Growth Factor II (IGF-II) Receptor From an IGF-II-Producing Cell Line, and Generation of an Antibody Which Both Immunoprecipitates and Blocks the Type 2 IGF Receptor

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SUMMARY: 18,54-SF cells, which secrete rat insulin-like growth factor II (rIGF-II), have abundant type 2 IGF receptors. We have purified the type 2 receptor from these cells by solubilization of crude membranes in Triton X-100, followed by chromatography on agarose-immobilized rIGF-II. A partially purified receptor preparation, obtained by chromatography of solubilized membranes over wheat germ agglutinin, was used to immunize a rabbit. The antibody generated both immunoprecipitates the type 2 receptor, and specifically inhibits IGF-II binding to a variety of rat tissues, including 18,54-SF cells, BRL-3A cells and placenta. The presence of abundant type 2 receptors on an rIGF-II-secreting cell line is consistent with an autocrine role for IGF-II in select cells. 

The somatomedins comprise a family of mitogenic peptides with major structural homology with proinsulin (1). In humans, two somatomedins have been identified, purified and sequenced: insulin-like growth factors (IGF) I and II (1,2). A rat IGF-II has also been identified, and found to have 94% sequence homology with human IGF-II (3). The metabolic and mitogenic actions of these growth factors are believed to be mediated by binding to specific receptors on target cell membranes (4). The insulin and IGF-I (type 1) receptors are structurally related, both migrating on electrophoretic gels with an apparent Mr >300,000 and consisting of disulfide-linked subunits (5). The IGF-II (type 2) receptor, on the other hand, migrates with an apparent Mr of 220-250,000, with no evidence of disulfide-linked subunits (5,6). This receptor also differs from the insulin and type 1 IGF receptor in its lack of affinity for insulin.

The biologic role of the type 2 receptor remains uncertain, despite extensive characterization by competitive binding studies, affinity cross-linking, and extraction and purification (5-8). Although the presence of abundant type 2 receptors in fetal and embryonic tissue, accompanied by high circulating levels of IGF-II in the fetus and infant, have suggested an important role for IGF-II and its receptor in fetal growth, this hypothesis remains unproven (9,10). Studies from Mottola and Czech (11) have indicated that the type 2 receptor is not involved in the stimulation of DNA synthesis in H-35 hepatoma cells, and recent studies from our laboratory demonstrate that the mitogenic action of IGF-II in human fibroblasts can be blocked by an antibody specifically directed at the IGF-I receptor. On the other hand, we have recently reported that two cell lines which secrete rIGF-II into conditioned media are also characterized by abundant type 2 receptors,

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transcriptionally regulated. Glucose addition to cells growing with ethanol as an energy and carbon source and exhibiting very low level of PDCmRNA elicited a rapid increase of PDCmRNA . However, the effect is not specific to glucose as galactose, glycerol and succinate are able to lead to PDCmRNA accumulation (4).

In the course of cloning experiments in yeast we have isolated an unknown gene whose expression is transcriptionally regulated by glucose. This gene has been called SRP (Serine rich protein) as the aminoacid composition of the protein deduced from the nucleotide sequence, is very rich in serine residues (in preparation). The preliminary results reported in this paper show the glucose positive modulation of the SRP mRNA. The nucleotide sequence of the promoter region is presented and compared to known promoter regions of glycolytic genes.

## METHODS

### Strains and Plasmids

The following strains were used in this study GRF18 (MATα his3-11 his3-15 leu 2-3 leu 2-112 can 1-100) and NC93spl (MATa ura3-373, ura3-251, ura3-328) (5).

Plasmid pLG669-Z is a gift of L. Guarente (6). Plasmid pR21A contained the SRP gene in a 5.1 kilobases (kb) EcoRI fragment of yeast DNA inserted at EcoRI site in pAT153. Plasmids were maintained and produced in E. coli HB101 (7).

Yeast culture, yeast transformation and plasmid constructions were carried out by standard methods (8,9).

