Multiple Regions of NSR1 Are Sufficient for Accumulation of a Fusion Protein within the Nucleolus

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Abstract. NSR1, a 67-kD nucleolar protein, was originally identified in our laboratory as a nuclear localization signal binding protein, and has subsequently been found to be involved in ribosome biogenesis. NSR1 has three regions: an acidic/serine-rich NH_2 terminus, two RNA recognition motifs, and a glycine/arginine-rich COOH terminus. In this study we show that NSR1 itself has a bipartite nuclear localization sequence. Deletion of either basic amino acid stretch results in the mislocalization of NSR1 to the cytoplasm. We further demonstrate that either of two regions, the NH_2 terminus or both RNA recognition motifs, are sufficient to localize a bacterial protein, β-galactosidase, to the nucleolus. Intensive deletion analysis has further defined a specific acidic/serine-rich region within the NH_2 terminus as necessary for nucleolar accumulation rather than nucleolar targeting. In addition, deletion of either RNA recognition motif or point mutations in one of the RNP consensus octamers results in the mislocalization of a fusion protein within the nucleus. Although the glycine/arginine-rich region in the COOH terminus is not sufficient to bring β-galactosidase to the nucleolus, our studies show that this domain is necessary for nucleolar accumulation when an RNP consensus octamer in one of the RNA recognition motifs is mutated. Our findings are consistent with the notion that nucleolar localization is a result of the binding interactions of various domains of NSR1 within the nucleolus rather than the presence of a specific nucleolar targeting signal.

THE majority of nuclear proteins contain nuclear localization sequences (NLSs)[1] that are required for their entry into the nucleus. The sequence fits the consensus Lys-Arg/Lys-X-Arg/Lys (Chelsky et al., 1989). Nuclear transport is saturable (Goldfarb et al., 1986), occurs by selective entry, and requires energy (Newmeyer et al., 1986; Markland et al., 1987; Newmeyer and Forbes, 1988; Richardson et al., 1988). Once nuclear proteins enter the nucleus, they are found in different subnuclear regions; their final destination is most likely defined by their structural and functional interactions with proteins or nucleic acids. Some examples are: the nuclear filament proteins (lamins) that play a role in nuclear cytoarchitecture are thought to attach to the nuclear envelope by binding to the surface of the inner nuclear membrane via a 54-kD protein (Bailer et al., 1991); transcription factors that activate expression of genes interact with specific DNA sequences; ribosomal proteins are found in the nucleolus where ribosomal DNA genes encoding ribosomal RNA (rRNA) are located. Although the notion of functional interactions being the major determinant of the localization of splicing or transcription factors is widely accepted, some have suggested that the localization of nucleolar proteins may be due to the presence of specific nucleolar targeting sequences (NOS) (Garcia-Bustos et al., 1991). Our manuscript addresses this question for the nucleolar protein NSR1, a nuclear signal binding protein in the yeast, *Saccharomyces cerevisiae.*

Transcription of ribosomal RNA and subsequent assembly of ribosomes has long been associated with the nucleolus. However, understanding how ribosome assembly is carried out and in what regions of the nucleolus the various steps take place has remained largely enigmatic. Seminal experiments in *Drosophila* showed that the transcription of a single rRNA gene is sufficient to organize a nucleolus, even if this gene is transcribed from a euchromatic region (Karpen et al., 1988). These studies suggest that all the machinery for processing the precursor rRNA, for attracting ribosomal proteins, and for recruiting proteins involved in assembly of the mature ribosome is available to the misplaced gene. Thus, it seems reasonable to propose that nucleolar proteins are recruited to the nucleolus on the basis of interactions required for their function, rather than by a specific targeting sequence. Indeed, reports from several different laboratories studying the sequences required for the localization of a vari-

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1. *Abbreviations used in this paper*: ASR, acidic serine region; DAPI, 4',6-diamidino-2-phenylindole; GAR, glycine-arginine rich; HTLV-1, human T cell leukemia virus type I; NLS, nuclear localization sequence; NOS, nucleolar targeting sequence; RRM, RNA recognition motif.

ety of proteins that function in the nucleolus (viral proteins, an amphibian and two mammalian nucleolar proteins) have found no common motif (Dang and Lee, 1989; Nosaka et al., 1989; Maeda et al., 1992; Peculis and Gall, 1992; Schmidt-Zachmann and Nigg, 1993). More importantly, there is evidence that some of the sequences identified are part of the functional domains of the various proteins.

The arginine-rich consensus motif: Arg/Lys-X-X-Arg-Arg-X-Arg-Arg is required for the nucleolar accumulation of the viral proteins human immunodeficiency virus (HIV-1) Tat and Rev, and the human T cell leukemia virus type I (HTLV-1) *rex*-encoded protein (Rex), and is sufficient to direct bacterial or cytoplasmic fusion proteins into the nucleolus (Siomi et al., 1988; Dang and Lee, 1989; Subramanian et al., 1991). However, further analysis identified the NOS of HIV Tat as necessary for its ability to transactivate genes expressed from the viral LTR (Hauber et al., 1989; Kuppuswamy et al., 1989; Ruben et al., 1989). Likewise, an alteration of the NOS in Rex abolishes both its nucleolar localization and its biological function (Nosaka et al., 1989). Therefore, the NOS likely targets these viral proteins to the nucleolus by serving as functional domains for these proteins to interact with other macromolecules within the nucleolus.

The arginine-rich consensus, however, is not present in endogenous nucleolar proteins that have been identified thus far, including: No38, a nucleolar protein found in amphibian oocytes; UBF, a mammalian nucleolar transcription factor required for ribosomal RNA gene expression; and nucleolin, a mammalian nucleolar protein involved in ribosome biogenesis.

A deletion of 24 amino acids in the COOH-terminal domain of No38 results in the inability of this protein to sort to the nucleolus (Peculis and Gall, 1992). However, the 24 amino acids are not sufficient to target a nonnucleolar protein to the nucleolus, suggesting that other domains of No38 may be required for proper nucleolar localization. Deleting of the RNA recognition motifs and glycine/arginine-rich domain prevented nucleolin from accumulation in the nucleolus, while the absence of the acidic NH_2-terminal region had no affect (Schmidt-Zachmann and Nigg, 1993). However, neither of the regions were sufficient to direct the cytoplasmic protein, pyruvate kinase, to the nucleolus. A similar analysis of UBF has shown that at least two regions are required for nucleolar accumulation, the HMG-box1 (necessary for rDNA binding), and an acidic area within the COOH terminus of the protein (Maeda et al., 1992). It was not determined if these two regions are sufficient to direct a nonnucleolar protein to the nucleolus.

