The *NSR1* Gene Encodes a Protein that Specifically Binds Nuclear Localization Sequences and Has Two RNA Recognition Motifs

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Abstract. We previously identified a protein (p67) in the yeast, *Saccharomyces cerevisiae*, that specifically recognizes nuclear localization sequences. We report here the partial purification of p67, and the isolation, sequencing, and disruption of the gene (*NSR1*) encoding this protein. p67 was purified using an affinity column conjugated with a peptide containing the histone H2B nuclear localization sequence from yeast. Using antibodies against p67 we have cloned the gene for this protein. The protein encoded by the *NSR1* gene recognizes the wild-type H2B nuclear localiza- tion sequence, but does not recognize a mutant H2B sequence that is incompetent for nuclear localization in vivo. Interestingly, the *NSR1* protein has two RNA recognition motifs, as well as an acidic NH_2 terminus containing a series of serine clusters, and a basic COOH terminus containing arg-gly repeats. We have confirmed the nuclear localization of p67 by immuno- fluorescence and found that a restricted portion of the nucleus is highlighted. We have also shown that *NSR1* (p67) is required for normal cell growth.

N UCLEAR proteins contain one or more copies of a short amino acid sequence that directs them to the nucleus (Gerace and Burke, 1988). This sequence, termed a nuclear localization sequence (NLS)[1], is composed of mostly basic residues. A comparison of several such se- quences led Chelsky et al. (1989) to propose the consensus: Lys-Arg/Lys-X-Arg/Lys (Chelsky et al., 1989). Addition of an NLS to a cytoplasmic protein such as pyruvate kinase by gene fusion results in the redirection of the protein to the nu- cleus (Kalderon et al., 1984b). Although the nuclear enve- lope can act as a molecular sieve for nonnuclear proteins of the size 20 to 40 kD, an abundance of evidence suggests that nuclear proteins are selectively transported (Paine et al., 1975; Newport and Forbes, 1987; Gerace and Burke, 1988). Movement of nuclear proteins into the nucleus occurs in two stages: an energy-independent binding at the nuclear sur- face, followed by an ATP-dependent transport of the protein through the nuclear pore complex (Newmeyer et al., 1986; Markland et al., 1987; Richardson et al., 1988; Newmeyer and Forbes, 1988).

The selective transport of nuclear proteins and the pres- ence of specific sequences within nuclear proteins that guide them to the nucleus suggest a model whereby a protein receptor exists at the nuclear envelope or in the cytoplasm. This receptor would bind nuclear proteins and be involved in the initial recognition of the nuclear envelope by entering nuclear proteins. Synthetic peptides containing nuclear lo- calization signals, when conjugated to nonnuclear proteins, are capable of targeting them to the nucleus (Goldfarb et al., 1986; Lanford et al., 1986; Chelsky et al., 1989). Such signal-containing peptides have been used to identify pro- teins in a number of eucaryotic organisms that specifically recognize nuclear localization signals. These proteins are candidates for receptors involved in the initiation of nuclear transport.

Using chemical cross-linking, Adam et al. (1989) iden- tified two rat hepatocyte proteins (60 and 70 kD) that could bind a radioactively labeled peptide containing the nuclear localization sequence of SV-40 large tumor antigen (SV40 T-antigen). Further, specificity was demonstrated by the abil- ity of the unlabeled wild-type SV40 T-antigen peptide, but not a mutant, to abolish binding by the labeled SV40 T-anti- gen peptide. The location of the proteins was largely cyto- plasmic, but they could also be found in the nucleoplasm and nuclear envelope. This led the investigators to propose that shuttle proteins initiate nuclear transport by recognizing nu- clear proteins in the cytoplasm and then bringing them to the nuclear pore complex. Yamasaki et al. (1989) and Benditt et al. (1989) used peptides containing UV-activatable groups to detect other proteins in rat hepatocytes that recognize nu- clear localization signals. Yamaski et al. (1989) identified two proteins (70 and 100 kD) in the cytoplasm, and two others (140 and 55 kD) that were loosely associated with the nuclear envelope. Benditt et al. (1989) used purified nuclear envelopes and identified four proteins (56, 57, 65, and 74 kD). In HeLa cells, a single protein of 66 kD was found to specifically bind nuclear signal sequences (Li and Thomas, 1989).

Finally, in the yeast, *Saccharomyces cerevisiae*, Silver et al. (1989) and our group (Lee and Melese, 1989) have

identified proteins potentially involved in the initial binding step at the nuclear envelope. Our group used the technique of ligand blotting (Georgatos et al., 1987) to show that a nuclear envelope protein, p67, specifically recognizes synthetic peptides containing the yeast histone H2B (Moreland et al., 1987) or the SV40 T-antigen (Kalderon et al., 1984a, b) NLS. Silver et al. (1989) using a similar approach identified two signal-binding proteins of molecular weight 70 and 59 kD. Unlike the data reported in rat hepatocytes, cytoplasmic proteins that recognize nuclear localization sequences have not been detected in yeast (Silver et al., 1989; Lee and Melese, 1989).

A different approach to identifying a receptor for nuclear proteins was used by Yoneda et al. (1988) in which they raised antibodies against a peptide containing the sequence Asp-Asp-Asp-Glu-Asp (DDDED). Since the nuclear localization signal for SV40 T-antigen contains a series of lysines and arginines, it was reasoned that a receptor recognizing this protein would likely contain groups of an opposite charge that interacted electrostatically with the basic amino acids. In indirect immunofluorescence using antibodies raised against the acidic peptide they found a punctate staining of the nucleus in a number of different cell lines and, also observed, that the antibody blocked transport of nuclear proteins into the nucleus of HeLa cells. In further studies using affinity chromatography, with either the anti-DDDED antibody or nucleoplasmin, they have isolated a 69-kD protein thought to be a candidate for a nuclear envelope receptor (Imamoto-Sonobe et al., 1990).

Although the implication from the above data are that these various proteins are receptors for incoming nuclear proteins, none of the putative receptors have been directly linked to nuclear transport in vivo. We have partially purified one of these proteins, p67, and isolated the gene encoding this protein. We describe the isolation and disruption of the gene (NSR1) which encodes a protein that specifically recognizes the nuclear localization sequence of H2B. Surprisingly, the NSR1 protein contains two RNA-recognition motifs. The protein also has separate acidic and basic regions, and is located at the periphery of a region within the nucleus as visualized by indirect immunofluorescence. Although the NSR1 gene is not essential for cell viability, it is required for normal cell growth.

Materials and Methods

Plasmids

Most of the DNA manipulations and bacterial transformations were carried out according to the method of Sambrook et al. (1989). E. Coli strain XL1 was used for all transformations unless otherwise noted.

pWL67. 2.0-kb EcoRI insert from the positive λgt11 clone subcloned into the EcoRI site of pBluescript KS(−). The orientation is such that the SacI site in the multiple cloning site is at the 5′ end of the insert.

pWL50-1a. A YCp50 clone containing the entire NSR1 gene isolated from a genomic library.

pWL1. A 3.7-kb HindIII fragment containing the entire NSR1 coding region, cut from pWL50-1a, and cloned into the HindIII site of pBluescript KS(−). The orientation is such that the KpnI site in the multiple cloning site is at the 5′ end of the gene.

pBM272. EcoRI-BamHI fragment of YCp50 replaced with a 0.6-kb fragment containing the GAL1-GAL10 bidirectional promoter via sticky end ligation. The GAL1 promoter is oriented toward the BamHI site (gift from Janet Kurjan, University of Vermont, Burlington, VT).

pWL32. A 1.6-kb HincII-EcoRV fragment of pWL1 that contains the en-

tire coding region of NSR1, cloned into the EcoRV site of pBluescript KS(−) by blunt-end ligation. The orientation is identical to pWL67.

pWL10. A 1.6-kb BamHI-SalI fragment of pWL32 (BamHI and SalI are the sites in the multiple cloning site that flank the EcoRV site) inserted into the BamHI-SalI site of pBM272.

pWL3. The 0.4-kb EcoRI-XbaI fragment of NSR1 gene in pWL1 replaced by a 1.8-kb EcoRI-XbaI fragment containing the S. cerevisiae HIS3 gene.

pE17. One of a series of deletion clones used for DNA sequencing where the 5′ region of NSR1 was deleted from ∼100 bp upstream of the AUG.

Strains

Standard media preparation and yeast cell culture were carried out according to Sherman et al. (1986). Yeast transformation was done using the lithium acetate procedure of Ito et al. (1983).

BJ 2168: MATa trp1, leu2, ura3-52, prb1-1122, prc1-407, pep4-3
W303-1A: MATa ade2-1, can1-100, ura3-1, leu2-3,112, trp1-1, his3-11,15
W303: isogenic a/α diploid
LDY1: MATa/MATα +/+ +/+ +/+
    MATα/MATa arg11/arg11 cry1/cry1 his4Δ 5/his4Δ 29
    lys1-1(UAA)/lys1-1(UAA)
    +/+

Preparation of Yeast Nuclei and Subnuclear Fractions

Yeast nuclei were isolated from the protease-deficient haploid strain BJ2168 according to the method of Aris and Blobel (1989). Generation of nuclear envelopes and extraction of the envelopes with 8 M urea and 2 M KCl were carried out as described previously (Lee and Melese, 1989).

