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A new subclass of nucleoporins that functionally interact with nuclear pore protein NSP1

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NSP1 is a nuclear pore protein (nucleoporin) essential for cell growth. To identify the components that functionally interact with NSP1 in the living cell, we developed a genetic screen for mutants that are lethal in a genetic background of mutated, but not wild type *NSP1*. Fourteen synthetic lethal mutants were obtained, belonging to at least four different complementation groups. The genes of two complementation groups, *NSP116* and *NSP49*, were cloned. Like the previously described nucleoporins, these genes encode proteins with many repeat sequences. NSP116 and NSP49, however, contain a new repetitive sequence motif 'GLFG', which classifies them as a subclass of nucleoporins. NSP116 and NSP49, tagged with the IgG binding domain of protein A and expressed in yeast, are located at the nuclear envelope. These data provide *in vivo* evidence that distinct subclasses of nucleoporins physically interact or share overlapping function in nuclear pore complexes.

Key words: nuclear pore complex/nuclear transport/nucleoporin/synthetic lethality/yeast

Introduction

Nuclear pore complexes (NPCs) are supramolecular structures of the nuclear membrane (Unwin and Milligan, 1982; Akey, 1989; Reichelt *et al.*, 1990), which mediate nucleocytoplasmic transport of a variety of substrates including proteins, RNA and ribosomal subunits (for review, see Dingwall and Laskey, 1986; Garcia-Bustos *et al.*, 1991; Silver, 1991; Hurt *et al.*, 1992). In order to be transported into the nucleus, nuclear proteins need to carry short, generally basic nuclear localization sequences (NLSs) (Kalderon *et al.*, 1984). These NLSs mediate a two-step import pathway: binding of the karyophile to the nuclear pore followed by energy-dependent translocation through the pore channel (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988; Moore and Blobel, 1992).

Components of this nuclear import machinery have been identified using several different techniques. Soluble factors located in the cytoplasm, including NLS binding proteins, have been implicated as first players in the translocation process (Yamasaki *et al.*, 1989; Adam *et al.*, 1990; Newmeyer and Forbes, 1990; Adam and Gerace, 1991; Moore and Blobel, 1992; Stochaj and Silver, 1992). NLS binding proteins have been identified independently by affinity approaches and are candidates for import receptors, some of them being located mainly inside the nucleus (Lee and Mélèse, 1989; Silver *et al.*, 1989; Yamasaki *et al.*, 1989; Meier and Blobel, 1990). Accordingly, these NLS binding proteins are thought to shuttle between the cyto- and nucleoplasm. Recently, heat shock proteins were also suggested to have a specific role in nucleocytoplasmic transport (Jeoung *et al.*, 1991; Shi and Thomas, 1992).

At the level of the nuclear pore, which has a molecular size of 125 MDa (Unwin and Milligan, 1982; Akey, 1989; Reichelt *et al.*, 1990), only a few nuclear pore proteins have been cloned and sequenced. Among these is the mammalian nuclear membrane spanning protein gp210 (Wozniak *et al.*, 1989), which has been proposed to play a role in pore assembly (Wozniak *et al.*, 1989; Greber *et al.*, 1990) and nuclear transport (Greber and Gerace, 1992).

Besides this membrane protein, peripheral pore proteins called nucleoporins have been identified, such as mammalian p62 (Davis and Blobel, 1986; D'Onofrio *et al.*, 1988; Starr *et al.*, 1990; Carmo-Fonseca *et al.*, 1991; Cordes *et al.*, 1991) and the yeast NUP1 (Davis and Fink, 1990) and NSP1 (Nehrbass *et al.*, 1990) proteins. Interestingly, certain monoclonal antibodies directed against mammalian nucleoporins cross-react with several yeast nuclear pore proteins (Aris and Blobel, 1989). In higher eukaryotes, wheat germ agglutinin (WGA) binds to nucleoporins that carry *N*-acetyl glucosamine sugar residues on their protein backbone (Holt and Hart, 1986; Hanover *et al.*, 1987; Holt *et al.*, 1987). Since WGA (Finlay *et al.*, 1987; Yoneda *et al.*, 1987) and antibodies against nucleoporins (Featherstone *et al.*, 1988) inhibit nuclear transport both *in vivo* and *in vitro*, these proteins appear to play an essential role in the translocation process through the pore channel, Indeed, nucleoporins were shown to interact with a cytosolic factor required for nuclear protein import (Sterne-Marr *et al.*, 1992). However, as yet, the specific step in which they are involved remains unclear. At the molecular level, nucleoporin p62 appears to be associated with at least two other pore components named p58 and p54, this complex being required for nuclear transport in *in vitro* systems (Finlay *et al.*, 1991). Additional evidence also suggests that nucleoporins might be required for nuclear pore formation (Dabauvalle *et al.*, 1990; Mutvei *et al.*, 1992).

The yeast NSP1 is homologous to the higher eukaryotic nucleoporin p62 (Carmo-Fonseca *et al.*, 1991; Starr and Hanover, 1991), suggesting that these proteins perform a similar, evolutionarily conserved function. In particular, NSP1 and p62 show an analogous three-domain structure consisting of an amino-terminal and a central domain with many repeat sequences and a carboxy-terminal domain that is organized into hydrophobic heptad repeats (Hurt, 1990; Nehrbass *et al.*, 1990; Carmo-Fonseca *et al.*, 1991). The essential functional elements of NSP1 are located in the carboxy-terminal domain: this domain mediates assembly into the nuclear pore complex (Hurt, 1990) and mutations in it inhibit NSP1 function and cause the protein to be mislocalized to the cytoplasm (Nehrbass *et al.*, 1990). The

rest of the protein, i.e. the repetitive central and amino-terminal domains, is dispensable for cell growth.

Another yeast pore protein, NUP1 (Davis and Fink, 1990), shares with NSP1 many analogous repeat sequences (FSFG-motif) and further nuclear pore proteins appear to have this repeat sequence motif (Davis and Fink, 1990; Nehrbass *et al.*, 1990) (J.Loeb, personal communication).

In order to unravel the mechanism of nucleocytoplasmic transport at the level of the nuclear pore complex, further components have to be identified. A genetic system is particularly desirable in the view of the apparent complexity of the nuclear import machinery. We therefore set up a genetic approach in yeast to isolate synthetic lethal mutants of NSP1 that may interact or share overlapping functions with this nucleoporin. Here, we report on the identification of two novel proteins that belong to a new subclass of the nucleoporin family.

Results

A synthetic lethal approach to isolate components interacting with NSP1

Synthetic lethality was considered as a possible way to identify components that genetically interact with NSP1. Synthetic lethality may be caused by combining mutant alleles of two different genes which genetically have an overlapping function, whereas each individual mutant allele still gives viable cells. Synthetic lethality may thus provide genetic evidence that two proteins physically interact with each other or share overlapping function (Huffaker *et al.*, 1987; Bender and Pringle, 1991). For our genetic screen, we rationalized that a mutation within NSP1, which partly impairs its function but still allows cells to grow, could cause synthetic lethality if another pore component (e.g. NSP-X) that physically interacts or functionally overlaps with NSP1 becomes mutated (Figure 1).

In order to identify these synthetic lethal mutants of NSP1, we adopted *the ade2/ade3* red/white colony sectoring system in yeast. This visual assay was pioneered to monitor the fidelity of mitotic transmission of minichromosomes in yeast (Koshland *et al.*, 1985). Subsequently, this method has been used in synthetic lethal screens (Huffaker *et al.*, 1987; Bender and Pringle, 1991; Costigan *et al.*, 1992) including cloning yeast homologues of known higher eukaryotic genes (Kranz and Holm, 1990). The basis of the colony sectoring approach is as follows: a strain that is *ade2* (lacking a functional *ADE2* gene) forms red colonies since it accumulates a red intermediate during adenine biosynthesis. An *ade2/ade3* strain, however, reveals white colonies since the *ade3* mutation blocks the pathway prior to accumulation of the *ade2*-dependent intermediate. An *ade2/ade3* strain transformed with a plasmid containing a functional *ADE3* gene will form red colonies only if the plasmid is selected for. Under non-selective conditions, the *ADE3*-containing plasmid can be lost during colony growth on plate and red/white sectoring colonies will appear. Thus the *ade2/ade3* colony sectoring approach allows the visual monitoring of the loss of the *ADE3*-containing plasmid or the finding of red mutants which can, apparently, no longer loose the plasmid.