The idea that functional domains rather than nucleolar targeting signals determine subnuclear localization of a few viral, as well as higher eucaryotic nucleolar proteins, led us to examine the sequences involved in the nucleolar localization of NSR1, a yeast nucleolar protein originally defined by our laboratory as a nuclear signal binding protein and subsequently shown to be involved in pre-rRNA processing and proper ribosome assembly (Lee and Melese, 1989; Kondo and Inouye, 1992; Lee et al., 1992). NSR1 has three major regions: an acidic/serine-rich NH_2 terminus, two RNA recognition motifs (RRM), and a COOH terminus rich in glycine/arginine residues (GAR domain). The various regions of NSR1 were individually fused to a bacterial protein (β-galactosidase) to test whether they could support the accumulation of the hybrid protein within the nucleolus. Using this approach, we have identified two regions of NSR1 that were sufficient: the NH_2 terminus and both RRMs. Thus, our data are consistent with the view that multiple regions determine the presence of a protein in the nucleolus.

Materials and Methods

Construction of NSR1 Hybrid Derivatives

DNA manipulations and microbiological techniques were carried out according to the method of Sambrook et al. (1989). The plasmids used in all the β-galactosidase fusion constructs were derived from pLG669Z (Guarente and Ptashne, 1981). All constructs are shown in Fig. 1. In the initial construct, pN1Kpn (described as N), the CYC1 promoter in pLG669Z was replaced with a 1.3-kb XhoI-BamHI fragment containing the GAL1-GAL10 bidirectional promoter and a 663-bp KpnI-EcoRI fragment from pWL1 (Lee et al., 1991) coding for the NH_2-terminal residues 1–187 of NSR1. The GAL1 promoter is oriented in front of the *NSR1* fragment.

Previously described nested deletions of the NH_2 terminus of NSR1 in pWL1, spanning to amino acid 187, were cloned in front of the β-galactosidase gene by modifying the KpnI site of pN1Kpn to a SphI site using linkers (pN1Sph). The EcoRI site at amino acid 187 was modified to a BamHI site using linkers. A partial digestion with AluI deleted the small portion of the first RRM (spanning residues 169–187) from NΔ91 and NLS:SphI-AluI. 1–82/NLS was made using a NSR1 COOH-terminal nested deletion of pWL1 by fusing residues 1–82 to the region of NSR1 containing NLS1 and NLS2, residues 134–168. NΔ39 was obtained by PCR of residues 39–187 using the *NSR1* fragment from pN1Kpn as template. 39–82/NLS was made using the same method as 1–82/NLS by fusing residues 39–82 to 134–168.

Oligonucleotide-directed in vitro mutagenesis was done based on the method described by Kunkel (1985). The following oligonucleotides were used to generate mutant sequences using the *NSR1* fragment from pNIKpn as template: deletion of NLS1 (residues 139–142) AGAGTCTAACGAT-(deletion)-TCTGAGGACGCC; deletion of NLS2 (residues 159–162) GAGTCTTCCAAC-(deletion)-AATGAAGAAACC. NSR1ΔKKRK was constructed by replacement of a 60-bp AccI-BamHI fragment from NΔKKRK by a 1.0-kb AccI-BamHI fragment from pWL1 containing the distal portion of *NSR1*.

β-galactosidase fusion constructs lacking the NH_2 terminus of NSR1 were made by fusing both NLS1 and NLS2 of NSR1 to either the RRM or the GAR domain using a nested deletion of pWL1 that deletes amino acid residues 1–133 (Fig. 1 *B*, 134-414). NLS/RRM1+2 deletes the sequences after an internal BstII site in *NSR1*. NLS/RRMΔ2 deletes the sequences after a HphI site. Both the BsteII and HphI sites, respectively, were modified to BamHI sites using linkers, and the resulting SphI-BamHI fragments were cloned into pN1Sph. Using NLS/RRM1+2, NLS/RRMΔ1 was generated by deleting the sequences between two internal AccI sites. Point mutations in the RNP consensus octamer of RRM1 were made by oligonucleotide-directed in vitro mutagenesis, described above, using the oligonucleotide AAGAGGTACCGATAGATCTC*AA*GGTT*TG*GGTTACGT. The nucleotide changes are underlined and italicized. Parallel constructs of NLS/RRM1+2 containing only *NSR1* sequences were made by insertion of a stop codon after amino acid 367. To obtain NLS/GAR, the XbaI site in *NSR1* was modified to a MboI site using BamHI linkers, and a 224-bp MboI fragment containing the GAR domain was isolated and inserted into pNISph behind the amino acid residues 134–187 of NSR1.

Strains and Media Preparation

The haploid strains W303-1A (*Mat a, ade2-1, can1-100, ura3-1, leu 2-3, 112, trp 1-1, his3-11,15*) or WLY353 (same as W303-1A except *nsr1::HIS3*) (Lee et al., 1992), were used in all experiments. Either W303-1A or WLY353, harboring pWL10 (Lee et al., 1991), was used as a control strain for wild-type NSR1 protein expression. 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).