Antibody Preparation and Immunoblotting

Antibodies were raised against the insoluble fraction (pellet) from a 8 M urea extraction of nuclear envelopes. The pellet was resuspended in deionized water, mixed with Freunds complete adjuvant, and injected subcutaneously into male New Zealand white rabbits. All subsequent booster injections were prepared with Freund's incomplete adjuvant. Booster injections were given 4 wk after the initial injection, and then at 2-wk intervals. Bleeding of the animals was done 10 d after the booster injections. Antibody response was analyzed on immunoblots (described below) of nuclei and nuclear envelopes using different dilutions of the crude serum collected from the rabbits.

For immunoblotting, samples were first separated on 10.5% SDS-polyacrylamide gels (Laemmli, 1970) and then electrophoretically transferred to two nitrocellulose filters. After transfer, one filter was stained with india ink to visualize the proteins (Harlow and Lane, 1988) and the other filter was probed with primary antibody. After incubation with the primary antibody (diluted in the incubation buffer, 15 mM Tris-HCl, pH 7.3, 150 mM NaCl, 0.1% Tween 20, 0.1% gelatin) at room temperature overnight, the filters were washed three times with the incubation buffer, and then incubated with peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Richmond, CA) diluted 1:2,500 in incubation buffer for 3 h at room temperature. The blots were washed as before, placed in 50 mM Tris-HCl, pH 7.3, 150 mM NaCl and developed by the addition of 30 mg 4-Chloro-1-Naphthol dissolved in 10 ml methanol plus 50 µl of 30% H_2O_2 as substrates of the peroxidase.

Ligand Blotting

Ligand blotting was performed as described in a previous publication (Lee and Melese, 1989) using H2B peptide-human serum albumin (HSA) conjugates. Recognition of the peptide conjugates was carried out as previously described using rabbit anti-HSA antiserum (Cappel Laboratories, Cochranville, PA) as the primary antibody and peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories) as the secondary antibody (Lee and Melese, 1989).

Peptide Synthesis and Preparation of Peptide-HSA Conjugates

The H2B peptides were synthesized by Janis Young at the UCLA Peptide Synthesis Facility (Los Angeles, CA). A 15-mer was made that contained 13 amino acids of the yeast histone H2B sequence, including the nuclear localization sequence (underlined in the sequence below) and two additional residues, a tyrosine for ^{125}I-labeling and a cysteine for conjugation reactions. A mutant H2B peptide was also synthesized that substituted methio-

nine for lysine-31. The sequences of the peptides were: wild-type, NH_2-Ser-Thr-Asp-Gly-Lys-Lys[31]-Arg-Ser-Lys-Ala-Arg-Lys-Glu-Tyr-Cys-COOH; and mutant, identical peptide except for replacement of lysine-31 by methionine.

Peptides were coupled to HSA with m-maleimidobenzoyl-N-hydroxysuccinimide ester (Sigma Chemical Co., St. Louis, MO) as the coupling reagent (Green et al., 1982; Lee and Melese, 1989).

Partial Purification of p67 by Affinity Chromatography

The wild-type H2B peptide was conjugated to Affi-gel 102 (Bio-Rad Laboratories) through the cross-linker m-maleimidobenzoyl-N-hydroxysuccinimide ester. 0.5 ml of Affi-gel 102 (15 μmol-free amino group/ml) was washed three times with 100 mM sodium phosphate buffer (pH 7.2) and then incubated with 2.8 mg m-maleimidobenzoyl-N-hydroxysuccinimide ester (8.2 μmol) in 0.1 ml dimethylformamide at room temperature for 30 min. The excess cross-linker was removed by washing five times with 50 mM sodium phosphate buffer (pH 6.0). 5 mg of wild-type H2B peptide in 1 ml PBS were added to the gel and the mixture was allowed to shake at room temperature for 4 h. At the end of the incubation, the solution was removed and 10 μl β-mercaptoethanol in 5 ml PBS was added to block the unreacted cross-linker. After incubation overnight, the gel was washed with TBS and then packed into a 1-ml syringe column (0.4 cm × 2.5 cm).

A 2 M KCl extract of yeast nuclear envelopes was dialyzed against buffer P (150 mM NaCl, 10 mM Tris-HCl, pH 7.5) overnight in a cold room with two changes of buffer. The dialyzed extract was then passed through the wild-type H2B peptide-Affigel column at a flow rate of 0.25 ml/min. The column was washed with 2 ml buffer P and the proteins that remained bound to the column were eluted with 2 M NaCl, 10 mM Tris-HCl, pH 7.5. Finally, the column was washed with 2 ml 8 M urea. 0.5-ml fractions were collected from the column and OD_{280} was measured for each fraction. The flow-through and eluted fractions were collected and proteins in the same fractions were precipitated with 20% TCA at 4°C overnight and analyzed.

Purification of Anti-p67 Antibody

p67 was partially purified by the wild-type H2B peptide-Affigel column. The proteins in the 2 M NaCl eluted fraction were separated by a 10.5% SDS-polyacrylamide gel and then electrophoretically transferred to a nitrocellulose filter. The nitrocellulose blot was stained with 0.2% Ponceau S, 3% TCA, 3% sulfosalicylic acid to visualize the proteins. The strip of nitrocellulose filter that contained p67 was excised. Anti-p67 antibody was affinity purified from the crude antiserum raised against the insoluble fraction left after 8 M urea extraction of yeast nuclear envelopes according to published procedures (Pringle et al., 1989).

Immunofluorescence Microscopy

Indirect immunofluorescence was performed using a modification of published procedures (Pringle et al., 1989). Cells were grown to ~1 × 10⁷ cells/ml. Ultrapure EM grade formaldehyde (16%) was added directly to the cells in growth medium to a final concentration of 4%. Cells were fixed at room temperature for 2 h and then washed twice with solution A (100 mM potassium phosphate buffer, pH 6.5; 1% β-mercaptoethanol; 1.2 M sorbitol) and resuspended to 1 × 10⁸ cell/ml in solution A containing protease inhibitors: 1 μg/ml each of leupeptin, pepstatin, chymostatin, 1 mM ε-amino caproic acid, and 0.5 mM phenylmethylsulfonylfluoride. Lyticase (Sigma Chemical Co.) was added to a final concentration of 50 U/OD cells and cells were digested at 30°C for ~6.5–10 min. Spheroplasts were washed once with the same solution and resuspended to 1 × 10⁸ cells/ml. They were then pipetted onto polyethylenimine-coated round coverslips in a 24-well dish (Corning Glass Works, Corning, NY). After 30 min, the coverslips were washed gently with PBS and then incubated with blocking buffer (1% BSA, 0.05% Non-idet 40, PBS, protease inhibitors) for 1 h at room temperature. First antibody incubation was done at room temperature in solution A without the β-mercaptoethanol but with protease inhibitors for 1 h. The coverslips were washed four times in PBS and incubated with FITC-conjugated affinity purified goat anti-rabbit IgG (Jackson Labs), diluted 1:300 in solution A minus β-mercaptoethanol, for 1 h at room temperature. They were washed as before, incubated with a 1:1000 dilution of 1 mg/ml DAPI in solution A for 10 min, and washed again in PBS. The coverslips were mounted on slides with 90% glycerol containing 1 mg/ml p-phenylene diamine buffered to pH 8.0. Slides were viewed with a microscope (Optiphot; Nikon Inc., Garden City, NY) Technical Pan 2145 film was used for all photomicroscopy.

Immunoscreening of λgt11 Library

Affinity-purified anti-p67 antibody was used to screen a yeast genomic library made from S. cerevisiae DNA (Young and Davis, 1983) by established procedures (Snyder et al., 1987) using the E. coli strain Y1090. Positive plaques were detected by a biotinylated secondary antibody and an avidin-biotinylated peroxidase complex (Vector Laboratories, Burlingame, CA). Possible positive clones were plaque purified and rescreened with antibody. Overproduction of the β-galactosidase fusion protein from positive clones using lysogenic strain Y1089 was performed as described (Snyder et al., 1987). The fusion protein was detected by immunoblotting with anti-p67 antibody.

Recovery of Full-length NSR1 Clone

To obtain the 5′ end of the NSR1 gene, a yeast genomic library carrying 10–30-kb inserts in the single copy vector YCp50 (a gift from Mark Rose) was screened by colony hybridization (Sambrook et al., 1989). The 2.0-kb EcoRI insert in the positive λgt11 clone was cloned into the EcoRI site of pBluescript KS(−) to make plasmid pWL67. The 2.0-kb fragment was band isolated from pWL67 and radiolabeled DNA probe was synthesized from this fragment using random oligonucleotide primers. Four positive clones were found among the 3,000 colonies screened. By restriction mapping, three of them were found to extend past the 5′ end of the NSR1 gene. A 3.7-kb HindIII fragment containing the whole NSR1 gene was isolated from one of these YCp50 clones and subcloned into pBluescript KS(−) to make pWL1.

