Characterization of YDJ1: A Yeast Homologue of the Bacterial dnaJ Protein

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Abstract. The YDJ1 (yeast dnaJ) gene was isolated from a yeast expression library using antisera made against a yeast nuclear sub-fraction termed the matrix lamina pore complex. The predicted open reading frame displays a 32% identity with the sequence of the *Escherichia coli* heat shock protein dnaJ. Localization of YDJ1 protein (YDJ1p) by indirect immunofluorescence reveals it to be concentrated in a perinuclear ring as well as in the cytoplasm. YDJ1p cofractionates with nuclei and also microsomes, suggesting that its perinuclear localization reflects association with the ER. YDJ1p is required for normal growth and disruption of its gene results in very slow growing cells that have pleiotropic morphological defects. Haploid cells carrying the disrupted YDJ1 gene are inviable for growth in liquid media. We further show that a related yeast protein, SIS1, is a multicopy suppressor of YDJ1.

THE heat shock family of proteins (hsps) have become central to the study of protein folding, transport and assembly in both eukaryotic and prokaryotic systems (for reviews see Lindquist and Craig, 1988; Rothman, 1990; Schlesinger, 1990). Functional studies of heat shock proteins have been most productive in bacteria and yeast because of the genetic maleability of these organisms. In *Escherichia coli*, for example, three of the heat shock proteins; dnaK (the prokaryotic analogue of hsp70), dnaJ, and grpE have been shown to function in a variety of cellular processes including protein folding (Gaitanaris et al., 1990), proteolysis (Strauss et al., 1988), phosphorylation (Itikawa et al., 1989) and also in replication of λ (Georgopoulos et al., 1990) and P1 phage (Wickner, 1990).

In the yeast *Saccharomyces cerevisiae*, there are at least nine hsp70 proteins (for reviews see Lindquist and Craig, 1988), some of which are cytoplasmic while others are targeted to specific organelles such as the mitochondria (SSC1) and endoplasmic reticulum (KAR2). Translocation of nascent proteins into either organelle requires hsp70 proteins on both sides of the membrane (Deshaies et al., 1988; Chirico et al., 1988; Kang et al., 1990; Vogel et al; 1990). Their action, in the broad sense, appears to lie in their ability to behave as "chaperones," by binding unfolded (usually nascent) polypeptides until they can be unloaded at a specific site for transport or assembly (for reviews see Pelham, 1988; Neupert et al., 1990).

Transport across the ER membrane in yeast also requires an integral membrane protein, SEC63, which shares limited homology with the bacterial dnaJ protein (Sadler et al., 1989). SEC63 was identified in two different genetic screens, one which identified mutants in translocation into the ER (Rothblatt et al., 1989) and one for nuclear protein localization (Sadler et al., 1989). While it remains unclear how an integral ER protein may affect nuclear import, the requirement for eukaryotic proteins homologous to dnaK and dnaJ in protein translocation is consistent with the function of these proteins in assembly in prokaryotes.

The proliferation of specialized hsp70 homologues in yeast, compared to bacteria, might suggest similar proliferation in dnaJ proteins. In this report we characterize a yeast homologue of the bacterial dnaJ protein which is required for normal cellular growth.

Materials and Methods

Cloning of the YDJ1 Gene

A yeast genomic library with an average insert size of 5 kb in λ Zap phage (Stratagene, La Jolla, CA) was screened with the matrix lamina pore complex (MLPC)[1] antisera (see below) at a concentration of 1:200 using standard methods. 40 positive clones were isolated and amplified. Samples of each were converted to phagemids and subjected to a hybridization analysis to assess sequence similarity. In this way the 40 clones were reduced to 10 groups (termed A-J) with clones in each group sharing identical sequence. One of these groups (group E, containing five phage members, each having identical sequence) encoded the YDJ1 open reading frame. The upstream region of YDJ1 was cloned by the integration excision method of Roeder and Fink (1980). The whole YDJ1 open reading frame (ORF) was subcloned (as an EcoRI-NdeI fragment) into a pBluescript based vector containing the yeast HIS3 gene to form the plasmid pBH3E49 (see Fig. 1 *B*). This plasmid was linearized with CvnI and was used to transform the wild-type yeast diploid strain W303. His^+ colonies were picked, checked for integration by Southern blot analysis and genomic DNA prepared. This DNA was cleaved with NdeI, ligated (at a concentration of 10 $\mu g ml^{-1}$), and used to transform *E. coli*. Ampicillin-resistant colonies were picked and plasmid DNA purified. The resulting plasmid (pAV2) was checked by restriction digestion and the upstream region of YDJ1 sequenced. Standard procedures detailed in Sambrook et al. (1990) were used for all subcloning, preparation

1. *Abbreviations used in this paper*: MLPC, matrix lamina pore complex; ORF, open reading frame.

of competent cells, transformation of *E. coli*, and restriction digestion. DNA sequencing was performed by the dideoxy method using Sequenase (United States Biochemical, Cleveland, OH) according to the manufacturer's instructions. Amplification of phage, conversion to phagemids and Exonuclease III/mung bean nuclease digestions of pE26 were performed according to instructions from Stratagene, the supplier of the λ Zap phage.

Preparation of Antisera and Affinity Purification

MLPC antisera was prepared by injection of both native and SDS solubilized MLPC samples (prepared according to Allen and Douglas, 1989) into four New Zealand white rabbits. The rabbits were boosted a total of four times and sera harvested 11 d after injection.

Monospecific antibodies were affinity purified from the MPLC antisera as follows. An overnight culture of *E. coli* strain BB4 grown in LB supplemented with 0.2% maltose, 1 mM $MgSO_4$, and 12.5 $\mu g ml^{-1}$ tetracycline was diluted 1:10 in the same medium and grown for 1 h at 37°C. An amount of a specific phage stock (titrated for confluence) was added to 200 μl of bacterial culture and incubated at 37°C for 15 min, after which 3 ml of molten NZY top agar (0.5% NaCl, 0.2% $MgCl_2$, 0.5% yeast extract, 1% casamino acids, 0.7% agarose) was added (prewarmed to 42°C), and the mixture plated out on LB agar. The plates were incubated at 42°C for ~4 h or until small plaques appeared. The plates were then overlayed with nitrocellulose filter circles (soaked with 10 mM IPTG and air dried). The plates were incubated overnight at 37°C. Filters were removed from the plates, washed in PBS, and blocked with 1× PBS, 3%BSA for 1 h at room temperature and then washed once in PBS, once with PBS, 0.5% NP-40, and once with PBS. Filters were then incubated with antisera diluted in blocking solution (diluted 1:10–1:50, which had been previously preadsorbed against filters containing BB4 plus noninduced phage or a different phage stock) for 1–2 h at room temperature. The antisera was removed, and the filters washed extensively in PBS, 0.05% Tween-20 before elution of antibodies by incubation with 0.1 M glycine pH 2.7 for 15 min. The elution buffer was removed and immediately neutralized by addition of a 10th vol of 1 M TrisHCl pH 8.0. Affinity-purified antibodies were concentrated ~10-fold using concentrators (Centricon 30; Amicon Corp., Danvers, MA).

Specific YDJ1 antisera was prepared after a trpE-YDJ1 fusion protein was constructed in the pATH3 vector using an EcoRI-CvnI fragment covering the first 600 bp of the YDJ1 open reading frame (Fig. 1 *A*, *(ii)*). This plasmid, termed pTEEC, was transformed into *E. coli* strain RR1 and transformants selected by their ability to express a 59-kD fusion protein upon indoleacrylic acid induction (Dieckman and Tzagoloff, 1985). The fusion protein partitioned into an *E. coli*–insoluble fraction (Dieckman and Tzagoloff, 1985) from which it was gel purified by SDS-PAGE and subsequent electroelution. Purified trpE-YDJ1 fusion protein was injected subcutaneously into two New Zealand white rabbits (250 μg per rabbit) with Freund's complete adjuvant (1:1 ratio). Three subsequent boosts using 50 μg of purified fusion protein were performed with one test and one final bleed 14 d after the second and third boosts, respectively. YDJ1 antibodies were affinity purified against the trpE-YDJ1 antigen as follows. An *E. coli*–insoluble fraction containing the trpE-YDJ1 fusion protein was fractionated in a 10% SDS–polyacrylamide gel and transferred to nitrocellulose. A strip containing the fusion protein was cut out after its detection by Ponceau S (0.1% in 3% TCA) staining. This strip was processed exactly according to Lillie and Brown (1987) using undiluted serum. Affinity-purified antibodies were eluted from the filter and concentrated as above.