To screen for synthetic lethals of NSP1, a haploid *ade2/ade3* yeast strain with a disrupted chromosomal *NSP1* gene was constructed; this strain called RW24 is

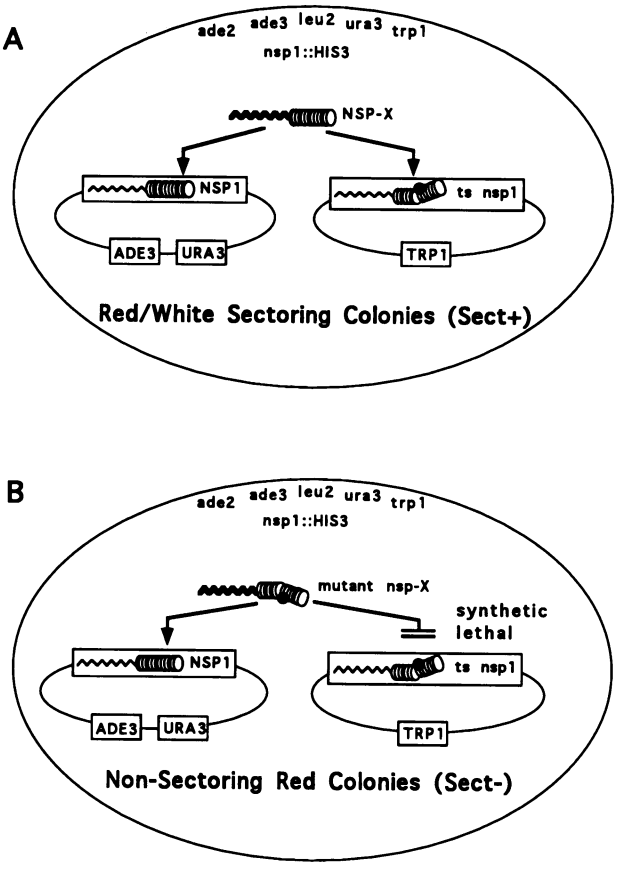

Fig. 1. A red/white sectoring colony assay to identify synthetic lethal mutants of *NSP1*. (**A**) The hypothetical protein NSP-X can genetically interact with both a wild type *NSP1*$^+$ and a mutated *nsp1*^{ts} (the mutation is indicated by a black circle) at the permissive temperature of 30°C. Both *NSP1* alleles are provided on plasmids in a *nsp1::HIS3* disrupted strain. Grown on SD (−trp +ura), the screening strain can loose the wild type *NSP1-ADE3-URA3*-containing plasmid. This event is indicated by the appearance of white sectors (Sect$^+$) in the colony due to the *ade2/ade3* genotype. (**B**) A mutated nsp-X still can interact with wild type *NSP1*, but no longer with mutated *nsp1*^{ts} at 30°C (synthetic lethality). The mutated strain cannot loose the *ADE3*-containing plasmid and non-sectoring red colonies (Sect$^-$) are formed.

complemented by two different plasmid-linked *NSP1* alleles (Table I): (i) the wild type *NSP1*$^+$ gene on a first *ADE3/URA3*-containing plasmid; (ii) a mutated, but still functional *NSP1* gene (*nsp1*^{ts}) on a second plasmid lacking the *ADE3* marker (Figure 1A). The mutant *nsp1* allele chosen for the screen harbours a single amino acid substitution leucine (640) to serine (L640S) within the carboxy-terminal domain. Cells carrying only *nsp1*^{ts} completely stop growing at 37°C (Figure 2), while they still grow at permissive temperature (30°C). Although growth of strain *nsp1*^{ts} (L640S) is slower at 30°C compared with *NSP1*$^+$ strain, the tester strain RW24 displays a distinct red/white sectoring phenotype if grown at this temperature (Figure 3A): it can indeed afford to loose the *ADE3* plasmid carrying wild type *NSP1*$^+$ since it still can grow at 30°C with the mutant *nsp1* gene alone.

At least four complementation groups give rise to synthetic lethality with NSP1

The tester strain RW24 (Table I) carrying both the *NSP1*$^+$ and *nsp1*^{ts} allele on the two different plasmids was randomly mutagenized by UV light to mutate genes which

Table I. Yeast strain construction and growth conditions

Strain	Genotype
RS453	*MATa/α ade2/ade2 his3/his3 trp1/trp1 leu2/leu2 ura3/ura3*
CH1462	*MATα ade2 ade3 leu2 ura3 his3 can1*
TF4	*MATa ade2 leu2 ura3 trp1 HIS3::nsp1* (pSB32-LEU2-NSP1); TF4 is a haploid progeny with disrupted *nsp1::HIS3* derived from strain RS453
R24	*MATa ade2 ade3 leu2 ura3 trp1 HIS3::nsp1* (pCH1122-URA3-ADE3-NSP1)
RW24	*MATa ade2 ade3 leu2 ura3 trp1 HIS3::nsp1* (pCH1122-URA3-ADE3-NSP1) [pRS414-TRP1-nsp1^{ts} (L640S)]
RW24*	*MATa ade2 ade3 leu2 ura3 trp1 HIS3::nsp1* (pCH1122-URA3-ADE3-NSP1) [pRS414-TRP1-nsp1^{ts} (L640S)]; a Sect$^-$ red mutant derived from the synthetic lethal screen, retransformed with pSB32-LEU2-nsp1^{ts} (L640S), which restored red/white sectoring
SL32	*MATa ade2 ade3 leu2 ura3 trp1 HIS3::nsp1 nsp116-32* (pCH1122-URA3-ADE3-NSP1) [pRS414-TRP1-nsp1^{ts} (L640S)]
SL392	*MATa ade2 ade3 leu2 ura3 trp1 HIS3::nsp1 nsp49-392* (pCH1122-URA3-ADE3-NSP1) [pRS414-TRP1-nsp1^{ts} (L640S)]
VD1	*MATa/α ade2/ade2 his3/his3 trp1/TRP1::nsp49/NSP49 leu2/leu2 ura3/ura3*

can give rise to synthetic lethality. Synthetic lethals of *nsp1*^{ts} should be identified on plate as red, non-sectoring colonies (Sect$^-$ phenotype), in which cells cannot afford to loose the *NSP1*$^+$ gene on the *ADE3*-containing plasmid (see also Figure 1B). Among 90 000 screened colonies, 14 red Sect$^-$ colonies were obtained that fulfilled the requirements of being synthetic lethals (SL) in a genetic background of *nsp1*^{ts}, but not wild type *NSP1* (Table II and Figure 3B): (i) all 14 Sect$^-$ mutants (SL10 to SL392) fail to grow on SDC plates containing 5-fluoro-orotic acid (5-FOA), a drug that kills cells with a functional *URA3* gene (Boeke *et al.*, 1984); this is independent proof that non-sectoring strains cannot loose the plasmid with the *NSP1*$^+$ gene, which contains, aside from the *ADE3* marker, the *URA3* gene (Figure 3C). (ii) all 14 Sect$^-$ mutants transformed with a new plasmid containing the *NSP1*$^+$ gene, but lacking the *ADE3* marker, regain the red/white sectoring phenotype and re-grow on 5-FOA containing plates (Figure 3C). (iii) all 14 Sect$^-$ mutants transformed with a new plasmid containing the *nsp1*^{ts} (L640S) allele still form red colonies and do not grow on 5-FOA containing plates (Fig. 3C).

The Sect$^-$ phenotype of these 14 mutants therefore appears to be due to an extragenic mutation rather than an intragenic lethal mutation of the plasmid-linked *nsp1*^{ts} gene or a genomic integration of the *ADE3* gene.

One Sect$^-$ mutant SL32 (Tables I and II) was crossed to an *ade2/ade3* tester strain; the segregation pattern of the synthetic lethal phenotype in haploid progeny was indicative of a single gene (data not shown). In addition, the diploid cross, which is heterozygous for the *SL32* allele and homozygous for disrupted chromosomal *NSP1*, can grow with plasmid-linked *nsp1*^{ts} alone. This shows that the synthetic lethal mutation can be complemented by the corresponding wild type allele enabling us to clone the corresponding gene.

The non-sectoring strain SL32 was transformed with a yeast genomic DNA library inserted into a single copy

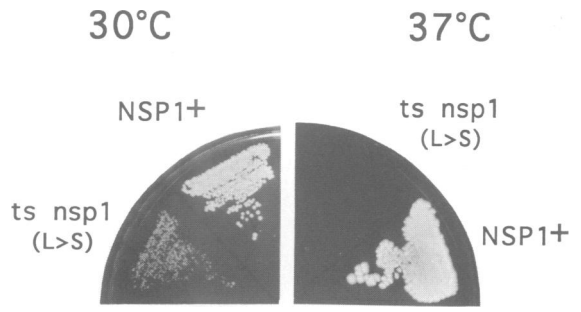

Fig. 2. A single amino acid exchange within the essential NSP1 carboxy-terminal domain leads to a temperature-sensitive phenotype. Strain R24 (*HIS3::nsp1*, pCH1122-URA3-ADE3-NSP1) was transformed with plasmid pRS414-TRP1-nsp1^{ts} (L640S) or pRS414-TRP1-NSP1 (NSP1$^+$). Transformants which lost plasmid pCH1122-URA3-ADE3-NSP1 (forming white colonies) were plated on YPD and incubated for 2 days at 30°C or 3 days at 37°C.

Table II. Complementation groups of synthetic lethal mutants of *NSP1*

Synthetic lethal (SL) strain	Transformed with pUN100 plasmids containing		
	NSP116	NSP49	pUN100-45
SL10	sectoring		
SL25	sectoring		
SL32	sectoring	red	red
SL126	sectoring		
SL299	sectoring		
SL369	sectoring		
SL33	red	sectoring	red
SL125	red	sectoring	red
SL273	red	sectoring	red
SL278	red	sectroing	red
SL392	red	sectoring	red
SL373	red	red	sectoring
SL363	red	red	red
SL370	red	red	red

Synthetic lethal mutants derived from the screening strain RW24 (called SL10 etc.) were grouped in different complementation groups (*NSP116, NSP49* and pUN100-45). For two of them, SL363 and SL370, the corresponding wild type genes were not cloned so far.