DNA Sequencing

A series of unidirectional nested deletions of pWL1 were made in both orientations using exonuclease III according to the procedure of Sambrook et al. (1989). The deletion series was transformed into the bacterial strain XL1. Dideoxy sequencing was carried out using a Sequenase kit (USB) and gel electrophoresis was done as described previously (Sanger et al., 1980). Sequence editing and analysis were performed using the Genbank databank version 63, "fast a" program (Pearson and Lipman, 1988).

In Vitro Synthesis of the NSR1 Protein

pE17, a clone that contains the full coding sequence of NSR1 behind the T3 promoter in pBluescript, was linearized at the 3′ end of the gene and the transcription of NSR1 was carried out according to published procedures but using T3 RNA polymerase (Melton et al., 1984). The product of the transcription was confirmed on a 0.8% formaldehyde agarose gel along with ribosomal RNA standards of known size from Neurospora crassa (kindly provided by C. R. McClung, Dartmouth College, Hanover, NH).

After the transcription, the RNA was directly translated in the presence of [³⁵S]methionine (Amersham Corp., Arlington Heights, IL), using a reticulocyte lysate system (Promega Biotec, Madison, WI) according to the procedure provided by the manufacturer. Products from the translation reaction were diluted with an equal volume of Laemmli sample buffer containing cold methionine (8.0 mM) and the samples were run on 10 or 10.5% SDS-polyacrylamide mini gels at 20 mAmps. After electrophoresis, the gel was fixed in 30% methanol/10% acetic acid, and incubated in an autoradiography enhancer, Entensify (New England Nuclear, Boston, MA). Fluorography was carried out by exposing the gel to film (XAR-5; Eastman Kodak Co., Rochester, NY) with a Lightening Plus intensifying screen (Picker Corp., Highland Heights, OH) at −80°C for 12 h. Molecular weight standards were ¹⁴C-labeled by reductive methylation using [¹⁴C]formaldehyde (ICN Radiochemicals, Irvine, CA) (Jentoff and Dearborn, 1979; Fisher et al., 1982).

Expression of the NSR1 Protein from the GAL1 Promoter

The yeast strain W303-1A was transformed to URA^+ with either pBM 272 or pWL10. To induce overexpression, cells were grown overnight in liquid synthetic medium containing 2% raffinose and lacking uracil. They were then diluted 1:10 in the same medium and grown to an OD_{600} of 0.3. 2% galactose (induced) or 2% glucose (repressed) were added. Cells were grown for 5 h, pelleted, and resuspended in Laemmli buffer (containing 6 M urea and 0.5 mM PMSF) for analysis on 10.5% SDS-polyacrylamide gels. Immunoblots and ligand blots were carried out after transfer of the proteins to nitrocellulose filters.

Disruption of the NSR1 Gene and Tetrad Analysis

The chromosomal copy of the *NSR1* gene was replaced with a partial *NSR1* deletion (*nsr1Δ::HIS3*) by the one-step gene disruption method (Rothstein, 1983). The 0.4-kb EcoRI-XbaI fragment of the *NSR1* gene (aa186-aa324) in pWL1 was cut and replaced by the 1.8-kb EcoRI-XbaI fragment containing the *S. cerevisiae HIS3* gene from the plasmid pUC18-*HIS3#*1 (Alex Tzagoloff, Columbia University, gift). A linear 2.9-kb HincII-StuI fragment containing the *HIS3* gene flanked by sequences of the *NSR1* gene (0.6 kb at 3' end and 0.5 kb at 5' end) was isolated and used to transform the diploid *his⁻* strain, W303. The *HIS⁺* transformants were checked by Southern analysis to confirm that the interrupted gene was integrated into the homologous *NSR1* locus. Two of the transformants which contained one wild-type copy and one disrupted copy of the *NSR1* gene (*NSR1/nsr1Δ::HIS3*) were sporulated, the tetrads dissected and analyzed according to the procedure of Sherman et al. (1986).

Results

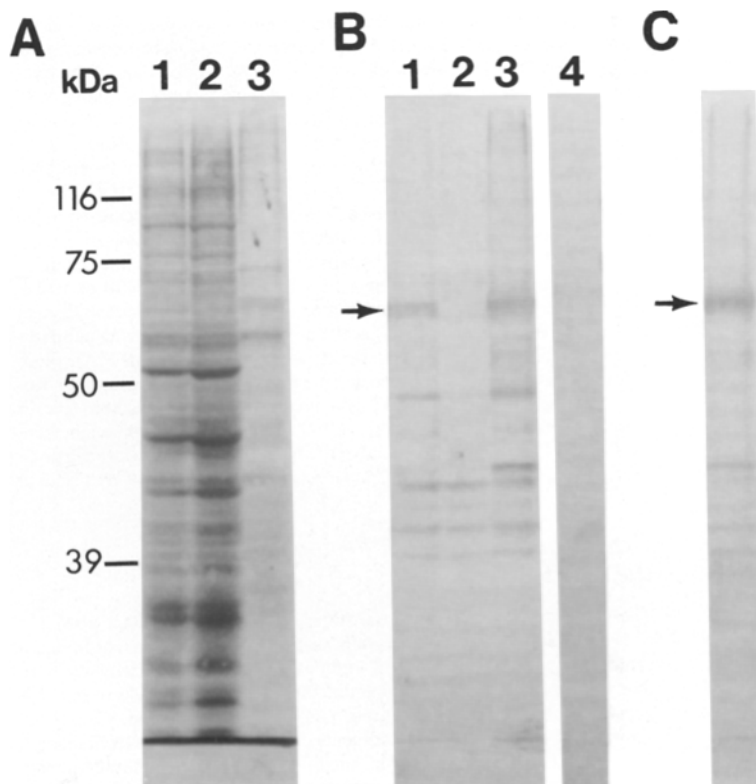### Partial Purification of p67 by Affinity Chromatography

We have previously identified a nuclear envelope protein, p67, that specifically binds synthetic peptides containing the yeast histone H2B or SV40 T-antigen nuclear localization sequence (Lee and Melese, 1989). This characteristic was exploited to purify p67 from yeast nuclear envelopes. Envelopes were prepared and then extracted with 2 M KCl, and the extract was dialyzed against a low-salt buffer (Fig. 1 *A*, lane *1*). The solubilized proteins were then passed over an Affi-gel column conjugated with a peptide containing the wild-type H2B NLS (see Materials and Methods). Although most of the solubilized proteins flowed through the column (Fig. 1 *A*, lane *2*), p67 remained tightly bound and was only eluted after the addition of a buffer containing 2 M NaCl (Fig. 1 *A*, lane *3*). The same column fractions were analyzed on ligand blots for the presence of p67, using the wild-type

(Fig. 1 *B*, lanes *1–3*) and mutant (Fig. 1 *B*, lane *4*) H2B-HSA peptide conjugate as probes, which confirmed the presence of the NLS-binding activity in the 2 M NaCl eluant. p67 was highly purified using this approach, but not to homogeneity (Fig. 1 *A*, lanes *1–3*).

