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An osmotic-remedial, temperature-sensitive mutation in the allosteric activity site of ribonucleotide reductase in *Neurospora crassa*

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Abstract An osmotic-remedial, temperature-sensitive conditional mutant (*un-24*) was generated by *R*epeat *I*nduced *P*oint mutation (RIP) from a cross between a wild-type *N. crassa* strain and a strain carrying a ≈250-kb duplication of the left arm of linkage group II (LGII). The mutation was mapped to the duplicated segment, within 2.6 map units of the heterokaryon incompatibility locus *het-6*. DNA transformation identified a 3.75-kb fragment that complemented the temperature-sensitive phenotype. A large ORF within this fragment was found to have a high degree of sequence identity to the large subunit of ribonucleotide reductase (RNR) from diverse organisms. Conserved amino acids at the active site and the allosteric activity sites are also evident. An unusual feature of the Neurospora sequence is a large insertion near the C-terminus relative to otherwise homologous sequences from other organisms. Three transition mutations, indicative of RIP, were identified in the N-terminal region of the temperature-sensitive mutant allele. One of these mutations results in a non-conservative amino acid substitution within the four-helix bundle that is important in the allosteric control of ribonucleotide reductase activity. This substitution appears to disrupt proper folding of the allosteric activity site during synthesis of the protein.

Key words *N. crassa* · Osmotic-remedial temperature-sensitive mutation · Ribonucleotide reductase · Repeat-induced point mutation (RIP) · Hydroxyurea

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Introduction

Ribonucleotide reductase is essential for the de novo synthesis of DNA (reviews in: Reichard 1993; Eklund et al. 1997; Jordan and Reichard 1998). The class I ribonucleotide reductases convert nucleoside diphosphates (NDPs) into the respective deoxynucleoside diphosphates (dNDPs), and are conserved amongst diverse organisms. The active form of the class I enzyme is a tetramer comprised of a large subunit dimer, referred to as R1 or M1, and a small subunit dimer, R2 or M2 (Wright et al. 1990; Reichard 1993). Each R2 subunit in these reductases contains two iron ions associated with a tyrosine residue. It is proposed that a long-range electron transfer occurs from the tyrosine radical in R2 along the protein to the active site of nucleotide reduction located in R1 (Nordlund and Eklund 1993; Eckberg et al. 1996). In addition to an active site, each large subunit contains two allosteric regulation sites by which a balance of dNTPs is maintained in the cell for DNA synthesis (Eklund et al. 1997; Eriksson et al. 1997). Overall activation or inhibition of the enzyme occurs when ATP or dATP, respectively, binds to the allosteric activity site located in the N-terminal region of the large subunit. Substrate specificity is regulated by nucleotides interacting at the allosteric specificity site. Nucleotide effector binding at the allosteric activity and specificity sites appear to influence domain stability and subunit interaction within the tetramer, and thereby coordinate enzymatic function (Eriksson et al. 1997).

Ribonucleotide reductase is of considerable interest in the health sciences and biotechnology. Since ribonucleotide reduction can become the rate-limiting step in

DNA synthesis, the enzyme is important for the regulation of cell division and cell proliferation, and is of interest, therefore, as a potential target for antimicrobial, antiviral and antitumor drugs. It has also been implicated in immunological diseases. The high levels of dATP and dGTP that are associated with immune dysfunction in humans with adenosine deaminase (ADA) or purine nucleoside phosphorylase (PNP) deficiencies are thought to result in feedback inhibition of ribonucleotide reductase and selective interference with lymphocyte viability and function (review in Hershfield and Mitchell 1995).

In this study, we generated a temperature-sensitive mutation, designated *un-24*, in the large subunit of ribonucleotide reductase in *Neurospora crassa*. Strains that carry *un-24* grow at near wild-type rates below 30° C but do not grow when temperatures exceed 34° C, except under conditions of high osmolarity. *un-24* was isolated in a series of crosses performed to induce mutations in a specific chromosomal region by RIP (*R*epeat *I*nduced *P*oint mutations) (Selker 1990). Crosses were made between the translocation-strain T(*II→III*)*AR18* [hereafter referred to as T(*AR18*)] and normal strains in order to generate partial diploid progeny. Such duplication (Dp) strains are generally barren in crosses, although occasionally a few progeny can be recovered. RIP mutations have been shown to occur in genes within duplicated chromosome regions when such duplication strains are crossed to structurally wild-type strains (Perkins et al. 1997). We located the wild-type sequence (*un-24*$^{+}$) within a stretch of 3.75 kb by RFLP analysis of progeny with crossovers in the vicinity of *un-24* and on the basis of complementation of the temperature-sensitive phenotype by transformations with wild-type DNA. Sequencing of the complementing DNA fragment revealed an ORF with a high level of identity to the large subunit of type I ribonucleotide reductases from a diverse group of organisms. Amino acids that are essential for function at the active site and the two allosteric activity sites are also conserved in the *N. crassa* protein. The protein sequence inferred from this gene in *N. crassa* is unusual in having a long, inserted region near the C-terminus relative to other ribonucleotide reductases. A comparison of *un-24* and wild-type *un-24*$^{+}$ DNA sequences revealed three GC → AT transition mutations near the N-terminus of the temperature-sensitive allele. One of these mutations results in a non-conservative amino acid substitution. This mutation occurs in a region which, in the *Escherichia coli* homolog, folds into a four-helix bundle that makes up the allosteric regulation site of ribonucleotide reductase. Our data suggest that at restrictive temperatures folding is disrupted during protein synthesis due to this mutation in the large subunit of ribonucleotide reductase of *un-24* strains and that this results in growth arrest.

Materials and methods

Strains and culture conditions.

N. crassa strains used in this study are listed in Table 1. Cultures were grown and maintained on 1 × Vogel's salts (Vogel 1964), 1.5% sucrose, 1.5% agar, and 1 × growth supplements, where necessary (Davis and de Serres 1970). Crosses were performed on synthetic cross medium (Westergaard and Mitchell 1947). Growth rate measurements were performed at 20, 25, 30, 34 and 39° C in growth tubes as described by Jacobson et al. (1995). To examine growth rate as a function of osmotic pressure, *un-24* and wild-type strains were grown on petri plates containing Vogel's medium plus supplements, to which sorbitol, mannitol, glycerol, glucose or KCl was added at concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1, 2 and 3 M.

Table 1 *N. crassa* strains used in this study

Genotype[a]	Strain designation	Origin[b]
Standard wild-type *A*	74-OR23-1V*A*	FGSC 2489
Standard wild-type *a*	74-ORS-6*a*	FGSC 4200
T(II → III)AR18 a	*T(AR18)*	FGSC 2644
Dp (II → III)AR18 A	DJ258-4	Cross 1
T(II → VI)P2869 A	*T(P2869)*	FGSC 1828
un-24; morph *a*	DJ517-1	Cross 2
un-24 a	DJ826-1	Cross 3
un-24 het-6^{OR} *A*	DJ949-90	Cross 4 (FGSC 7354)
ro-7 un-24 het-6^{OR} *a*	DJ960–2	Cross 5
het-6^{PA} *het-C* PA *pyr-4*; *inl a*	RLM58-18	Smith et al. (1996)
ro-7 un-24 het-6^{OR} *het-C*^{OR}; *trp-1 A*	C5-44	Cross 7
un-24; *trp-1*; *inl a*	C8c-164 and -165	Cross 8
ro-7 un-24	DJ986-1	D.J. Jacobson
trp-1; *am a*	1847*trp-1*; *am a*	FGSC 1847
ro-7 het-6^{OR} *pyr-4 a*	DJ793-3	D.J. Jacobson
cyh-1; *het-6*^{OR} *het-C*^{OR} *pyr-4 A*	RLM57-30	R.L. Metzenberg
het-6^{SP} *thr-2 A*	DJ959-1	D.J. Jacobson

[a] Abbreviations after Perkins et al. (1982): *a* and *A*, mating types; *am*, amination deficient; *inl*, inositol requirement; *cyh-1*, cycloheximide resistant-1; *het-6*, heterokaryon incompatibility-6, *het-C*, heterokaryon incompatibility-C (the superscript OR indicates the Oak Ridge allele and PA the Panama allele); *pyr-4*, pyrimidine-4; *ro-7*, ropy-7; *thr-2*, threonine-2; *trp-1* (tryptophan-1). *un-24* and "morph" are described in this study. Note that the *het-6*^{SP} allele, from the Spurger background, is equivalent to *het-6*^{PA} in being incompatible with *het-6*^{OR}, strains carry OR alleles at the other *het* loci

[b] FGSC, Fungal Genetics Stock Center (Department of Microbiology, University of Kansas Medical Center, Kansas City). For Crosses 1–8, see Table 2

Hydroxyurea (Aldrich) was used as an inhibitor of ribonucleotide reductase activity.

DNA isolation

N. crassa genomic DNA was isolated by the method of Oakley et al. (1987). Plasmid and cosmid DNAs were isolated by the alkaline lysis procedure with or without further purification in CsCl-ethidium bromide gradients (Sambrook et al. 1989), by passage through Qiagen columns (Qiagen) or with the Circle Prep Spin-Midi Kit (Bio 101) as recommended by the manufacturers.

DNA hybridization

Genomic DNAs were digested with restriction endonucleases (New England Biolabs), subjected to electrophoresis through 0.8% agarose in 1 × TAE (0.04 M Tris-acetate, 0.001 M EDTA), and transferred to Hybond N membrane according to the membrane manufacturer's recommendations (Amersham). The Orbach/Sachs Neurospora genomic library in the cosmid pMOcosX (Orbach 1994) was obtained from the Fungal Genetics Stock Center (FGSC, Department of Microbiology, University of Kansas Medical School, Kansas City, Kan.), and handled using standard protocols (Vollmer and Yanofsky 1986). Bacterial colony DNA transfers were done by the method outlined in Sambrook et al. (1989). Probe DNAs were labeled using either T7 QuickPrime (Pharmacia) with [α-^{32}P]dCTP (Amersham) or with fluorescein-dUTP (ECL, Amersham), or using Klenow enzyme with digoxigenin (DIG)-dUTP (Boehringer Mannheim). Hybridization conditions and washes were done according to the membrane manufacturers' protocols. Hybridization signals were visualized following exposure to Kodak X-OMAT or Kodak Biomax MR film.