Microscopic Methods

Cells were prepared for indirect immunofluorescence as follows. Mid or late log phase yeast cultures were fixed by direct addition of a 10th volume of a 37% formaldehyde solution, and rolled at room temperature for 2 h. The cells were washed twice in water then resuspended in SH buffer (1.2 M sorbitol and 20 mM Hepes; pH 7.2). Fixed cells were digested with 2–3 mg/ml^{-1} zymolyase (20T; ICN Biomedicals, Inc., Irvine, CA) until they appeared dark by phase-contrast microscopy through a 20× objective. The spheroplasts were washed three times in SH buffer. Fixed spheroplasts were allowed to settle onto wells of an eight well polylysine coated (0.01% [wt/vol]) slide for 5 min. Excess and unattached cells were removed by aspiration and the cells allowed to dry for 5 min before postfixation by immersion of the slide into methanol (−20°C) for 5 min. The methanol was removed by aspiration and the wells blocked by addition of 20 μl of 3% BSA in PBS, 0.05% Tween 20, and 0.1% thimerosal for 20 min at room temperature in a moist humid chamber. The blocking solution was aspirated and a solution containing primary antibody (diluted into 0.1% BSA in PBS, 0.05% Tween 20, and 0.1% thimerosal) in 20 μl was added to each well and incubated in a moist humid chamber for at least 1 h at room temperature. Primary antibody solutions were then aspirated and the slides washed eight times in antibody dilution buffer before addition of secondary antibody (fluoroscein or rhodamine tagged depending on source of primary). Conditions of secondary antibody incubation and subsequent washing were as above and the wells allowed to dry slightly before addition of a small drop (2–5 μl) of mounting media (1 mg/ml^{-1} *p*-phenylenediamine, pH 8.0, in 90% glycerol) plus 0.5 $\mu g/ml^{-1}$ DAPI to each well. A coverslip was attached and the slide was ready for viewing.

For viewing yeast nuclei without further immunological analysis, yeast cultures were made 90% ethanol, 0.5 $\mu g/ml^{-1}$ DAPI, and incubated for 5 min at room temperature. The ethanol-fixed cells were washed once in water and resuspended in the original volume before attaching to polylysine coated slides. A Nikon Optiphot epifluorescent microscope fitted with UV, FITC, and rhodamine filters and Nomarski (DIC) optics was used for microscopy. Visualization and photography was performed using 100× objectives. Kodak T-Max 400 35 mm film was used for all photomicroscopy.

Yeast Methods

See Table I for genotype of strains used in this study. Growing conditions, media, preparation of genomic DNA, sporulation, and tetrad dissection were performed according to the methods of Sherman et al. (1986). Transformation was according to the method of Ito et al. (1983). Yeast whole cell extracts were prepared by glass bead lysis using RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM TrisHCl, pH 8.0) according to the method of Harlow and Lane (1980).

Biochemical Subfractionation

Yeast cell nuclei were prepared according to the method of Kalinich and Douglas (1989). Matrix lamina pore complexes were prepared according to the method of Allen and Douglas (1989). Subfractions enriched in mitochondria, microsomes, and soluble proteins were prepared as follows. 4 liters of yeast strain BJ926 (the gift of E. Jones, Carnegie Mellon University, Pittsburgh, PA) were grown to mid-log phase, washed once in water and resuspended in 30 ml of 100 mM $TrisSO_4$ pH 9.4, 10 mM DTT for 10 min at room temperature. The cells were then washed once in SH buffer (1.2 M sorbitol, 20 mM Hepes, pH 7.2) and resuspended in 100 ml of the same buffer containing 1.2 mg oxalyticase (40,000 U/mg^{-1}; Enzogenetics, Corvallis, OR) and gently shaken for 40 min at 30°C for conversion of cells to spheroplasts. The spheroplasts were washed once in SH buffer before resuspension in lysis buffer (20 mM Hepes, pH 7.2, 500 mM sucrose, 1 mM DTT, 3 mM Mg acetate, 1 mM EGTA, 1 mM EDTA, 0.5 mM PMSF plus protease inhibitor cocktail [~1 $\mu g ml^{-1}$ each of leupeptin, chymostatin, aprotinin, and pepstatin]). The following steps were performed at 4°C unless otherwise stated. The spheroplasts were homogenized with a dounce homogenizer (18 strokes), and the whole cell homogenate was centrifuged at 10,000 *g* for 10 min. The supernatant was saved and the pellet rehomogenized and recentrifuged as above. The supernatant from the first and second spins were pooled and used in the preparation of microsomes (see below). Mitochondria were prepared as follows (Estey, L., and M. G. Douglas, unpublished results). The pellet was rehomogenized in SEM buffer (10 mM MOPS, pH 7.2, 250 mM sucrose, 1 mM EDTA, and 0.5 mM PMSF), then spun at 3,000 *g* to remove cell debris. The mitochondria were washed three times in SEM buffer by pelleting at 10,000 *g* for 10 min followed by rehomogenization (two strokes in the dounce homogenizer) and recentrifugation. After three washes, the mitochondrial pellet was resuspended in 0.5 ml SEM buffer and layered on a 20% (vol/vol) Percoll gradient (in SEM buffer, preformed by centrifugation at 25,000 *g* for 12 min after the method of Yaffe, 1991). The mitochondria were banded in the gradient by centrifugation at 27,250 *g* for 20 min in a rotor (Model HB4; Sorvall Instruments, Newton, CT). The mitochondria, which banded in the bottom third of the tube, were recovered from the gradient and concentrated by pelleting at 10,000 *g* for 10 min followed by resuspension in SEM buffer.

Microsomes were prepared from the 10,000 *g* supernatant essentially according to the method of Hansen et al. (1986). The 10,000 *g* supernatant, as prepared above, was layered on top of an 18 ml 30% Percoll gradient (in lysis buffer) and centrifuged at 76,000 *g* in a rotor (Ti 42.2; Beckman Instruments, Inc., Palo Alto, CA) for 50 min at 4°C. Two closely spaced turbid bands were removed and pooled according to the method of Hansen et al. (1986) and concentrated by pelleting in a rotor (Ti75; Beckman Instruments, Inc.) at 100,000 *g* for 1 h at 4°C. The microsomes were resuspended in 20 mM Hepes, pH 7.2, 250 mM sucrose, 1 mM DTT, and stored at −80°C. Soluble proteins were isolated by centrifuging an aliquot of the 10,000 *g* supernatant at 100,000 *g* for 1 h at 4°C. The supernatant from this spin corresponds to the soluble protein fraction.

Table I. Yeast Strains Used in this Study

Strain	Genotype	Source
W303	*MATa/α ade2-1 leu2-3.112 his3-11.15 trp1-1 ura3-1 can1-100*	Lab stock
ACY1	*MATα ade2-1 leu2-3.112 his3-11.15 trp1-1 ura3-1 can1-100 ydj1-1::HIS3*	This study
ACY3	*MATα ade2-1 leu2-3.112 his3-11.15 trp1-1 ura3-1 can1-100 ydj1-1::HIS3, pGYDJ(YDJ1, URA)*	This study
ACY4	*MATα ade2-1 leu2-3.112 his3-11.15 trp1-1 ura3-1 can1-100 ydj1-1::HIS3, pCB338(SIS1, URA3)*	This study
LDY1	*MATa/MATa + / + + / + + / + lys1-1(UAA)/lys1-1(UAA)* *MATα/MATα arg11/arg11 cry1/cry1 his4d5/his4d29 + / +*	L. Davis
BJ926	*MATa/α trp1/+ his1/+ prc1-126/− pep4-3/− prb1-1122/− can1/− gal2/−*	E. Jones

The protease digestion experiment shown in Fig. 8 was performed using whole cell homogenate prepared by homogenization of spheroplasts as detailed above but in the absence of protease inhibitors. The whole cell homogenate was centrifuged at 575 *g* to remove unbroken cells and frozen at −80°C before use. Protease digestions were performed at room temperature in the presence or absence of 0.4% Triton X-100 with 25 $\mu g/ml^{-1}$ proteinase K. Digestion was allowed to proceed for 30 min and stopped by the addition of 1 vol of lysis buffer plus 4 mM PMSF. Control samples were removed before protease addition and diluted with 1 vol of lysis buffer as above. Samples were then boiled in the presence of sample buffer before SDS-PAGE and immunoblotting.

Miscellaneous

Clamped homogenous electric field (CHEF) gel analysis was performed as follows. Whole yeast chromosomes (in agarose plugs prepared from the strain YNN295, obtained from Bio-Rad Laboratories, Richmond, CA) were fractionated in a 1% agarose gel in 0.5× TBE in a CHEF II DR gel system according to the manufacturer's instructions. The gel was stained with ethidium bromide (0.5 $\mu g/ml^{-1}$) and the DNA nicked by subjection to UV light (at 254-nm wavelength) for 2 min. The DNA was then transferred by standard capillary action to Hybond-N nylon membrane (Amersham Corp., Arlington Heights, IL), and hybridized using an EcoRI-CvnI fragment of the YDJ1 ORF. Standard procedures for Southern blotting were performed according to Sambrook et al. (1990). Probes were labeled with ^{32}P-labeled dCTP using the random priming method (see Sambrook et al., 1990). Standard procedures for SDS gel analysis and Western blotting (using a semi-dry transfer cell; Bio-Rad Laboratories) were performed (see Harlow and Lane, 1988). Searches of nucleic acid and protein sequence data bases were performed using the GCG software on a Vax computer.

