Purification and Characterization of _Saccharomyces cerevisiae_ DNA Damage-responsive Protein 48 (DDRP 48)*

(Received for publication, July 23, 1992)

Shijie Sheng and Sheldon M. Schuster‡

From the Department of Biochemistry and Molecular Biology, College of Medicine, University of Florida, Gainesville, Florida 32610-0245

A yeast protein was purified from wild type _Saccharomyces cerevisiae_ (S. cerevisiae) to near homogeneity using an ethanolamine affinity chromatography procedure. The N-terminal amino acid sequencing and the amino acid composition analyses identified this protein as the product of the second open reading frame of _S. cerevisiae_ DNA Damage-responsive gene 48 (DDRP 48) (Treger, J. M., and McEntee, K. (1990) Mol. Cell. Biol. 10, 3174–3184). The first methionine residue encoded by the translation starting codon was not present in the mature protein which is designated as DDRP 48. DDRP 48 was found to be a negatively charged and highly hydrophilic glycoprotein. The glycosidase cleavage analyses suggested that DDRP 48 was mainly N-linked-glycosylated. The apparent molecular mass of DDRP 48 was estimated to be approximately 65 kilodaltons. DDRP 48 was found able to hydrolyze ATP and GTP yielding PP₃. The Kₘ values for ATP and GTP are 0.29 mM and 0.58 mM, respectively. The Western blot analysis demonstrated that DDRP 48 was expressed to various concentrations in different _S. cerevisiae_ strains. Increased DDRP 48 abundance was observed after yeast cells carrying the wild type RAD 52 gene were exposed to either ethylmethane sulfonate or heat shock treatments. After similar DNA-damaging treatments, however, no significant inductions of DDRP 48 were found in a rad 52 mutant strain. These observations are consistent with the predictions resulting from previous studies on transcriptional regulation of the DDR gene (Maga, J. A., McClanahan, T. A., and McEntee, K. (1986) Mol. & Gen. Genet. 205, 276–284; McClanahan, T., and McEntee, K. (1986) Mol. Cell. Biol. 6, 90–96).

Living organisms respond to environmental stresses such as heat shock, x-ray or UV light irradiation, and DNA alkylating chemical treatments in numerous ways. Three major inducible pathways in the prokaryote _Escherichia coli_ have been identified: the SOS response (1), the adaptive response to alklylation (2, 3), and the adaptive response to oxidative damages (4). A general feature in these responses is the elevated transcription of a set of genes that are involved in DNA repair (5, 6), mutagenesis (7), and inhibition of cell division (8), etc. When the ascomycete _Neurospora crassa_ was exposed to sublethal doses of UV, x-ray, or nitrous acid, they showed an enhanced ability to rescue lethally irradiated cells when fused into a heterokaryon (9). This observation is considered to be the first evidence of eukaryotic inducible responses to environmental stresses. Since then, more evidence has been obtained that similar inducible responses also exist in other eukaryotic systems. Several classes of eukaryotic inducible genes have been identified. Yet, in comparison with the well established _E. coli_ inducible response pathways, the regulation of the eukaryotic inducible response system is still a mystery, and the functions of those inducible genes and their products remain unclear.

_Saccharomyces cerevisiae_ has been used as a model for studying the regulation of eukaryotic inducible responses to environmental stresses. Several groups of inducible genes in _S. cerevisiae_ have been isolated and characterized. Among them are heat shock genes, DNA damage-inducible (DIN) genes, and DNA damage-responsive (DDR) genes (10–16). The results of previous studies demonstrate that the transcriptions of some inducible genes are preferentially elevated by certain kinds of environmental stresses. For example, heat shock genes YG102 and YG100 are activated by heat shock, but not by 4-nitroquinoline-1-oxide (NQO) treatment, and DNA damage-inducible gene DIN 1 can be induced by chemical mutagen, but not by thermal stress. More complex transcriptional regulation patterns have been observed with two DDR genes: DDR A2 and DDR 48. Ten to fifteen-fold more transcripts of DDR A2 and DDR 48 are obtained when the cells are exposed to either heat shock or NQO treatment (17).