PCR

PCR reactions were performed using the Expand High Fidelity kit (Boehringer Mannheim) or *Taq* DNA polymerase (Gibco/BRL) in an Amplitron II Thermolyn Thermocycler (Barnstead/Thermolyn, Dubuque, Iowa). Oligonucleotide primers were synthesized with an Applied Biosystems 390 PCR-Mate DNA synthesizer located at the Carleton University Biology Department. Primer sequences are listed in the legend to Fig. 4. DNA was denatured for 2 min at 94° C, and reactions were cycled 25 times as follows: 15 s at 94° C, 30 s at 55° C, 1 min/1.5 kb DNA at 68° C. This thermal cycle regime was followed by 10 min at 72° C. The samples were then kept at 4° C until use. Reaction products were visualized by agarose gel electrophoresis as described above. PCR products were purified as recommended in the High Pure PCR Product Purification kit (Boehringer Mannheim).

Plasmid vectors, ligation and cloning

Ligations were done with T4 DNA ligase as recommended by the manufacturer (Gibco/BRL). Vectors used were pIBI76 (The Clonar Biosystem, IBI), pMOcosX (Orbach 1994), pCRII (TA Cloning kit, Invitrogen), pOKE103 (a gift from R.L. Metzenberg, contains *N. crassa pan-2*$^{+}$ as a selectable marker in *pan-2*$^{-}$ strains), pUC18 (Gibco/BRL), pMP6 (Orbach 1994) and pCSN44 (Staben et al. 1989). *E. coli* DH5α cells (Gibco/BRL) and Inv F′ cells (Invitrogen) were transformed using an *E. coli* Pulser electroporator as recommended by the manufacturer (BioRad).

Spheroplasts and DNA transformations

Spheroplasts were prepared with Novozym 234 (lot 4859, Interspex Products, Foster City, Calif.) by methods similar to that of Schweizer et al. (1981), as modified by Royer and Yamashiro (1992). Spheroplasts were subsequently treated for transformation as described by Akins and Lambowitz (1985). A 100-µl aliquot of spheroplast suspension (8.0×10^7 spheroplasts/ml) was mixed with 0.6–2 µg of transforming DNA. Co-transformations were with 300 ng pCSN44 (hyg^{R}) and 0.6–2 µg of test DNA. Transformed spheroplasts of *un-24* strains were selected at 37° C on regeneration plates without hygromycin B.

DNA sequencing

DNA sequencing was carried out with [α-^{35}S]dATP and a T7 Sequenase kit (Amersham Life Sciences) on a Model S2 sequencing apparatus (Life Technologies) by the dideoxy chain-termination method of Sanger et al. (1977). Sequencing through GC-rich regions employed dITPs (Amersham). Between 1 and 2.5 µg of plasmid DNAs, or 1.4 µg of PCR product, was used for sequencing. Oligonucleotide primers used for DNA sequencing were synthesized as above and sequences are given in the legend to Fig. 4. The cDNA sequence of the 5′ end of ribonucleotide reductase was obtained through the Neurospora Genome Project (NGP) database (Clone ID# NC5G9, Nelson et al. 1997). The cDNA sequence of the 3′ end of the coding region was obtained in the following way. First-strand cDNAs from 74-OR23-1V*A* (a gift of P.J. Vierula) were used as template in a PCR amplification with primer P11 and the 3′ RACE adapter primer (5′-GACTCGAGTCGACATCG-3′; Frohman 1990). A second round of PCR used the nested primers P8 and P6 (see Fig. 4). This PCR product was then cloned into PCRII and sequenced with universal and internal primers. Databank searches were done in Swiss-Prot (Bairoch and Apweiler 1997) and the Saccharomyces Genome Database (Stanford Genomic Resources, Stanford University, Calif.). Amino acid sequence alignments were done with Clustal V (Higgins et al. 1992) on a Power Macintosh 7200/PC.

Results

Derivation of *un-24*

Table 2 lists the crosses used and progeny selected during the derivation of *un-24*. The cross of *T(AR18)a* with

Table 2 Pedigree of the *un-24* mutants

Cross	Progeny selected
1. *T(II → III)AR18 a* × 74-OR23-1VA	DJ258-4 *Dp(II → III)AR18 A*
2. DJ258-4 *Dp(II → III)AR18 A* × 74-ORS-6*a*	DJ517-1 *un-24*; morph *a*
3. DJ517-1 *un-24*; morph *a* × 74-OR23-1V*A*	DJ826-1 *un-24 a*
4. DJ826-1 *un-24 a* × *T(P2869)A*	DJ949-90 *un-24 het-6*^{OR} *A* (FGSC 7354)
5. DJ949-90 *un-24 het-6*^{OR} *A* × DJ793-3 *ro-7 het-6*^{OR} *pyr-4 a*	DJ960-2 *ro-7 un-24 het-6*^{OR}*a*
6. DJ960–2 *ro-7 un-24 het-6* ^{OR}*a* × DJ959-1 *het-6*^{SP} *thr-2 A*	First mapping cross
7. 1847 *trp-1*; *am a* × DJ986-1 *ro-7 un-24 A*	C5-44 *trp-1*; *ro-7 un-24 A*
8. C5-44 *trp-1*; *ro-7 un-24 het-6* ^{OR} *het-C* ^{OR} *A* × RLM58-18 *het-6*^{PA} *het-C*^{PA} *pyr-4 inl a*	Second mapping cross; C8c-164, -165

the wild-type strain 74-OR23-1V*A* (Cross 1, Table 2) produced 25% white, non-viable, ascospores. The remaining mature, black, ascospores were incubated on complete medium at 34° C for 2 days. Progeny with duplications were recognized by their colonial growth form (Perkins and Barry 1977). The cause of anomalous growth of homozygous *Dp*(*AR18*) strains is unknown, but it is less severe than in *Dp*(AR18) strains that are heterozygous at *het-6* (Mylyk 1975).

Although wild-type growth rates were never achieved, 15 of 17 partial diploid progeny displayed an increase in growth rate with elevated levels of conidiation as compared to the original *Dp*(*AR18*) offspring after 8–10 days incubation at 25° C. The "escape" morphology was stable through repeated subculture. These 15 "escape" *Dp*(*AR18*) progeny were crossed to *A* or *a* wild-type strains. Two of these crosses produced spores after 6 weeks, with the remaining crosses being completely barren. Cross 2 (Table 2) yielded one perithecium containing 25% aborted white (hyaline) ascospores; 33 black ascospores were transferred to complete medium, heat-shocked, and incubated at 25° C. After 5 days, the spores that germinated gave 24 viable progeny showing various morphologies: four with wild-type-like growth rates and abundant conidiation, 14 with colonial growth [resembling the *Dp*(*AR18*) morphology], two with inhibited growth and dark brown pigmentation, and four with fine, sparse mycelium appressed to the agar and no aerial hyphae. After continued growth, the 14 Dp-like progeny "escaped" and began to conidiate.

The 14 escaped and four conidiating progeny with wild-type growth rates were screened for auxotrophic and temperature sensitive mutations. One of the four original fast-growing progeny, DJ517-1*a*, was temperature sensitive, growing rapidly at 25° C but showing no growth at 38° C. This mutant was prototrophic, and was therefore provisionally called *un-24*, a temperature-sensitive, conditional mutation affecting an unknown function. The morphology of strain DJ517-1*a* at 25° C was also distinctive. Aerial hyphae were short and conidiation was limited to the surface of the colony; this morphology was similar to that of the peach (*pe*) mutant (Perkins et al. 1982).

Linkage mapping of *un-24*

The cross DJ517-1*a* × 74-OR23-1V*A* (Cross 3, Table 2) demonstrated that the *peach*-like morphology and the temperature sensitivity segregated independently, such that four distinct categories of progeny were produced: 20 parental wild-type; 23 parental *peach*-like morphology, temperature-sensitive; 21 *peach*-like morphology; and 18 wild-type morphology, temperature sensitive. The *peach*-like morphology segregated independently of the temperature-sensitive phenotype and of the duplicated *T*(*AR18*) region, but was linked to mating type (25% recombination). The recovery of a morphological mutation on LGI is unexplained since this position should not be subject to duplication-driven mutation events.

One of the 18 temperature-sensitive strains with wild-type morphology, DJ826-1, was investigated further. Crosses with both *T*(*AR18*)*A* and *T*(*II* → *VI*)*P2869A* (Cross 4, Table 2) revealed that the temperature-sensitive mutation in strain DJ826-1 maps to LGII and segregates in a 1:2 ratio (mutant to wild-type), indicating that *un-24* is covered by, and recessive to, its wild-type allele in the duplication progeny (data not shown). Linkage of *un-24* to *het-6* was established in two separate three-point crosses (Crosses 6 and 8, Table 2). For the first mapping cross, DJ949-90 *un-24 A* (FGSC 7354, from Cross 4, Table 2) was crossed to a marked strain of Oak Ridge *het-6* genotype (Cross 5, Table 2) to yield the strain *ro-7 un-24 het-6*^{OR} (DJ960-2*a*), which was then crossed to a marked Spurger *het-6* genotype, DJ959-1 *het-6*^{SP} *thr-2 A* (Cross 6, Table 2). For the second mapping cross, C5-44 *trp-1*; *ro-7 un-24 het-6*^{OR} *het-C*^{OR} *A* was selected from 1847 progeny of the cross *trp-1*; *am a* × DJ986-1 *ro-7 un-24 het-6*^{OR} *het-C*^{OR} *A* (Cross 7, Table 2). C5-44 was then crossed with RLM58-18 *het-6*^{PA} *het-C*^{PA} *pyr-4*; *inl a* (Cross 8, Table 2). Cross 6 gave four out of 151 progeny that were initially scored as recombinant for *un-24* and *het-6*. At least one of these progeny was subsequently found to be non-recombinant, and was probably mis-scored because of the osmotic remedial characteristic of *un-24* (see below). Cross 8 gave no recombinants among 220 progeny scored. These mapping crosses indicate that *un-24* is closer than 2.6 map units and centromere distal to *het-6* (Fig. 1). Strains C8c-164 and -165 (both *un-24*; *trp-1*; *inl a*) were selected from Cross 8 for use in subsequent experiments.