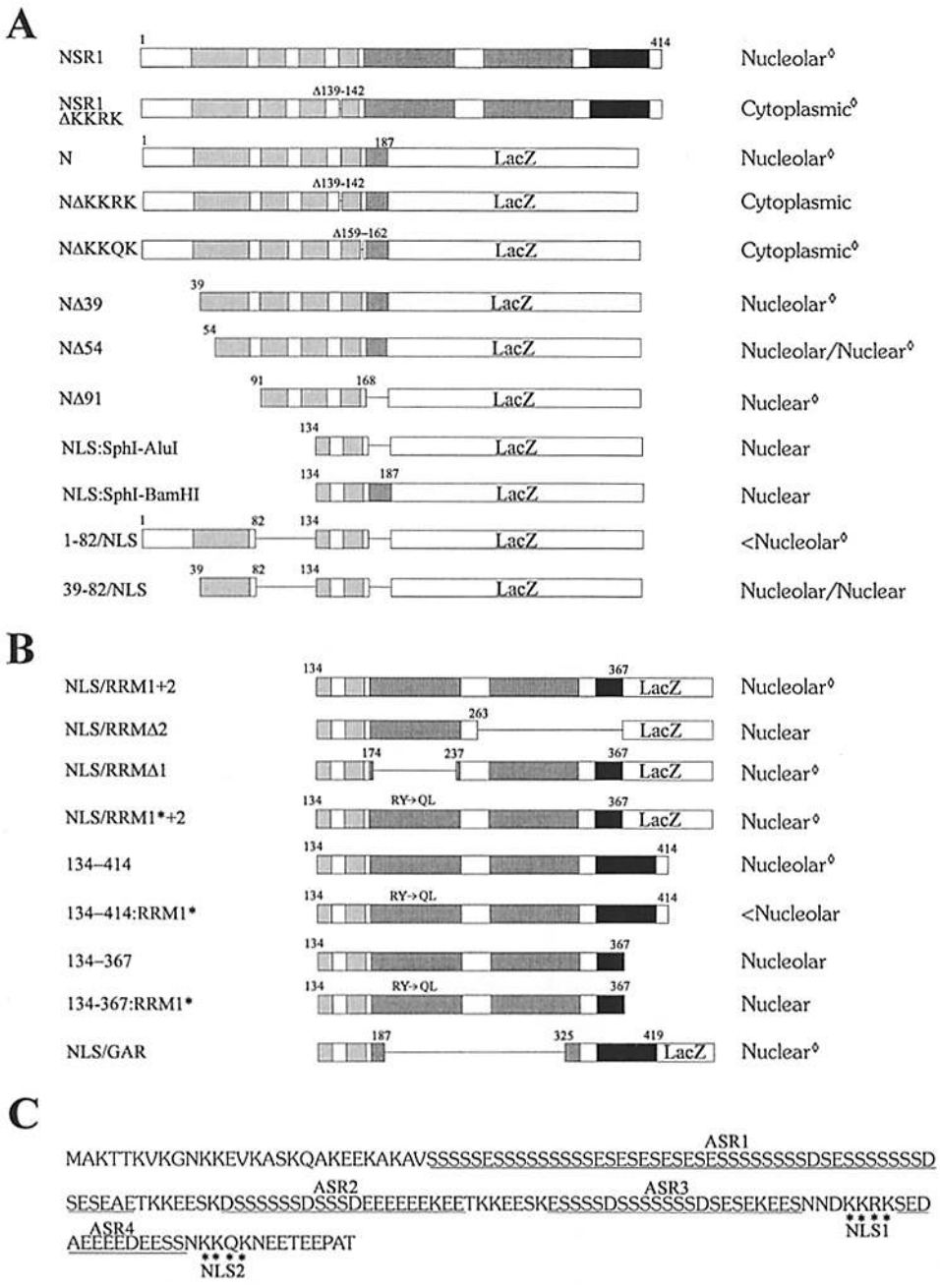


Figure 1. (*A*) and (*B*) Hybrid contructs containing NSR1 deletions fused to the β-galactosidase gene and NSR1 deletion mutants containing only *NSR1* sequences. The intracellular distribution of all the constructs are shown. The protein domains of the deduced *NSR1* sequence are displayed on top. The first four light gray boxes represent the acidic/serine-rich regions (ASR1–4); two shaded boxes in the middle denote the two RRMs; the GAR domain is shown as a black box. The sizes of the boxes are roughly proportional to their length in the protein. Small internal deletions and point mutations in several constructs are displayed directly above the affected domains. An * next to RRM1 indicates the amino acid changes Arg(R)→Gln(Q), Tyr(Y)→Leu(L), as described in Results. ◊ Indicates constructs in which immunofluorescence data are shown. Immunofluorescence data are not shown for the other constructs in which similar results were obtained. (*C*) The deduced amino acid sequence of the NH_2 terminus of NSR1. ASR1–4 are underlined. NLS1 and NLS2 are denoted by four asterisks (*).

Expression of NSR1 Hybrid Proteins and Deletion Constructs Containing Only NSR1 Sequences from the GAL1 Promoter; Preparation of Whole Cell Lysates and Subcellular Fractionations

The yeast strain W303-1A was transformed to *URA+* with the plasmids containing the β-galactosidase fusion constructs. NSR1 deletion constructs containing only *NSR1* sequences were transformed into the *nsr1−* strain, WLY353. To induce expression, cells were first grown overnight in liquid synthetic media containing 2% raffinose and lacking uracil to OD_{600} 0.3. They were then switched into rich media+2% raffinose media (YPR) for 2 h, and 2% galactose (induced) or 2% glucose (repressed) were added. Cells were grown for 5 h and harvested in exponential phase. In the case of strains containing the NSR1 deletion constructs, since these proteins are greatly overexpressed, at 2 h after galactose induction, 1% glucose was added to decrease expression; cells were grown for an additional 3 h and harvested. Cells were washed with 10 mM Tris/1 mM EDTA, pH 8, containing protease inhibitors (0.5 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM aminocaproic acid). Whole cell lysates were prepared by dissolving cells in Laemmli buffer (containing 6 M urea and 0.5 mM PMSF). Subcellular fractionations were carried out using a method developed by Baker et al. (1988) to obtain pure cytosol. Fractions were then dissolved in Laemmli buffer. Samples from both these procedures were analyzed on a 10.5% SDS-polyacrylamide gel and transferred onto nitrocellulose paper as described previously (Lee et al., 1991).

Immunoblotting and Immunofluorescence Microscopy

Immunoblotting was performed as described by Lee et al. (1991). The primary antisera used were either monoclonal anti-β-galactosidase antibody (Promega Corp., Madison, WI) at 2 μg/ml or 1:200 dilution of an affinity-purified polyclonal antibody against NSR1.