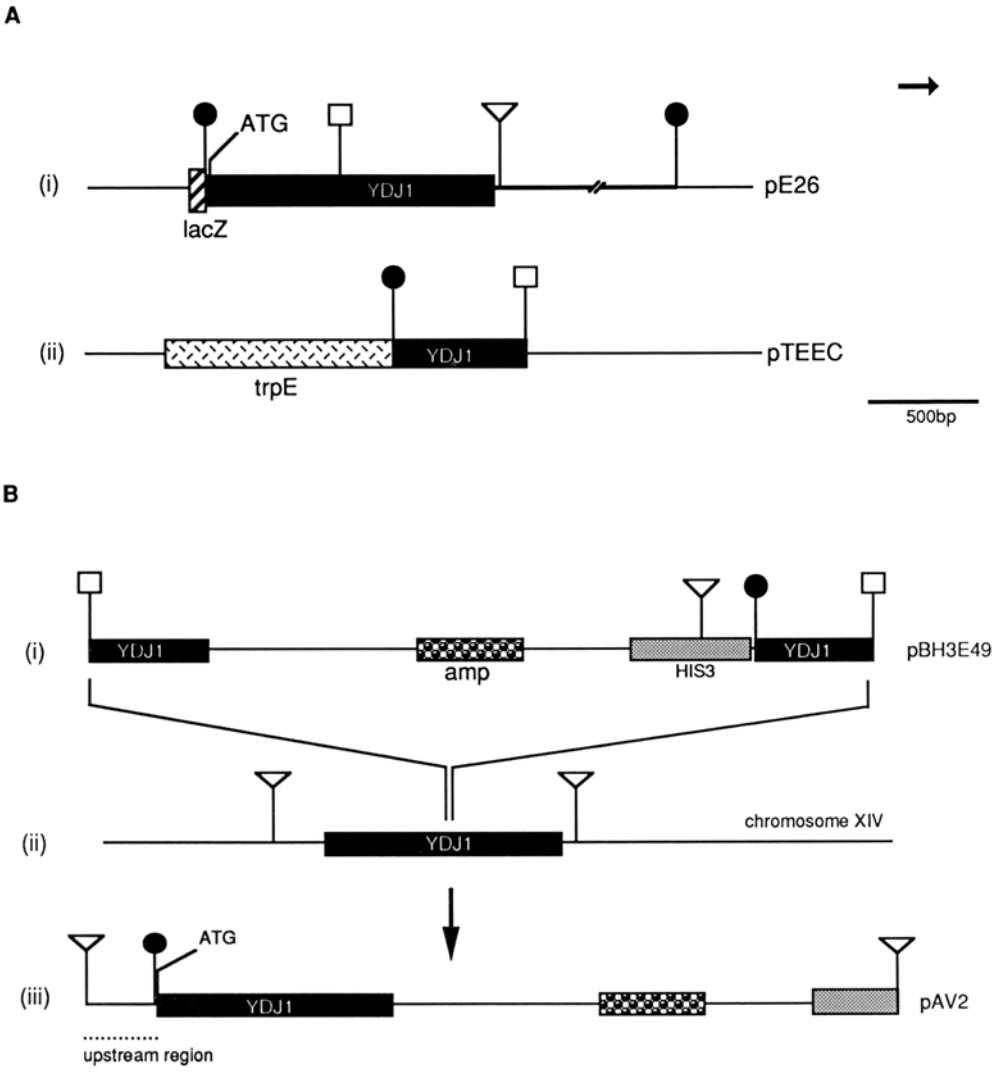

Figure 1. (*A* (*i*)) Restriction map of YDJ1 open reading frame in the plasmid pE26. (*A* (*ii*)) Cartoon showing the portion of the YDJ1 open reading frame used to construct the trpE fusion protein. (*B*) Integration/excision cloning of YDJ1 upstream region. The plasmid pBH3E49 (*B* (*i*)) was constructed and integrated into the host chromosome (*B* (*ii*)) after which the upstream region was excised along with plasmid sequences with NdeI (*B* (*iii*)) to form the plasmid pAV2. (*Solid circles*) EcoRI; (*open boxes*) CvnI; (*inverted* (*triangle*) NdeI. Filled boxes denote genes.

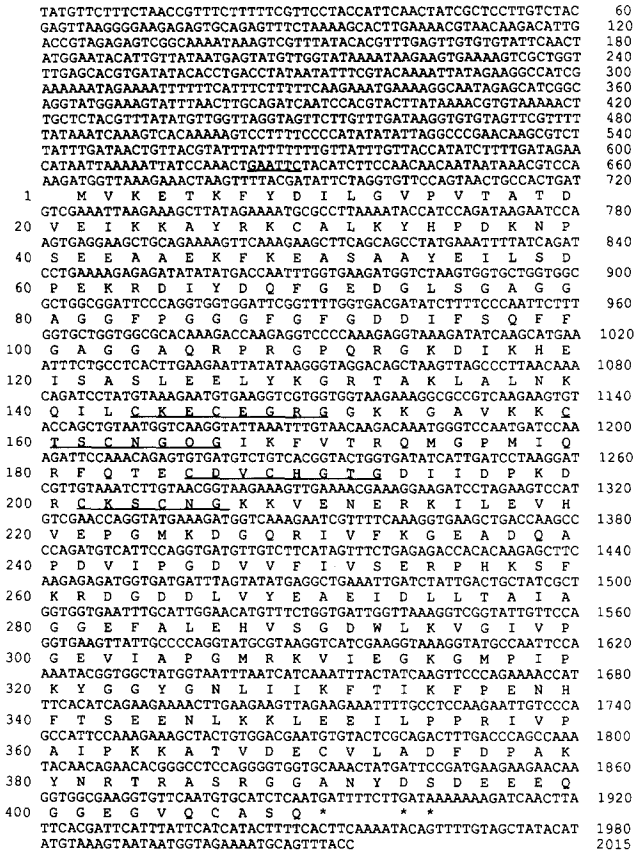

Figure 2. Complete gene sequence of YDJ1 and predicted open reading frame. DNA bases are numbered in the right-hand column and amino acids in the left-hand column. The one letter amino acid code is used. The cysteine rich repeated regions are underlined (see text). Stars denote stop codons. These sequence data are available from EMBL/GenBank/DDBJ under accession number X56560.

Results

Isolation of the YDJ1 Gene

A yeast nuclear sub-fraction (the matrix lamina pore complex [MLPC]; Allen and Douglas, 1989) was used to generate an antisera in rabbits, which was then used to probe a yeast expression library in the Lambda Zap phage. In a single screening, 40 positive clones were isolated, which were reduced into 10 distinct groups based upon cross-hybridization.

To determine which clones specifically encoded MLPC proteins, we affinity-purified monospecific antibodies from the crude serum using beta-galactosidase–yeast fusion proteins from the previously isolated phage. This procedure used lysates of phage infected bacteria induced for fusion protein expression with IPTG. These lysates were adsorbed onto nitrocellulose filters and incubated with crude serum, at suitable dilution, which had been depleted by previous incubation with non-induced lysate (or a different phage stock). Antibodies which bound to the lysate were eluted and concentrated. These affinity-purified antibodies were then used to determine intracellular localization of the cloned gene product, in wild-type yeast cells, by indirect immunofluorescence after Western blot analysis to establish their monospecificity.

For the clones in group "E," affinity-purified antibodies reacted on Western blots of yeast nuclear and MLPC lysates with a major band at 46 kD (and one minor band at 49 kD, see below). These antibodies were also used to assess the intracellular localization of the antigen in fixed yeast spheroplasts by indirect immunofluorescence. In this experiment, the antibodies decorated the nuclear envelope in a concentrated fashion as well as the cytoplasm (see below). These data suggested that the clones of group E potentially encoded a protein of the MLPC.

Preliminary DNA sequence data indicated that all five group E phages were identical clones, with an ORF in frame with the lacZ gene. After conversion of the phage to double stranded phagemids, one of them (pE26) was chosen for further DNA sequencing after creation of a series of nested deletions with Exonuclease III and mung bean nucleases (data not shown). The ORF of pE26 (see Fig. 1 *A*) was in frame with the lacZ gene as mentioned above, requiring independent cloning of upstream sequences. This was achieved using the integration/excision method (Roeder and Fink, 1980; see Fig. 1 *B*) and a full-length clone including upstream sequences was isolated. The complete DNA sequence is shown in Fig. 2 as well as the predicted amino acid sequence for the ORF, which is 409 amino acids long, with a calculated molecular mass of 44.6 kD. The gene was localized to chromosome XIV by hybridization analysis of a chromosome blot after fractionation of whole yeast chromosomes in a CHEF gel apparatus (data not shown).

YDJ1 Is a Yeast Homologue of dnaJ

A search of the NBRF protein sequence data banks revealed a close homology between the predicted amino acid sequence of the open reading frame of this yeast gene and bacterial dnaJ proteins. The percent identity is 32 and 39% along the entire length of *E. coli* (Ohki et al., 1986; Bardwell et al., 1986) and *Mycobacterium tuberculosis* (*M. tub*; Lathigra et al., 1988) dnaJ proteins, respectively, hence the name YDJ1 (yeast dnaJ) (see Fig. 3 for comparison). In common with bacterial dnaJ proteins YDJ1 contains a glycine/phenylalanine rich region stretching for ~30 amino acids positioned ~80 amino acids from the NH_2 terminus. Starting at amino acid 143 is a cysteine rich region (*underlined* in Fig. 2), which is organized into a series of direct repeats of the sequence CxxCxGxG (where x is typically either a charged or polar amino acid), repeated four times (the final G is missing from the fourth repeat of YDJ1, but is present in both sequenced bacterial dnaJ proteins). Further inspection of these repeats in YDJ1 reveals that they may be further organized into two series of direct repeats of the sequence $CxxCxGxG(x)_8CxxCxGxG$. These two repeats are separated by 18 amino acids. The organization of this cysteine rich region is similar to that found in the family of zinc finger proteins (Berg, 1986, 1990). They do not conform, however, to the exact consensus sequence commonly found in "zinc finger" DNA binding proteins (Thiesen, 1990).