Growth rate of *un-24* strains

A comparison of wild-type and *un-24* growth rates at temperatures between 20 and 39° C is depicted in Fig. 2. DJ949-90 *un-24 A* showed near wild-type growth rates at temperatures between 20 and 25° C. Growth rates at 30° C of wild-type and *un-24* strains were significantly different by Analysis of Covariance ($P \leq 0.001$). Since the growth rate of *un-24* strains at 34 and 39° C was zero, they could not be compared to wild type by this statistical test. At 30° C, conidial germination is slower in *un-24* strains but conidia are produced at about the same time as in wild-type strains.

Growth of *un-24* strains at 37° C was occasionally noted on the glass above the agar surface in culture tubes and in partially desiccated petri dishes. This observation suggested that *un-24* might belong to the class of osmotic remedial mutants (R.L. Metzenberg, personal communication; Hawthorne and Friis 1964; Metzenberg 1968) and this was tested as shown in Fig. 3. Temperature sensitivity of *un-24* strains could be nearly, or completely overcome by amending the culture medi-

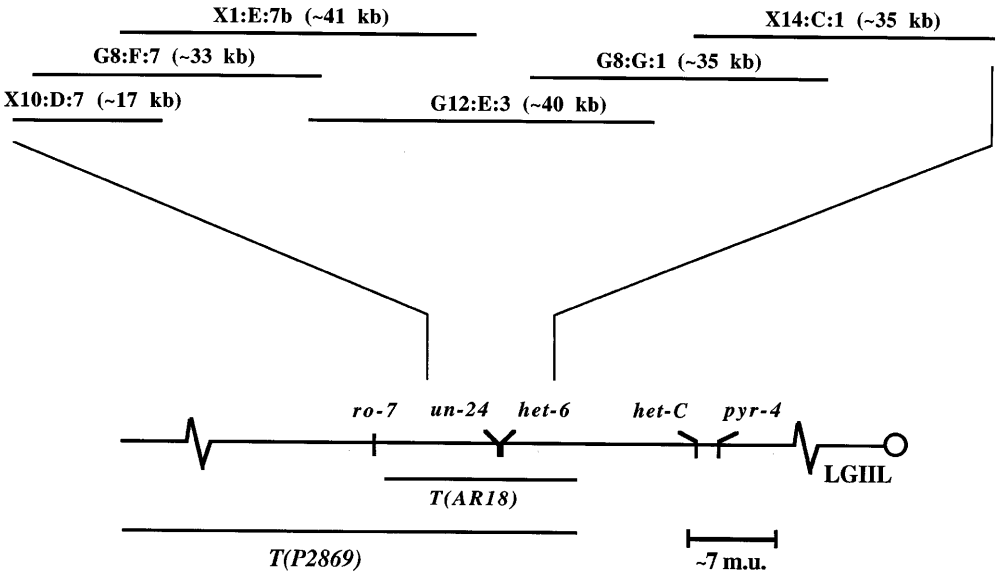


Fig. 1 Linkage map and cosmids in the vicinity of *un-24*. The relative positions and insert sizes of cosmids are shown at the *top*. The scale bar at the bottom *right* refers to the approximate map unit distance for the genetic markers shown on LGII (Perkins et al. 1982). The translocated regions of *T(AR18)* and *T(P2869)* *below* the linkage map

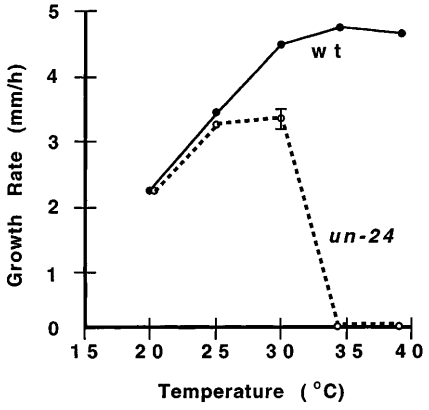


Fig. 2 Growth rates of 74-OR-23-1V *A* (wt) and DJ949-90 *un-24 A* (*un-24*) *N. crassa* strains at 20, 25, 30, 34 and 39° C. Standard error bars do not exceed the width of data points except in the case of the *un-24* strain grown at 30° C, as shown

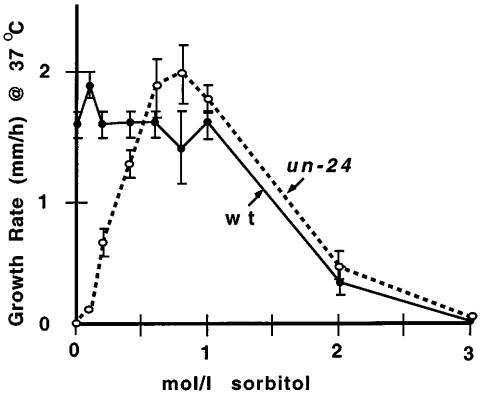


Fig. 3 Growth of the temperature-sensitive *un-24* strain in medium of high osmolarity. Growth rates were determined for 74-OR-23-1V*A* (wt, *continuous line*) and C8c-164 *un-24*; *trp-1*; *inl A* (*un-24*, *dashed line*) grown on petri plates on Vogel's medium with tryptophan and inositol and supplemented with the indicated concentrations of sorbitol. *Bars* represent standard errors (n = 3)

um to include 0.6 to 1 M sorbitol. Similar growth responses were observed following the addition of glycerol, mannitol, glucose and KCl, except that optimum growth of the *un-24* strain at 37° C was obtained on about 0.2 M KCl.

Localization of *un-24* by RFLP analysis

The position of *un-24* was determined with respect to a set of overlapping cosmids that span the translocated region in strain *T(AR18)* (Smith et al. 1996). Overlapping cosmids in the *un-24* region are shown in Fig. 1 together with the relative positions of genetic markers used in the region. The strategy adopted to analyze crossovers in the vicinity of *un-24* was as follows. It was determined that strains RLM58-18 and C5-44 differed in RFLP patterns within the *T(AR18)* translocated region. The RFLP pattern in strain RLM58-18 is designated as the Panama (PA) type, that of C5-44 as the Oak Ridge (OR) type. These two strains also differed at the genetic markers *ro-7*, *un-24*, *het-6*, *het-C*, and *pyr-4* (Table 1). The genotypes of 220 progeny from a cross between these two strains (Cross 8, Table 2) were determined for these five genetic markers on LGII, in addition to the unlinked markers *inl* and *trp-1*. RFLP patterns were determined for progeny with crossovers between *ro-7* and *pyr-4* using the cosmid probes X10:D:7, G8:F:7, G12:E:3, X1:E:7b, G8:G:1 and X14:C:1 (Fig. 1). Crossover points were evident along the contiguous set of cosmids in some C8c progeny as a switch-over in RFLP pattern, from one parental type to the other, and by the occurrence of a hybrid RFLP pattern (OR/PA) at the crossover point. RFLPs in two of the progeny (C8c-106 and C8c-353) indicated that *un-24* was located within, or centromere-proximal to, the region of overlap between G12:E:3 and G8:G:1. A hybrid RFLP pattern was also detected with cosmid X14:C:1 in C8c-32, which

indicated *un-24* was within or centromere-distal to this cosmid. These crossover points indicated that *un-24* lies within the region covered by G8:G:1 and X14:C:1.

Complementation of *un-24*

Tests for complementation of the temperature sensitivity were carried out by transforming strain C8c-165 with cosmids localized in and around the region identified by RFLP analyses as containing *un-24*. Initially, transformants were regenerated at 30° C on plates containing hygromycin and then subcultured and incubated at 37° C to test for growth at the restrictive temperature. The largest numbers of transformants (about 600/μg DNA) were observed when G12:E:3 and G8:G:1 cosmids were used as transforming DNAs. These transformants grew at wild-type rates at 37° C, unlike colonies transformed with other cosmids in the region or with pMP6 (hyg^R control), in which growth was inhibited at 37° C. Subsequently, we found that hygromycin selection was not necessary and that complementation of *un-24* could be selected for directly at 37° C.

Localization of *un-24* within G8:G:1 and G12:E:3

Approximately 11 kb of DNA is shared by cosmids G12:E:3 and G8:G:1 (Fig. 1). The left portion of cosmid G8:G:1, including the region that overlaps cosmid G12:E:3, was subcloned into the pMOcosX vector using a *Not*I site within the insert DNA and the *Not*I sites in the vector (Orbach 1994) to give the subclone TLP-1-31. Restriction enzyme digestions prior to transformations indicated that *Eco*RI and *Mbo*I did not reduce *un-24* complementation by TLP-1-31 DNA. An 11.5-kb *Eco*RI fragment of TLP-1-31, cloned as p31Eco16 in the vector pOKE103, was shown to complement *un-24*, as did a 4.8-kb *Mbo*I-*Eco*RI fragment cloned in pOKE103 and designated as pECO16M.