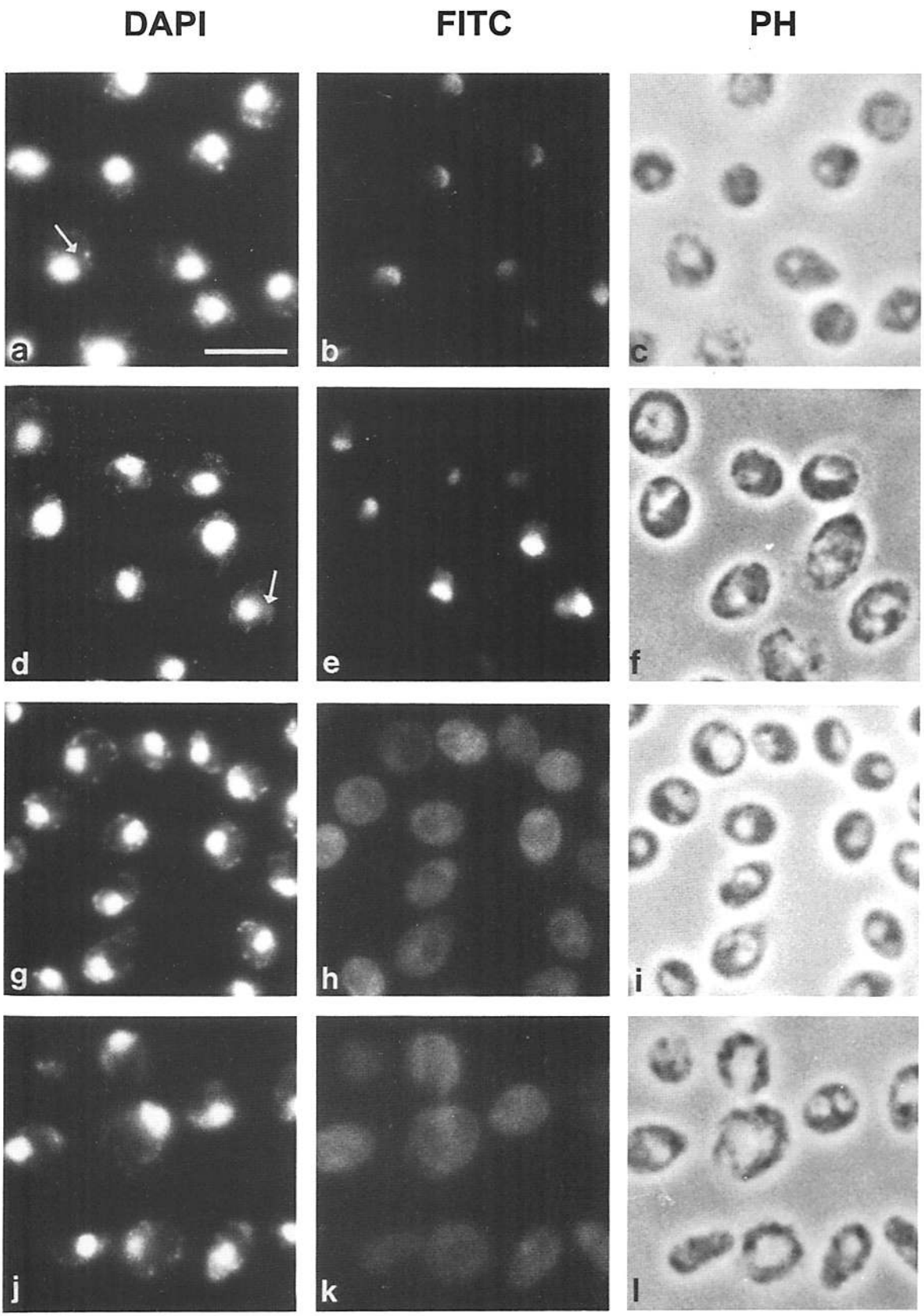


Figure 2. Immunofluorescence localization of NSR1, N, NΔKKQK, and NSR1ΔKKRK. The constructs are shown in Fig. 1 *A*. Indirect immunofluorescence was performed on the yeast strains WLY353 (the *nsr1-* strain) expressing NSR1ΔKKRK and W303-1A expressing NSR1 or the hybrid proteins, as described in Materials and Methods. Antibody against NSR1 was used to detect the localization of NSR1 in a wild-type haploid strain, W303-1A (*a–c*), and the NSR1 deletion mutant lacking residues 139–142 (NLS1), NSR1ΔKKRK (*j–l*). Anti-β-galactosidase antibody was used to detect the intracellular distribution of the hybrid proteins, N (*d–f*) and NΔKKQK (*g–i*). *Arrows* are used to indicate the position of the nucleolar region within the nucleus. DAPI staining of DNA (*a*, *d*, *g*, *j*); FITC staining (*b*, *e*, *h*, *k*); phase contrast (PH) (*c*, *f*, *i*, *l*). Bar, 2 μm.

After 3-h galactose induction, cells were prepared for indirect immunofluorescence by following published procedures for spheroplasting using Zymolase 100T (ICN Biomedicals, Inc., Costa Mesa, CA) (Pringle et al., 1989). First antibody incubations were performed overnight at 4°C using a monoclonal anti-β-galactosidase antibody diluted at 6.8 μg/ml in blocking buffer, or using a 1:30 dilution of an affinity-purified polyclonal antibody against NSR1. As secondary antibody, FITC-conjugated affinity-purified goat anti-mouse IgG diluted at 6.8 μg/ml, or FITC-conjugated affinity-purified goat anti-rabbit IgG diluted at 4.5 μg/ml, was used to visualize the cellular localization of the proteins. In the case of 1-82/NLS construct where the hybrid protein was underexpressed, a tertiary antibody, FITC-conjugated affinity-purified donkey anti-goat IgG (goat anti-mouse, goat anti-rabbit, and donkey anti-goat; Jackson ImmunoResearch Labs., Inc., West Grove, PA) diluted at 4.5 μg/ml was used. 1 μg/ml of DAPI was used to visualize nuclear staining. Slides were viewed with a fluorescence microscope (Optiphot; Nikon Inc., Garden City, NY). Kodak TMAX 400 film was used for all photomicroscopy.

Cells containing the NSR1 deletion constructs, in contrast, were harvested after 5 h in 2% galactose/1% glucose, as discussed in the previous section, since overexpression of the proteins resulted in protein levels greatly higher than the expression of NSR1 or the hybrid proteins by immunoblotting analysis, and by indirect immunofluorescence, total cell staining was observed (data not shown).

Results

NSR1 Contains a Bipartite NLS

Since NSR1 is a nucleolar protein (Fig. 2, *a–c*), we wondered whether the subnuclear distribution of the protein could be attributed to a specific nucleolar signal, or to two overlapping or distinct signals for its nuclear versus nucleolar localization.

Between the NH_2-terminal domain and the domain containing the RNA recognition motifs are two putative NLS's, (^{139}KKRKS and ^{157}KKQK), that fit the highly basic consensus sequence for an NLS assigned by Chelsky et al. (1989). To determine if either of the NLSs were functional, we tested whether they were necessary for the localization of a NSR1/β-galactosidase fusion protein. A hybrid protein was constructed that contained the NH_2 terminus of NSR1 (including the KKRK and KKQK sequences) and the bacterial protein, β-galactosidase. By indirect immunofluorescence, this protein was found in the nucleolus (Fig. 2, *d–f*). Deletion of either the KKRK or the KKQK sequence by in vitro mutagenesis resulted in mislocalization of the hybrid protein to the cytoplasm (Fig. 2, *g–i*), suggesting that NSR1 contains a bipartite NLS (Robbins et al., 1991).

A deletion of the KKRK sequence was also made in the NSR1 protein, and like the hybrid proteins, was found to be cytoplasmic. NSR1 is highly stable in the cytoplasm when it is mislocalized as analyzed by both immunofluorescence (Fig. 2, *j–l*) and cellular fractionation (data not shown).

A Combination of Different Regions within the NH_2 Terminus, Rather Than a Specific Sequence, Is Required for Nucleolar Localization

NSR1 has three well defined regions: an acidic/serine-rich NH_2 terminus, a middle region containing two RNA recognition motifs, and a COOH-terminal region containing a sequence abundant in arginine/glycine repeats. We decided to test each region separately for its ability to target β-galactosidase to the the nucleolus.

Our assay for determining nucleolar localization was indirect immunofluorescence. In yeast, the nucleolus forms a crescent that lines the nuclear envelope and occupies a sizeable volume of the nucleus. In some visual planes the nucleolus will wrap around the nucleus such that viewed from above it will appear to lay on top of the nucleus. In a field of cells, those having this orientation will show overlap of the DNA (stained by DAPI) and the nucleolar antigen (stained by FITC). In most cases the orientation of the cells will be such that the nucleolar antigen will not overlap the DNA, and because the nucleolar region is not stained well by DAPI, the two staining patterns will be nearly independent. Our criterion for nucleolar staining is that most of the cells in a particular field show distinct FITC and DAPI staining, while in the case of nuclear staining all cells in the field show an overlap between DAPI and FITC staining.