Further analysis of p67 and isolation of the gene for this protein would be facilitated by an antibody reagent. Since we had previously shown that p67 is only partially extracted from nuclear envelopes with 8 M urea (Lee and Melese, 1989), we tested an antiserum raised against an insoluble fraction left after extraction of yeast nuclear envelopes with 8 M urea, for recognition of p67. We found that antibodies recognizing this protein were present in this crude antiserum (Fig. 1 *C*). To purify anti-p67 specific antibodies from the antiserum, we used the technique of affinity purification on nitrocellulose blots (Olmsted, 1981; Pringle et al., 1989). p67 was purified on the peptide affinity column, run on SDS-polyacrylamide gels, and then transferred to nitrocellulose filters. By using the affinity of the antibody for p67 immobilized on nitrocellulose filters, we were able to specifically bind and then elute the polyclonal antibodies against p67. A number of the lower molecular weight proteins observed in the 2 M NaCl eluted fraction of the peptide affinity column (Fig. 1 *A*, lane *3*) may be degradation products of p67 since they also bind wild-type H2B-HSA peptide conjugates on ligand blots (Fig. 1 *B*, lane *3*). However, our data do not rule out the possibility that additional signal-binding proteins are present in this preparation.
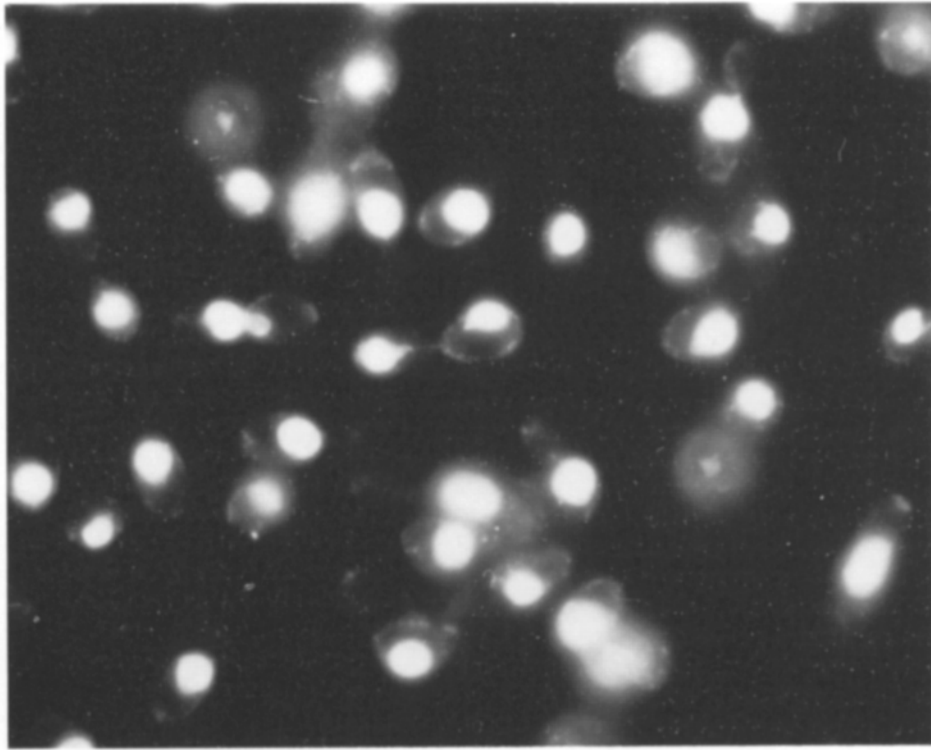
Immunofluorescence Using Affinity-Purified Antibodies to p67

To investigate the subcellular distribution of p67, indirect im-

Figure 1. Partial purification of p67 by affinity chromatography. (*A*) India ink staining of a nitrocellulose filter with different fractions from the affinity column. 2 M KCl extract from yeast nuclear envelopes (lane *1*) was dialyzed overnight against 150 mM NaCl, 10 mM Tris-HCl, pH 7.5 at 4°C, and then passed through a wild-type H2B peptide-Affigel column (0.4 cm × 2.5 cm) at a flow rate of 0.25 ml/min (lane *2*, flow through). The proteins bound to the column were eluted by addition of 2 M NaCl (lane *3*). Proteins in the different fractions were precipitated with 15% TCA, run on 10.5% SDS-polyacrylamide gels, and blotted to two nitrocellulose filiters. (*B*) Ligand blot of the other nitrocellulose filter. Lanes *1–3* are identical to that described in *A*, and were probed with the wild-type H2B-HSA conjugate. Lane *4* is also the eluant fraction but probed with the mutant H2B-HSA conjugate. (*C*) Immunoblot of the eluant fraction with a polyclonal antiserum raised against the insoluble fraction left after 8 M urea extraction of nuclear envelopes. An arrow indicates the position of p67.
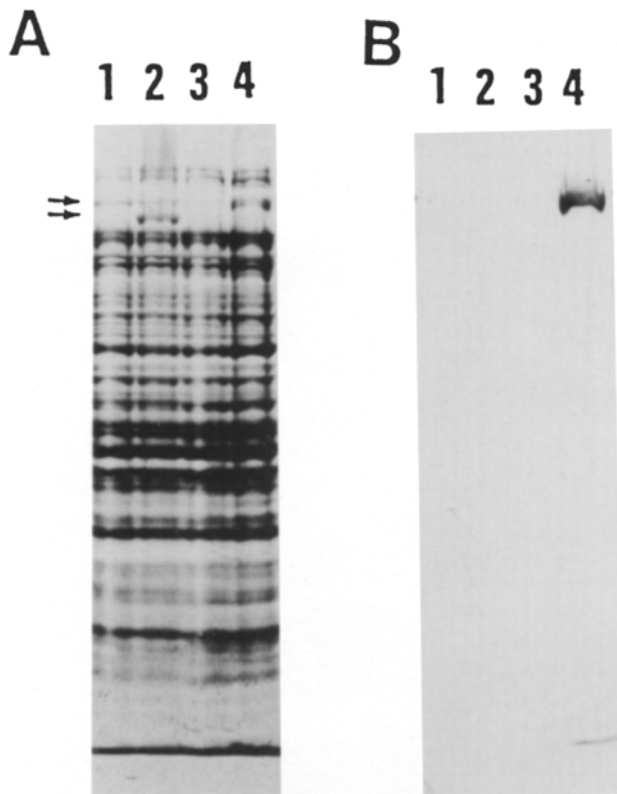
A

B
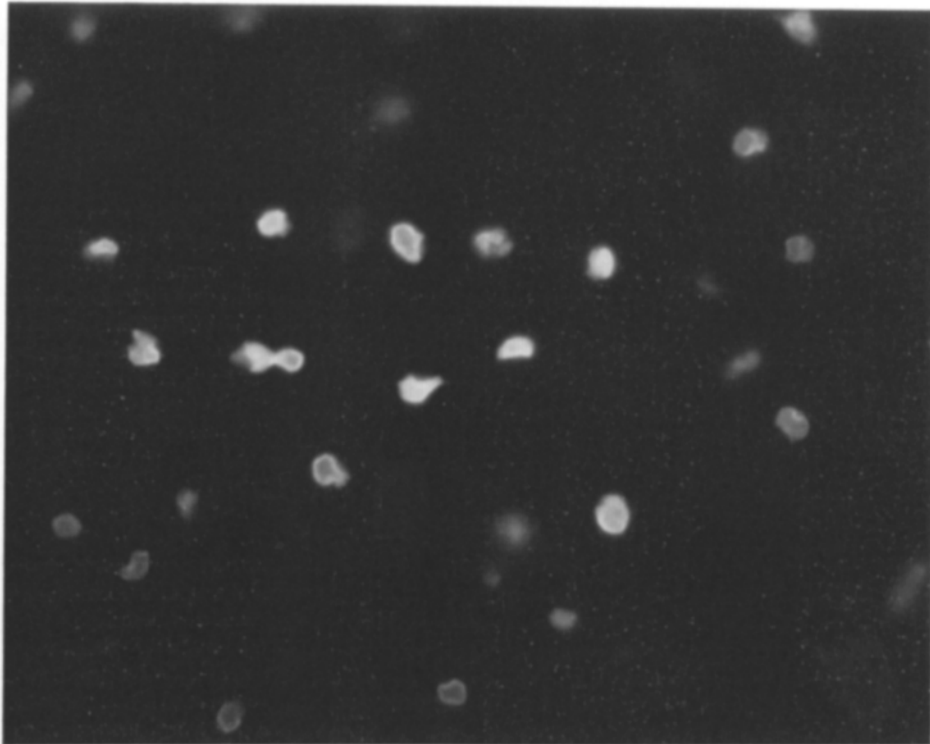
Figure 2. Immunofluorescence using anti-p67 antibody. Indirect immunofluorescence was performed on a tetraploid yeast strain LDY1 using the affinity purified anti-p67 antibody, as described in Materials and Methods. (*Top*) DAPI staining of DNA; (*Bottom*) FITC staining.

Figure 3. Expression of β-galactosidase-p67 fusion protein. (A) India ink–stained nitrocellulose filter shows the protein profiles of lysogens. (B) Immunoblot of another nitrocellulose filter probed with purified anti-p67 antibody. The nitrocellulose filter was transferred from the same SDS–polyacrylamide gel as in A. (Lanes 1 and 2) λgtl1 lysogen; (Lanes 3 and 4) the lysogen of λgtl1 containing the 2.0-kb EcoRI insert. β-galactosidase gene was induced by isopropyl-β-D-thiogalactopyranoside in lanes 2 and 4. Lanes 1 and 3 are uninduced. Arrows indicate the positions of β-galactosidase (116 kD) and β-galactosidase-p67 fusion protein (higher molecular weight than β-galactosidase).

the area stained by DAPI in immunofluorescence (Davis and Fink, 1990). These data suggest that p67 has an intranuclear location. More specifically, the p67 antibody highlights a restricted portion of the nucleus, with more intensity at peripheral regions (Fig. 2). Comparison of the staining of p67 with that of a known nucleolar antigen (p38; Aris and Blobel, 1989) shows similar but not identical staining (in double-labeling experiments the two antigens do not completely overlap, data not shown), which raises the possibility that p67 may be located at the nucleolus.