DNA sequencing was initiated in p31ECO16 and pECO16M and primers were designed based on these sequences. Figure 4 shows the locations of selected restriction enzyme recognition sites and oligonucleotide primers around the 4-kb region of p31ECO16 that complements *un-24*. Primer pairs were used to amplify PCR products from p31ECO16 DNA and were transformed into *un-24* spheroplasts to test for complementation of the temperature-sensitive phenotype. This led to the identification of a 3.75-kb region bounded by the primers P3 and P6 that restored growth in *un-24* strains. The P3/P6 PCR product was cloned into pCRII and is referred to as p3/6OR; p3/6OR gave an average 450 colonies/μg of transforming DNA.

un-24 is a temperature-sensitive mutation in the gene for the large subunit of ribonucleotide reductase

The DNA sequence of the P3/P6 fragment that complements *un-24* was determined and three potential ORFs were revealed (Fig. 4). Complementation of the temperature sensitivity of *un-24* required the largest of these three ORFs; P3/P9 PCR fragments, which carry all of ORFs 2 and 3 but are missing a portion of the largest ORF, did not complement *un-24*. The largest ORF (*un-24* ORF, Fig. 5) encodes 929 amino acids and extends over 2931 bp (Figs. 4 and 5). The product shows about 50% amino acid identity to the large subunit of ribonucleotide reductase in the eukaryotes human, mouse,

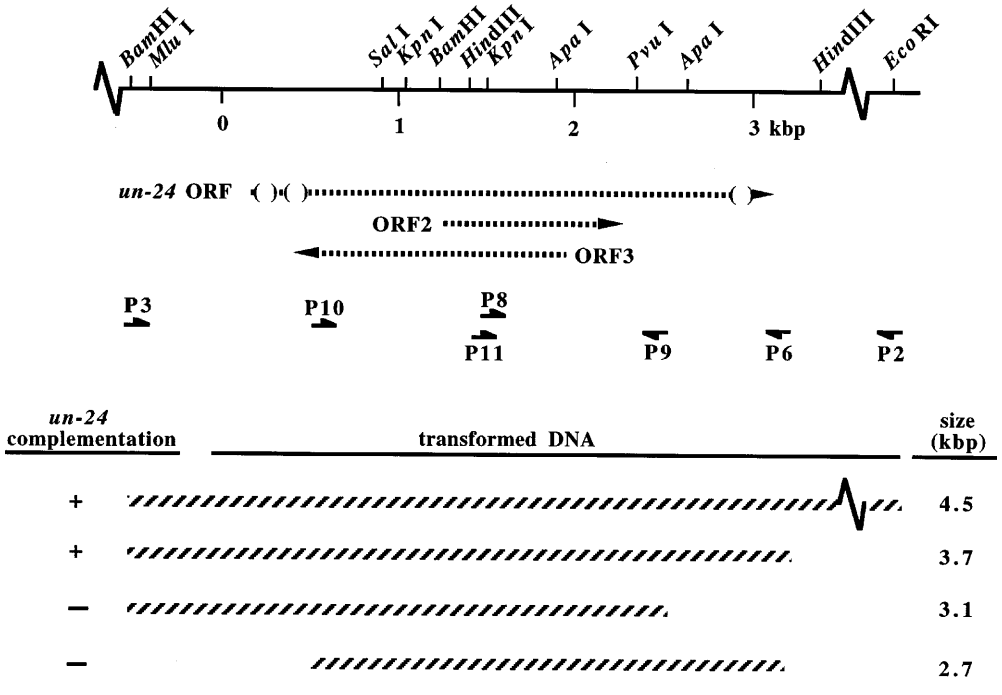


Fig. 4 Selected endonuclease recognition sites in a 4-kb segment of p31ECO16 that complements *un-24*. The *un-24* ORF with putative introns (*parentheses*), and ORFs 2 and 3, are shown *below* the map, with the positions of PCR primers used to identify the ORF that complements *un-24* indicated by the *arrowheads*. Complementation of the *un-24* phenotype by transforming DNA (PCR fragments) is indicated by a *plus*, lack of complementation by a *minus* (*bottom left*). The sequences of primers mentioned are: P2, 5′-CCTGAGGTGTATGAGGG-3′; P3, 5′-GGTGACACGGCGCTGTG-3′; P6, 5′-GTGCGGGCTTAACCGCTG-3′; P8, 5′-CCTTGCCTCTCTCGCCC-3′; P9, 5′-TTGCCCATGGTGGGTTCG-3′; P10, 5′-GGACGAGTTCGACTCGGC-3′; P11, 5′-CTCCGGATGAGGTTGCCG-3′

Saccharomyces cerevisiae, *Caenorhabditis elegans* and *Schizosaccharomyces pombe* (Fig. 6). The gene has three putative introns (Fig. 5) based on sequence comparisons with the *N. crassa* consensus 5′-donor (GˆGTAAGT), branch (CTRAC) and 3′-acceptor (WACAGˆ) sequences (Bruchez et al. 1993; Edelmann and Staben 1994). Splice sites were verified by analysis of cDNA sequences. The cDNA sequence of the 5′ region of this gene from the *Neurospora* Genome Project (NGP ID#NC5G9) database begins 179 bases upstream, and extends to position 684 of the genomic sequence presented in Fig. 5. While there are minor discrepancies between our sequence and that in the NGP, the splice sites of introns 1 and 2 are clear. We verified the splice sites for intron 3 (Fig. 5) by sequencing cDNA between positions 2510 and 3102. It is not likely that additional introns are present in this gene based on the conserved nature of the predicted amino acid sequence between positions 684 and 2510 (amino acid positions 147–760, Fig. 6). Introns 1, 2 and 3 are 69, 73 and 63 bp long, respectively. These sizes are within the range of 50 to 100 bases that is most often observed for introns in *N. crassa*

```
    CAAGTTTCGTGTTCTTCTGTTTGTCCCTCCATTTTGGTTGTCAATAACTGTACTTGTTCTTGTTCCCTCTTCTTCACCTT    80
    CTCCCCCACACATCAGACATTATGTATGTCAAGAAGCGCGgtatgttcacgactctcccttcctaccccaataccatcat   160
  1                      M  Y  V  K  K  R  D

    cacgcccaatgctaactctctcctcatagATGGCCGCCAGGAGCGCGTCCAGTTCGACAAGATCACAGCTCGTGTTTCGA   240
  8                               G  R  Q  E  R  V  Q  F  D  K  I  T  A  R  V  S  R

        A
    GGCTCTGTTATGGCCTCGACATGAACCATGTTGATCCTGTAGCCATTACACAAAAGGTCATCTCTGGCGTCTACGGTGGC   320
 25   L  C  Y  G  L  D  M  N  H  V  D  P  V  A  I  T  Q  K  V  I  S  G  V  Y  G  G

    GTCACCACAGCACAACTAGACGATCTGgtaagcaccttctccccagtccctctccttgtaccgattataaatcacgagct   400
 51 V  T  T  A  Q  L  D  D  L

    aacacataatctttccctagGCTGCCGAGACTGCCGCCTACATGACCGTCACCCACCCCGACTATGCCATCCTCGCCGCC   480
 60                      A  A  E  T  A  A  Y  M  T  V  T  H  P  D  Y  A  I  L  A  A

    CGTATCGCCGTGTCCAACCTCCACAAGCAGACCAAGAAGCAATGGTCGCTCGTTATCAGCGAGCTGTACAACTATGTCAA   560
 80 R  I  A  V  S  N  L  H  K  Q  T  K  K  Q  W  S  L  V  I  S  E  L  Y  N  Y  V  N

                                                          A   A
    CCCCAGGACCGGAAAGCACTCCCCCATGATCGCCAAGGACGTATACGAGTGTGTCATGCGCCACAAGGACGAGTTCGACT   640
107  P  R  T  G  K  H  S  P  M  I  A  K  D  V  Y  E  C  V  M  R  H  K  D  E  F  D  S

    CGGCAATTGTCTACGACCGTGACTTCAACTACCAATACTTCGGCTTCAAGACCCTCGAACGATCATACCTTCTTAAGCTC   720
134   A  I  V  Y  D  R  D  F  N  Y  Q  Y  F  G  F  K  T  L  E  R  S  Y  L  L  K  L

    GATGGCCAGATTGCTGAGCGTCCCCAGCACATGATCATGCGTGTTGCTGTCGGTATCTGGGGAGACGACGTTGAGCGGGT   800
160 D  G  Q  I  A  E  R  P  Q  H  M  I  M  R  V  A  V  G  I  W  G  D  D  V  E  R  V

    CATTGAGACTTACAACCTCATGTCCCTCAAGACCTTCACCCACGCTTCTCCCACCCTCTTCAACGCCGGTACTCCCCAGC   880
187  I  E  T  Y  N  L  M  S  L  K  T  F  T  H  A  S  P  T  L  F  N  A  G  T  P  Q  P

    CCCAGCTCTCTTCGTGCTTCCTGGTCGACATGAAGGAAGACAGTATCGAGGGCATCTACGATACCCTCAAGACCTGCGCC   960
214   Q  L  S  S  C  F  L  V  D  M  K  E  D  S  I  E  G  I  Y  D  T  L  K  T  C  A

    ATGATCTCTAAGATGGCTGGCGGTATCGGTCTCAACATCCACCGCATTCGTGCCACTGGCTCCTACATCGCTGGTACCAA  1040
240 M  I  S  K  M  A  G  G  I  G  L  N  I  H  R  I  R  A  T  G  S  Y  I  A  G  T  N

    CGGCACATCCAACGGTATCGTTCCCATGCTCCGTGTCTTCAACAACACCGCCCGCTACGTTGACCAGGGTGGCAACAAGC  1120
267  G  T  S  N  G  I  V  P  M  L  R  V  F  N  N  T  A  R  Y  V  D  Q  G  G  N  K  R

    GTCCTGGTGCTTTCGCCATCTACCTCGAGCCTTGGCACGCCGATGTCTTCGAGTTCCTTGACCTCCGCAAGAACCACGGT  1200
294   P  G  A  F  A  I  Y  L  E  P  W  H  A  D  V  F  E  F  L  D  L  R  K  N  H  G

    AAGGAAGAGGTGCGCGCTCGCGATCTCTTCCTTGCCCTCTGGATCCCCGATCTTTTCATGAAGCGTGTTGAGCAGAACGG  1280
320 K  E  E  V  R  A  R  D  L  F  L  A  L  W  I  P  D  L  F  M  K  R  V  E  Q  N  G

    CCAGTGGACTCTCATGTGCCCTCACGAGTGCCCTGGCCTTGCCGACGTTTACGGTGACGAGTTCGAAGCTCTGTACGAAA  1360
347  Q  W  T  L  M  C  P  H  E  C  P  G  L  A  D  V  Y  G  D  E  F  E  A  L  Y  E  K

    AGTACGAAAAGGAAGGCAAGGGTCGCAAGACCGTCAAGGCCCAGAAGCTTTGGTATGCCATCCTCGAGGCCCAGACTGAG  1440
374   Y  E  K  E  G  K  G  R  K  T  V  K  A  Q  K  L  W  Y  A  I  L  E  A  Q  T  E

    ACGGGCAACCCCTTCATGCTCTACAAGGATGCCTGCAACCGCAAGAGTAACCAGAAGAACCTGGGTACCATCCGCAGCTC  1520
400 T  G  N  P  F  M  L  Y  K  D  A  C  N  R  K  S  N  Q  K  N  L  G  T  I  R  S  S

    CAACCTGTGCACTGAGATTATCGAGTACTCTGCTCCGGATGAGGTTGCCGTTTGCAACCTTGCCTCTCTCGCCCTTTCCG  1600
427  N  L  C  T  E  I  I  E  Y  S  A  P  D  E  V  A  V  C  N  L  A  S  L  A  L  S  A

    CCTTTATCGACTACGAGAATGCCTCTTACGACTTCAAGAAGCTGCACGAGGTCACTCAAGTCGTCGTCCGCAACCTCAAC  1680
454   F  I  D  Y  E  N  A  S  Y  D  F  K  K  L  H  E  V  T  Q  V  V  V  R  N  L  N
```