A hybrid protein containing the NH_2 terminus of NSR1, (residues 1-187; including the nuclear localization sequences), fused to β-galactosidase was observed in the nucleolus as discussed in the previous section (Fig. 2, *d–f*). The nucleolar localization of the fusion protein, despite the absence of the RNA recognition motifs (RRMs) and the GAR domain, suggested that a nucleolar targeting sequence may exist within the NH_2 terminus of NSR1. We then carried out an extensive deletion analysis of the NSR1 NH_2 terminus to determine if a specific amino acid sequence was responsible for the correct localization of the hybrid protein. A series of existing deletions of the NH_2-terminal domain of NSR1, made during the sequencing of the protein, were used for this study (Lee et al., 1991). Expression of the hybrid proteins was confirmed by immunoblotting using anti-β-galactosidase antibody (Fig. 3).

The NSR1 NH_2 terminus is highly repetitive and contains four separate clusters of acidic/serine-rich residues (ASR or

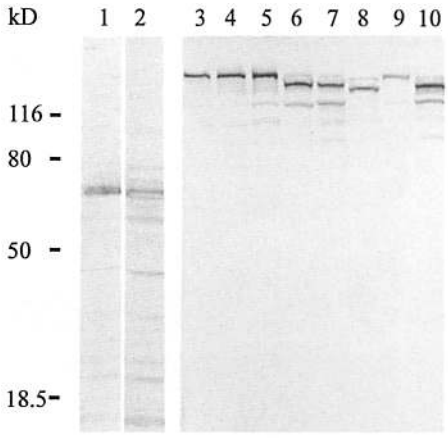
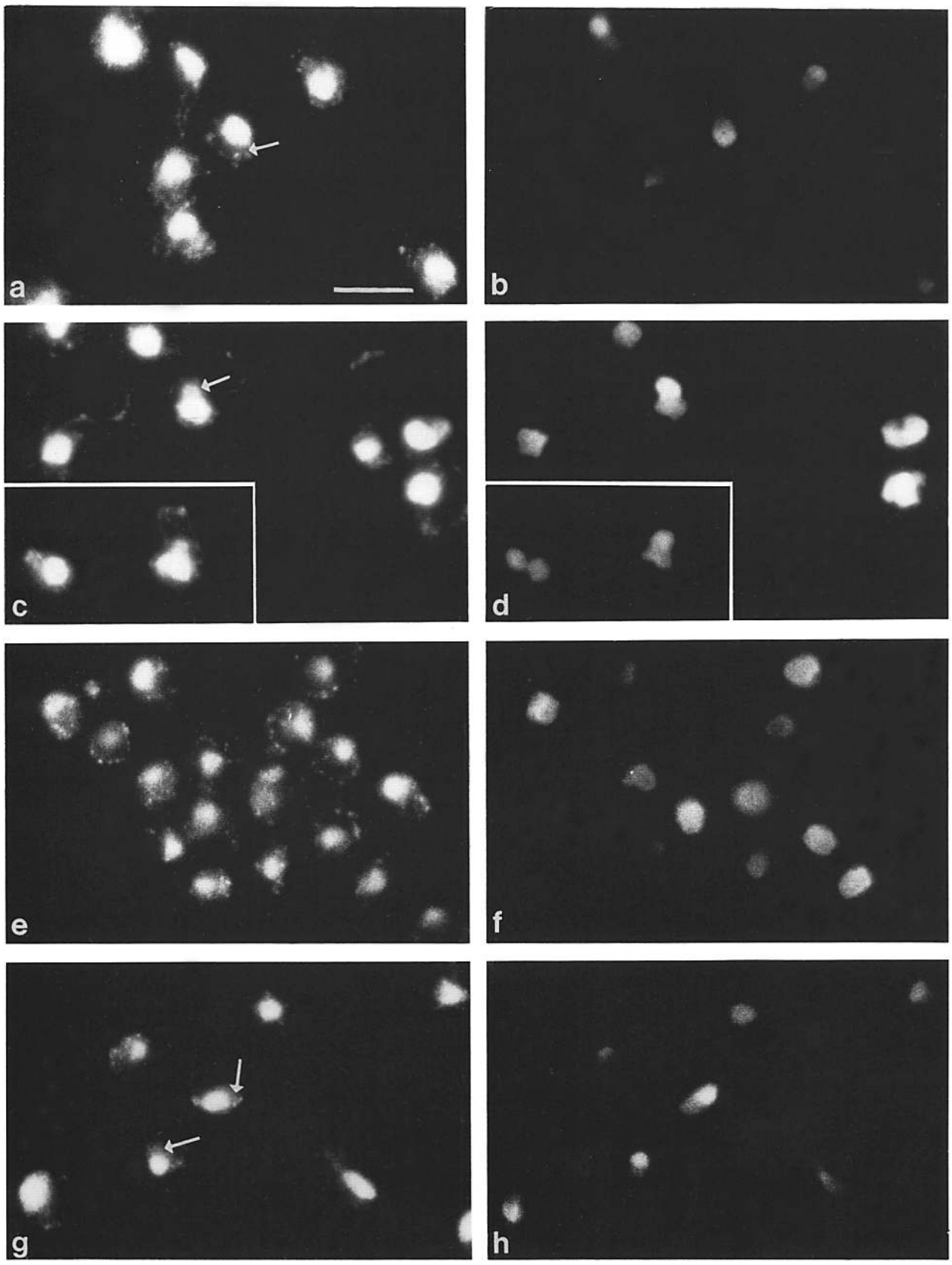


Figure 3. Expression of NSR1, NSR1ΔKKRK, and hybrid proteins containing deletions in the NSR1 NH_2 terminus under the GAL1 promoter. Whole cell lysates were prepared from the yeast strains WLY353 expressing NSR1ΔKKRK and W303-1A expressing NSR1 or the hybrid proteins. Proteins were separated on a 10.5% SDS-polyacrylamide gel and transferred onto two nitrocellulose filters. One filter was stained with india ink, and the second filter was blotted with anti-NSR1 antibody (lanes *1* and *2*) or anti-β-galactosidase antibody (lanes *3–10*), as described in Materials and Methods. The constructs are shown in Fig. 1 *A*. NSR1 (lane *1*); NSR1ΔKKRK (lane *2*); N (lane *3*); NΔKKRK (lane *4*); NΔKKQK (lane *5*); NΔ39 (lane *6*); NΔ54 (lane *7*); NΔ91 (lane *8*); 1-82/NLS (lane *9*); 39-82/NLS (lane *10*).


DAPI
FITC
a
b
c
d
e
f
g
h

acidic/serine regions 1–4, Fig. 1 *C*), defined by us, as a stretch of serines and acidic amino acids lacking any basic residues and containing consensus casein kinase II sites. In fact, NSR1 is phosphorylated in vitro by casein kinase II (unpublished results). Separating the first acidic/serine cluster from the second, and the second cluster from the third is the repeat TKKEESK. The third and fourth acidic/serine clusters are separated by one of the nuclear localization sequences, KKRK.