ARS/CEN plasmid (plasmid pUN100). Of the 7000 transformants, 12 regained the red/white sectoring phenotype. The pUN100 plasmids with genomic inserts were re-isolated from these sectoring transformants; nine of them contained the *NSP1* DNA, the remaining three had genomic inserts not related to *NSP1*. Although their insert size differed (ranging from 5.8−8 kb), they all revealed a similar restriction pattern indicative of a single gene locus.

The plasmid with the smallest insert (called pUN100-NSP116; 5.8 kb insert) was further analysed. If re-introduced into Sect$^-$ SL32, pUN100-NSP116 restored red/white sectoring and growth on 5-FOA plates (Figure 4). In addition, pUN100-NSP116 also suppressed the Sect$^-$ phenotype of a further five Sect$^-$ mutants (SL10, SL25, SL126, SL299 and SL369) suggesting that they belong to the same complementation group (Table I; complementation group *NSP116*; see also later).

Subsequently, the non-sectoring mutant SL392, which was not complemented by plasmid pUN100-NSP116 (Table II), was transformed with the yeast genomic plasmid library (see also above). pUN100 plasmids that exhibited Sect$^+$ suppressor activity, but did not carry the *NSP1* gene, were isolated and one plasmid, pUN100-NSP49, which has a

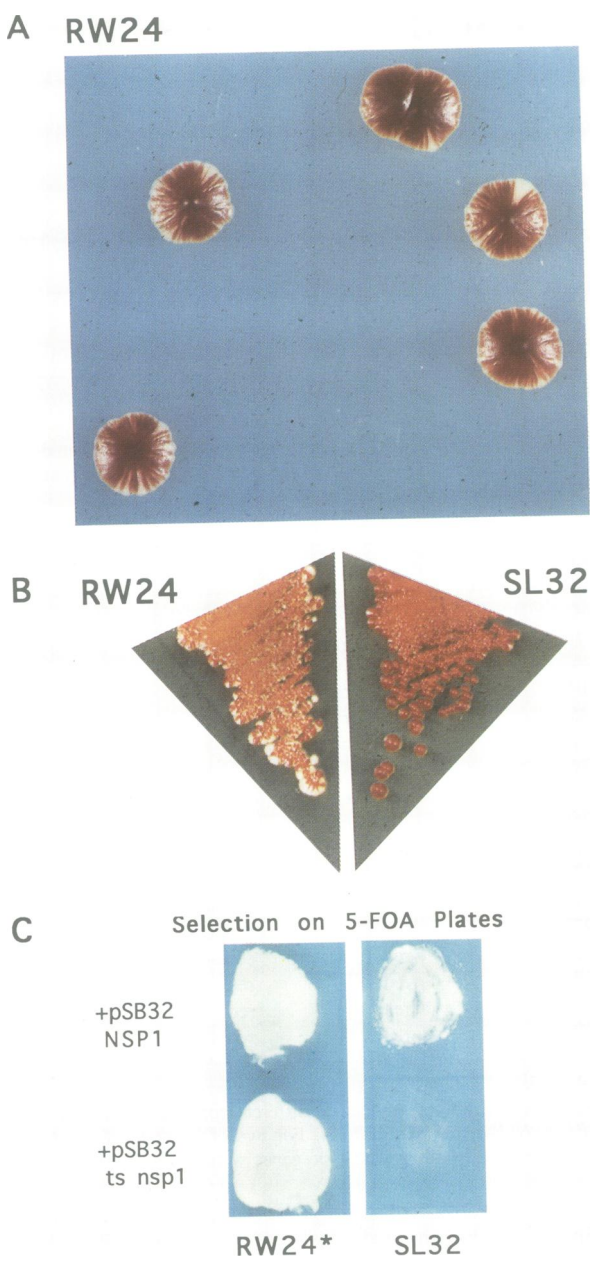

Fig. 3. SL32 is a synthetic lethal mutant in a genetic background of nsp1^{ts}. (**A**) RW24, derived from strain R24 (*HIS3::nsp1*, pCH1122-URA3-ADE3-NSP1) by transformation with plasmid pRS414-TRP1-nsp1^{ts} (L640S) was plated on SD (−trp +ura) plates and grown as single colonies at 30°C. The colonies exhibit a distinct red/white sectoring phenotype. (**B**) Synthetic lethal mutants, including SL32, were derived from the screening strain RW24 by UV mutagenesis. RW24 and SL32 cells were pre-grown from a single colony on a SD (−ura −trp) plate before being streaked on a YPD plate. SL32 uniformly forms red colonies whereas RW24 shows red/white sectoring. (**C**) The red Sect$^-$ strains RW24* and SL32 derived from RW24 and UV mutagenesis were transformed with pSB32-LEU2-NSP1 or pSB32-LEU2-nsp1^{ts} (L640S). They were grown on 5-FOA-containing SDC plates. RW24* grows on FOA when either *NSP1* or *nsp1*^{ts} (L640S) alleles are re-introduced indicating that the pCH1122-URA3-ADE3-NSP1 plasmid can be lost. Its Sect$^-$ phenotype is therefore due to an intragenic lethal mutation within the plasmid pRS414-TRP1-nsp1^{ts} (L640S). SL32 can grow on 5-FOA plates only if *NSP1* is reintroduced, but cannot grow on 5-FOA plates when transformed with the *nsp1*^{ts} (L640S) containing plasmid. This indicates that the Sect$^-$ phenotype of SL32 is caused by an extragenic synthetic lethal mutation.

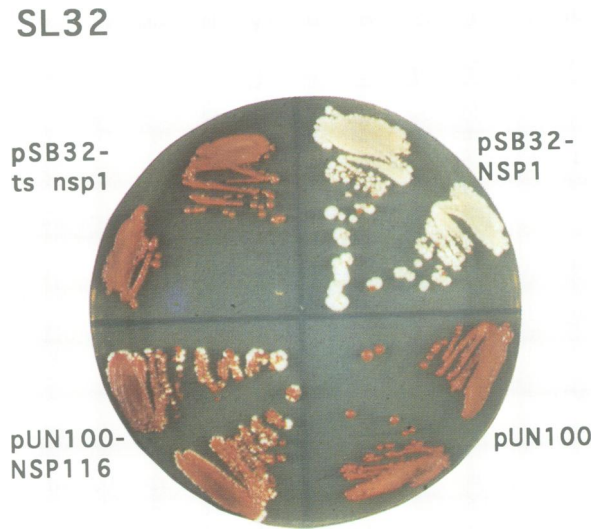

Fig. 4. Complementation of SL32 by the cloned wild type NSP116 gene. The synthetic lethal mutant SL32 was transformed with the indicated plasmids and transformants were selected on SD (−leu −trp +ura) plates. Two individual colonies derived from each transformation experiment were streaked on a YPD-plate and grown for 5 days at 30°C. pUN100-NSP116 and pSB32-NSP1 restore the Sect$^+$ phenotype, while the pUN100 vector without insert and pSB32-nsp1^{ts} do not. SL32 transformed with pSB32-LEU2-NSP1 shows mainly white colonies on YPD; this is due to the fact that transformants were pre-selected on SD (−leu −trp +ura), which favours loss of plasmid pCH1122-URA3-ADE3-NSP1 from SL32. SL32 transformed with pUN100-NSP116 shows less sectoring on YPD because it grows better with plasmid pCH1122-URA3-ADE3-NSP1 than with pRS414-TRP1-nsp1^{ts} (L640S) alone.

3.5 kb insert, was further characterized; it restored the sectoring phenotype in the original red mutant SL392 and in four other Sect$^-$ mutants (Table II). Plasmid pUN100-NSP49, however, did not suppress the Sect$^-$ phenotype of SL32 (Table II).

Finally, the gene of a third complementation group was cloned using SL373 as an acceptor strain. The corresponding complementing plasmid was called pUN100-45. This plasmid only complements SL373 (Table II); therefore, SL373 represents a rare complementation group within our SL mutant collection. The gene complementing SL373 has not been sequenced so far, but according to its restriction map it does not correspond to *NUP1* (Davis and Fink, 1990) or *NUP2* (J.Loeb, personal communication).

In summary, 12 out of 14 Sect$^-$ mutants isolated by the red/white colony sectoring approach, could be complemented by the cloned genes. By cross-complementation, these mutants could be arranged into at least four different complementation groups.