Isolation of a Gene Encoding p67

The affinity-purified antibody was used to screen a yeast genomic λgtl1 expression library (Snyder et al., 1987), and one positive clone was identified. Immunoblots of extracts from isopropyl-β-D-thiogalactopyranoside–induced lysogen were probed with the anti-p67 antibody and a positive response was observed (Fig. 3 B, lane 4). The λgtl1 clone had a 2.0-kb insert, and the expressed β-galactosidase fusion protein was ∼140 kD. Since we could obtain large amounts of the β-galactosidase-p67 fusion protein by overexpression from the lysogen containing the positive clone, this hybrid protein was used to affinity purify anti-p67 antibody for all further experiments.

The putative p67 gene with flanking regions was isolated from a yeast plasmid bank (Rose et al., 1987), excised from the plasmid and subcloned into pBluescript KS(−) to facilitate sequencing (see Materials and Methods). A series of nested deletions in this clone, pWL1, were made in both orientations. From the resulting sequence data, a single long–open reading frame was found capable of encoding a protein of predicted molecular weight 44,537 D (Fig. 4 A) and contained three distinct domains (Fig. 4 B; for discussion see section on RNA-recognition motifs). The gene was designated NSR1. Northern analysis of total mRNA from the yeast strain BJ2168 showed one band of ∼1.8 kb when probed with radioactively labeled DNA fragments synthesized from the 2.0-kb EcoRI insert from the λgtl1 clone (data not shown).

In Vitro Transcription and Translation of NSR1

A possible explanation for the discrepancy between the predicted molecular weight of the protein encoded by the NSR1 gene and p67 is that the protein is highly charged (see the discussion of the deduced amino acid sequence, below), which may cause the protein to migrate aberrantly on SDS–polyacrylamide gels. The migration of the human U1 70K protein, which has a predicted molecular weight of 52 kD but migrates on SDS–polyacrylamide gels at 70 kD, was shown to be the result of an arginine rich carboxy-terminal

munofluorescence was performed on a tetraploid yeast strain (LDY1) to facilitate visualization of nuclear structure (Fig. 2). The immunofluorescence analysis yielded the same results with antibody that had been affinity purified against p67 or a β-galactosidase-p67 fusion protein from a λgtl1 lysogen (see section below). Comparison of the p67 staining pattern with that of the DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI) shows that in most cells the p67 staining overlaps with, but does not extend beyond, the DAPI staining. A recently described yeast nuclear pore protein (NUP1) outlines a region of the nucleus distinctly larger than

Figure 4. Nucleotide and deduced amino acid sequence of NSR1. (A) Sequence of the NSR1 gene. The RNA-recognition motifs are shown by a single underline, and the RNP consensus octamers are indicated by a double underline. (B) The protein domains in the deduced NSR1 amino acid sequence. The hatched box represents the acidic NH2 terminus and contains the serine clusters. The RNA-recognition motifs are indicated by the open box, and include the RNP consensus octamer. The black box represents the COOH terminus that is comprised of mostly arginines and glycines. The size of the boxes are roughly proportional to the length they occupy in the protein. The thin black lines extending at the 5 and 3' end of the gene represent the untranslated regions. The thicker black lines, marked by ATG or TGA, mark the coding region for the gene. The arrows underneath the boxes represent the regions that were sequenced and point in the direction of sequencing. These sequence data are available from EMBL/GenBank/DDBJ under accession number X57185.

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A

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          10                30                50                70                90
GAAAATTTTCAATCCAATATTAAAAGTACTTAAGTGTAGCTGTTGCTGTCTGCACTTCCCAATCCATTGGTACCTTAAGTTATTTCCTTT

          110               130               150               170
CGTAGTATTTTTCTTACTTTTGCTTCCCAAAGACGAACTGTTAACCAATTTCGGATCACTCAACCCAGGCAGGATAAAATAAGATGGCTA
                                                                                  M  A  K

          190               210               230               250               270
AGACTACTAAAGTAAAAGGTAACAAGAAGGAAGTTAAGGCTTCCAAACAAGCCAAAGAAGAAAAAGCTAAGGCCGTCTCTTCCTCTTCCT
 T  T  K  V  K  G  N  K  K  E  V  K  A  S  K  Q  A  K  E  E  K  A  K  A  V  S  S  S  S  S

          290               310               330               350
CCGAATCTTCATCCTCATCTTCATCTTCATCTGAATCTGAATCTGAGTCTGAGTCTGAATCTGAATCTTCATCTTCATCTTCATCCTCTG
 E  S  S  S  S  S  S  S  E  S  E  S  E  S  E  S  E  S  E  S  S  S  S  S  S  S  D

          370               390               410               430               450
ATTCTGAATCCTCTTCTTCATCGTCTTCTGACAGCGAAAGTGAAGCTGAAACCAAGAAGGAAGAATCCAAGGATTCCTCTTCCTCTTCCT
 S  E  S  S  S  S  S  S  S  D  S  E  S  E  A  E  T  K  K  E  E  S  K  D  S  S  S  S  S

          470               490               510               530
CTGACTCTTCTTCCGACGAAGAAGAAGAAGAAGAAAAAGAAGAAACCAAGAAGGAAGAATCAAAAGAATCTTCTAGCTCTGATTCATCCT
 D  S  S  S  D  E  E  E  E  E  E  K  E  E  T  K  K  E  E  S  K  E  S  S  S  S  D  S  S  S

          550               570               590               610               630
CATCTTCATCTTCTGATAGCGAAAGCGAAAAGGAAGAGTCTAACGATAAGAAACGTAAATCTGAGGACGCCGAAGAAGAAGAAGACGAAG
 S  S  S  D  S  E  S  E  K  E  E  S  N  D  K  K  R  K  S  E  D  A  E  E  E  E  D  E  E

          650               670               690               710
AGTCTTCCAACAAGAAGCAAAAAAATGAAGAAACCGAAGAACCAGCTACTATTTTCGTTGGTAGACTATCGTGGTCTATTGATGACGAAT
 S  S  N  K  K  Q  K  N  E  E  T  E  E  P  A  T  I  F  V  G  R  L  S  W  S  I  D  D  E  W

          730               750               770               790               810
GGTTGAAGAAGGAATTCGAACACATCGGTGGTGTCATTGGTGCCAGAGTTATTTATGAAAGAGGTACCGATAGATCTCGTGGTTATGGTT
 L  K  K  E  F  E  H  I  G  G  V  I  G  A  R  V  I  Y  E  R  G  T  D  R  S  R  G  Y  G  Y

          830               850               870               890
ACGTTGATTTTGAAAACAAATCTTATGCTGAAAAGGCCATTCAAGAAATGCAAGGTAAGGAAATTGATGGTAGACCAATCAACTGTGATA
 V  D  F  E  N  K  S  Y  A  E  K  A  I  Q  E  M  Q  G  K  E  I  D  G  R  P  I  N  C  D  M

          910               930               950               970               990
TGTCCACAAGCAAGCCAGCTGGTAACAACGATCGTGCCAAGAAATTCGGTGATACCCCATCTGAACCATCTGACACTTTGTTCTTGGGTA
 S  T  S  K  P  A  G  N  N  D  R  A  K  K  F  G  D  T  P  S  E  P  S  D  T  L  F  L  G  N

          1010              1030              1050              1070
ACTTATCTTTCAATGCTGACAGAGACGCTATTTTCGAATTATTCGCTAAACACGGTGAAGTTGTTTCCGTCCGTATCCCAACACATCCAG
 L  S  F  N  A  D  R  D  A  I  F  E  L  F  A  K  H  G  E  V  V  S  V  R  I  P  T  H  P  E

          1090              1110              1130              1150              1170
AAACTGAACAACCAAAAGGTTTCGGTTATGTTCAATTCTCCAACATGGAGGACGCCAAGAAGGCTCTAGACGCTTTACAAGGTGAATACA
 T  E  Q  P  K  G  F  G  Y  V  Q  F  S  N  M  E  D  A  K  K  A  L  D  A  L  Q  G  E  Y  I

          1190              1210              1230              1250
TTGACAACGACCAGTTAGATTAGACTTCTCTTCTCCAAGACCAAACAACGATGGTGGTCGTGGCGGTAGCCGTGGTTTTGGTGGTCGTG
 D  N  R  P  V  R  L  D  F  S  S  P  R  P  N  N  D  G  G  R  G  G  S  R  G  F  G  G  R  G

          1270              1290              1310              1330              1350
GCGGTGGTCGTGGCGGTAACCGTGGATTCGGTGGTCGTGGTGGCGCTCGCGGTGGCCGTGGCGGTTTCAGACCATCTGGTTCTGGTGCTA
 G  G  G  R  G  G  N  R  G  F  G  G  R  G  G  A  R  G  G  R  G  G  F  R  P  S  G  S  G  A  N

          1370              1390              1410              1430
ATACTGCTCCATTGGGCAGATCAAGAAATACCGCTTCTTTCGCTGGTTCAAAGAAAACATTTGATTAATGAGAAAATGAAATGAATTTCA
 T  A  P  L  G  R  S  R  N  T  A  S  F  A  G  S  K  K  T  F  D  *