### RNA extraction and electrophoretic analysis.

RNAs were extracted from yeast cells grown to an early log phase in complete medium which consisted of 1% yeast extract (Difco), 2% peptone (Difco) and 2% glucose, 2% raffinose or 3% glycerol/ethanol. Formaldehyde-denatured RNA samples were electrophoresed on agarose gels (1.5%) in 2.2M formaldehyde, 20mM MOPS, 5mM sodium acetate, 1mM EDTA buffer as described by Maniatis et al. (9). RNA were transferred to nitrocellulose filter according to the method described by Thomas (10). The 0.6 kb PvuII fragment of SRP gene and the 1.7 kb BamHI fragment containing the entire HIS3 gene (11) were [$^{32}$P]-labeled by nick-translation (12). Hybridization conditions were as described by Sherman et al. (8).

### Sequence determination.

DNA sequence was determined by the dideoxy nucleotide chain termination method of Sanger et al.(13) or by the chemical method of Maxam and Gilbert (14).

### β-galactosidase activity.

Single colonies of yeast transformants were grown according to Yocum et al.(15). β-galactosidase assays were carried out by the method of Guarente (16). All assays were done in duplicate from three independant transformants.

## RESULTS AND DISCUSSION

The SRP gene has been cloned and sequenced (to be published elsewhere). Its main characteristics are, 1- the presence of 8 tandem repeats in the coding region of the nucleotide sequence, these repeats

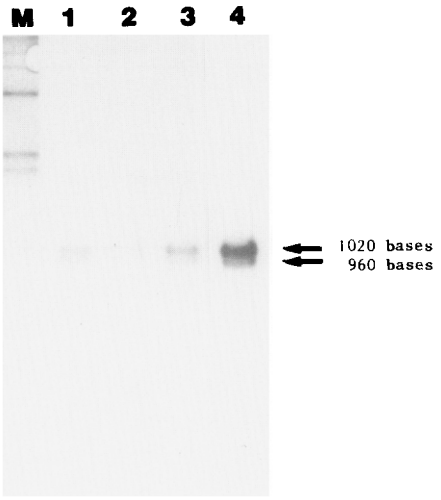



Fig. 1. Northern blot analysis of SRP gene.
10 µg of total RNA were size fractionated on 1.5% agarose gels containing formaldehyde tranferred to a nitrocellulose filter and hybridized. The probe was the 0.57 kb PvuII fragment of the SRP gene. Molecular weight markers were λ/HindIII fragments (lane M). Growth conditions were YP glucose (lane 1 and 4), YP glycerol/ethanol (lane 2), YP raffinose (lane 3); cells were collected in the exponential phase (lanes 2,3,4) or in the plateau phase (lane 1).

being also apparent in the amino-acid sequence, 2- amino-acid composition deduced from the nucleotide sequence is very unusual. The protein is very rich in serine and alanine residues (24.8% and 20.9% total amino-acid respectively).

To analyse transcriptional modulation elicited by glucose, RNA were prepared from GRF18 yeast strain grown with different carbon sources and separated by electrophoresis. After transfer to nitrocellulose membrane mRNA were probed with the PvuII fragment which is located in the coding sequence. Transcripts from the SRP gene are relatively abundant as a strong signal was easily detectable after blotting only 10 µg of total RNA (Fig. 1). Two populations of transcripts were clearly seen when shorter exposure times were used. The size of the major one is about 1020 bases while the minor one is 960 bases size. Both transcript populations are the product of the SRP gene because genomic deletion of SRP leads to the complete absence of these transcripts (not shown).

The steady-state amount of SRP mRNA is regulated by the carbon source of the growth medium. In glycerol/ethanol or raffinose containing medium the relative amount of mRNA (table I) were estimated to be 10 and 20% of the glucose control experiment, respectively (Fig. 1 lane 2 and 3). Specific mRNA is also less abundant (table I) when cell cultures reach the plateau phase compared to exponential phase (Fig.1 lane 1). This effect