Fig. 5 (Contd.)

```
     AAGATCATTGACATCAACCACTACCCCGTTAAGGAGGCCCACAACAGCAACATGCGTCACCGTCCCATTGGTGTCGGTGT  1760
480  K  I  I  D  I  N  H  Y  P  V  K  E  A  H  N  S  N  M  R  H  R  P  I  G  V  G  V

     TCAGGGTCTTGCTGATGCTTTCTTGGCTCTCCGCATGCCCTTCGATTCTGACGCGGCTAGCAAGCTCAACATCCAGATTT  1840
507   Q  G  L  A  D  A  F  L  A  L  R  M  P  F  D  S  D  A  A  S  K  L  N  I  Q  I  F

     TCGAGACCATCTACCACGCCGCTCTTACTGCCTCTTGCCAGCTCGCCAAGGAGCAGGGCCCGTACGCCACCTACGAAGGC  1920
534    E  T  I  Y  H  A  A  L  T  A  S  C  Q  L  A  K  E  Q  G  P  Y  A  T  Y  E  G

     TCACCCGTCTCCCAAGGAATCCTCCAATACGACATGTGGAACGTCACCCCTACCAACCTCTGGGACTGGACTGCTCTCAA  2000
560  S  P  V  S  Q  G  I  L  Q  Y  D  M  W  N  V  T  P  T  N  L  W  D  W  T  A  L  K

     GGCGGACATCAAGAAGTACGGTGTTCGCAACAGTCTGCTGCTTGCTCCTATGCCCACTGCTTCCACTTCTCAGATTCTTG  2080
587   A  D  I  K  K  Y  G  V  R  N  S  L  L  L  A  P  M  P  T  A  S  T  S  Q  I  L  G

     GCAACAACGAGTGCTTCGAGCCTTACACCTCTAACATCTACCAACGACGTGTGCTCGCCGGCGAGTTCCAGGTCGTCAAC  2160
614    N  N  E  C  F  E  P  Y  T  S  N  I  Y  Q  R  R  V  L  A  G  E  F  Q  V  V  N

     CCCTGGCTTCTCAGGGATCTTGTGGAGATGGGCCTTTGGTCTGATGCCATGAAGAACCGCATCATTGCTGAGGGCGGTTC  2240
640  P  W  L  L  R  D  L  V  E  M  G  L  W  S  D  A  M  K  N  R  I  I  A  E  G  G  S

     TATCCAGAACATCCAAAGCATTCCCAATGACATCAAGGCTCTCTATAAGACCGTCTGGGAGATTTCTCAGCGTACCATTG  2320
667   I  Q  N  I  Q  S  I  P  N  D  I  K  A  L  Y  K  T  V  W  E  I  S  Q  R  T  I  V

     TCAAGATGGCTGCCGATCGTGGTGCCTTCATTGATCAGTCCCAGTCCCTCAACATTCACATGCGCGAACCCACCATGGGC  2400
694    K  M  A  A  D  R  G  A  F  I  D  Q  S  Q  S  L  N  I  H  M  R  E  P  T  M  G

     AAGATTACCAGCATGCATTTCGCCGGCTGGAAGATGGGTCTCAAGACCGGCATGTACTACCTCCGTACGCAGGCTGCTGC  2480
720  K  I  T  S  M  H  F  A  G  W  K  M  G  L  K  T  G  M  Y  Y  L  R  T  Q  A  A  A

     TCAGCCCATCCAGTTCACTGTAGACCAGGAGGCTCTCAGGGCCACGGATGACCGAGTGGCCCCAGCCCACTCGGGTCTTA  2560
747   Q  P  I  Q  F  T  V  D  Q  E  A  L  R  A  T  D  D  R  V  A  P  A  H  S  G  L  K

     AGAAGCGCTCCCCTCCCGCTGGCACTTATACGTCTATCGTGTTGAGGGAGAACACTTCGGGCCCCCGCCCGTATGCCCAG  2640
774    K  R  S  P  P  A  G  T  Y  T  S  I  V  L  R  E  N  T  S  G  P  R  P  Y  A  Q

     ACTGGTGTTAGTGGCACTAGCACACCCATCGGCACCAGGGATGTTCCCACACCCGCCAGCACACCTCCTCCCACTGAGGT  2720
800  T  G  V  S  G  T  S  T  P  I  G  T  R  D  V  P  T  P  A  S  T  P  P  P  T  E  V

     TCCTGAAACTCTTGTACAGAGTGACAACAGGCCCCGTCCACTTGTCTCGCCCGCCAAGAGTGCCGGTTTCAAGGCCGATT  2800
827   P  E  T  L  V  Q  S  D  N  R  P  R  P  L  V  S  P  A  K  S  A  G  F  K  A  D  L

     TGCCCGAGCCTGAGAGCCCCAAGGCTCTGGCCACTGACCCGATTGTTAAGACCGAGGACATTGGCTCACCGCTGCTGGAG  2880
854    P  E  P  E  S  P  K  A  L  A  T  D  P  I  V  K  T  E  D  I  G  S  P  L  L  E

     AGAAAGGAGGGCCAGAATGAGGACGTGGACGAAGATAGCCAGGAGAGGGACGAGAATATCTACTCTAATGCTCCACTTTC  2960
880  R  K  E  G  Q  N  E  D  V  D  E  D  S  Q  E  R  D  E  N  I  Y  S  N  A  P  L  S

     TGAGCAACAAGTTGCTGCTTgtaagtgacaagacttagcccctaccagaccttgagacacacactaaccacatcaaataa  3040
907   E  Q  Q  V  A  A  C

     cagGCGCCTGGAATCCCGGTGCCGACCCCTCCTCCTGCGAGATGTGCAGCGGTTAAGCCCGCAC                  3104

914       A  W  N  P  G  A  D  P  S  S  C  E  M  C  S  G  *
```

Fig. 5 DNA sequence of the wild-type *un-24*$^+$ gene and deduced amino acid sequence of its product. Three nucleotide substitutions detected in *un-24* are given *above* the complete DNA sequence. Upstream and downstream sequences are given in *italics*, introns in *lower case*. Nucleotide numbers are given on the right, amino acid number on the left. The GenBank accession number for this sequence is AF171697

(Edelmann and Staben 1994). The position of intron 1 is identical to that of an intron in the homologous *rir1* (*cdc22*$^+$) gene of *S. pombe* (Fernandez Sarabia et al. 1993). The distances from the branch sites to the 3′-acceptor sites are 13, 17 and 15 nucleotides, for introns 1, 2 and 3, respectively; these are within the observed range of 6–29 nucleotides (Bruchez et al. 1993). After removal of these putative introns, the ORF shows about 52% identity to *RIR1* of *S. pombe*.

A comparison of the *un-24* and *un-24*$^+$ sequences revealed three point mutations at nucleotide positions 247, 613 and 618 of *un-24* (Fig. 5). The first mutation, at position 247, is a G → A transition which would result in a cysteine being replaced by a tyrosine. The mutation at position 613 is the result of a G → A transition that would convert a valine to an isoleucine. The G → A transition mutation at position 618 changes a methionine codon to an isoleucine codon in *un-24*. Transition mutations associated with duplicated sequences are consistent with a RIP-mediated origin of the *un-24* mutant allele.