Sequence analysis of NSP116 and NSP49

Plasmid pUN100-NSP116 contained a 5.8 kb genomic insert with two internal *Hin*dIII restriction sites. Smaller restriction fragments of this insert were subcloned into yeast vectors and tested for complementation of the Sect$^-$ mutant SL32 (Figure 5). None of these fragments possessed a Sect$^+$ complementing activity. Therefore, DNA on either side of the two internal *Hin*dIII restriction sites is essential to rescue synthetic lethality of SL32; accordingly, the DNA of the 5.8 kb insert was sequenced (Figure 6A). As deduced from

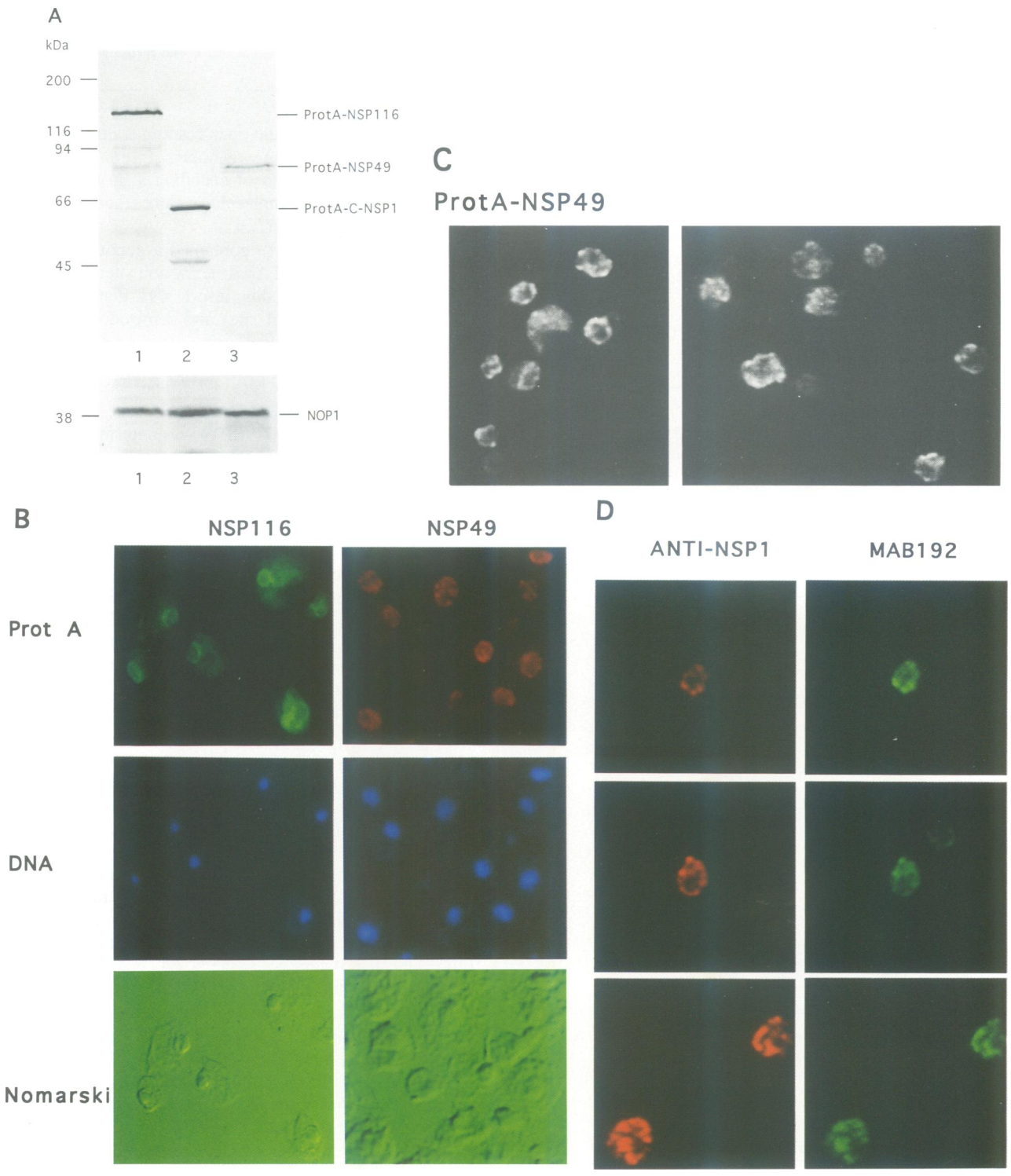

Fig. 8. Expression and localization of NSP116 and NSP49 proteins. Construction of protein A−*NSP116* and protein A−*NSP49* fusion genes, their expression in yeast strain RS453 and analysis of their subcellular location by immunofluorescence is described in Materials and methods. (**A**) Immunoblot analysis. Whole cell extracts were prepared from yeast strains expressing protein A−NSP116 (lane 1), protein A−C-NSP1 (lane 2) and protein A−NSP49 (lane 3). Equivalent amounts of extract from each strain were analyzed by SDS−PAGE and immunoblotting using an IgG−horseradish peroxidase conjugate for detection. The position of the three protein A fusion proteins is indicated. The immunoblot was also probed with an anti-NOP1 antibody and the corresponding area of the immunoblot containing the NOP1 protein is shown in the lower panel. The molecular weights of a protein standard are indicated. (**B**) Immunofluorescence analysis. The subcellular location of protein A−NSP116 and protein A−NSP49 was analyzed by immunofluorescence using rabbit anti-chicken IgG as first antibody followed by goat anti-rabbit IgG coupled to fluorescein (for NSP116) and Texas Red (for NSP49). Cells were also stained for DNA using Hoechst 33258 and viewed in the Nomarski optics. A ring-like staining of the nuclear periphery typical for nucleoporins is observed for the two fusion proteins. (**C**) Confocal immunofluorescence localization of protein A−NSP49. Haploid yeast cells with disrupted *NSP49* and functionally expressing protein A−NSP49 were analysed by immunofluorescence using rabbit anti-chicken IgG as first antibody followed by goat anti-rabbit IgG coupled to Texas Red. A ring-like and punctate staining of the nuclear envelope is seen. (**D**) Colocalization of NSP1 and the 'GLFG' nucleoporins as seen by confocal immunofluorescence microscopy. Yeast cells were double labelled with affinity-purified anti-NSP1 antibodies derived from immune serum EC10-2 (rabbit) and mAb192 (mouse) which reacts with the 'GLFG' nucleoporin subfamily including NSP116 and NSP49 (Wente *et al.*, 1992).

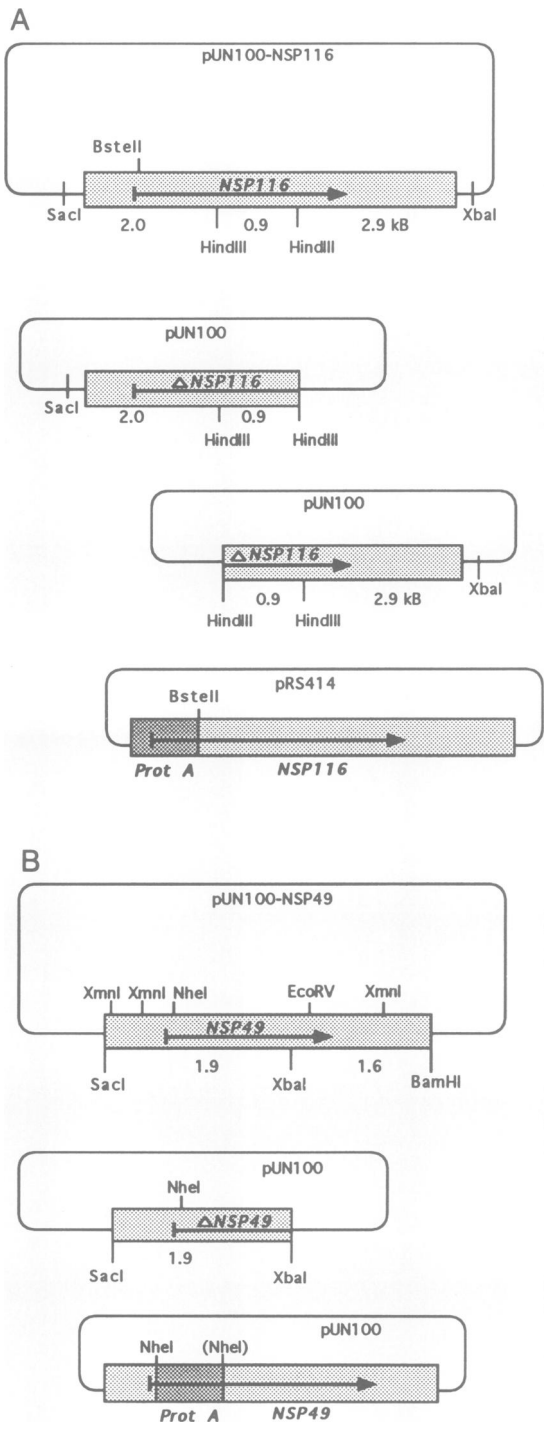

Fig. 5. Restriction map of *NSP116* and *NSP49* constructs. (**A**) Restriction map of plasmid pUN100-NSP116. The 5.8 kb yeast genomic DNA insert contains the gene encoding *NSP116*. The open reading frame of *NSP116* is indicated by an arrow and diagnostic restriction sites are shown. Restriction fragments of the 5.8 kb insert were subcloned into pUN100 as indicated. They did not restore the Sect$^+$ phenotype of SL32. A fusion gene between two IgG binding domains derived from *S.aureus* protein A under the control of the *NOP1* promoter and the *NSP116* open reading frame joined at the *Bste*II site was inserted into pRS414. (**B**) Restriction map of plasmid pUN100-NSP49. The 3.5 kb yeast genomic insert contains the *NSP49* gene. The position of the *NSP49* open reading frame within the insert is indicated by an arrow and restriction sites are given. A 2.2 kb *Sac*I–*Xba*I restriction fragment encoding only a truncated NSP49 protein was subcloned into pUN100 as indicated. It did not suppress the Sect$^-$ phenotype of SL392. A fusion gene between four IgG binding domains derived from *S.aureus* protein A and the *NSP49* open reading frame was constructed by inserting the corresponding protein A DNA into the unique *Nhe*I site at the 5′ end of the *NSP49* gene.

the DNA sequence, a single open reading frame was found within the complementing genomic insert of plasmid pUN100-NSP116, capable of encoding a protein of 1113 amino acids or 116 kDa molecular weight (Figure 6A). The gene was designated *NSP116*.