          1450              1470              1490              1510              1530
ATTTCAATTTTTTCTCTTTTTACGTTAATTACTATATTCCATTTTTGAGGAAAAATTTGGTCTATAATATTTTGTGTACATTAGTAAGTA

          1550              1570              1590              1610
AATAGGATACATTCTTAAACCTTTCATTCACCATCTCTATTTGCCAACTTTTCTTCGAATGGCTTACTCTTTTTTTTTTTCACGATGAGAT

          1630              1650              1670              1690     1707
GAGATCGCTAGATACGGAAGATTACAAGGCCTTGGAAAATACTCAAAAAATTTCAGTAATATGAAATGAATATCTAAATAAAAGCTT
```

B

ATG   RNA–Binding Domains   TGA

200bp
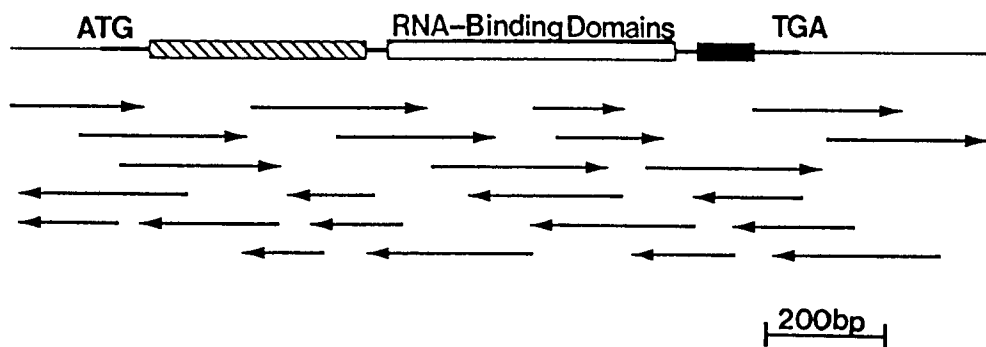
domain (Query et al., 1989). To test whether *NSR1* encodes a protein that migrates on polyacrylamide gels at 67 kD, the gene was transcribed and the mRNA translated in vitro.

The *NSR1* gene was cloned downstream of the T3 promoter in pBluescript and was transcribed using T3 RNA polymerase (see Materials and Methods). Different aliquots of the transcription reaction mixture containing the mRNA were then added to a reticulocyte lysate in the presence of [^{35}S]methionine. A major radioactively labeled band was observed following SDS–polyacrylamide gel electrophoresis of the translation products (Fig. 5, lanes *2–7*). The protein band migrated along with the BSA standard, and was ∼66–68 kD. We infer from these results that the gene we had cloned encodes a protein of molecular weight 44,537 D, but which migrates at 67 kD on SDS–polyacrylamide gels.

The NSR1 Protein has Two RNA-recognition Motifs

The nucleotide and deduced amino acid sequence of *NSR1* are shown in Fig. 4 *A*. The protein can be roughly divided into three different domains (Fig. 4 *B*). The NH$_2$ terminus contains five stretches each containing approximately seven serine residues flanked by asparatic or glutamic acid, as well as two stretches containing eight consecutive acidic amino acid residues. There is also a KKRKS amino acid sequence that could represent a nuclear localization sequence. The middle 45% of the protein consists of two ∼80 amino acid RNA-recognition motifs (Query et al., 1989; see Discussion), and the last 20% of the protein is rich in glycine and arginine residues.

Expression of NSR1 under the GAL1 Promoter

To provide further evidence that *NSR1* encodes a protein that binds nuclear targeting signals, we placed the gene under control of the inducible galactose promoter on plasmid pWL10. Yeast cells containing a chromosomal copy of *NSR1* but also harboring plasmid pWL10 (Fig. 6 *A*, lanes *3* and *4*) were induced to express the cloned *NSR1* gene by growth on galactose. As a control, the same yeast strain was transformed with the control plasmid pBM272, containing the GAL1 promoter but not the *NSR1* gene (Fig. 6 *A*, lanes *1* and *2*). Immunoblots of whole-cell lysates from cells containing pBM272 or pWL10, either induced (Fig. 6 *A*, lanes *2* and *4*) or uninduced (Fig. 6 *A*, lanes *1* and *3*), were probed with anti-p67 antibody (Fig. 6 *A*, immunoblot). In control or uninduced cells only one band at 67 kD was detected. However, after 5 h of growth on galactose, both this protein band and a new band of slightly greater mobility were observed (Fig. 6 *A*, lane *4*). In ligand blots of uninduced, induced, and control cells, both the original protein band and the new protein band bind the wild-type H2B-HSA peptide conjugate, but not a mutant H2B-HSA peptide conjugate.

A genetic polymorphism could explain the difference in electrophoretic mobility between the endogenous p67 (W303-1A host strain) and the galactose induced *NSR1* gene product. In other words, the W303-1A strain and the S288C strain (from which the full-length *NSR1* gene was isolated) contain different forms of p67. To test this possibility we performed the following experiment. Whole-cell lysates from the W303-1A strain that had been transformed with pWL10 and also from BJ2168 (a strain that is congenic with S288C and where p67 was first identified and subsequently purified) were ana-
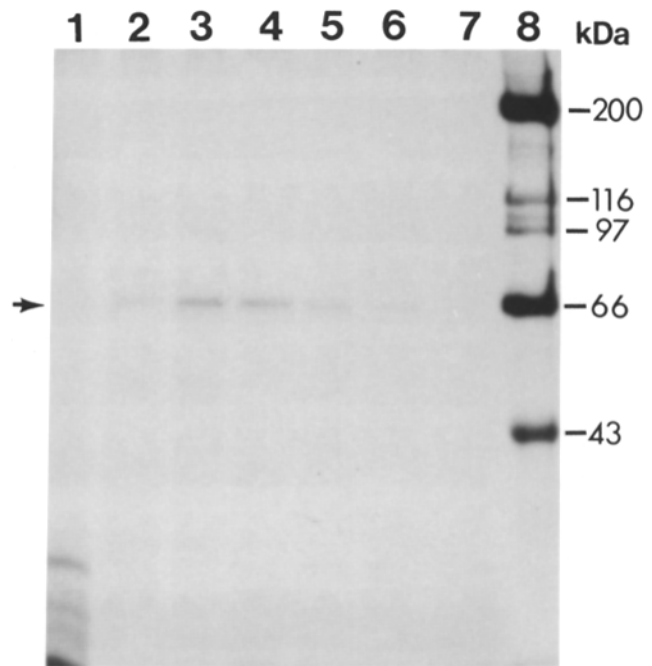
Figure 5. In vitro translation of *NSR1* using a reticulocyte lysate system. *NSR1* was cloned into pBluescript behind the T3 promoter and transcribed using T3 RNA polymerase. Different aliquots of the transcription mix were then translated in the presence of [^{35}S]methionine using a reticulocyte lysate. The figure is a fluorograph of a 10.5% SDS–polyacrylamide gel showing the translation product. Different amounts of transcription mix were used in the translation reaction. Lanes *1–4* had 0, 2, 1, and 0.5 μl of the transcription mix, respectively. Note the inhibition at high mRNA concentration. In lanes *5* through *7* the transcription mix was diluted 10-fold and the same aliquots added as in lanes *2–4*. (Lane *8*) ^{14}C-labeled high molecular weight standards. p67 is marked by an arrow.
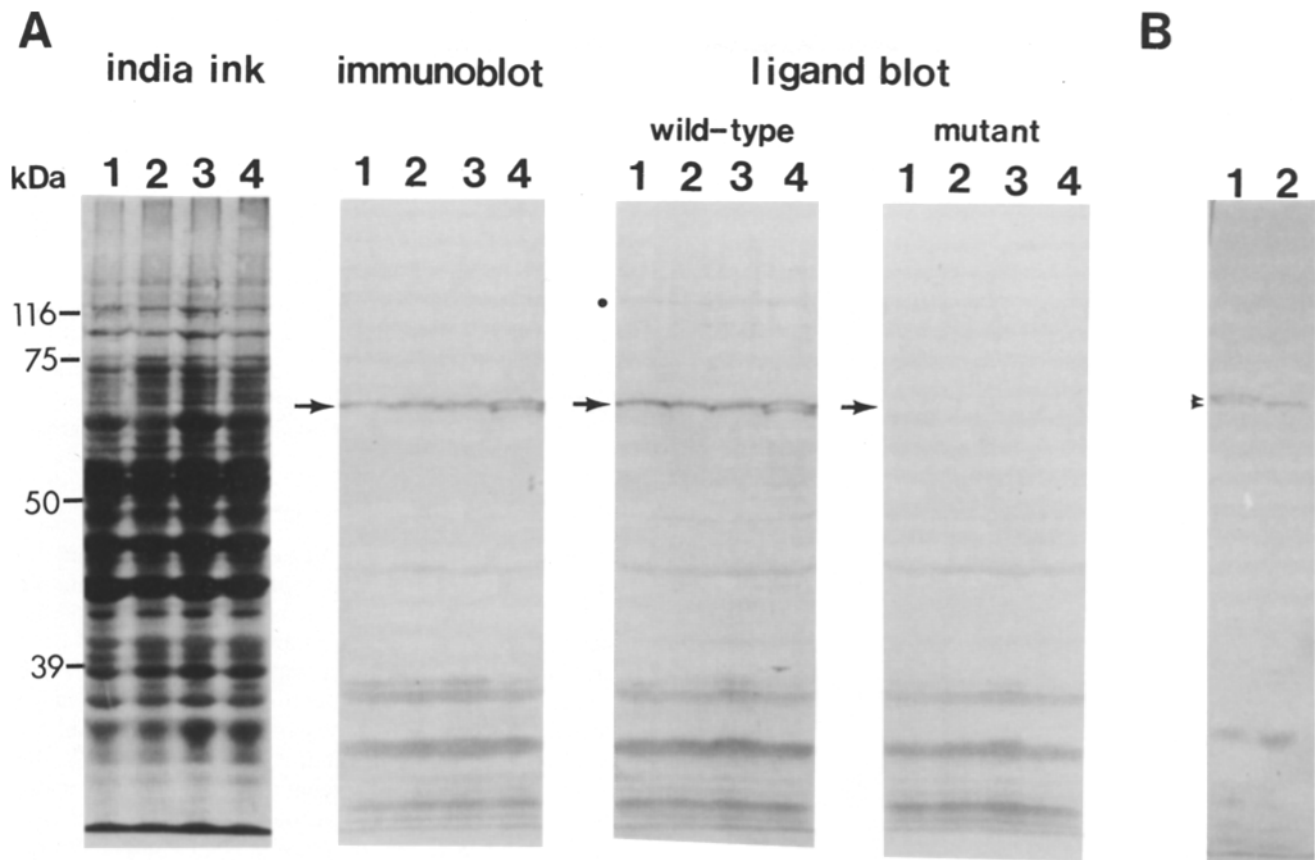
lyzed by immunoblotting with anti-p67 antibody. The BJ2168 strain contained one protein band that clearly co-migrated with the galactose induced *NSR1* gene product in gels (Fig. 6 *B*, lane *2*). Additionally, in immunoblots and ligand blots, we found that S288C also contains the faster migrating form of p67 (data not shown). We conclude from these data that p67 and the *NSR1* gene product are the same protein.

NSR1 Is Required for Normal Cell Growth

Approximately one-third of the *NSR1* gene, encoding most of the RNA-recognition motifs, was deleted and replaced with the *S. cerevisiae* selectable marker *HIS3*. A fragment carrying the deletion allele (*nsr1Δ::HIS3*) was isolated from plasmid pWL3 and then transformed into diploid strain W303. By homologous recombination, the deletion allele replaced a copy of the wild-type *NSR1* gene in the diploid and made the diploid *HIS*+ (Rothstein, 1983). Stable and viable *HIS*+ transformants were produced that exhibited no measurable growth defect. Southern analysis of several diploids showed that the disrupted copy was integrated at the *NSR1* locus and had replaced the resident gene (data not shown).

Two independent heterozygous diploids (*NSR1/nsr1Δ::HIS3*) were sporulated, and 24 tetrads of each were analyzed. In all cases four viable spores were recovered from each tetrad;

A india ink   immunoblot   ligand blot   **B**

                                 wild-type   mutant

kDa 1 2 3 4   1 2 3 4   1 2 3 4   1 2 3 4   1 2

116—

75—

50—

39—