Table I : Relative steady state amounts of SRP mRNA

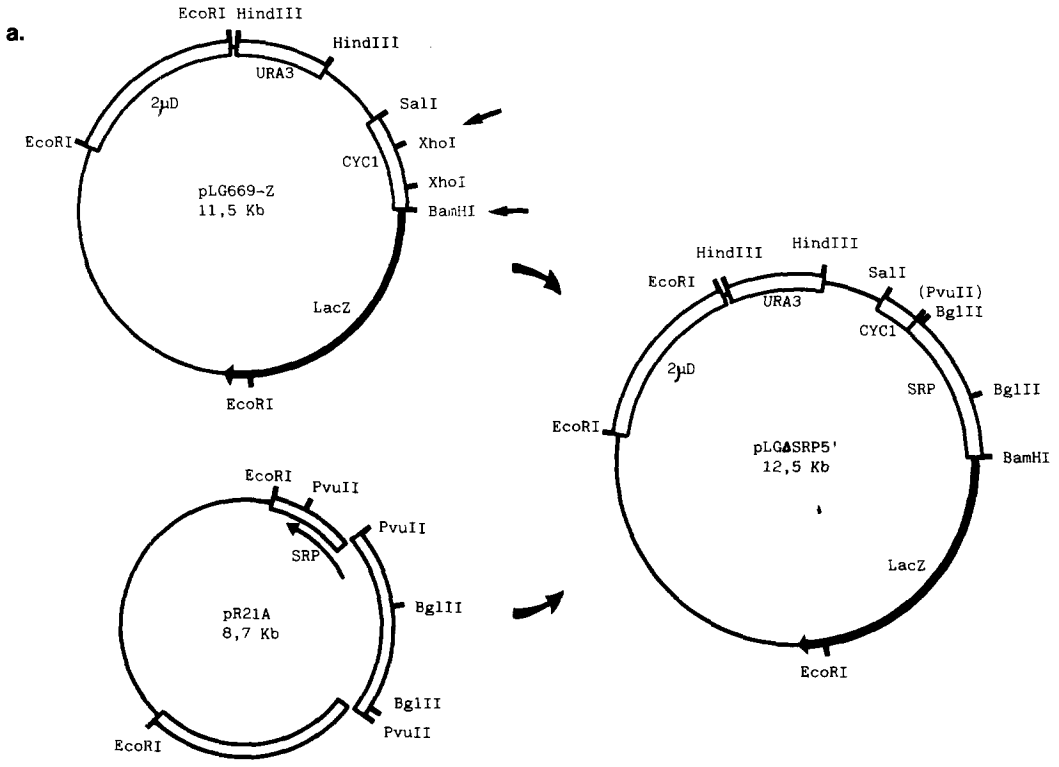
| Growth phase | exp[a] | | exp | | exp | | plateau | |
|---|---|---|---|---|---|---|---|---|
| Carbon source | Glycerol/ethanol | | Raffinose | | glucose | | glucose | |
| | N[b] | %[c] | N | % | N | % | N | % |
| RNA 2.3 kb | 1 | | 1 | | 1 | | 1 | |
| RNA SRP | 0.4 | 10 | 0.7 | 18 | 3.9 | 100 | 0.8 | 20 |
| RNA HIS3 0.7 kb | 0.4 | 95 | 0.4 | 95 | 0.42 | 100 | 0.32 | 75 |

a) exp : exponential
b) N : normalized amount
c) % : refers to standard growth conditions (2% glucose, exponential phase).
Northern blot of fig.1 was rehybridized with the HIS3 probe (1.7 kb BamHI fragment). Beside the HIS3 mRNA (0.7 kb) the HIS3 probe detects a 2.3 kb mRNA and a 1.2 kb mRNA. After scanning with a Vernon densitometer the areas under the peaks were determined by weighting and the relative amounts of RNA were normalized to the amount of 2.3 kb mRNA.

could be probably related to the depletion of glucose in the culture medium at the end of exponential growth.

To demonstrate further that SRP gene is positively modulated by glucose, the 5' flanking region of the SRP coding region has been inserted in a lacZ plasmid in such a way that it can control the expression of lacZ and the β-galactosidase activity was determined after transformation of yeast. The construction of plasmid pLGΔSRP5' is described in fig. 2. Plasmid pLG669-Z with lacZ under CYC1 promoter control was the source of lacZ gene. The promoter region of SRP was purified from plasmid pR21A as a 2.2 kb PvuII fragment and ligated to the blunt-ended 10.6 kb BamHI-XhoI fragment of plasmid pLG669-Z. The first 58 amino-acids of SRP protein were put in phase with lacZ reading frame when the CYC1 promoter was substituted for the SRP promoter to give the final plasmid pLGΔSRP5'. The fusion junction is shown in fig. 2b.

Plasmid pLGΔSRP5' was introduced in yeast NC93spl, $URA^+$ transformants were isolated and assayed for β-galactosidase activity in induction or repression conditions. Cells were inoculated into minimal or complete medium containing either 2% glucose or 3% glycerol/ethanol as a carbon and energy source. Cultures were grown until early exponential phase (OD 578 nm = 1) washed once with water and β-galactosidase activities determined as described in Methods. Control experiments were done




b.