Plasmid pUN100-NSP49 contained a genomic insert of ~3.5 kb with an internal *Xba*I restriction site. If a smaller restriction fragment of this insert was generated (e.g. internally cut at the *Xba*I site) and reintroduced into the corresponding SL mutants, no complementation was obtained (Figure 5). This indicates that DNA on either side of the *Xba*I restriction site is crucial for the Sect$^+$ suppressing activity. The nucleotide sequence of the 3.5 kb insert of plasmid pUN100-NSP49 was determined (Figure 6B). As deduced from the DNA sequence, a single open reading frame was found, encoding a 472 amino acid long protein (Figure 6B). Since its predicted molecular weight is 49 kDa, the gene was named *NSP49*. One gene copy of *NSP49* was disrupted in a diploid yeast strain by the one step gene replacement procedure (Rothstein, 1983); a diploid transformant heterozygous for *NSP49* (one *NSP49* allele has been destroyed by gene replacement using *TRP1* as a selectable marker; see also Materials and methods) was sporulated and asci with tetrad spores were dissected. A 2:2 segregation for viability was found and the *TRP1* marker (indicative of the *NSP49* gene disruption) co-segregated with the lethal spores. This demonstrates that *NSP49* is an essential gene in yeast. However, if the strain heterozygous for *NSP49* was transformed with the *NSP49* gene or the protein A–*NSP49* fusion gene (see also Figure 5) present on a single copy number *ARS/CEN* plasmid, four tetrad spores could be recovered (4:0 segregation), two of the spores containing the *TRP1* marker (indicative of the disrupted *NSP49* gene) as well as the plasmid carrying respectively the *NSP49* or the protein A–*NSP49* fusion gene (data not shown).

The *KEM1/DST2* gene (Kim *et al.*, 1990; Dykstra *et al.*, 1991) is located 5′ adjacent to the *NSP49* encoding sequence and thus allows the mapping of *NSP49* to the left arm of chromosome VII. An unknown open reading frame adjacent to the *DST2* gene was already noticed (Dykstra *et al.*, 1991), which we now know to be *NSP49*. Due to a few sequencing errors in the published DNA sequence corresponding to the *NSP49* gene locus (Dykstra *et al.*, 1991), the entire and correct amino acid sequence of NSP49 could not be deduced. However, gene replacement within the unknown open reading frame adjacent to *KEM1/DST2* resulted in a 2:2 spore viability (Dykstra *et al.*, 1991). This is independent proof that *NSP49* is an essential gene.

Northern analysis was performed to detect whether *NSP116* and *NSP49* are transcribed genes. Transcripts of ~3.5 and 1.8 kb in size with similar expression levels were found for *NSP116* and *NSP49*, respectively (data not shown).

Comparison of the NSP116 and NSP49 inferred protein sequences with those in the protein sequence data banks using FASTA comparison programs revealed no striking homology to known protein sequences. A closer inspection of the protein sequence revealed that NSP116 is organized in three

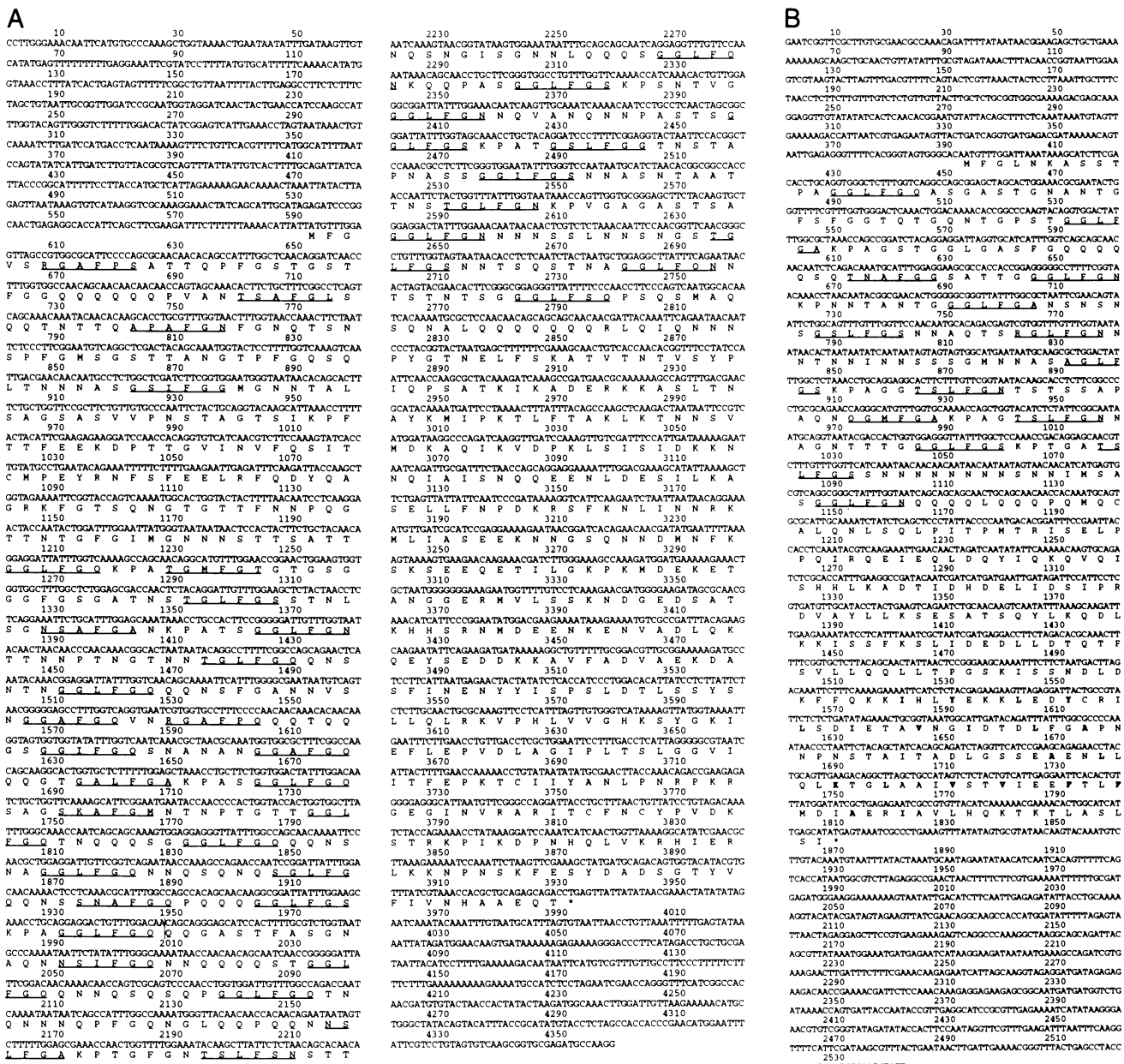

Fig. 6. DNA and deduced amino acid sequence of *NSP116* (**A**) and *NSP49* (**B**). Cloning of the genomic DNA encoding the nucleoporins NSP116 and NSP49 by complementation of SL mutants and their DNA sequence analysis are described in Materials and methods. The hexapeptide repeats found in the amino-terminal and central repetitive domains of NSP116 and NSP49 are underlined. Like in the NSP1 carboxy-terminal domain, heptad repeats were found in the second half of the NSP49 carboxy-terminal domain. The corresponding hydrophobic amino acids at position 1 and 4 are marked in bold.

different domains: the most conspicuous is a highly repetitive middle domain consisting of 37 tandemly repeated sequence units varying in length but all having the consensus hexapeptide motif 'GGLFGQ' in common (Figure 7). The amino-terminal domain also has four short repeat sequences, but these are more degenerate. The repetitive amino-terminal and middle domains are separated by a short non-repetitive spacer sequence enriched in acidic and basic residues. Finally, the carboxy-terminal domain is non-repetitive and consists of 378 amino acids (Figure 6A).

Interestingly, NSP49 exhibits 14 tandemly repeated sequence units of varying length in its amino-terminal half, but all share a core hexapeptide very similar to that found in NSP116 (Figure 7). No sequence homology exists between the carboxy-terminal domains of NSP49 and NSP116. However, the second half of the NSP49 carboxy-terminal domain shows a heptad repeat pattern with hydrophobic amino acids at position 1 and 4 of a 7-residue long repeating sequence, which is similar to the heptad repeats within the NSP1 carboxy-terminal domain (Hurt, 1990). Therefore, this part of the protein could be involved in coiled coil protein interaction (Figure 6B).