Figure 6. Expression of the *NSRI* gene under the GAL1 promoter. (*A*) Yeast strain W303-1A was transformed with either pBM272 (a plasmid containing the GAL1 promoter; lanes *1* and *2*) or pWL10 (pBM272 containing the *NSRI*-coding region regulated by the GAL1 promoter; lanes *3* and *4*). The cells were grown in synthetic medium lacking uracil and containing 2% raffinose to an OD_{600} of 0.3. Glucose or galactose was then added to a final concentration of 2% and the cells allowed to continue growing for 5 h. Whole-cell lysates were made by dissolving cells in Laemmli buffer plus 6 M urea and run on 10.5% SDS-polyacrylamide gels (lanes *1* and *3*, glucose; and lanes *2* and *4*, galactose). Separated proteins were transferred to two nitrocellulose filters and one filter was stained with India ink. The second filter was cut into three sections, and one section was blotted with affinity-purified anti-p67 antibody. The other two sections were subjected to ligand blotting using a peptide containing the wild-type or mutant nuclear localization sequence of H2B conjugated to HSA. The position of p67 is noted with an arrow. A dot marks the higher molecular weight protein band that also recognizes the wild-type H2B peptide in the ligand blot. (*B*) Immunoblot comparing whole-cell lysates from strain W303-1A harboring pWL10 and grown in galactose medium (lane *1*) with strain BJ2168 (lane *2*). Arrowheads indicate the position of both forms of the *NSRI* protein.

two of which grew abnormally slow (Fig. 7). The slow-growing colonies were always *HIS⁺*. These results indicate a role for the *NSRI* gene in cell growth. Southern analysis of the haploid histidine prototrophs showed that the disrupted copy of the *NSRI* gene (*nsr1Δ::HIS3*) had replaced the chromosomal copy of the gene. Immunoblots of whole-cell lysates from a set of four viable spores, using affinity-purified antibodies against native p67, showed that the *NSRI* protein (p67) was only present in the *his⁻* haploids (Fig. 7, immunoblot). Further analysis of the *nsr1Δ::HIS3* and *NSRI* haploids on ligand blots, using the wild-type H2B-HSA conjugate as a probe, showed that a protein band with an apparent molecular weight of 67 kD was only present in the *his⁻* colonies (Fig. 7, ligand blot). These results confirm that the cloned *NSRI* gene encodes p67, identified previously as the major protein specifically recognizing nuclear localization sequences on ligand blots. Another protein with a molecular weight of ~110 kD, present in *NSRI* and *nsr1Δ::HIS3* strains, was observed to specifically bind the ligand (Fig. 6 *A* and Fig. 7, ligand blot, represented by a dot).

Discussion

NSRI Encodes a Signal-binding Protein

In a previous study, we identified a protein (p67) in yeast nuclear envelopes that specifically bound nuclear localization sequences (Lee and Melese, 1989). We have partially purified p67, as well as isolated, sequenced, and disrupted the gene, *NSRI*, encoding this protein. Although the predicted molecular weight of the *NSRI* protein is only 44,537 D, we have shown that when it is transcribed and translated in vitro it migrates on SDS-polyacrylamide gels at ~67 kD. From the deduced amino acid sequence the protein is highly charged at both the NH_2 and COOH terminus, and this high charge density most likely accounts for the aberrant migration. In addition, the protein contains two RNA-recognition motifs and specifically recognizes nuclear localization sequences, therefore, the *NSRI* protein is a candidate for a receptor at the nucleus that may be involved in both RNA and protein transport.

Immunofluorescence analysis suggests that the majority of the *NSR1* protein is intranuclear, and is possibly at the nucleolus. *NSR1* protein may also be present at the nuclear pore complex and was not detected because of a lack of sensitivity of the immunofluorescent assay. We plan to carry out immuno-electron microscopy to further define its location within the nucleus. If *NSR1* is located at the nucleolus, it will not be the first time that a nucleolar protein has been functionally implicated in nuclear transport. Borer et al. (1989) have proposed that nucleolin and another abundant nucleolar protein (B23/No38) are involved in the transport of ribosomal proteins into the nucleus. Another putative nucleolar shuttling protein was recently described by Meier and Blobel (1990). They showed that a rat hepatocyte protein of 140 kD, that specifically recognizes a peptide containing the nuclear localization sequence of SV40 T-antigen on ligand blots, is immunolocalized to the nucleolus. Most likely the protein that Meier and Blobel (1990) have identified is identical to another 140-kD NLS-binding protein previously found in rat hepatocytes (Yamasaki et al., 1989; see Introduction; and Meier and Blobel, 1990).
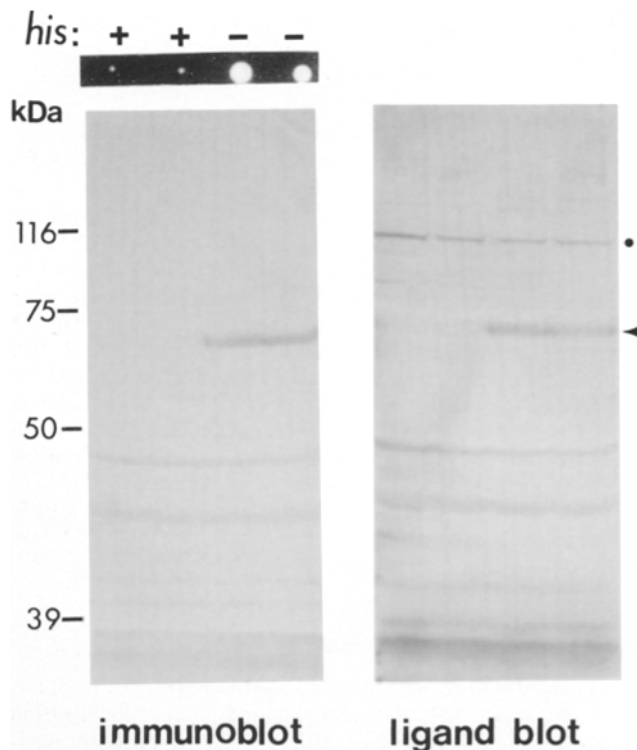
Figure 7. Analysis of tetrads from the *NSR1/nsr1Δ::HIS3* diploid. Tetrads obtained after sporulation of the *NSR1/nsr1Δ::HIS3* diploid strain were dissected and the spores grown on plates containing rich medium for 3 d at 30°C. Haploid colonies from one tetrad are shown in the figure. The colonies were replicated onto plates containing complete medium lacking histidine to test for a *HIS⁺* phenotype. Whole-cell lysates from these four haploids were analyzed by immuno- and ligand blots as described in Fig. 6. The lanes on the immunoblot directly correspond to the haploid strains shown above. The lanes on the ligand blot correspond to the identical haploid strains shown above the immunoblot. An arrowhead indicates the position of p67, and a dot marks the higher molecular weight protein band that also specifically recognizes the NLS of the yeast histone H2B.

Expression of the *NSR1* gene under the GAL1 promoter in strain W303-1A revealed a genetic polymorphism of the *NSR1* gene product. W303-1A and the strain from which the full length *NSR1* gene was cloned (S288C) possess different forms of the *NSR1* protein as evidenced by the position of the proteins on immunoblots probed with anti-67 antibody. The fortuitous size difference between endogenous p67 in the W303-1A strain and the cloned *NSR1* gene product has allowed us to prove that the *NSR1* protein, expressed in vivo, binds a wild-type, but not a mutant, NLS.

It is clear from our data that p67 is not overproduced under the inducible galactose promoter, suggesting that the level of the protein is tightly regulated by the cell. Overexpression of the *NSR1* protein could lead to its immediate degradation, similar to that observed for ribosomal proteins (Warner, 1989).

NSR1 Contains Two RNA-recognition Motifs

There are three regions containing distinct motifs within the *NSR1* protein. Each may have a separate function within the cell. In searching Genbank database, we found that the clustering of serine residues in the NH₂-terminus has been reported in vitellogenin, a food reserve protein. In both frog and chicken, vitellogenin is the precursor of yolk proteins (lipovitellins I and II, and phosvitin) that are essential for early development (Nardelli et al., 1987). Unlike *NSR1*, the stretches of serines in vitellogenin are not flanked by acidic residues. The presence of two acidic amino acid stretches in the NH₂ terminus raises the possibility that the acidic region may form electrostatic interactions with the basic amino acid residues that comprise the nuclear localization sequence. Such an interaction was proposed by Yoneda et al. (1988), and formed the basis for their identification of a 69-kD protein as a putative NLS receptor (see Introduction). Whether or not this interaction forms the total basis for the recognition of the nuclear localization sequence will require further investigation.