In addition to environmental stresses, some _S. cerevisiae_ genomic elements, their products, or both, are involved in the regulation of stress-inducible gene expressions. RAD genes, required to repair UV-irradiated DNA in vivo, play some indirect roles in the transcriptional regulations of DDR A2 and DDR 48 genes (18). Normally, untreated wild type _S. cerevisiae_ cells do not produce a detectable amount of DDR A2 transcripts. The induction of DDR A2 transcription by NQO or _N_ -methyl- _N_ -nitro- _N_ -nitrosoguanidine (MNNG) seems to require the presence of a wild type RAD 3 gene. A higher abundance of DDR A2 transcripts is observed in untreated _S. cerevisiae_ rad 6 and rad 52 mutants. When these two mutants are exposed to NQO or MNNG, the increase of DDR A2 transcripts demonstrates a normal dose dependence. Detectable DDR 48 transcripts, on the other hand, are found in all untreated _S. cerevisiae_ strains tested. After the exposure

---

* This work was supported by Grant CA28725 from The National Cancer Institute of the National Institutes of Health, Department of Health and Human Services. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M36110.

‡ To whom correspondence and reprint requests should be addressed. Tel.: 904-392-8408; Fax: 904-392-8588.

1 The abbreviations used are: NQO, 4-nitroquinoline-1-oxide; MNNG, _N_ -methyl- _N_ -nitro- _N_ -nitrosoguanidine; PNGase F, _N_ -glycosidase F; POGase, _O_ -glycosidase; EMS, ethylmethane sulfonate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; MES, 2-( _N_ -morpholinol)ethanesulfonic acid.
to NQO of MNNG, the transcription of DDRP 48 increases in both wild type and rad 3 mutant cells. Although similar stress treatments also activate DDRP 48 transcription in rad 6 and rad 52 mutant S. cerevisiae, the induction is severely reduced compared to that in the wild type strains.

In 1990, the detailed structure of the DDRP 48 gene was determined (19). The entire DDRP 48 gene contains two overlapping open reading frames, each of which encodes a protein with a molecular mass of approximately 45 kilodaltons. Gene fusion experiments have demonstrated that only the second open reading frame is expressed in vivo. The deduced protein sequence contains multiple repeats of the peptide sequence Ser-Asn-Asn-X-Asp-Ser-Tyr-Gly where X is either Asn or Asp. Since similar repeated sequences are found throughout the primary structure, the protein is, therefore, predicted to be extremely hydrophilic. The function of the protein encoded by DDRP 48 (DDRP 48) remains unclear, although the viability studies of diploid yeast cells containing disrupted DDRP 48 genes (ddr 48) suggest that DDRP 48 may be involved in S. cerevisiae spontaneous mutations or recovery from mutations.

The purification of DDRP 48 was an unexpected outcome from our effort to purify recombinant human asparagine synthetase expressed in S. cerevisiae. Initially, a yeast protein with an apparent molecular mass of approximately 75 kDa was often co-purified with the recombinant enzyme by a monoclonal antibody affinity chromatography procedure (20). This protein was identified as yeast DDRP 48. In this paper, the purification and enzymatic characterizations of DDRP 48 are described. The expression of DDRP 48 in several S. cerevisiae strains cultured under different conditions are also reported.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—Yeast culture media was purchased from Difco. Cyanobrome-activated Sepharose 4B resin and PD-10 desalting columns were purchased from Pharmacia LKB and asparagine synthetase was produced by Kel Farm (Alachua, FL). Pyrophosphate assay reagents and other chemicals were purchased from Boehringer Mannheim. Protein concentration assay dye and desalting columns were purchased from Pharmacia LKB N-glycosidase F (PNGase F) and 4-galactosidase (POrase) were purchased from Boehringer Mannheim. Protein concentration assay dye and protein electrophoresis reagents were purchased from Bio-Rad. The polyclonal antiserum against both DDRP 48 and recombinant human asparagine synthetase was produced by Kel Farm (Alachusa, FL). Pyrophosphate assay reagents and other chemicals were purchased from Sigma.