The last four amino acids of YDJ1p bear the sequence CASQ (single letter code) which fit the "CaaX" motif common to proteins that become farnesylated. The CaaX motif corresponds to a consensus sequence (where C is cysteine, a is usually an aliphatic amino acid and X is any amino acid) that is found at the carboxy terminus of *ras* proteins (and some other GTP-binding proteins), nuclear lamins, and a

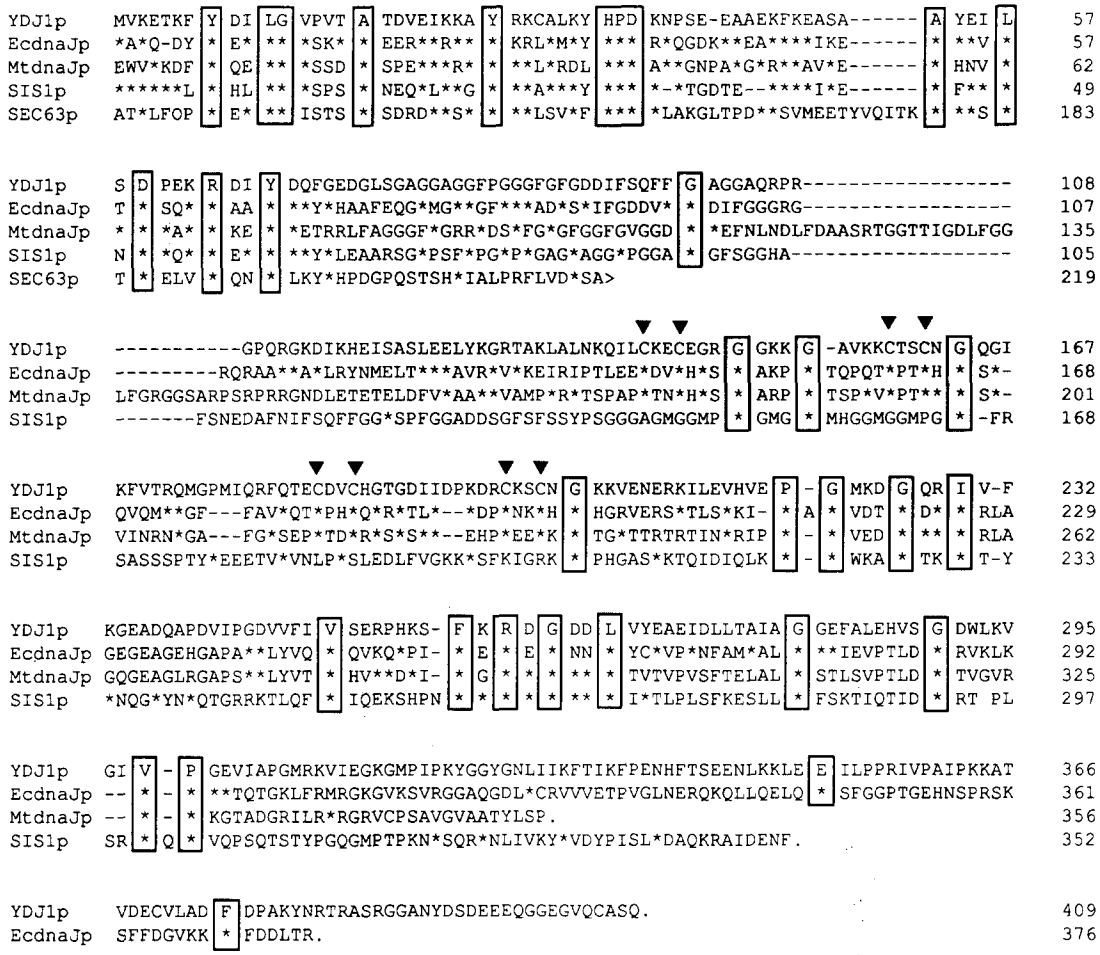

Figure 3. Alignment of YDJ1 with homologous proteins from bacteria (*E. coli* and *M. tub* dnaJ) and yeast (*S. cerevisiae* SEC63 and SIS1). A star beneath the YDJ1 sequence denotes a conserved amino acid. Boxed regions denote conserved identity for all aligned protein sequences. Dashes have been inserted to assist alignment. Numbering is from the initiation methionine codon in each case. Only the amino acids 120–219 of the SEC63 sequence are shown. Inverted arrowheads denote conserved cysteine residues between YDJ1 and bacterial dnaJ proteins.

yeast mating type pheromone (see Glomset et al., 1990, for review).

YDJ1 shares homology with a number of other yeast proteins also related to dnaJ. These include the integral membrane protein of the ER:SEC63 (Sadler et al., 1989), and SIS1, a suppressor of the yeast gene SIT4 (Arndt et al., 1989; Luke et al., 1991). In Fig. 3, the predicted amino acid sequence of YDJ1 has been aligned with bacterial dnaJp sequences and the yeast proteins SIS1 and SEC63. The most conserved region between all five aligned proteins is in first 80 NH_2 terminus amino acids (except in SEC63 where this region begins at amino acid 120). YDJ1p and SIS1p are especially well conserved in this region, sharing 66% identity over the first 68 amino acids. Interestingly, SIS1p does not contain the cysteine repeated regions common to YDJ1 and bacterial dnaJ proteins.

One significant difference that we have observed between YDJ1 and dnaJ is that YDJ1 expression is not heat shock inducible. There was no accumulation of YDJ1 above normal levels 20 min after a 10°C heat shock as assessed by Western blot analysis of whole cell extracts (data not shown).

YDJ1 Localizes to the Nuclear Envelope and Cytoplasm

To further characterize YDJ1, we generated a polyclonal antiserum against a trpE-YDJ1 fusion protein in rabbits (see Materials and Methods and Fig. 1 *A* (*ii*) for construct). Antibodies were affinity purified from the fusion protein and used to assess the intracellular localization of YDJ1p by indirect immunofluorescence. The results of this experiment are shown in Fig. 4 along with data using the original affinity-purified antibody from the MLPC sera (as described above). Shown in Fig. 4, *A* and *B* are a single cell from the strain LDY1; a tetraploid strain whose cells have enlarged nuclei (Davis and Fink, 1990). The DAPI staining in Fig. 4 *A* clearly reveals the large nucleus, as well as the mitochondrial DNA distributed in the cytoplasm. The fluorescence in Fig. 4 *B* reveals the localization of YDJ1p in the same cell; it appears to be distributed evenly throughout the cytoplasm and concentrated in a ring around the nucleus. Similar data was recorded using the same antisera against haploid cells overproducing YDJ1 from a galactose inducible promoter

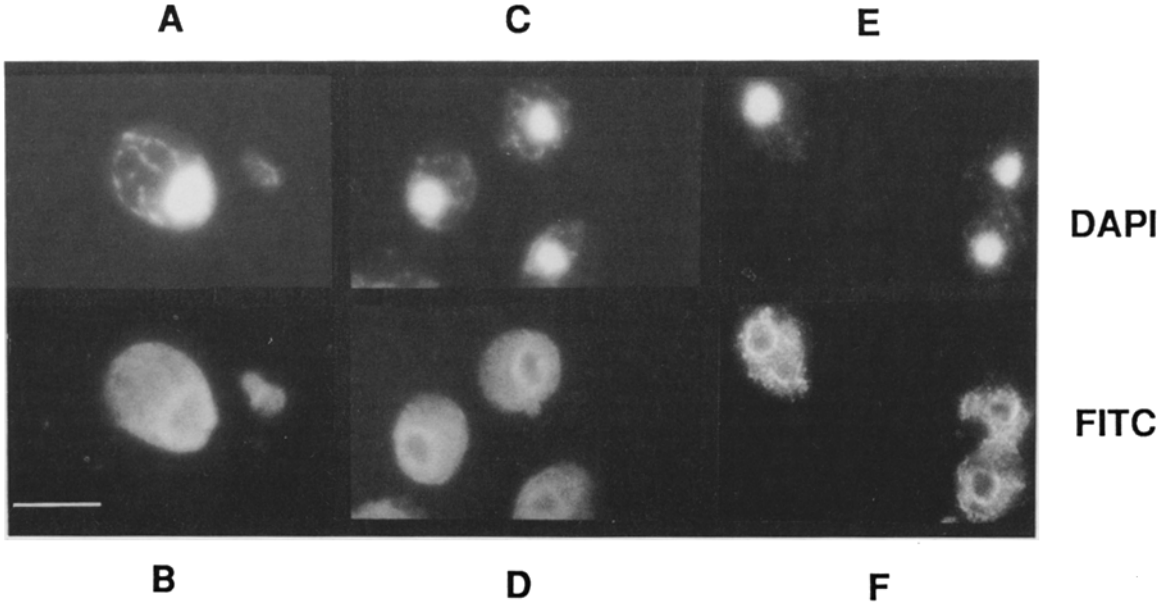

Figure 4. Intracellular localization of YDJ1 by indirect immunofluorescence. (*A*, *C*, and *E*) DAPI staining of DNA. (*B*, *D*, and *F*) Decoration of YDJ1 antibodies in the same cells. The cells shown in *A* and *D* are from the tetraploid strain LDY1, in *C* and *D* from the strain ACY3 which overexpresses YDJ1, and in *E* and *F* from the wild-type strain SEY621. The cells in *D* were decorated with primary antibodies that were affinity purified from the trpE-YDJ1 serum while those in *F* were decorated with affinity-purified YDJ1 antibodies from the MLPC serum. YDJ1 antibodies were visualized using a goat anti-rabbit secondary antibody tagged with FITC. Bar, 10 μm.