Initially, we decided to delete the beginning of the NSR1 NH_2 terminus just prior to ASR1 because it was highly basic and such regions had been proposed to be involved in the nucleolar localization of other proteins (Dang and Lee, 1989). However, the loss of amino acids 1–39 (NΔ39) from the NSR1 NH_2 terminus still resulted in the nucleolar localization of the hybrid protein (Fig. 4, *a* and *b*). Deletion of an additional 15 amino acids (NΔ54), which removed approximately half of ASR1 resulted in the hybrid protein being distributed between the nucleolus and the nucleus (Fig. 4, *c* and *d*). Complete removal of ARS1 was accomplished by deleting an additional 37 amino acids (NΔ91), and resulted in predominately nuclear staining, though exclusion from the nucleolus was not observed (Fig. 4, *e* and *f*). It was interesting that despite the continued presence of the three remaining acidic/serine clusters (ASR2–4), total removal of ASR1 resulted in nuclear rather than nucleolar localization of the hybrid protein.

Since the constructs above, except for the one lacking residues 1–91, all contain a small part of one of the RNA recognition motifs, we could not rule out the possibility that the observed nucleolar localization was due solely to the incomplete RRM. However, addition of this small region of the RRM to a hybrid protein that is nuclear does not confer the ability to localize to the nucleolus (see Fig. 1 *A*, NLS:SphI-AluI versus NLS:SphI-BamHI; immunofluorescence data not shown).

To test if ASR1 is sufficient for β-galactosidase to be maintained in the nucleolus, a hybrid protein (1–82/NLS) composed of the first 82 amino acids of the NSR1 NH_2 terminus (containing the entire ASR1) and the NSR1 NLS within residues 134–168 fused to β-galactosidase was made, and was predominantly nucleolar (Fig. 4, *g* and *h*). Unfortunately, to maintain the internal bipartite NLS, ASR4 must be included in the hybrid protein. However, since a fusion protein with ASR2–4 is not found in the nucleolus, we assume that this region is not required for nucleolar localization.

We were surprised to find that when we deleted residues 1–39 in the shortened construct containing ASR1 and ASR4, it dramatically lowered the amount of β-galactosidase in the nucleolus (see Fig. 1 *A*, 39-82/NLS; immunofluorescence data not shown). We had already shown that residues 1–39 are dispensable if the rest of the NH_2 terminus is present,

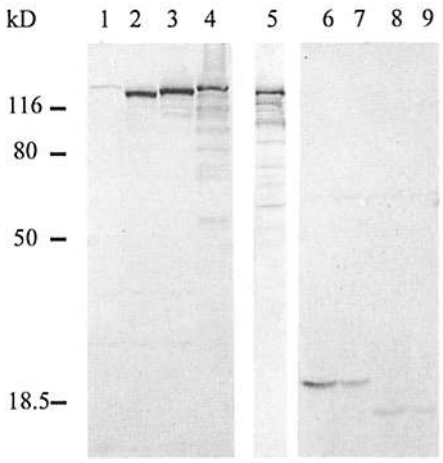


Figure 5. Expression of hybrid proteins lacking the NH_2 terminus but containing the NLS of NSR1 with either the RRMs or the GAR domain and deletion mutants containing only *NSR1* sequences. Whole cell lysates were prepared from the yeast strains W303-1A expressing the hybrid proteins or WLY353 expressing the NSR1 deletion mutants. The proteins were resolved on a 10.5% SDS–polyacrylamide gel, transferred onto two nitrocellulose filters, and one filter was stained with india ink. The second filter was blotted with anti-β-galactosidase antibody (lanes *1–5*) or antibody against NSR1 (lanes *6–9*). The constructs are shown on Fig. 1 *B*. NLS/RRM1+2 (lane *1*); NLS/RRMΔ2 (lane *2*); NLS/RRMΔ1 (lane *3*); NLS/RRM1*+2 (lane *4*); NLS/GAR (lane *5*); 134-414 (lane *6*); 134-414:RRM1* (lane *7*); 134-367 (lane *8*); 134-367:RRM1* (lane *9*).

but these residues clearly become important for nucleolar localization in the shortened construct that contains the first 82 amino acids of the NSR1 NH_2 terminus and residues 134–168 of NSR1 containing the NLS. We interpret this as an indication that residues 1–39 have the ability to enhance binding within the nucleolus as compensation for the loss of binding interactions normally facilitated by the distal half (82–168) of the NH_2 terminus. Thus, different combinations of regions within the NH_2 terminus can result in the ability of β-galactosidase to reside in the nucleolus.

Both RNA Recognition Motifs Are Required for the Nucleolar Localization of NSR1

To assess whether the other regions of NSR1 (the RRMs and the GAR domain) could influence the subnuclear localization of β-galactosidase, hybrid proteins lacking the NH_2 terminus but containing the NLS of NSR1 (residues 134–168) with either the RRMs or the GAR domain, were made. The constructs were transformed and expressed (Fig. 5), as described in Materials and Methods. The RRM fusion containing both RRMs was found in the nucleolus even in the absence of the NH_2 terminus (Fig. 6, *c* and *d*).

Figure 4. Immunofluorescence localization of NΔ39, NΔ54, NΔ91, and 1-82/NLS. Indirect immunofluorescence was performed on the wild-type haploid strain, W303-1A, expressing the hybrid proteins. Anti-β-galactosidase antibody was used to detect the intracellular distribution of the β-galactosidase fusion constructs NΔ39 (*a* and *b*), NΔ54 (*c* and *d*), NΔ91 (*e* and *f*), and 1-82/NLS (*g* and *h*), as described in Materials and Methods. The small inset shown in *d* was added to more clearly show the difference in staining between NΔ54 versus NΔ39 and NΔ91. *Arrows* are used to indicate the position of the nucleolar region within the nucleus. DAPI staining of DNA (*a*, *c*, *e*, *g*); FITC staining (*b*, *d*, *f*, *h*). Bar, 2 μm.