Protein A-tagged NSP116 and NSP49 fusion proteins are located at the yeast nuclear envelope

We previously showed that NSP1 carrying the IgG binding domain from *Staphylococcus aureus* protein A (Moks *et al.*, 1987) is functional in yeast and is correctly localized at the

NSP116

GGLFGQ	KPA
TGMFGT	GTGSGGGFGSGATNS
TGLFGS	STNLSG
NSAFGA	NKPATS
GGLFGN	TTNNPTNGTNN
TGLFGQ	QNSNTN
GGLFGQ	QQNSFGANNVSN
GGAFGQ	VN
RGAFPQ	QQTQQGS
GGIFGQ	SNANAN
GGAFGQ	QQGT
GALFGA	KPAS
GGLFGQ	SAG
SKAFGM	NTNPTGTT
GGLFGQ	TNQQQSG
GGLFGQ	QQNSNA
GGLFGQ	NNQSQNQ
SGLFGQ	QNS
SNAFGQ	PQQQ
GGLFGS	KPA
GGLFGQ	QQGASTFASGNAQN
NSIFGQ	NNQQQQST
GGLFGQ	QNNQSQSQP
GGLFGQ	TNQNNNQPFGQNGLQQPQQN
NSLFGA	KPTGFGN
TSLFSN	STTNQSNGISGNNLQQQS
GGLFQN	KQQPAS
GGLFGS	KPSNTVG
GGLFGN	NQVANQNNPASTS
GGLFGS	KPAT
GSLFGG	TNSTAPNASS
GGIFGS	NNASNTAATTNS
TGLFGN	KPVGAGASTSA
GGLFGN	NNNSSLNNSNGS
TGLFGS	NNTSQSTNA
GGLFQN	NTSTNTSG
GGLFSQ	PSQSMAQSQNALQQQQQQQ

NSP49

GGLFGQ	ASGASTGNANTGFSFGGTQTGQNTGPST
GGLFGA	KPAGSTGGLGASFGQQQQQSQ
TNAFGG	SATTG
GGLFGN	KPNNTANTG
GGLFGA	NSNSNS
GSLFGS	NNAQTS
RGLFGN	NNTNNINNSSSGMNNAS
AGLFGS	KPAGG
TSLFGN	TSTSSAPAQN
QGMFGA	KPAG
TSLFGN	NAGNTTTG
GGLFGS	KPTGA
TSLFGS	SNNNNNNNNSNNIMSAS
GGLFGN	QQQQLQQQPQMQ

NSP1

AGAFGT	GQSTFGFNNSAPNNTNNANSSI
TPAFGS	NNTG
NTAFGN	SNPTSNVFGSNNSTTNTFGSNSAG
TSLFGS	SSAQQTKSNGTAGGNTFGS
SSLFNN	STNSNTT
KPAFGG	LNFGGGNNTTPSSTGNANTS
NNLFGA	TANAN

NUP1

SDIFGA	NAASGSNSNVTNP
SSIFGG	AGGVPTTSFGQPQSAPNQMGMGTNNGMSMG
GGVMAN	RKIARMRHSKR

Fig. 7. Comparison of the hexapeptide repeats within NSP116, NSP49, NSP1 and NUP1. The amino acid sequence of the repetitive central domain of NSP116 [residue glycine (204) to glutamine (735)] and NSP49 [residue glycine (13) to glutamine (249)] were arranged as hexapeptide repeats flanked by sequences of variable length. As consensus motif, the hexapeptide GGLFGQ, is typically found in this domain. For comparison, a similar, but more degenerate hexapeptide repeat is found in the amino-terminal domain of NSP1 (residue glycine 33 to asparagine 175) (Hurt, 1988) and carboxy-terminal domain of NUP1 (residue serine 1005 to arginine 1076) (Davis and Fink, 1990).

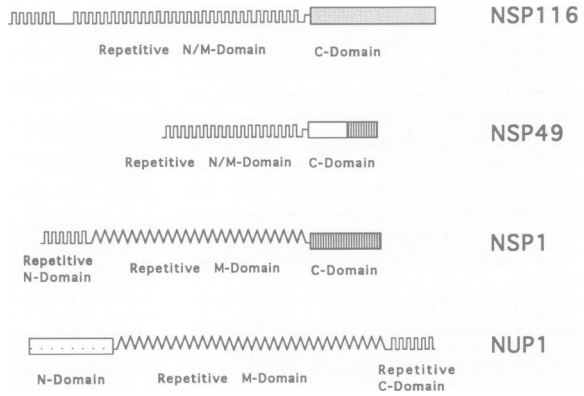

Fig. 9. Domain organization of NSP116 and NSP49 in comparison to NSP1 and NUP1. The domain organization of NSP116, NSP49, NSP1 and NUP1 is schematically shown. The repetitive amino-terminal and middle (N/M) domains of NSP116 and NSP49 contain many repeat sequences with the consensus motif GLFG, different from the FSFG consensus sequence previously described in the middle repetitive domains of NSP1 and NUP1 (Davis and Fink, 1990). However, this GLFG motif can be found within the amino-terminal (N-) domain of NSP1 and the carboxy-terminal (C-) domain of NUP1. All four proteins exhibit specific non-repetitive sequences which are found in the corresponding carboxy-terminal (NSP116, NSP49, NSP1) or amino-terminal (NUP1) domains. The vertical lines within the NSP1 and NSP49 carboxy-terminal domains indicate regions that can potentially form coiled-coil interactions.

nuclear pores (P.Grandi and E.C.Hurt, manuscript in preparation). In a similar way, NSP116 and NSP49 were tagged with this IgG binding domain (see also Figure 5) and the fusion genes were expressed in yeast. Total cell homogenates were analysed by SDS−PAGE and immunoblotting using IgG−horseradish peroxidase conjugates as a probe to detect the fusion proteins: on the immunoblot, protein A−NSP116, protein A−NSP49 and protein A−C-NSP1 are detected as single bands of the expected molecular weight (plus some minor breakdown products) and no cross-reaction with endogenous yeast proteins is seen (Figure 8A). Subtraction of the mass contributed by the protein A moiety allows calculation of an apparent molecular weight of 128 kDa for NSP116 and 50 kDa for NSP49.

The subcellular localization of the fusion proteins was determined by indirect immunofluorescence (Figure 8B). NSP1 tagged with protein A shows a characteristic ring-like staining at the nuclear periphery (P.Grandi, unpublished data) similar to authentic NSP1 (Nehrbass *et al.*, 1990). Immunofluorescence staining of protein A−NSP116 was ring-like at the nuclear periphery, resembling the NSP1 immunolabeling. Similarly, protein A−NSP49 gave a punctate nuclear envelope staining as well (Figure 8B), which is best seen by confocal microscopic analysis (Figure 8C). As a control, cytosolic dihydrofolate reductase from mouse tagged with protein A reveals a cytoplasmic location (data not shown). Finally, double immunofluorescence of yeast cells using anti-NSP1 antibodies and monoclonal mAb192, which recognizes GLFG repeat containing nucleoporins (Wente *et al.*, 1992) reveals a co-localization of NSP1 and the 'GLFG' nucleoporins at the nuclear periphery (Figure 8D). In conclusion, immunofluorescence reveals that NSP116/NSP49 co-localize with NSP1 at the nuclear envelope.

Furthermore, subcellular fractionation of yeast cells expressing protein A−NSP49 and protein A−NSP116 was performed yielding a nuclear pellet and post-nuclear supernatant. Both fusion proteins were enriched in the nuclear fraction together with NSP1 and NOP1 and depleted from the post-nuclear supernatant in which cytoplasmic hexokinase was recovered (data not shown).

Discussion

In this work, we applied a genetic approach to (i) identify novel nucleoporins and (ii) detect a genetic network of interaction between NSP1 and a distinct subgroup of nuclear pore proteins. This shows that genetic study of the nuclear pore complex, a huge organelle-like assembly of 125 MDa, can be used to unravel structure−function relationships of nuclear pore components.

Following an unbiased genetic screen, 11 out of 14 synthetic lethal mutants map within two genes that encode proteins resembling nucleoporins such as NSP1, NUP1 and p62 in their modular domain structure (Figure 9) (Davis and Fink, 1990; Nehrbass *et al.*, 1990; Carmo-Fonseca and Hurt, 1991). However, the repeats in NSP116 and NSP49 differ from those found in the known nucleoporins. The 'FSFG' repeat motif separated by highly charged sequences is characteristic for yeast NSP1 and NUP1 (Davis and Fink, 1990; Nehrbass *et al.*, 1990). In contrast, NSP116 and NSP49 contain another core repeat motif, 'GLFG', which is separated by mainly uncharged peptide sequences of variable length. A similar, but more degenerate, repeat motif is found in the repetitive amino-terminal domain of NSP1 (Hurt, 1988) and carboxy-terminal domain of NUP1 (Davis and Fink, 1990) (see also Figure 7). Thus, NSP116 and NSP49 are structurally related to NSP1 and NUP1 (Figure

9). Interestingly, > 50% amino acid sequence identity is found between NSP116 and NSP49 within 150 amino acids of the central repetitive domain. This makes it likely that this modular domain is derived from a common ancestral gene which gave rise to several nuclear pore proteins during evolution.