Turnover time of the UN-24 protein

When *un-24* strains are grown at room temperature in growth tubes and then transferred to 37° C, they exhibit increased growth rates for up to 1 day followed by a gradual decrease until growth arrest occurs at about 2 days after the transfer to 37° C (Fig. 7). This suggests that either the available supply of deoxynucleotides, or functional ribonucleotide reductase, persists for up to 2 days at the elevated temperature, enabling DNA synthesis and an increase in growth rate as seen in wild-type strains (Fig. 2). It should be possible to distinguish

between these two possibilities by adding hydroxyurea to the growth tubes at the time they are transferred to the restrictive temperature. Hydroxyurea is thought to specifically inhibit ribonucleotide reductase activity by destabilizing the iron center of the R2 subunit and thus destroying the tyrosine free radical that initiates the long-range electron transfer to the active site of nucleotide reduction (reviewed in Wright et al. 1990). If the brief increase in growth-rate is due to a deoxynucleotide pool then the same response should occur in the presence of hydroxyurea. When 0.1 M hydroxyurea is added to the growth tubes at the time they are shifted to the restrictive temperature, the 1-day period of increased growth rate does not occur (Fig. 7). This suggests that the growth-rate increase that follows transfer to 37° C requires functional ribonucleotide reductase. Together, these results also indicate that functional UN-24, formed at permissive temperatures, remains in the mycelium for a period of a few days and is gradually replaced by mis-folded enzyme newly synthesized at restrictive temperatures.

Discussion

In this study, a temperature-sensitive conditional mutant was generated through a cross between *Dp*(*AR18*) and wild-type. The mutation mapped to within the translocated segments of both *T*(*AR18*) and *T*(*II* → *VI*)P2869, within 2.6 map units of the *het-6* locus on LGIIL, and was complemented by transformation with a 3.75-kb PCR product. A long ORF in this region associated with *un-24* complementation shows striking similarity to the large subunit of class I ribonucleotide reductases, the best characterized of which is from *E. coli*. This enzyme is essential for DNA synthesis, replacing the hydroxyl at the C-2′ position of ribonucleoside diphosphate with a hydrogen, to yield deoxyribonucleoside diphosphate (Reichard 1993). In addition to good overall alignment with other large subunits of ribonucleotide reductase, the predicted protein in Neurospora possesses several important conserved amino acid residues identified in

Fig. 6 (Contd.)

```
                                                Y
crassa          M------YVKKRDGRQERVQFDKITARVSRLCYGLDMNHVDPVAITQKVI  44
human           M------HVIKRDGRQERVMFDKITSRIQKLCYGLNMDFVDPAQITMKVI
mouse           M------HVIKRDGRQERVMFDKITSRIQKLCYGLNMDFVDPAQITMKVI
cerevisiae      M------YVYKRDGRKEPVQFDKITARISRLCYGLDPKHIDAVKVTQRII
elegans         MQRYNSTYVVKRDGRKEDVHFDKITSRIQKLSYGLNMDFVDPVAVAIKVI
vacvirus        M------FVIKRNGYKENVMFDKITSRIRKLCYGLNTDHIDPIKIAMKVI
pombe           M------FVYKRDGRQEKVAFDKITARVSRLCYGLDSDHVDPVEITQKVI
Ecoli           MNQ--NLLVTKRDGSTERINLDKIHRVLDWAAEGLHNVSISQVELRSHI-  47
                *       * ** *  *    ***         **

crassa          SGVYGGVTTAQLDDLAAETAAYMTVTH-PDYAILAARIAVSNLHKQTKKQ  93
human           QGLYSGVTTVELDTLAAETAATLTTKH-PDYAILAARIAVSNLHKETKKV
mouse           QGLYSGVTTVELDTLAAETAATLTTKH-PDYAILAARIAVSNLHKETKKV
cerevisiae      SGVYEGVTTIELDNLAAETCAYMTTVH-PDYATLAARIAISNLHKQTTKQ
elegans         SGLYKGVTTVELDNLAAETAASMTTQH-PEYALLAARIAVSNLHKKTNKV
vacvirus        QGIYNGVTTVELDTLAAEIAATCTTQH-PDYAILAARIAVSNLHKETKKL
pombe           SGVYPGVTTIELDNLAAETAATMTTKH-PDYAILAARIAVSNLHKQTEKV
Ecoli           -QFYDGIKTSDIHETIIKAAADLISRDAPDYQYLAARLAIFHLRKKAYGQ  96
                   * *  *           *       * *   **** *   * *
                                           II
crassa          WSLVISELYNYVNPRTGKHSPMIAKDVYECVMRHKDEFDSAIVYDRDFNY 143
human           FSDVMEDLYNYINPHNGKHSPMVAKSTLDIVLANKDRLNSAIIYDRDFSY
mouse           FSDVMEDLYNYINPHNGRHSPMVASSTLDIVMANKDRLNSAIIYDRDFSY
cerevisiae      FSKVVEDLYRYVNAATGKPAPMISDDVYNIVMENKDKLNSAIVYDRDFQY
elegans         FSEVMKTLHEFHHPHTGKHAPMISDETWAIIEKNADKLNSAIVYDRDYSY
vacvirus        FSEVMEDLFNYVNPKNGKHSPIISSITMDIVNKYKDKLNSVIIYERDFSY
pombe           FSTVVQQLHDYVNPKTDKPAPMISDKIYDIVMKHKDELDSAIIYDRDFTY
Ecoli           FEP--PALYDHVVKMVEM-GKYDNHLLEDYTEEEFKQMDTFIDHDRDMTF 143
                     *                                   *   **

crassa          QYFGFKTLERSYLL--KLDGQIAERPQHMIMRVAVGI-----WGDDVERV 186
human           NYFGFKTLERSYLL--KINGKVAERPQHMLMRVSVGI-----HKEDIDAA
mouse           NYFGFKTLERSYLL--KINGKVAERPQHMLMRVSVGI-----HKEDIDAA
cerevisiae      SYFGFKTLERSYLL--RINGQVAERPQHLIMRVALGI-----HGRDIEAA
elegans         TYFGFKTLERSYLL--KINKEIVERPQQMLMRVSIGI-----HGDDITSA
vacvirus        NYFGFKTLEKSYLL--KINNKIVERPQHMLMRVAVGI-----HQWDIDSA
pombe           NFFGFKTLERSYLL--RIDGKVAERPQHMIMRVAVGI-----HGEDIEAA
Ecoli           SYAAVKQLEGKYLVQNRVTGEIYESAQFLYILVAACLFSNYPRETRLQYV 193
                     * **  **          *  *     *

crassa          IETYNLMSLKTFTHASPTLFNAGTPQPQLSSCFLVDMKEDSIEGIYDTLK 236
human           IETYNLLSERWFTHASPTLFNAGTNRPQLSSCFLLSMKDDSIEGIYDTLK
mouse           IETYNLLSEKWFTHAPPTLFNAGTNRPQLSSCFLLSMKDDSIEGIYDTLK
cerevisiae      LETYNLMSLKYFTHASPTLFNAGTPKPQMSSCFLVAMKEDSIEGIYDTLK
elegans         IETYNLMSERYMTHASPTLFNSGTCRPQMSSCFLLTMSEDSILGIYDTLK
vacvirus        IETYNLLSEKWFTHASPTLFNAGTSRHQMSSCFLLNMIDDSIEGIYDTLK
pombe           IETYNLMSQRYFTHASPTLFNAGTPRPQLSSCFLVTMKDDSIEGIYDTLK
Ecoli           KRFYDAVSTFKISLPTPIMSGVRTPTRQFSSCVLIE-CGDSLDSINATSS 242
                   *   *        *      *   * *** *     **   *  *
```

RNR from *E. coli* (Fig. 6; Ormö and Sjöberg 1996; reviewed in Eklund et al. 1997).

In *S. cerevisiae* there are two forms of the large subunit of ribonucleotide reductase, *RNR1* and *RNR3* (Elledge and Davis 1990) which are about 80% identical at the amino acid level; *RNR1* is essential for mitosis and is tightly regulated during the cell cycle, whereas *RNR3* is non-essential. Transcription of both genes is induced by DNA damage. Based on Southern analyses, we detect only one form of this gene in *N. crassa*. The inferred amino acid sequence encoded by *un-24* in *N. crassa* is more similar to *RNR1* (64% identity) than to *RNR3* (62% identity). To our knowledge, *un-24* from *N. crassa* is the first gene encoding the large subunit of ribonucleotide reductase to be characterized from a filamentous fungus, and is of interest as the only sequenced temperature-sensitive mutation available in the large subunit of a ribonucleotide reductase from a eukaryote.