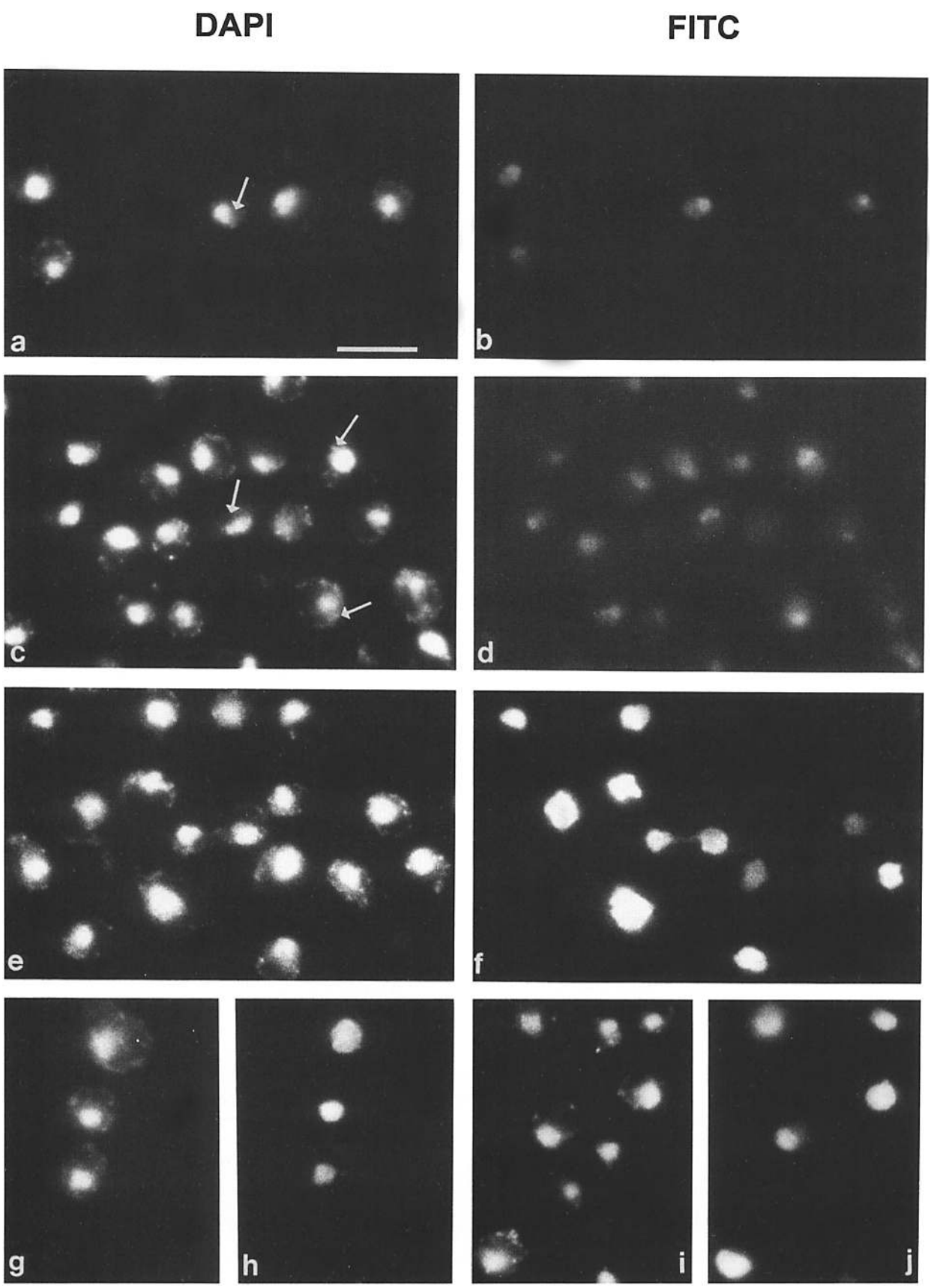


Figure 6. Immunofluorescence localization of 134-414, NLS/RRM1+2, NLS/RRMΔ1, NLS/RRM1*+2, and NLS/GAR. Indirect immunofluorescence was performed on WLY353 expressing the NSR1 deletion mutant, 134-414, which lacks the NH_2-terminal residues 1–133, and on W303-1A expressing the hybrid proteins, as described in Materials and Methods. Antibody against NSR1 was used to detect

Deletion of the second RRM, leaving only the first RRM (RRM1), resulted in localization of the hybrid protein to the nucleus (see Fig. 1 *B*, NLS/RRMΔ2; immunofluorescence data not shown). To ensure that the amino acid requirements for nucleolar localization were not contained within the second motif, a deletion of the first RRM, leaving only the second RRM (RRM2), was constructed and was also found in the nucleus as analyzed by indirect immunofluorescence (Fig. 6, *e* and *f*). Although RRMs are defined as loose consensus sequences that extend over an 80 amino acid area, the RNP consensus octamer lies within this region and is highly conserved. The alignment of RRM sequences by Kenan et al. (1991) indicate the conserved amino acids in RNP1 at positions 52, 54, and 56. An Arg52→Gln change in the U1-A protein was found to abolish RNA binding (Nagai et al., 1990). Based on these observations, we decided to mutate the first RNP consensus octamer (*Arg-Gly-*Tyr-Gly-Tyr-Val-Asp-Phe) changing *Arg52→Gln and *Tyr54→Leu. A hybrid protein containing the mutated RNP1 and the wild-type RNP2 fused to β-galactosidase accumulated in the nucleus (Fig. 6, *g* and *h*).

Our next series of experiments was directed at asking if the subnuclear localization of NSR1 itself would be similar to the NSR1/β-galactosidase hybrid protein if the same deletions and mutations of the RRMs were made. To observe the localization of these mutant NSR1 proteins, they were expressed in an *nsr1-* strain and their cellular localization was detected using antibody against NSR1. NSR1 lacking only the NH_2 terminus, like the bacterial fusion protein, was still located in the nucleolus (Fig. 6, *a* and *b*, and Fig. 1 *B*, 134-414). However, the NSR1 protein carrying the identical point mutations within RNP1 was still predominately nucleolar, not nuclear (see Fig. 1 *B*, 134-414:RRM1*; immunofluorescence data not shown). This result was unexpected given the strong nuclear accumulation of the fusion protein carrying the same mutation. The major difference in the two constructs was that the GAR domain was present in the NSR1 protein but absent from the fusion protein. When an additional NSR1 construct was made that lacked approximately two thirds of the GAR domain, but still contained the point mutations in RNP1, it now accumulated in the nucleus (see Fig. 1 *B*, 134-367:RRM1*; immunofluorescence data not shown).

Despite the fact that the presence of the GAR domain was able to compensate for a mutation in RNP1, it was not sufficient, when fused to β-galactosidase, to localize the hybrid protein to the nucleolus (Fig. 6, *i* and *j*).

Discussion

NSR1 is a nucleolar protein which was originally identified by a ligand blot analysis in a search for proteins that specifically recognized nuclear localization sequences (Lee and Melese, 1989; Lee et al., 1991). The protein has since been shown to be involved in preribosomal RNA processing as well as in the correct assembly of ribosomal subunits (Kondo and Inouye, 1992; Lee et al., 1992). Although the ligand blot data may suggest that NSR1 is involved in the nuclear transport pathway, we have yet to define its role in vivo.

A number of nucleolar proteins have been proposed to be involved in the transport of proteins into the nucleus by acting as receptors that continuously shuttle between the nucleus and cytoplasm (Borer et al., 1989; Meier and Blobel, 1992). A putative shuttling protein must have a binding site for nuclear proteins and also a separate binding site for interacting with the nuclear pore proteins. NSR1 has a bipartite NLS. The presence of a functional NLS within NSR1 may seem incongruous with the protein having a role in the nuclear transport pathway. However, most proteins that are structurally or functionally associated with the nucleus have an NLS or in some cases use the NLS of another protein (Schuster et al., 1986; Booher et al., 1989). Notably, deletion of part of the bipartite NLS within NSR1 results in a stable cytoplasmic form of NSR1, a property expected for a shuttling receptor.

Additionally, we have shown that multiple regions are likely to determine the subnuclear localization of NSR1: either different combinations of residues in the NSR1 NH_2 terminus, or the RRMs, when fused to a bacterial protein, are sufficient to allow the hybrid protein to accumulate in the nucleolus.