In conclusion, NSP1 and NUP1 can be grouped as a subclass of nucleoporins sharing the 'FSFG' peptide motif in their central repetitive domains, whereas NSP49 and NSP116 (and additional proteins; see also Wente *et al.*, 1992) belong to another nucleoporin subfamily, because of their characteristic 'GLFG' repeat motif.

NSP116 and NSP49 tagged with the IgG binding domain of *S. aureus* protein A reveal a punctate immunolabelling at the rim of the nuclear envelope. This labelling pattern is typical of a nuclear pore labelling and does not differ from the immunofluorescence staining of NSP1 (Nehrbass *et al.*, 1990) and other known nucleoporins (Aris and Blobel, 1989; Davis and Fink, 1990). In fact, double immunofluorescence of yeast cells reveals a co-localization of NSP1 and the 'GLFG' nucleoporins at the rim of the nuclear envelope. Therefore, NSP49 and NSP116 may not only functionally overlap, but also physically interact with NSP1 in nuclear pores. Thus genetics may be a powerful way to detect a complex network of interactions between various members of the nucleoporin family in the nuclear pore complex.

No sequence homology exists in the non-repetitive carboxy-terminal domains of NSP1, NSP116 and NSP49. However, these domains could play an important role in the function of individual nucleoporins. Although the mutations giving rise to synthetic lethality have not been mapped so far, we have indirect evidence that the carboxy-terminal domains of NSP49 and NSP116 participate in a genetic interaction with the NSP1 carboxy-terminal domain: deleting part of the carboxy-terminal domain from either NSP116 or NSP49 no longer complements synthetic lethality in SL32 or SL392, respectively (see also Figure 5). In agreement with this observation, the NSP49 carboxy-terminal domain contains several heptad repeats with hydrophobic residues mainly at position 1 and 4 of a 7-residue long peptide sequence (Figure 6B). In analogy to NSP1, this part of NSP49 could be involved in nuclear envelope targeting, assembly into the nuclear pore complex or interaction with other pore constituents (Hurt, 1990).

It is intriguing that starting with a mutated NSP1 carboxy-terminal domain we pick up proteins with repeat sequences in the genetic screen. Therefore, genetics has revealed that repetitive and non-repetitive elements are characteristic of the structures of nucleoporin family members and not unique to NSP1 and NUP1 proteins. It is interesting to speculate that redundant repetitive and unique non-repetitive domains of nucleoporins form a functional entity in the nuclear pore complex. However, other nucleoporins, such as NUP1 (Davis and Fink, 1990) and NSP2 (Nehrbass *et al.*, 1990), were so far not found in our genetic screen.

NSP116 and NSP49 were repeatedly isolated in the screen for synthetic lethal mutants; other complementation groups, however, were found only once, suggesting that the screen is not yet saturated. Other rare complementation groups may not encode nucleoporins. Sequence analysis of genes belonging to these complementation groups may give us additional clues as to how NSP1 is involved in pore structure and function.

Our data are best explained by a model in which NSP1 is part of a structure that interacts with other nucleoporins, two of which are NSP116 and NSP49. These nucleoporins may assemble into a higher order structure at the pore, which is inhibited if more than one partner carries a mutation in the corresponding interaction domain. Alternatively, the various nucleoporins could be part of individual subcomplexes that perform overlapping functions. As a consequence, mutations in more than one of these subcomplexes could severely impair function and, for example, result in inhibition of nucleocytoplasmic transport or nuclear pore assembly. We can now use the synthetic lethal mutants to address this type of question.

Materials and methods

Yeast strains and media

Standard yeast rich and synthetic media were used (Sherman *et al.*, 1986). YPD plates contained 2% agar, 2% glucose, 1% yeast extract and 2% bacto-peptone; if used for red/white colony sectoring assays, YPD plates contained 2% agar, 4% glucose, 0.5% yeast extract and 2% bacto-peptone. For optimal red colour development in the screen for synthetic lethal mutants, SD or SGal plates (2% agar, 2% glucose or galactose, 0.7% yeast nitrogen base) were used, which contained the standard amount of nutrients (Sherman *et al.*, 1986), but adenine was reduced to 1.7 μg/ml. SDC (+5-FOA) plates contained 2% agar, 2% glucose, 0.7% yeast nitrogen base, the complete set of nutrients (Sherman *et al.*, 1986) including 50 mg/l uracil and 1 mg/ml 5-fluoro-orotic acid (Boeke *et al.*, 1984). Diploid yeasts were sporulated by growing them first for 1 day on YPD and further 3 days on YPA plates (2% agar, 1% yeast extract, 2% bacto-peptone and 1% potassium acetate). For tetrad analysis, sporulated diploids were incubated for 10 min with cytohelicase, before dissection of the ascus on YPD plates. Transformation of yeast was performed by the lithium acetate method (Itoh *et al.*, 1983) or electroporation (Becker and Guarente, 1990).

Construction of strain RW24 used to screen for synthetic lethal NSP1 mutants was done as follows: strain CH1462 (Kranz and Holm, 1990) transformed with plasmid pCH1122-URA3-ADE3-NSP1 and strain TF4 carrying disrupted chromosomal *nsp1::HIS3* and pSB32-LEU2-NSP1 were mated on selective SD (−leu −ura) plates and resulting diploids were sporulated on YPA plates. After tetrad analysis haploid progeny were recovered, which were *ade2 ade3 leu2 ura3 trp1 HIS3::nsp1* and contained the plasmids pCH1122-URA3-ADE3-NSP1 and pSB32-LEU2-NSP1, and therefore exhibited a red/white colony sectoring phenotype on SD (−leu) or YPD plates. As expected, cells derived from this haploid progeny, which lost the pSB32-LEU2-NSP1 plasmid, formed red colonies and one such red colony was transformed with either plasmid pRS414-TRP1-nsp1^{ts} (L640S) yielding the tester strain RW24 or, for control reasons, with plasmid pRS414-TRP1-NSP1. RW24 exhibits a distinct red/white sectoring phenotype on SD (−trp +ura) plates (see also Figure 3A and B), but only if grown under permissive conditions (below 32°C). White colonies derived from strain RW24 showed temperature-sensitive growth inhibition at 37°C (see also Figure 2), because they only contained the *nsp1*^{ts} (L640S).

Plasmids

The following plasmids were used in this study: pSB32, *ARS1/CEN4* plasmid with the *LEU2* marker; pUN100, *ARS1/CEN4* plasmid with the *LEU2* marker (Elledge and Davis, 1988); pRS414, *ARS1/CEN4* plasmid with the *TRP1* marker; pCH1122, YCp50 derivative (*ARS1/CEN4*) with the *URA3* marker and *ADE3* for red/white colony sectoring (Kranz and Holm, 1990); pCH1122-URA3-ADE3-NSP1, the complete *NSP1* gene was inserted as a 3.5 kb *Bam*HI fragment (Hurt, 1988) in the *Bam*HI site of pCH1122; pSB32-LEU2-NSP1 and pRS414-TRP1-NSP1, respectively pSB32 and pRS414 containing a *Sph*I/*Hin*dIII restriction fragment encoding the *NSP1* carboxy-terminal domain under the control of the *ADH1* promoter (Nehrbass *et al.*, 1990); pSB32-LEU2-nsp1^{ts} (L640S) or pRS414-TRP1-nsp1^{ts} (L640S), respectively pSB32 and pRS414 containing a *Sph*I/*Hin*dIII restriction fragment encoding the mutated *NSP1* carboxy-terminal domain (L640S) under the control of the *ADH1* promoter (U.Nehrbass and E.C.Hurt, manuscript in preparation); pUN100-NSP116, containing a 5.8 kb genomic DNA insert encoding NSP116; pUN100-NSP49, containing a 3.5 kb genomic DNA insert encoding NSP49; pUN100-45, containing a 10 kb genomic DNA insert encoding the wild type gene of SL373.

Generation of the nsp1^{ts} (L640S)

Generation of *nsp1*^{ts} alleles will be described in detail elsewhere (U.Nehrbass and E.C.Hurt, manuscript in preparation). Briefly, mutagenesis of single-stranded *NSP1* DNA encoding the carboxy-terminal domain under the *ADHI* promoter (Nehrbass *et al.*, 1990) and inserted into the polylinker region of pBluescript II KS (Promega) was done with bisulfit. Mutagenized *NSP1* DNA was ligated into the *Bam*HI/*Hin*dIII site of pSB32. In total, four *nsp1*^{ts} mutant alleles were obtained. To map the mutation within *NSP1* causing the temperature sensitivity, the DNA sequence of the mutant gene was determined by dideoxy sequencing. One *nsp1*^{ts} mutant allele had a single amino acid substitution within the carboxy-terminal domain changing leucine (640) into serine which gave rise to temperature-sensitive growth inhibition at 37°C, but allowed growth below 35°C.