(strain ACY3) in Fig. 4, *C* and *D*. In these cells, YDJ1 antibodies clearly decorate the nuclear rim in a concentrated manner. Also, the fluorescence is brighter in the cytoplasm than in the nuclear interior. No fluorescence was observed when these affinity-purified antibodies were used to stain yeast cells lacking YDJ1 (stain ACY4; see below), indicating that the nuclear rim/cytoplasmic decoration is specific for YDJ1 (data not shown).

YDJ1-specific antibodies were also affinity purified from the MLPC antisera using the lacZ-YDJ1 fusion protein (as expressed by the phage isolated from the lambda Zap library, as described above). The pattern of fluorescence displayed by these antibodies (Fig. 4 *F*) is similar to that found with the affinity-purified trpE-YDJ1 antibodies, but with more intense nuclear rim decoration and a more patchy cytoplasmic distribution. Such differences may be attributed to raising the original MLPC serum against native as well as denatured proteins (affinity-purified MLPC antibodies were also prepared against native rather than SDS-denatured proteins).

The immunofluorescent localization of YDJ1p suggests it is present in the cytosol but is also enriched at the nuclear envelope. We further characterized this enrichment by preparing nuclei from yeast spheroplasts according to the method of Kalinich and Douglas (1989). This method used hypotonic lysis of spheroplasts followed by centrifugation through glycerol ficoll gradients to remove nuclei and membranes from soluble components (see *soluble* and *nuclei* lanes in Fig. 5). The nuclei were then treated for preparation of the MLPC (Allen and Douglas, 1989); a procedure that involves digestion with nucleases, and washes with 2% Triton X-100 and 1 M NaCl to remove chromatin, membranes, and salt-soluble components. Samples from the various pellets (Fig. 5, *nuclei* and *mlpc* lanes) and supernatants (*soluble*, *triton*, and *salt wash* lanes) were then separated in an SDS–polyacrylamide gel (Fig. 5 *A*), and the distribution of YDJ1 monitored by Western blot analysis (Fig. 5 *B*). In this experiment YDJ1p associates predominantly with the nuclear fraction. Further treatments of the nuclear fraction with nucleases (data not shown) and Triton X-100 (2%) fail to extract YDJ1p (Fig. 5 *B*), but treatment with 1M NaCl, however, does result in partial (~50%) extraction.

These data suggest that YDJ1p is a component of the MLPC, a complex mixture of proteins that was used to generate the antisera with which the YDJ1 gene was cloned. Previous electron microscopic characterization of the yeast MLPC (Allen and Douglas, 1989) suggests that it is enriched in nuclear pore complexes and other nuclear material. Nuclear pore proteins also localize to the nuclear envelope, but display a more punctate or patchy pattern in indirect immunofluorescence experiments (see Davis and Fink, 1990) rather than the smooth rim staining seen by YDJ1 antibodies. Indeed, the pattern of fluorescence displayed by YDJ1 antibodies is closer to that found for ER proteins, e.g., KAR2 (Rose et al., 1989) and ERD1 (Hardwick et al., 1990).

The cofractionation of YDJ1p with nuclei is consistent with its presence at the nuclear envelope, but it is also consistent with a possible localization at the ER, which is contiguous with the nuclear envelope. We investigated this possibility by performing additional fractionation experiments designed to enrich for intracellular membrane fractions; specifically mitochondria, microsomes, and also soluble proteins present in the postmicrosomal supernatant (see Materials and Methods). Equal amounts of protein from each fraction were separated by SDS-PAGE and immunoblotted for YDJ1 (Fig. 6 *A*). Identical immunoblots were probed with the integral mitochondrial inner membrane pro-

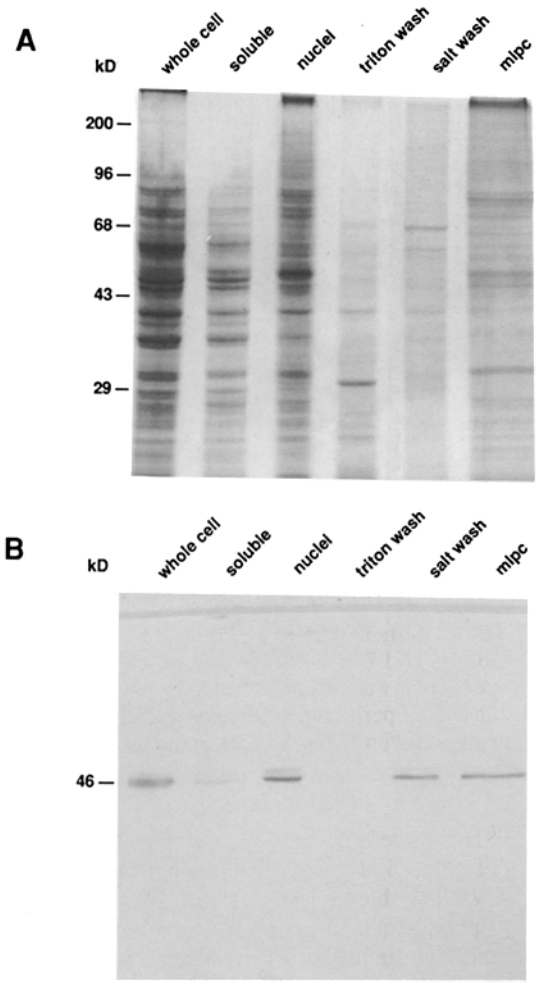

Figure 5. Cofractionation of YDJ1p with nuclei and MLPC. (*A*) SDS-PAGE of various fractions resulting from subcellular fractionation and isolation of MLPC. Approximately equal amounts of supernatants (*soluble*, *triton wash*, and *salt wash* lanes) and pellets (*nuclei* and *mlpc* lanes) were fractionated in a 12% polyacrylamide gel and stained with Coomassie blue R250. (*B*) A duplicate of the gel shown in *A* was transferred to nitrocellulose and probed with YDJ1 antisera (prepared against the trpE-YDJ1 fusion protein). The band at 46 kD corresponds to YDJ1. Crude serum was used at 1:3,000 dilution.

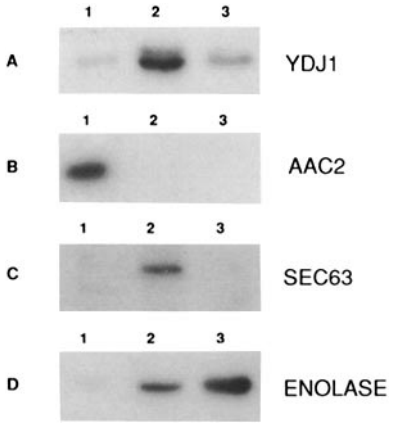

Figure 6. Partitioning of YDJ1p between membrane bound and soluble fractions. Mitochondria (lane *1*), microsomes (lane *2*) and soluble proteins (lane *3*) were isolated from yeast cells. Equal amounts (15 μg) of protein were fractionated by SDS-PAGE and immunoblotted for YDJ1 (*A*), the mitochondrial protein AAC2 (*B*), the integral ER membrane protein SEC63 (*C*), and the soluble enzyme enolase (*D*). Only the relevant sections of the immunoblots are shown in the panels. The relative molecular size of these proteins in the SDS gel is 32 kD for AAC2, 73 kD for SEC63, and 45 kD for enolase. All of these antisera were monospecific except anti-SEC63, which recognized two other proteins, one of which partitioned exclusively with the mitochondria and one exclusively in the soluble fraction.

tein AAC2 (Fig. 6 *B*), the integral ER membrane protein SEC63 (Fig. 6 *C*) and the soluble enzyme Enolase (Fig. 6 *D*) as controls. YDJ1p is clearly enriched in the microsome containing fraction, but is also present in the soluble fraction and to a lesser extent in the mitochondrial fraction. Equal amounts of protein from the different fractions were loaded in each lane of Fig. 6. However, in similar experiments (data not shown) when these data are normalized to the total recovery of proteins in each fraction, >80% of YDJ1p is found in the supernatant fraction (corresponding to lane *3* in Fig. 6). The amount of soluble YDJ1p recovered may depend on the method of its preparation. Thus, ~20% of YDJ1p remains associated with the microsomal fraction under conditions of homogenization, while the remaining YDJ1p is found almost exclusively in the soluble fraction.