**Cell Cultures**—The names, genotypes, and sources of the S. cerevisiae strains used in this study are listed in Table I. The growth condition for yeast cells in liquid media was 30 °C with vigorous shaking. In the DDRP 48 expression studies, single colonies of yeast cells on solid media were picked to inoculate 10 ml of YPD or yeast extract (30 °C with vigorous shaking) and harvested after another 30 min of incubation at 30 °C. While the first 2 aliquots of yeast cultures were being treated by heat shock or EMS, cells in the third set of aliquots were allowed to grow for another 30 min at 30 °C before the cells were harvested. The protein extraction was performed as follows (24): the cell pellets were washed with 1 ml of deionized distilled water. After 30 s of centrifugation at 3000 x g and 4 °C, the cell pellets were resuspended in 0.5 ml of cell lysis buffer (50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM β-aminoprophosphine) and mixed with 0.5 ml of ice-cold, acid-washed glass beads (0.45-0.5 mm in diameter). The cell disruption was complete after 6 cycles of vortexing (15 s) and cooling (5 min on ice). Clear cell extracts were collected after a 15-min centrifugation at 10,000 x g and 4 °C.

**Table I**

| Yeast S. cerevisiae strains | Genotypes | Sources |
|----------------------------|-----------|---------|
| AB116                      | a, leu2, trp1, ura3-52, prb1-1122, pep4-3, psc407, cis+ | Dr. Phil Barr (Chiron Corp.) |
| AB116/pBS24GAS1            | p, prc407, trp1, ura3-52 | Sheng et al. (20) |
| BJ2168                     | a, leu2, trp1, ura3-52, prb1-1122, pep4-3, psc407 | Yeast Genetic Shock Center (Berkeley, CA) |
| BI2168/47216/VTXAS1        | a, leu2, trp1, ura3-52, prb1-1122, pep4-3, psc407 | van Eyke and Schuster (21) |
| SEY 2102                   | a, leu2-3, 112, his4-519, gal2, sec2-2a9 | Professor Michael Douglas (Univ. of Texas, Southwestern Medical Center at Dallas) |
| AVY-4                      | Derived from SEY 2102 by insertion of LEU2 into ATP2 (atp2-165/LEU2) | Available in authors’ laboratory |
| AVY-4/103                  | Derived from SEY 2102 by insertion of LEU2 into ATP2 (atp2-165/LEU2) | Ramos and Wiaux (22) |
| MH4                        | L-Asparagine auxotroph (asn a, asn b) mutant derived from wild type strain Σ1278b | Originally from Bauer and Burgers (23) |
| JN984                      | a, leu2, trp1, his7, ade1, rad52:leu2, ise2 | Available in authors’ laboratory |
| MB4/YAS                    | L-Asparagine protoporphyrin |
phate-buffered saline buffer was lower than 0.1 μg/ml as detected by the method of Bradford (28). DDRP 48 was eluted with 0.1 M Na2CO3, (pH 10.6) at a flow rate of 0.25–0.30 ml/min. The eluate was collected in 3-ml fractions. Fractions with protein concentrations higher than 50 mg/ml were pooled together, and solid ammonium sulfate was added to a final saturation of 60%. Following centrifugation at 25,000 X g for 30 min, the precipitated protein was redissolved in a minimum volume of enzyme buffer (50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA (pH 8.0), 1 mM dithiothreitol, and 20% (v/v) glycerol). Excess ammonium sulfate was removed by chromatography through a PD-10 column, and the desalted protein was finally stored in the enzyme buffer at a concentration of 1.0–1.5 mg/ml.

**DDRP 48 Protein N-Terminal Sequencing and Amino Acid Composition Analysis—** Ethanolamine affinity-purified DDRP 48 was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (25), and electroblotted onto polyvinylidene difluoride membranes with 10 mM MES buffer (pH 6.0, containing 1 mM dithiothreitol, and 20% (v/v) glycerol). Excess ammonium sulfate was removed by chromatography through a PD-10 column, and the desalted protein was finally stored in the enzyme buffer at a concentration of 1.0–1.5 mg/ml. DDRP 48 was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (25), and electroblotted onto polyvinylidene difluoride membranes with 10 mM MES buffer (pH 6.0, containing 20% methanol) at 340 mA and 4 °C for 90 min. Two layers of polyvinylidene difluoride membranes were used to prevent the loss of protein due to overtransfer. The proteins blotted on the membranes were stained with Coomassie Brilliant Blue R-250 (26), and the DDRP 48 band was excised for sequencing. The protein N-terminal sequencing was performed by the Protein Chemistry Core Facility of the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida, using an Applied Biosystems 473A Protein Sequencer and 610A software for data analysis.