To verify that the mutation in *un-24* was caused by RIP, the wild-type and *un-24* alleles were mapped, cloned and sequenced. RIP mutations (G:C to A:T transitions) occur in duplicated loci prior to meiotic DNA replication in hyphae of Ascomycetes (Selker 1990). In addition, cytosine nucleotides often become methylated. A comparison of *un-24* and wild-type *un-24*$^{+}$ DNA sequences in the P3/P6 region revealed three GC → AT transition mutations at positions corresponding to amino acids 26, 124 and 125 (Fig. 5). All three mutations are within the first 220 residues of the N-terminus, which form a 12-helix allosteric activity site in the R1 subunits of the *E. coli* homolog (Uhlin and Eklund 1994; Eriksson et al. 1997; review in Eklund et al. 1997). The valine to isoleucine substitution at position 124 is a conservative change; isoleucine is present at this same position in the homolog from *C. elegans* (Fig. 6). Similarly, the conservative change from methionine to isoleucine at position 125 is probably not disruptive. This position can evidently be occupied by several different amino acids, including methionine, leucine, glutamate, asparagine and phenylalanine, in other organisms (Fig. 6). The change from cysteine to tyrosine at amino acid position 26, however, may be

Fig. 6 (Contd.)

```
crassa      TCAMISKMAGGIGLNIHRIRATGSYIAGTNGTSNGIVPMLRVFNNTARYV 286
human       QCALISKSAGGIGVAVSCIRATGSYIAGTNGNSNGLVPMLRVYNNTARYV
mouse       QCALISKSAGGIGVAVSCIRATGSYIAGTNGNSNGLVPMLRVYNNTARYV
cerevisiae  ECALISKTAGGIGLHIHNIRSTGSYIAGTNGTSNGLIPMIRVFNNTARYV
elegans     QCALISKSAGGIGLNVHKIRATGSVIAGTNGTSNGLIPMLRVYNNTARYV
vacvirus    RCALISKMAGGIGLSISNIRASGSYISGTNGISNGIIPMLRVYNNTARYI
pombe       MCAMISKTAGGIGINIHNIRATGSYIAGTNGTSNGIVPMIRVYNNTARYV
Ecoli       AIVKYVSQRAGIGINAGRIRALGSPIRGGEAFHTGCIPFYKHFQTAVKSC 292
                      ***     **  ** * *      *  *

crassa      DQGGNKRPGAFAIYLEPWHADVFEFLDLRKNHGKEEVRARDLFLALWIPD 336
human       DQGGNKRPGAFAIYLEPWHLDIFEFLDLKKNTGKEEQRARDLFFALWIPD
mouse       DQGGNKRPGAFAIYLEPWHLDIFEFLDLKKNTGKEEQRARDLFFALWIPD
cerevisiae  DQGGNKRPGAFALYLEPWHADIFDFIDIRKNHGKEEIRARDLFPALWIPD
elegans     DQGGNKRPGAFAIYLEPWHADIFEFVSLRKNTGPEEERARDLFLALWIPD
vacvirus    DQGGNKRPGVMAIYLEPWHSDIMAFLDLKKNTGNEEHRTRDLFIALWIPD
pombe       DQGGNKRPGAFAAYLEPWHADVMDFLELRKTHGNEDFRAREMFYALWIPD
Ecoli       SQGGV-RGGAATLFYPMWHLEVESLLVLKNNRGVEGNRVRHMDYGVQINK 341
             ***  * *        **              * *  * *       *

crassa      LFMKRVEQNGQWTLMCPHECPGLADVY---GDEFEALYEKYEKEGKGRKT 383
human       LFMKRVETNQDWSLMCPNECPGLDEVW---GEEFEKLYASYEKQGRVRKV
mouse       LFMKRVETNQDWSLMCPNECPGLDEVW---GEEFEKLYESYEKQGRVRKV
cerevisiae  LFMKRVEENGTWTLFSPTSAPGLSDCY---GDEFEALYTRYEKEGRGK-T
elegans     LFMKRVEKDQEWSLMCPCECPGLDDCW---GEEFEALYAKYEAEGRVRKT
vacvirus    LFMKRVKDDGEWSLMCPDECPGLDNVW---GDEFERLYTLYERERRYKSI
pombe       LFMQRVERNEQWTFFCPNEAPGLADVW---GDEFVALYEKYEKENRGRRS
Ecoli       LMYTRLLKGEDITLFSPSDVPGLYDAFFADQEEFERLYTKYEKDDSIRKQ 391
            *   *          *   ***        **  **  **

crassa      -VKAQKLWYAILEAQTETGNPFMLYKDACNRKSN-QKNLGTIRSSNLCTE 431
human       -VKAQQLWYAIIESQTETGTPYMLYKDSCNRKSN-QQNLGTIKCSNLCTE
mouse       -VKAQQLWYAIIESQTETGTPYMLYKDSCNRKSN-QQNLGTIKCSNLCTE
cerevisiae  -IKAQKLWYSILEAQTETGTPFVVYKDACNRKSN-QKNLGVIKSSNLCCE
elegans     -VKARKLWEHIVSNQIETGLPYITYKDAANRKSN-QQNLGTIKCSNLCTE
vacvirus    -IKARVVWKAIIESQIETGTPFILYKDACNKKSN-QQNLGTIKCSNLCTE
pombe       -LPAQKVWYAILQSQVETGNPFMLYKDSCNRKSN-QKNVGTIRCSNLCTE
Ecoli       RVKAVELFSLMMQERASTGRIYIQNVDHCNTHSPFDPAIAPVRQSNLCLE 441
              *             **       *  *  *           **** *

crassa      IIEYSAP--------DEVAVCNLASLALSAFIDYENA----SYDFKKLHE 469
human       IVEYTSK--------DEVAVCNLASLALNMYVT-SEH----TYDFKKLAE
mouse       IVEYTSK--------DEVAVCNLASLALNMYVT-PEH----TYDFEKLAE
cerevisiae  IVEYSAP--------DETAVCNLASVALPAFIETSEDGKTSTYNFKKLHE
elegans     IIEYSAP--------DEIAVCNLASIALNRYVT-PEK----KFDFVKLAE
vacvirus    IIQYADA--------NEVAVCNLASVALNMFVI-DGR-----FDFLKLKD
pombe       IVEYSSP--------DEVAVCNLASVALPTFIK--DG----KYNFQKLHD
Ecoli       IALPTKPLNDVNDENGEIALCTLSAFNLGAINNLDE-----------LEE 480
            *               * * * *    *                  *
```

responsible for the temperature-sensitive nature of the *un-24* mutant. In the *E. coli* RNR homolog, this position is occupied by alanine and is within one of four α-helixes that form a bundle similar to that found in thermolysin (Uhlin and Eklund 1994; Eriksson et al. 1997). This alanine is situated at the base of a cleft where three of the four helixes converge. It is within this cleft that ATP or dATP bind and, respectively, activate or deactivate the enzyme. The important features of the *E. coli* allosteric activity site (Eriksson et al. 1997) are also conserved in the *N. crassa* homolog (Fig. 6). The cysteine at position 26 in this domain in *N. crassa* (which is altered to a tyrosine in the *un-24* form) is conserved in an evolutionarily diverse group of organisms and could be important in hydrogen bonding or disulfide bridge stabilization. Perhaps thermal destabilization of the N-terminal allosteric activity site results in a loss of proper function at the distantly located active site of this essential enzyme. Alternatively, tyrosine has a large side group compared to that of cysteine and the substitution found in *un-24* may impede proper folding or packing of the α-helixes at the base of the cleft at restrictive temperatures. Another possibility is that the conformational change at elevated temperature may mimic the normal allosteric inhibition of this enzyme by dATP. However, the recessive nature of *un-24* in *un-24*/*un-24*$^{+}$ heterokaryons or partial diploids suggests that the inability of *un-24* mutants to grow at restrictive temperatures is not due to a loss of feedback inhibition and uncontrolled oversynthesis of dNDPs. Therefore, loss of ATP feedforward activation or loss of catalytic activity seem to be more satisfactory explanations.

Osmotic remediation may work by reducing the water content of the cell and thereby changing the conformation of an enzyme into an active form (Hawthorne and Friis 1964; Metzenberg 1968). This mechanism is consistent with our observation that restoration of growth of *un-24* strains at restrictive temperatures occurs during water stress, either on the addition of solutes to the growth medium or by partial desiccation. We also observed that *un-24* strains exhibit a brief increase in growth rate following a shift from a permissive to a