The enrichment of YDJ1p in the microsome fraction raised the possibility that it could be localized to the ER lumen. We addressed this question by assessing the protease sensitivity of YDJ1p under conditions where the lumenal protein KAR2 would be digested in the presence, but not in the absence, of Triton X-100. The detergent treatment solubilizes the microsomal membranes which protect KAR2p from protease digestion. In Fig. 7, KAR2p is protected from protease digestion only in the absence of 0.4% Triton X-100, but not in its presence (the band of slightly lower mobility seen in the KAR2 panel in the presence of detergent and protease is a degradation product). YDJ1p, however, is fully degraded by proteinase K even in the absence of Triton X-100 (Fig. 7) indicating that it resides outside of the ER lumen.

We have also observed that YDJ1 antibodies (crude and affinity purified) decorate a band at 49 kD as well as the normal 46-kD band. The former is always less intense (Fig. 5 *B*, *nuclei* lane and Fig. 6 *A*, lane *2*) and was judged YDJ1 specific by its disappearance (alone with the 46-kD band) in whole cell extracts of cells depleted in YDJ1 (see below, Fig. 10). This band may represent a posttranslationally modified version of YDJ1p.

YDJ1 Is Required for Normal Growth

To determine whether YDJ1 was essential for cell viability we constructed a haploid yeast strain disrupted for YDJ1. This was achieved by constructing a version of YDJ1 in vitro that had a wild-type HIS3 gene replacing 280 bp of the YDJ1 ORF (Fig. 8 *A*). A restriction fragment containing the disrupted YDJ1 gene was then transformed into a diploid wild type yeast strain auxotrophic for histidine (W303), and resulting His^+ colonies were picked. Integration of the mutant gene into one of the wild-type alleles was monitored by

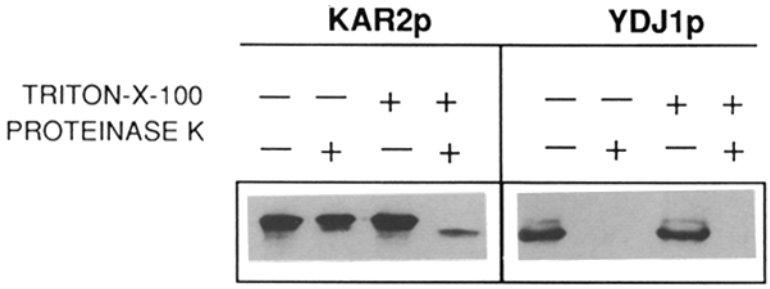

Figure 7. Protease sensitivity of YDJlp. Whole cell homogenates were digested with 25 $\mu g \cdot ml^{-1}$ proteinase K in the presence or absence of 0.4% Triton X-100 for 0 or 30 min as indicated. Equal amounts of protein from the digests (+/− Triton X-100) were fractionated by SDS-PAGE (10%) and immunoblotted for KAR2 or YDJ1 antibody as indicated.

Southern blot analysis (Fig. 8 *B*). Heterozygous diploids for YDJ1 were then induced to undergo meiosis and sporulation. The resultant spores were dissected and their growth monitored after 3 d on rich media. 38 out of 40 dissected tetrads scored 4:0 for growth, but with two large and two small colonies (see Fig. 8 *C*). The small colonies were all His^+ while the large colonies were His^- (data not shown), indicating that YDJ1 disruption cosegregated with the slow growth phenotype. Haploid cells containing the disrupted chromosomal copy of YDJ1 could form small colonies upon restreaking, but were inviable for growth in liquid media. YDJlp is required, therefore, for normal growth. Similar data was obtained using a complete gene deletion mutant (data not shown).

The limited viability of *YDJ1* cells made it possible to further characterize the consequences of YDJlp loss on cellular morphology. For these experiments, the strain carrying the disruption of YDJ1 was transformed with a plasmid carrying the YDJ1 gene under control of the inducible GAL1-10 promoter (pGYDJ). Haploid cells carrying the plasmid and the disruption of YDJ1 were selected for further study after sporulation of the transformed diploid. Such cells (termed ACY3) expressed YDJ1 at approximately three times above the wild-type level when grown in media containing galactose. To deplete YDJlp, cells were switched to media substituting dextrose for galactose, thereby repressing the GAL1-10 promoter, and grown for an additional 24 h.

Shown in Fig. 9, *A* and *C* is a representative sample of ACY3 cells grown in YP galactose after ethanol fixation and DAPI staining for visualization of nuclei. Such cells are similar in size to wild-type cells and have normal nuclear morphology. After depletion of YDJlp by growth in YP dextrose, however, the cells become much larger (Fig. 9 *B*). In addition, these cells were often observed with large buds (Fig. 9, *C* and *D, central and lower right cells*). Close inspection of these cells reveals that nuclear DNA is present in either the putative mother cell or the bud, but rarely in both. Occasionally, remnants of nuclear DNA were observed in the neck between the mother and bud (Fig. 9 *D, arrow*). Such remnants are characteristic of the terminal phenotype associated with the karyogamy defect in *kar1* cells (Rose and Fink, 1986).

The nuclear morphology of YDJlp-depleted ACY3 cells was further studied by indirect immunofluorescence, using monoclonal antibodies against the nucleolar protein NOP1 (A66, the kind gift of J. Aris; see Aris and Blobel, 1988). This antibody specifically decorates the nucleolus that has been characterized in yeast as a crescent-shaped structure that lies against one wall of the nuclear envelope, occupying approximately one-third of the nuclear volume (see Aris and Blobel, 1988). The pattern of fluorescence of ACY3 cells grown in galactose (i.e., overexpressing YDJ1) with A66 antibody is shown in Fig. 9 *F*, and its DAP1 counterpart shown in Fig. 9 *E*. These antibodies decorate the nucleus in a heterogeneous manner, with greater fluorescence observed toward one end of the nucleus (compare Fig. 9, *A* and *F*). In the YDJ1 depleted cells shown in Fig. 9, *H* and *J*, however, the nucleolus appears highly distorted and spread out, occupying an apparent volume at least equal to or greater than that of the nucleus (Fig. 9, *G* and *I*). In Fig. 9 *G* DAPI staining reveals nuclear DNA to be present at the neck between mother cell and bud with virtually no DAPI staining in the putative bud. NOP1 decoration of the same cells, however, reveals this protein to be present in both putative mother and daughter, suggesting that these cells are defective in chromosome, but not nuclear migration during mitosis.

Besides changes in nuclear morphology, cells depleted in YDJlp contain unusually large vacuoles, often encompassing almost the entire cell volume (data not shown). The morphology of cells depleted in YDJlp by GAL1-10 promoter repression has similar morphology to haploid cells carrying the YDJ1 gene disruption.

SIS1 Complements for YDJ1

The high degree of similarity between YDJ1 and SIS1 amino acid sequences (see Fig. 3) led us to ask whether overexpression of SIS1 could suppress the slow growth phenotype in YDJ1-disrupted cells (the ACY1 strain). We therefore transformed a YDJ1 heterozygous diploid strain with a yeast 2μ based episomal plasmid containing a URA3 gene and the SIS1 gene under control of its own promoter (pCB338, a gift from K. Arndt). This plasmid is multicopy in yeast, resulting in greater accumulation of SIS1 gene product relative to its chromosomal single copy counterpart. Transformants containing the plasmid were selected for uracil prototrophy and grown in rich media (YPD) before meiotic induction. The resulting spores were then dissected and the growth phenotype monitored.

After 3 d growth we found that the dissected tetrads gave two different phenotypes. Out of 31 tetrads that scored 4:0 for growth, 58% grew with a phenotype identical to the parent YDJ1 heterozygous diploid (i.e., two large and two small colonies). 42%, however, grew with four large colonies. After replating these cells for their auxotrophy, we found that all dissected tetrads that grew with four large colonies contained the plasmid with the SIS1 gene (i.e., they scored 4:0 for Ura^+ and 2:2 for His^+). Those that grew with two large and two small colonies, however, were all Ura^-, but scored 2:2 for His^+, i.e., those cells had lost the SIS1 containing plasmid before meiosis. Those haploid cells that were Ura^+