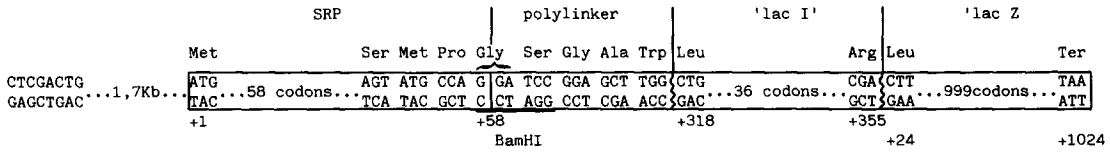



Fig. 2. a) Construction of plasmid pLGΔSRP5'.
b) Details of the fusion junction created by construction of pLGΔSRP5'.

with yeast transformed with pLG669-Z in which β-galactosidase is under the control of CYC1 promotor and then sensitive to glucose repression.

Results are shown in table II. β-galactosidase activities are higher when complete medium was used for the cultures but essentially similar results were obtained with minimal and complete medium. Plasmid pLGΔSRP5' was able to produce 26 and 1 units of enzyme activity with minimal medium containing glucose and glycerol, respectively. In the same growth conditions control experiment carried out with pLG669-Z showed 10 and 103 units of enzyme activities with glucose and glycerol containing media, respectively.

If we define a regulation index as the ratio of enzyme activities between induced and repressed conditions, SRP promoter was able to

Table II : β-galactosidase expression of fusion plasmids

| plasmid | minimal medium | | | rich medium | | |
|---|---|---|---|---|---|---|
| | glucose | glycerol | regulation index | glucose | glycerol | regulation index |
| pLGΔSRP5' | 26 | 1 | 26 | 68 | 8 | 8.5 |
| pLG669-Z | 10 | 103 | 10.3 | 37 | 503 | 13.6 |

Yeast strain NC93spl was transformed with pLGΔSRP5' or pLG669-Z and β-galactosidase assays performed after growth on minimal or rich media containing 2% glucose or 3% glycerol. Assays were done in duplicate from three independent transformants.

elicit an induction index of 26 while CYC1 promoter gave an index of 10.3 when cells were grown in minimal medium. In complete medium the regulation indexes for SRP and CYC1 promotors were 8.5 and 13.6, respectively.

400 bp of the 5' flanking region of the SRP gene have been sequenced (fig.3). Strong similarities with other known strong yeast promoters were noted. They include a TATA box located at -128 through -123, a PyAAPu sequence coinciding with each initiation site and specifically a CAAG box for the major initiation site (not shown). The region between the CAAG box and the translation initiating ATG codon is highly AT rich (75%). Moreover this area presents important homologies with the strong yeast promoters described by Dobson et al.(17) and more specifically with glycolytic gene promoters (18). The main characteristic is a

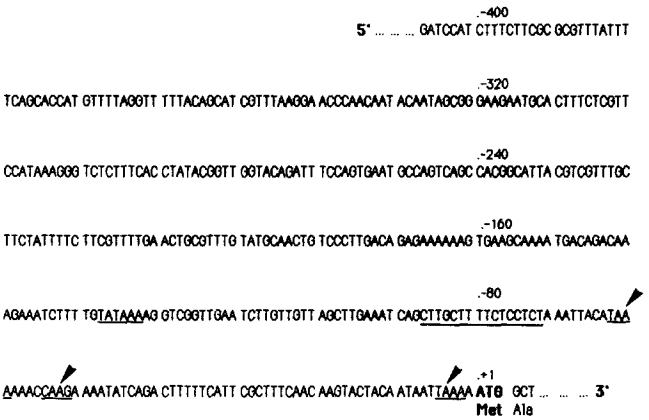



Fig. 3. Nucleotide sequence of the SRP promoter region . ▼: transcription initiation site.

pyrimidine stretch of 16 nucleotides located 16 bp upstream of the major transcription initiation site. These structural charateristics of the promoter region of the SRP gene are in agreement with the large amount of SRP transcipts that was detectable.

The determination of 5' sequence(s) responsible for the positive glucose regulation is now in progress in this laboratory.

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