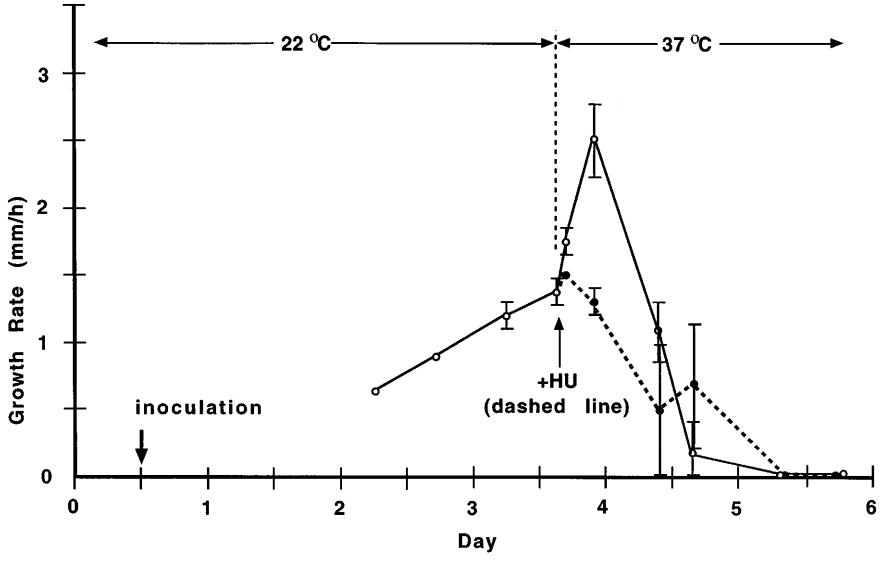
Fig. 6 (Contd.)

```
crassa      VTQVVVRNLNKIIDINHYPVKEAHNSNMRHRPIGVGVQGLADAFLALRM-  518
human       VTKVVVRNLNKIIDINYYPVPEACLSNKRHRPIGIGVQGLADAFILMRY-
mouse       VTKVIVRNLNKIIDINYYPIPEAHLSNKRHRPIGIGVQGLADAFILMRY-
cerevisiae  IAKVVTRNLNRVIDRNYYPVEEARKSNMRHRPIALGVQGLADTFMLLRL-
elegans     VTKVITRNLNKIIDVNYYPVEEARNSNMRHRPIGLGVQGLADCFMLMRY-
vacvirus    VVKVIVRNLNKIIDINYYPIPEAEISNKRHRPIGIGVQGLADAFILLNY-
pombe       VVKVVTRNLNKIIDVNYYPVPEARRSNMRHRPVGLGVQGLADAFFALRL-
Ecoli       LAILAVRALDALLDYQDYPIPAAKRGAMGRRTLGIGVINF--AYYLAKHG  528
                  * *    *   **   *       *    **

crassa      -PFDSDAASKLNIQIFETIYHAALTASCQLAKEQGPYATYEGSPVSQGIL  567
human       -PFESAEAQLLNKQIFETIYYGALEASCDLAKEQGPYETYEGSPVSKGIL
mouse       -PFESPEAQLLNKQIFETIYYGALEASCELAKEYGPYETYEGSPVSKGIL
cerevisiae  -PFDSEEARLLNIQIFETIYHASMEASCELAQKDGPYETFQGSPASQGIL
elegans     -PFTSAEARDLNKRIFETIYYAALEASCELAELNGPYSTYEGSPVSKGQL
vacvirus    -PFDSLEAQDLNKKIFETIYYGALEASCELAEKEGPYDTYVGSYASNGIL
pombe       -PFESAGAKKLNIQIFETIYHAALEASCEIAQVEGTYESYEGSPASQGIL
Ecoli       KRYSDGSANNLTHKTFEAIQYYLLKASNELAKEQGACPWFNETTYAKGIL  578
                  *  *    ** *      **   *   *           * *

crassa      QYDMWNVTPTNL------WDWTALKADIKKYGVRNSLLLAPMPTASTSQI  611
human       QYDMWNVTPTDL------WDWKVLKEKIAKYGIRNSLLIAPMPTASTAQI
mouse       QYDMWNVAPTDL------WDWKPLKEKIAKYGIRNSLLIAPMPTASTAQI
cerevisiae  QFDMWDQKPYGM------WDWDTLRKDIMKHGVRNSLTMAPMPTASTSQI
elegans     QFDMWGVTPTDQ------CDWATLRKKIAKHGIRNSLLMAPMPTASTAQI
vacvirus    QYDLWNVVPSDL------WNWEPLKDKIRTYGLRNSLLVAPMPTASTAQI
pombe       QYDMWNVNPTDL------WDWAELKEKIAKHGIRNSLLVAPMPTASTSQI
Ecoli       PIDTYKKDLDTIANEPLHYDWEALRESIKTHGLRNSTLSALMPSETSSQI  628
              *                  *  *   *   * ***   * **     **

crassa      LGNNECFEPYTSNIYQRRVLAGEFQVVNPWLLRDLVEMGLWSDAMKNRII  661
human       LGNNESIEPYTSNIYTRRVLSGEFQIVNPHLLKDLTERGLWHEEMKNQII
mouse       LGNNESIEPYTSNIYTRRVLSGEFQIVNPHLLKDLTERGLWNKEMKNQII
cerevisiae  LGYNECFEPVTSNMYSRRVLSGEFQVVNPYLLRDLVDLGIWDEGMKQYLI
elegans     LGNNESIEPYTSNIYSRRVLSGDFQIVNPHMLKDLVERGLWTDEMKNRLI
vacvirus    LGNNESVEPYTSNIYTRRVLSGEFQVVNPHLLRVLTERKLWNDEIKNRIM
pombe       LGFNECFEPYTSNMYQRRVLSGEFQIVNPWLLKDLVERDLWNEDMKNKLV
Ecoli       SNATNGIEP----------------------PRGYVSIKASKDGILRQVV  656
                   **

crassa      AEGGSIQNIQSIPNDIKALYKTVWEIS-QRTIVKMAADRGAFIDQSQSLN  710
human       ACNGSIQSIPEIPDDLKQLYKTVWEIS-QKTVLKMAAERGAFIDQSQSLN
mouse       ACNGSIQSIPEIPDDLKQLYKTVWEIS-QKTVLKMAAERGAFIDQSQSLN
cerevisiae  TQNGSIQGLPNVPQELKDLYKTVWEIS-QKTIINMAADRSVYIDQSHSLN
elegans     ANNGSIQNIDGIPSDIKELYRTVWEIS-QKDIIEMAADRGAYIDQSQSLN
vacvirus    ADGGSIQNT-NLPEDIKRVYKTIWEIP-QKTIIKMAADRGAFIDQSQSMN
pombe       MLDGSIQAIPEIPQDLKDLYKTVWEIS-QKTVIDYAADRGPFIDQSQSLN
Ecoli       PDY----------EHLHDAYELLWEMPGNDGYLQLVGIMQKFIDQSISAN  696
                         *   **                 **** * *
```

Fig. 6 Predicted amino acid sequence of the large subunit of ribonucleotide reductase from *N. crassa* aligned with homologs (Swiss-Prot, Bairoch and Apweiler 1997) from human (P23921), mouse (P07742), *Saccharomyces cerevisiae RNR1* (P21524), *Caenorhabditis elegans* (Q03604), Vaccinia virus (P12848), *Schizosaccharomyces pombe* (P36602) and *Escherichia coli* (P00452). Predicted amino acid alterations in *un-24* are given *above* the *N. crassa* wild-type sequence at positions 26, 124 and 125. An *asterisk below* the sequence indicates amino acid identity across all entries. Amino acid position is given on the right for *N. crassa* and *E. coli*. Conserved amino acids at the active site (*E. coli* positions 225, 437, 439, 441, 462, 730 and 731), P-loop region (*E. coli* positions 294 to 300) and allosteric activity site (*E. coli* positions 7, 9, 10, 17, 21, 22, 55 and 91) are indicated in *bold*

```
crassa      IHMREPT---MGKITSMHFAGWKMGL-KTG---MYYLRTQAAAQPIQFTV 753
human       IHIAEPN---YGKLTSMHFYGWKQGL-KTG---MYYLRTRPAANPIQFTL
mouse       IHIAEPN---YGKLTSMHFYGWKQGL-KTG---MYYLRTRPAANPIQFTL
cerevisiae  LFLRAPT---MGKLTSMHFYGWKKGL-KTG---MYYLRTQAASAAIQFTI
elegans     IHMAKPS---YAGITSMHFYGWKKGL-KTG---MYYLRTKPAVNAVQFTV
vacvirus    IHIADPS---YSKLTSMHFYGWSLGL-KTG---MYYLRTKPASAPIQFTL
pombe       IHLKDPS---YGKITSMHFYGWKKGL-KTG---MYYLRTMAASAAIKFTV
Ecoli       TN-YDPSRFPSGKVPMQQLLKDLLTAYKFGVKTLYYQNTRDGAEDAQ--D 743
                  *                       * *    **  *

crassa      DQEALRATDDRVAPAHSGLKKRSPPAGTYTSIVLRENTSGPRPYAQTGVS 803
human       NKEKLKDKEKVSKEEEEKER----------------------------
mouse       NKEKLKDKEKALKEEEEKER----------------------------
cerevisiae  DQKIADQATENVADISNLKRPSYMPSSASYAASDFVPAAVTANATIPSLD
elegans     DKNALKTNQQA-------------------------------------
vacvirus    DKDKIK------------------------------------------
pombe       DPVALRARNEESNEENKKPVIKNGKA-----------------------
Ecoli       DLV---------------------------------------------- 746

crassa      GTSTPIGTRDVPTPASTPPPTEVPETLVQSDNRPRPLVSPAKSAGFKADL 853
human       ----------------------------------------------------
mouse       ----------------------------------------------------
cerevisiae  SSSEASREASPAPTGSHSLTKGMAELNVQESKVEVPEVPAPTKNEEKA--
elegans     ----------------------------------------------------
vacvirus    ----------------------------------------------------
pombe       ----------------------------------------------------
Ecoli       ----------------------------------------------------

crassa      PEPESPKALATDPIVKTEDIGSPLLERKEGQNEDVDEDSQERDENIYSNA 903
human       ----------------------------------------------------
mouse       ----------------------------------------------------
cerevisiae  ---------------------------------APIVDDEETEFDIYNSK
elegans     ----------------------------------------------------
vacvirus    ----------------------------------------------------
pombe       ---------------------------------EISAEPTKEEIDIYNEK
Ecoli       ----------------------------------------------------

crassa      PLSEQQVAACAWNPGADPSSCEM--CSG                       929
human       ---NTAAMVCS---LENRDECLM--CGS
mouse       ---NTAAMVCS---LENREECLM--CGS
cerevisiae  VIA------CA---IDNPEACEM--CSG
elegans     ---ETPATVAE---SQD-EGCLM--CSG
vacvirus    -----PLVVC------DSEICTS--CSG
pombe       VLA------CS---IKNPEACEM--CSA
Ecoli       ------------PSIQDDGCESGACKI                       761
                              *    *
```

Fig. 7 Growth-rate response of C8c-164 *un-24*; *trp-1*; *inl A* grown at 22 °C for 3 days before shifting to 37 °C and further incubating in the presence (*dashed line*) or or absence (*solid line*) of 0.1 M hydroxyurea (HU). *Bars* represent standard errors (n = 4)



restrictive temperature but that this does not occur in the presence of the inhibitor hydroxyurea (Fig. 7), suggesting that active forms of ribonucleotide reductase persist in the mycelium for a period of up to about 2 days.

The large subunit of ribonucleotide reductase from *N. crassa* is strikingly similar to those in diverse life forms, except for a variable region from about amino acid position 766 of *N. crassa* to a conserved block of ten amino acids at the C-terminus (Fig. 6). In this region, the *N. crassa* protein has a long, inserted sequence with respect to the other examples shown in Fig. 6. Interestingly, there are at least two forms of this variable region in different *N. crassa* strains, as revealed by restriction site differences in the 3′ region of the gene, that are correlated with *het-6* incompatibility function (Mir-Rashed, Jacobson and Smith, unpublished). In the three-dimensional structure of *E. coli* R1 (Uhlin and Eklund 1994), the C-terminus is situated near the active site, close to regions that interact during R1 dimerization. We are now investigating whether this variable region near the C-terminus of the large subunit has a secondary function in non-self recognition in *N. crassa*.

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