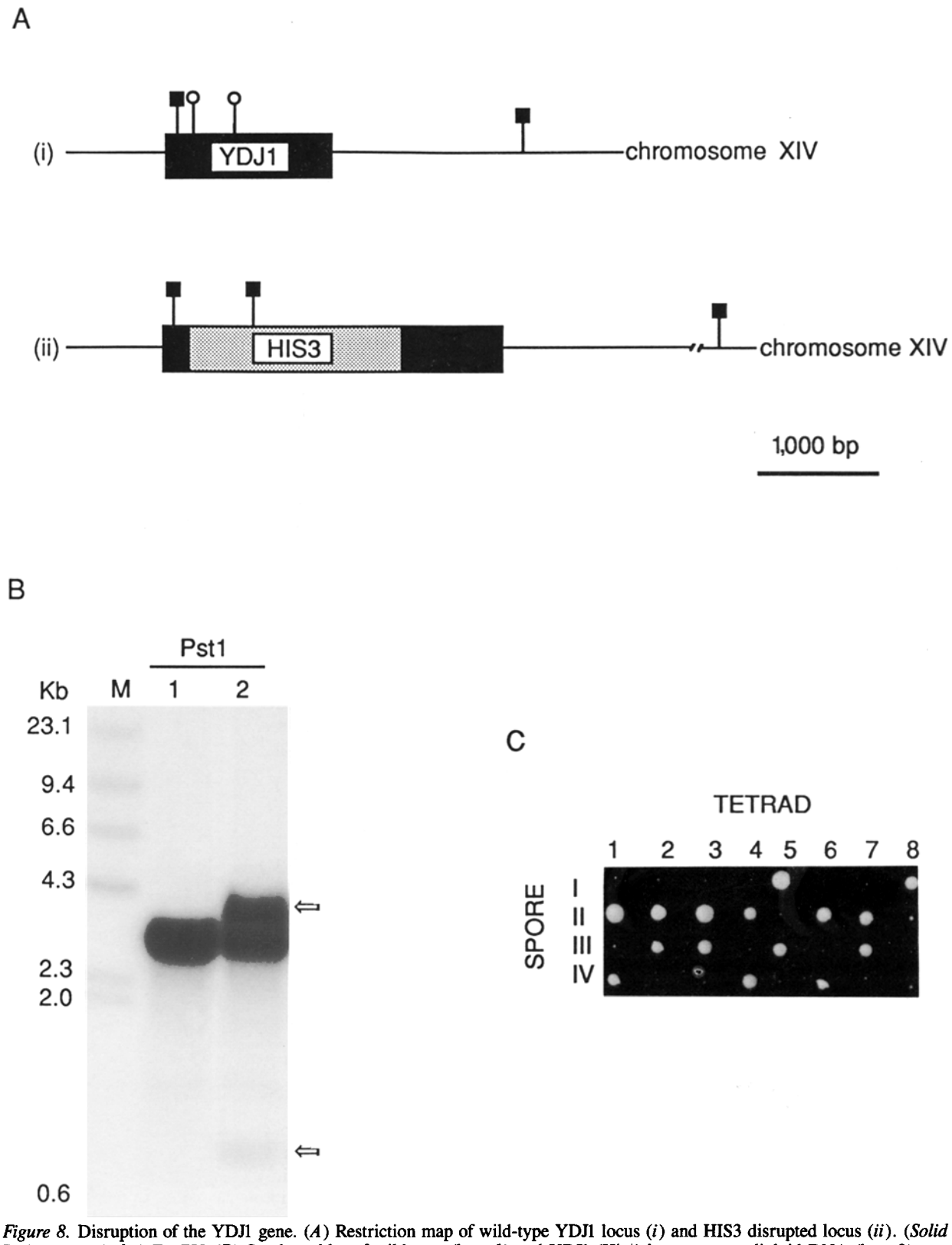

Figure 8. Disruption of the YDJ1 gene. (*A*) Restriction map of wild-type YDJ1 locus (*i*) and HIS3 disrupted locus (*ii*). (*Solid squares*) PstI; (*open circles*) EcoRV. (*B*) Southern blot of wild-type (lane *1*) and YDJ1 (His^+) heterozygous diploid DNA (lane *2*) cut with PstI and probed with EcoRI–CvnI fragment from the YDJ1 open reading frame. Arrows denote bands expected as a result of HIS3 integration. (*C*) Dissection of tetrads after sporulation of YDJ1 heterozygous diploid. Tetrads are numbered across and spores from each tetrad are numbered down (*Roman numerals*).

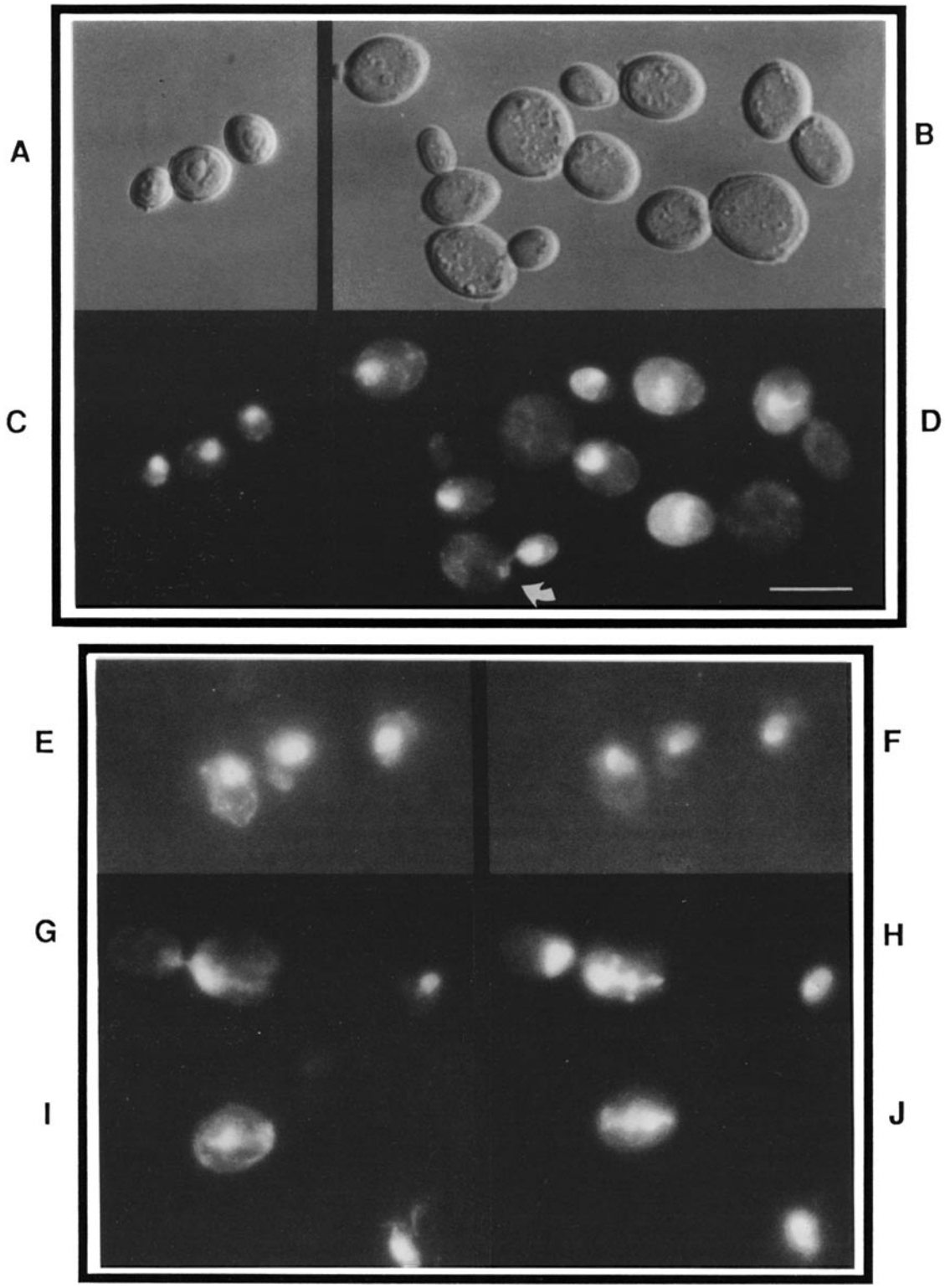

Figure 9. Microscopic characterization of strain ACY3 under conditions of YDJ1p overexpression and depletion. ACY3 cells were grown in media using galactose (*A*, *C*, *E*, and *F*) or dextrose (*B*, *D*, and *G–J*) as the carbon source. In *A–D*, cells were ethanol fixed and stained with DAPI then viewed by Nomarski (*A* and *B*) or UV (*C* and *D*) for visualization of DNA. (*E–J*) Cells were formaldehyde fixed and prepared for immunofluorescence by staining with DAPI and monoclonal antibody A66, then viewed through a UV filter for visualization of DNA (*E*, *G*, and *I*) or rhodamine for visualization of the nucleolar protein NOP1 (*F*, *H*, and *J*). Monoclonal antibody A66 was the gift of Dr. J. Aris. Bar, 10 μm.