A hybrid protein constructed by a fusion of the NH_2 terminus of NSR1 with β-galactosidase was located in the nucleolus as analyzed by indirect immunofluorescence. An extensive analysis of the residues responsible for the accumulation of the hybrid protein in the nucleolus showed that the largest of four acidic/serine-rich clusters (ASR1) was sufficient. However, the amino acids in all four ASRs are highly repetitive (aspartic, glutamic, and serine residues) and it is unlikely that a specific sequence within ASR1, that is absent in ASR2–4, would provide a unique signal for nucleolar targeting.

ASR1 has the longest stretch of serine residues and could potentially be highly phosphorylated in vivo. However, ASR2–4 together should also be a highly acidic region and yet, curiously, it is not sufficient for β-galactosidase to accumulate in the nucleolus. Perhaps ASR1 is specifically required for proper structural and functional interactions of NSR1 with other proteins within the nucleolus. Other data in our laboratory indicate that ASR1 is also the prime region for recognition of NLS peptide-conjugates in ligand blots (unpublished data).

Although the NH_2 terminus of NSR1 is sufficient to localize β-galactosidase to the nucleolus, it is not necessarily required. A construct containing the two RNA recognition motifs and lacking the NH_2-terminal domain is also found in the nucleolus. We found that deletion of either RRM resulted in the nuclear location of the fusion protein. Additionally, two point mutations in the RNA consensus octamer (RNP) of RRM1 resulted in the nuclear location of both a hybrid protein (NLS/RRM1*+2) and NSR1 lacking the NH_2-terminal and GAR domains (134-367:RRM1*).

the localization of 134-414 (*a* and *b*). Anti-β-galactosidase antibody was used to detect the intracellular distribution of the hybrid proteins NLS/RRM1+2 (*c* and *d*), NLS/RRMΔ1 (*e* and *f*), NLS/RRM1*+2 (*g* and *h*), and NLS/GAR (*i* and *j*). *Arrows* are used to indicate the position of the nucleolar region within the nucleus. DAPI staining of DNA (*a*, *c*, *e*, *g*, *i*); FITC staining (*b*, *d*, *f*, *h*, *j*). Bar, 2 μm.

The observation that in the absence of the NH_2 terminus both RRMs are required for the proper nucleolar localization of a hybrid protein as well as NSR1 itself may imply, that like nucleolin (Bourbon et al., 1983; Herrera and Olson, 1986; Ghisolfi et al., 1990), NSR1 recognizes a larger RNA, possibly ribosomal RNA. The disruption of NSR1, as well as GAR1, NOP1, U3, and U14 result in improper processing of preribosomal RNA (Li et al., 1990; Hughes and Ares, 1991; Tollervey et al., 1991; Girard et al., 1992; Lee et al., 1992). Unlike these proteins, NSR1 does not coimmunoprecipitate small nucleolar RNAs (snoRNAs). On the basis of the presence of snoRNAs immunoprecipitating with GAR1, NOP1, and SSB1, it has been suggested that these proteins may form a snoRNP particle, similar to a small nuclear RNP (snRNP) particle, but involved with processing of preribosomal RNA rather than pre-mRNA (Tollervey et al., 1991; Girard et al., 1992). Nucleolin, an abundant mammalian nucleolar protein, is not thought to be a snoRNP protein and has been found to bind rRNA in vitro (Bourbon et al., 1983; Herrera and Olson, 1986). Nucleolin has also been shown to shuttle between the nucleus and cytoplasm (Borer et al., 1989), and it has been proposed to play a role in the nucleocytoplasmic transport of ribosomal proteins. Nucleolin and NSR1 share a similar organization of domains, and both have been shown to bind SV-40 T-antigen type NLSs in vitro (Xue et al., 1993) and to be involved in ribosome biogenesis (Bugler et al., 1982; Bourbon et al., 1983; Lee et al., 1992). Data on the localization of nucleolin corroborate our findings with NSR1 in that a nucleolar targeting signal was not identified. Unlike NSR1, the RNA recognition motifs were not sufficient to target nonnucleolar proteins to the nucleolus (Schmidt-Zachmann and Nigg, 1993).

Finally, the highly basic residues interspersed with aromatic amino acids that constitute the GAR domain have been suggested to bind RNA or proteins (Ghisolfi et al., 1992*a*). In the studies by Ghisolfi et al., a polypeptide corresponding to the nucleolin GAR domain was synthesized in *Escherichia coli* (Ghisolfi et al., 1992*b*). The authors demonstrated that the GAR domain is required for the efficient binding of RNA by the RNA recognition motifs, although it does not contribute to the specificity of the interaction. We have shown that the native RRMs of NSR1 in the absence of the GAR domain are capable of allowing a fusion protein to accumulate in the nucleolus. Point mutations in the RNP1 of a hybrid protein (NLS/RRM1*+2) or in NSR1 lacking both the NH_2 terminus and the GAR domain (134-367:RRM1*), result in the distribution of these proteins to the nucleus. In other experiments we have also shown when the RNP octamer in RRM1 is mutated, the GAR domain is necessary for the nucleolar accumulation of NSR1 lacking the NH_2 terminus. These results support the idea that the GAR domain coupled with the mutant RRM are capable of stronger binding interactions within the nucleolus than either can carry out alone.

Ghisolfi et al. also suggest that the GAR domain may carry a nucleolar targeting signal since it has been found to date only in nucleolar proteins (Ghisolfi et al., 1992*a*). However, our results clearly show that the GAR domain itself is unable to direct a fusion protein to the nucleolus. A hybrid protein constructed by making a fusion of the GAR domain with β-galactosidase was located in the nucleus.

In conclusion, nucleolar signals were first suggested to be extended NLSs that contained longer arrays of basic amino acid stretches (Dang and Lee, 1989). However, extensive mutational analysis of a number of proteins found in the nucleolus have been carried out and no consensus nucleolar targeting sequence, or NOS, has as yet been identified. Additionally, the NOSs of the viral proteins, HIV Tat and the Rex protein of HTLV-1, and those of the higher eukaryotic nucleolar proteins UBF (a nucleolar transcription factor) and nucleolin (involved in ribosome biogenesis) were found to be functional domains necessary for their cellular activity (Hauber et al., 1989; Kuppuswamy et al., 1989; Nosaka et al., 1989; Ruben et al., 1989; Maeda et al., 1992; Schmidt-Zachmann and Nigg, 1993). We have demonstrated that all three regions of the NSR1 protein, depending on the circumstances, can contribute to its accumulation in the nucleolus. Our findings, and the lack of other common sequences between nucleolar proteins, lend increased support to the idea that unlike NLSs, nucleolar targeting does not occur via a specific consensus sequence. Like transcription factors that form protein complexes and bind to DNA, and proteins involved in the nuclear cytoarchitecture, the subnuclear accumulation of nucleolar proteins appears to occur through specific binding interactions with other proteins and/or nucleic acids.

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