Isolation of synthetic lethal (SL) mutants of NSP1

Before mutagenesis, the tester strain RW24 was grown in liquid Gal (−ura −trp) medium to $OD_{600\ nm}$ of 0.8. About 1.5 × 10^6 cells were plated on 100 SGal (−trp +ura +leu +lys +his + 1.7 μg/ml adenine) plates (15 cm diameter) and UV irradiated (λ = 256 nm) on plate for 30 s. Cells were incubated at 30°C. About 90 000 cells survived the mutagenesis, most of them forming colonies with a distinct red/white sectoring phenotype (generally seen after 5−7 days). Non-sectoring red colonies were picked (~ 2000 in the first screen) and restreaked on SD (−trp +ura +leu +lys +his +1.7 μg/ml adenine) plates. For ~ 30 clones, the non-sectoring phenotype (Sect$^-$) was found stable in the second screen and these clones continued to give uniformly red colonies. These red clones were also Sect$^-$ on YPD plates (4% glucose), which gave a more intense red colour compared with SD plates. After test transformations with plasmids pSB32-LEU2-NSP1 or pSB32-LEU2-nsp1^{ts} (L640S), 14 red synthetic lethal (SL) mutants remained.

Cloning of NSP116 and NSP49

A yeast genomic DNA library inserted into plasmid pUN100 (Elledge and Davis, 1988) was used to clone the wild type genes of the various synthetic lethal mutants of NSP1. A detailed description of this genomic plasmid library will be given elsewhere (R.Jansen, manuscript in preparation). SL32 was grown in 500 ml SD (−trp −ura) medium to $OD_{600\ nm}$ of 1. Cells were then transfered into 3 l fresh YPD medium and grown for further 7 h to $OD_{600\ nm}$ of 0.3. After centrifugation, the cells were successively washed at 4°C in 500 ml and 50 ml double distilled water followed by 15 ml 1 M sorbitol. Finally, cells were resuspended in 6 ml of ice-cold 1 M sorbitol and mixed with 8 μg of genomic yeast DNA library in plasmid pUN100; this was incubated for 20 min on ice before cells were transformed in 40 μl aliquots by electroporation (1.5 kV, 25 μF, 200 Ω; Bio-Rad Gene Pulser) according to (Becker and Guarente, 1990). Immediately after the pulse, 1 ml of ice-cold 1 M sorbitol was added to the cuvette. The suspension was removed from the cuvette and kept on ice until plating. Electroporated cells were collected by centrifugation and plated on 50 SD (−leu −trp +ura +ade +his +lys) plates containing 1 M sorbitol. This was incubated at 30°C for 3 days until colonies became visible. In total, ~ 7000 transformants were obtained. Since the colonies remained small on the sorbitol-containing plates and red colour appearance was weak, colonies were transferred onto nitrocellulose membrane filters and grown twice on fresh SD (−leu −trp +ura +ade +his +lys) plates and twice on YPD plates, which gave optimal screening conditions. By this procedure, 12 transformants were obtained which regained the red/white sectoring phenotype. Plasmid DNA (pUN100 plasmids with yeast genomic inserts) from complemented Sect$^+$ SL transformants was recovered by purifying total yeast DNA and re-transformation of competent *Escherichia coli* DH5 cells by electroporation and selection on ampicillin-containing Luria broth plates (Strathern and Higgins, 1990). The recovered plasmids were characterized by restriction digestion. According to their restriction map, nine of them contained genomic inserts corresponding to *NSP1*, while three were characterized as independent clones of *NSP116*. Similarly, *NSP49* was cloned by complementation using *SL392* as acceptor strain for the plasmid genomic library.

DNA sequencing, gene disruption and northern analysis

The 5.8 kb genomic insert containing *NSP116* and the 3.5 kb genomic insert corresponding to the *NSP49* gene were cut with several restriction enzymes and subfragments covering the entire length of the genomic inserts were subcloned in pBluescript KS. DNA sequence analysis was done for both strands according to Sanger *et al.* (1977) using either pBluescript M13 universal and reverse primers (Promega) or, once DNA sequence data from the inserts were available, oligonucleotide primers which annealed within the subcloned inserts ('primer walking'). DNA and deduced amino acid sequences of *NSP116* and *NSP49* were analyzed by the GCG programs. The molecular weight was determined by using the program PEPTIDESORT. Amino acid sequence comparisons were done using FASTA or BESTFIT (Devereux *et al.*, 1984).

For disruption of the chromosomal *NSP49*, the cloned *NSP49* gene was inserted as 3.5 kb *Sac*I−*Bam*HI restriction fragment (see also Figure 5) into Bluescript II KS (Promega). It was cut with restriction enzymes *Nhe*I and *Eco*RV, which released most of the *NSP49* coding sequence from the plasmid, but left at the 5′ and 3′ end sufficient genomic DNA for homologous recombination. The *TRP1* gene (isolated as a *Nhe*I−*Sma*I fragment) was joined to the previously cut *Nhe*I/*Eco*RV sites yielding the null allele *nsp49::TRP1*, which was excised from the plasmid as linear DNA and used to transform the diploid strain RS453 and selection on SDC (−trp) plates according to (Rothstein, 1983). Trp1$^+$ transformants were characterized for correct integration of *nsp49::TRP* at the *NSP49* locus by PCR analysis. RS453 diploids heterozygous for *NSP49* were sporulated and tetrad analysis was performed.

Northern analysis was done as described earlier using *NSP116*- and *NSP49*-specific DNA probes (Schimmang *et al.*, 1989).

Epitope tagging of NSP116 and NSP49 with protein A

The IgG binding domain from *S.aureus* protein A (Moks *et al.*, 1987) was initially used as an epitope to tag the essential carboxy-terminal domain of NSP1. Functional expression of the protein A−C-NSP1 fusion construct in yeast and immunolocalization will be described elsewhere (P.Grandi and E.C.Hurt, manuscript in preparation). In a similar way, NSP116 and NSP49 were tagged with the protein A epitope. The DNA encoding two synthetic IgG binding domains (designated Z) from protein A (Moks *et al.*, 1987) was fused in-frame to the coding sequence of *NSP116* at a blunt-ended *Bst*EII site; thereby, the first 58 amino acids of NSP116 were replaced by 116 amino acids from the protein A. The protein A−NSP116 fusion protein was placed under the control of the NOP1 promoter (Schimmang *et al.*, 1989) including an ATG start codon. The protein A−NSP116 fusion gene was inserted into plasmid pRS414 and transformed into strain RS453 by selection for TRP$^+$ transformants. The DNA encoding four synthetic IgG binding domains from protein A was fused in frame to the coding sequence of NSP49 at the unique *Nhe*I site as outlined in Figure 5, thereby keeping the authentic *NSP49* promoter. The protein A−NSP49 fusion gene was inserted into plasmid pUN100 and introduced into strain RS453 by selection for Leu$^+$ transformants.

For protein expression, transformed cells were grown in selective SD medium to $OD_{600\ nm}$ of 1 and total cell extracts were analysed by SDS−PAGE and immunoblotting using directly IgG coupled to horseradish peroxidase. For localization of NSP116 and NSP49, yeast cells expressing the protein A fusion proteins were fixed in 3% formaldehyde for 1 h, spheroplasted with zymolyase 100 000 and processed for immunofluorescence as described earlier (Nehrbass *et al.*, 1990). Rabbit anti-chicken IgG, which binds to the protein A moiety (Medac, Hamburg, Germany), was used as first antibody in a 1:50 dilution followed by goat anti-rabbit IgG coupled to fluorescein (for NSP116) or Texas Red (for NSP49) in a 1:50 dilution. As control, protein A fused to mouse dihydrofolate reductase or yeast NOP1 were expressed in yeast cells and their subcellular localization was analyzed (T.Berges, unpublished results). Double immunofluorescence was performed using affinity-purified anti-NSP1 antibodies made in rabbit (EC10-2; 1:10 dilution) and monoclonal antibody mAb192 (1:5 dilution) recognizing nucleoporins NUP116 and NUP49 (Wente *et al.*, 1992). Second antibodies were goat anti-rabbit IgG coupled to Texas Red (for NSP1) and goat anti-mouse IgG coupled to fluorescein (for mAb192), both in a 1:50 dilution. Confocal microscopical analysis was done as outlined earlier (Hurt *et al.*, 1992) using Fleischmann's yeast tetraploid strain (Aris and Blobel, 1989). Subcellular fractionation and isolation of nuclei was done as previously described (Hurt *et al.*, 1988).

GenBank accession number

The accession numbers for the sequences reported in this paper are X68108 for NSP116 and X68109 for NSP49.

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for protein A, and R.Davis (Stanford University, Stanford, CA, USA) for plasmid pUN100. We are grateful to S.Wente and G.Blobel (The Rockefeller University, New York) for communicating results prior to publication and providing monoclonal antibody mAb192. We thank I.Mattaj for critically reading the manuscript. V.D. was recipient of an EMBO Long Term Fellowship. E.C.H. was the recipient of a grant from the Deutsche Forschungsgemeinschaft.

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Note added in proof

During the course of this work we learned that NSP49 and NSP116 are identical to NUP49 and NUP116 previously identified as novel nuclear pore proteins (Wente *et al.*, 1992).