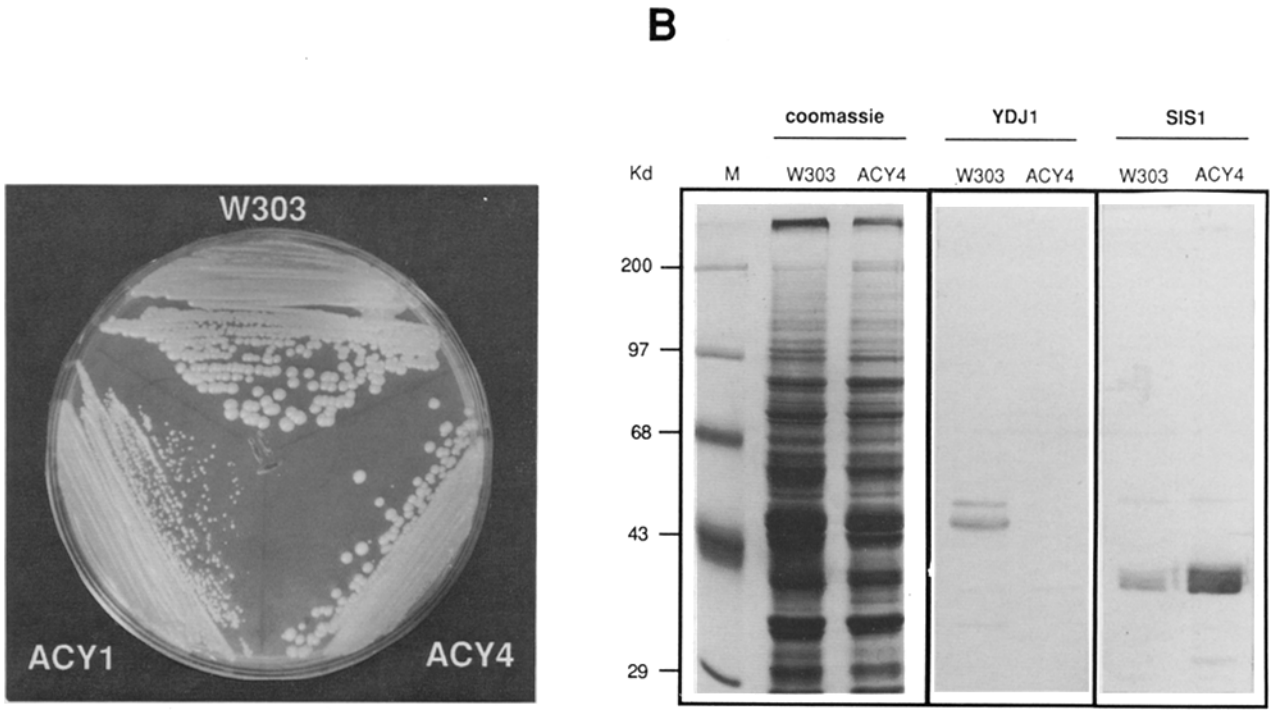

Figure 10. (*A*) Haploid cells from the strain W303 (wild type), ACY1 (YDJ1 disrupted), and ACY4 (ACY1 plus pCB338) were streaked onto a YPD plate and incubated at 30°C for 3 d before photography. (*B*) Whole cell extracts from the strain W303 and ACY4 were fractionated on a 10% PAGE. Two duplicate gels were run, transferred to nitrocellulose, and probed with antibodies against YDJ1 or SIS1 as indicated in the figure. pCB338 and SIS1 antibodies were gifts of Dr. K. Arndt.

and His$^+$ (i.e., disrupted for YDJ1 but contained SIS1 in high copy number) were designated ACY4. In Fig. 10 *A*, haploid cells from WT, ACY1, and ACY4 strains have been streaked for single colonies for a qualitative comparison of their growth rate. The cells from the ACY4 strain grow faster than those of ACY1 (and form larger colonies), but not as fast as wild-type cells; their doubling time in liquid media was calculated to be ~one-third that of wild-type cells.

These data indicate that overexpression of SIS1 can at least partially compensate for the loss of YDJ1p (Fig. 10 *A*) and may be the reason why the YDJ1 gene–disrupted haploid cells show limited viability. To confirm that ACY4 contains SIS1 in high copy number, we monitored the amount of YDJ1p and SIS1 by Western blot analysis (Fig. 10 *B*). The immunoblots shown in Fig. 10 *B* reveal that whole cell extracts from ACY4 cells contain excess SIS1p (approximately five times more) in the absence of YDJ1p.

Discussion

YDJ1p is one of several yeast proteins related to the bacterial protein dnaJ. Its primary amino acid sequence closely resembles dnaJ, indicating that secondary and tertiary structures are probably conserved.

The region of closest homology between YDJ1 and its yeast/bacterial homologues is at the NH_2-terminal 70 amino acids of the protein (see Fig. 3). YDJ1p and SIS1p are especially well conserved at the NH_2 terminus, sharing 11 of the first 13 amino acids. Both genes reside on the same chromosome (*XIV*) (see Luke et al., 1991). The partial suppression of the *YDJ1* growth phenotype by SIS1 overexpression suggests a functional relationship between these two proteins.

The major difference between YDJ1p and SIS1p is in the middle portion of the protein; only YDJ1 retains the cysteine-rich repeats occurring in dnaJ. These repeats are very similar to those found in zinc finger proteins and while a zinc finger model is easy to assemble from this primary sequence, there are other structural interpretations (for example see Schwabe et al., 1990). It is also clear that proteins containing such repeats are not all nuclear and/or DNA binding proteins (for review see Berg, 1990). A more modest prediction may be that such regions will bind Zn^{2+}, and while this is unproven for YDJ1, *E. coli* dnaJ is a Zn^{2+} binding protein (Liberek, K., and C. Georgopoulos, unpublished data).

The combined biochemical (Figs. 5–7) data suggests that YDJ1p is a predominantly cytoplasmic protein that also associates with microsomal membranes. Association with the ER probably accounts for the perinuclear decoration displayed by YDJ1 antibodies in the indirect immunofluorescent experiments shown in Fig. 4. Although the full extent of ER distribution in yeast is unknown, most of it appears to be at the nuclear envelope. This is suggested by the predominantly perinuclear decoration displayed by antibodies specific for lumenal ER proteins, e.g., KAR2 (Rose et al., 1989) and ERD1 (Hardwick et al., 1989), and the integral ER membrane protein, SEC62 (Deshaies and Schekman, 1991).

The protease sensitivity experiments shown in Fig. 7 demonstrate the freely accessible nature of YDJ1p to protease digestion, suggesting that it does not reside in the ER lumen. This is not surprising since it does not contain either a hydrophobic signal sequence at its amino terminus or the *S. cerevisiae* ER retention signal HDEL (single letter amino acid code), as does the lumenal protein KAR2p (see Rose et

al., 1989; Normington et al., 1989; Lewis et al., 1990). YDJ1p appears, therefore, to bind to the cytoplasmic face of the ER membrane.

The solubility properties of YDJ1p appear to depend on the method of cell lysis. The hypotonic lysis method for nuclear preparation renders most YDJ1p to the nuclear fraction, yet homogenization of spheroplasts yields up to 80% of the protein in the soluble fraction. One possible explanation for these data is that YDJ1p is only loosely associated with membranes, being more easily removed by the process of homogenization than hypotonic lysis. Whether such membrane association is a function of lipid modification at the putative CaaX box at the COOH terminus remains to be determined. We note, however, that *E. coli* dnaJ protein is also membrane bound (Zylicz et al., 1985), yet does not have the CaaX motif (see Fig. 3 for sequence comparison). Given the predominantly cytoplasmic and membrane bound localization of YDJ1p, it remains uncertain how it becomes associated with the MLPC, and what, if any, physiological role this may have.

Disruption of the YDJ1 gene results in pleiotropic defects in cell growth and organelle morphology. The mutation does not render the cells inviable for growth per se, although they are inviable for growth in solution. It is currently unclear why this should be the case. Cells grown at limiting YDJ1 display a much larger size and appear to arrest with large buds. These cells display aberrant nuclear morphology and are possibly defective in chromosome segregation. It is currently unclear whether these phenomena may be directly attributed to the function(s) of YDJ1p, and it is possible that some of these defects result indirectly as a result of encroaching cell death.

By comparison with the bacterial system and judging from the close homology between YDJ1 and dnaJ, we may deduce that some of the functions that dnaJ performs will be conserved. While these are not well characterized, it is thought that dnaJ performs tasks related to protein assembly and targeting. The most recent model is that dnaJ, in the presence of grpE, will stimulate the hydrolysis of dnaK bound ATP, thereby catalyzing the release of chaperone bound proteins for subsequent targeting or assembly (Sell et al., 1990). In bacteriophage λ DNA replication, dnaK, in the presence of dnaJ, is required for assembly and disassembly of a multiprotein complex that targets the *E. coli* helicase (dnaB) to the λ replication origin (Liberek et al., 1990). Many of the phenotypes displayed by a ts dnaJ allele at the nonpermissive temperature are identical to those found in dnak, grpE, and groEL mutants. These include defects in protein folding (Gaitanaris et al., 1990) and proteolysis (Strauss et al., 1988) as well as in λ replication. Mutants in dnaK and dnaJ are further found to be defective in phosphorylation of certain tRNA synthetases (Itikawa et al., 1989) and temperature sensitive for both DNA and RNA synthesis (Wada et al., 1982).

It is also clear, however, that dnaJ is not limited to interacting solely with dnaK, as shown by its interaction with both dnaB and λ P proteins. dnaJ also interacts with the RepA protein required for initiation of plasmid P1 replication (Wickner, 1990). Further work by Ohki et al. (1986) has demonstrated some specific functions for dnaJ that do not appear to involve dnaK; mutants in dnaJ specifically affect mRNA synthesis of some specific genes, notably β-galactosidase. Ohki et al. (1986) further demonstrate that ts dnaJ mutants arrest at a specific stage in the *E. coli* cell cycle.

At this time, we cannot propose a specific function for YDJ1. Its concentrated localization at the nuclear envelope may point to an involvement in the variety of processes that occur there, including protein assembly into the ER or the nucleus (as proposed for SEC63; Sadler et al., 1989).[2]

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