Interestingly, the deduced amino acid sequence of the central portion of *NSR1* contains two motifs previously designated as RNA recognition motifs (Query et al., 1989), and are homologous to those observed in for example, nucleolin, poly-A binding protein, and helix-destablization protein (Query et al., 1989). Each RNA-recognition motif encompasses ∼80-amino acid residues. Within the RNA recognition motifs is a smaller region containing eight highly conserved amino acids known as the RNP consensus octamer (Adam et al., 1986; Dreyfuss et al., 1988). This octamer has been observed in many proteins associated with RNA, although it is not yet known if this sequence binds directly to RNA. Both octamer sequences in *NSR1* (RGYGYVDF and KGFGYVQF) are identical for at least four out of eight residues of the 14 RNA-recognition motifs listed in Query et al. (1989). The best matches were six out of eight residues of the first (RKFGYVDF) and fourth (KGFGFVDF) RNA-recognition motif of nucleolin, the other two changes being conservative and found in the RNP consensus octamers of other proteins containing RNA-recognition motifs. The octamer in the second *NSR1* RNA-recognition motif also has only two conservative changes when compared with the RNP consensus octamer in the second RNA-recognition motif in poly-A–binding protein (KGFGFVHF). The surrounding subdomains in the 80-amino acid RNA-recognition motifs

are equally homologous to a number of different proteins containing RNA-recognition motifs.

The COOH-terminus of *NSR1* contains a series of arginine and glycine repeats, also found in a number of nucleolar and other proteins containing RNA-recognition motifs, e. g., mammalian fibrillarin and nucleolin, NOP1, helix-destabilization protein, and SSB1 protein (Jong et al., 1987; Christensen and Fuxa, 1988). These stretches have been proposed to be involved in protein–protein or nucleic acid–protein interactions (Jong et al., 1987; Christensen and Fuxa, 1988).

The roles of the proteins containing RNA-recognition motifs that are homologous to *NSR1* are quite different. Nucleolin has been presumed to function in ribosomal biogenesis; poly-A–binding protein binds mRNA and has been implicated in mRNA translation (Sachs and Davis, 1989; helix destabilization protein which binds both single stranded DNA and RNA, may be a component of heterogeneous nuclear ribonucleoproteins (Cobianchi et al., 1986), and SSB1, another single stranded DNA binding protein, also has a high homology to proteins in heterogeneous nuclear ribonucleoprotein particles (Jong et al., 1987). A possibility is that the *NSR1* protein has multifunctional roles in the cell. For example, it may be involved in ribosomal biogenesis, including RNA transport to the cytoplasm, as well as in the targeting of proteins to the nucleus. Investigators have shown that traffic through the nuclear pore complex is bidirectional (Dworetzky and Feldherr, 1988), and that wheat germ agglutinin (which binds to *N*-acetylglucosamine residues found in the glycoproteins of nuclear pores) not only inhibits the transport of proteins into the nucleus, but also the transport of 5S RNA, tRNA, mRNA, and pre-snRNAs (Baglia and Maul, 1983; Schröder et al., 1989).

If *NSR1* is a nucleolar protein, and is involved in ribosomal biogenesis, or RNA transport out of the nucleus, then an association between the pore complex and the nucleolus may be required to facilitate transport. The evidence for an interaction between the nucleolus and the nuclear envelope in a number of higher and lower eucaryotes has been reviewed by Bourgeois and Hubert (1988). There are also reports demonstrating a direct interaction between the nucleolar skeleton and the nuclear pore complex lamina in rat hepatocytes (Hubert et al., 1984) and human fibroblasts (Bourgeois et al., 1987).

Disruption of the NSR1 Gene Results in A Severe Growth Defect

Deletion of the middle region encoding the RNA-recognition motifs of the *NSR1* gene in the haploid strain results in a marked growth defect. Although we have not deleted the entire coding region, we have shown by immunoblotting with anti-p67 antibody that cell lysates of *nsr1*Δ::*HIS3* haploids do not contain the *NSR1* gene product. We do not have evidence to suggest that a new low molecular weight protein (possibly a truncated form of the *NSR1* protein) exists in this strain. The results suggest that the partial deletion of the gene abolishes the production of the *NSR1* protein. In ligand blots, the strain carrying a disrupted chromosomal copy of the *NSR1* gene is missing the major protein that specifically recognizes nuclear localization sequences in ligand blots. Again, no new lower molecular weight protein band exists in the ligand blot that specifically recognizes nuclear localization sequences. These data suggest that the ligand binding

property of the *NSR1* protein is not required for cell viability, although a severe growth defect is observed in the strain carrying the *NSR1* deletion. The nucleus of *nsr1*Δ::*HIS3* haploids is morphologically normal as viewed under the electron microscope (data not shown).

If the major protein responsible for binding to NLS sequences in ligand blots is the major receptor for nuclear proteins in vivo, the growth of the *nsr1*Δ::*HIS3* haploids suggest the possibility that other NLS-binding proteins are capable of functionally replacing the *NSR1* gene. In ligand blots of the *nsr1*Δ::*HIS3* haploids, some high molecular weight protein bands (the most noticeable at 110 kD) are still capable of recognizing the wild-type and not a mutant nuclear localization sequence. Indeed the growth defect observed in the disrupted strain may reflect the inability of these proteins to completely replace the *NSR1* protein.

Isolating the gene for a nuclear signal–binding protein will allow us to determine if a protein that specifically binds peptides containing nuclear localization sequences on ligand blots, functions as a receptor in the nuclear transport pathway. The signal-binding proteins exhibit the first criterion of a receptor involved in the initiation of nuclear transport, i. e., they recognize only one amino acid difference between peptides containing a wild-type nuclear localization sequence and a point mutant incapable of directing proteins to the nucleus in vivo. We are now in the position to test the importance of this protein in nuclear transport.

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