**DDRP 48 amino acid composition analysis was also performed by** the Protein Chemistry Core Facility of ICBR. Purified DDRP 48 was digested with 6 N HCl hydrolysis procedure. The quantities of the resulting amino acids were determined using a Beckman 6300 amino acid analysis system.

**Deglycosylation of DDRP 48—** Thirty micrograms of purified and desalted DDRP 48 (0.14 mg/ml) was denatured by boiling for 2 min in the presence of 1% SDS. Denatured DDRP 48 was cooled to room temperature and diluted with the deglycosylation buffer (20 mM sodium phosphate (pH 7.6), 10 mM sodium azide, 0.5% (v/v) Nonidet P-40) to a final concentration of 0.1 mg/ml. The resulting deglycosylation mixture was boiled for 2 min, cooled to room temperature, and then divided into three aliquots. DDRP 48 deglycosylation reactions were initiated by adding 4 units of PNGase F, 10 milliunits of POGase into each aliquot, respectively. The reaction mixtures were incubated at 37 °C for 50 h. SDS-PAGE sample buffer was used to terminate the reactions. One-third of each resulting mixture was used for SDS-PAGE analysis.

**Coupled Enzyme Activity Assay—**Sigma PP, assay reagents containing the enzymatic coupling system that utilizes PP, as a substrate and subsequently converts NAD+ to NADH (27) was used to determine the PP production catalyzed by DDRP 48. A vial of PP, assay reagents was reconstituted with 1 ml of deionized distilled water. The final DDRP 48 reaction mixtures of 100 μl contained 50 mM Tris-HCl (pH 7.0), 50 mM NaCl, 20 μl of reconstituted PP, assay reagent, 5 μg of purified DDRP 48, and substrates at various concentrations as indicated under “Results.” Upon the addition of DDRP 48 and quick vortex-mixing, the reaction mixtures were immediately transferred into quartz microcuvettes, and their absorbances at 340 nm (A340) at 37 °C were monitored on a Beckman DU-64 spectrophotometer for 20 min. The blanks were performed in a similar manner except that Mg2+ was excluded from the reaction mixtures. For each vial of PP, assay reagents, a standard curve was obtained using the rates of A340 decrease for pure PP, in DDRP 48 substrate mixtures. The productions of PP, in DDRP 48 reactions were calculated accordingly.

Protein concentrations were determined by the method of Bradford (28) using pure mouse immunoglobulin G to construct the standard curve. Protein molecular mass estimations by SDS-PAGE and protein Western blotting by polyclonal antisera were performed as described earlier (24, 29).

**RESULTS**

DDRP 48 was first isolated from yeast *S. cerevisiae* BJ2168/pVTXAS1 as a contaminant of recombinant human asparagine synthetase (20). When human asparagine synthetase was purified from the total cell-extractable protein by immunoadfinity chromatography, a yeast protein of an apparent molecular mass of approximately 75 kDa (previously designated as “YP 75”) was often co-purified. Such contamination could be eliminated by using an ethanolamine affinity pre-column procedure. Later, it was found that YP 75 could be specifically purified from crude yeast extract to near homogeneity (see Fig. 1, a and b) by one-step ethanolamine affinity chromatography. Approximately 500–800 μg of pure YP 75 was obtained from each liter culture of *S. cerevisiae* BJ2168. Purified YP 75 was highly hydrophilic and negatively charged. Isoelectric focusing gel electrophoresis of YP 75 indicated that the protein had an isoelectric point below 3.0 (data not shown). When it was electroblotted from SDS-PAGE gel onto polyvinylidene difluoride membranes for protein N-terminal sequencing, two layers of membranes were used. After a 90-min transfer at 340 mA at 4 °C, approximately 90% of YP 75 was blotted onto the second layer membrane. The sequence of the first 30 N-terminal amino acid residues of YP 75 was obtained by HPLC analysis (see Fig. 2) and compared to the known protein sequences in the genEMBL database. Except for the absence of the N-terminal methionine residue, this sequence was identical with that deduced from the nucleotide sequence of the second open reading frame of *S. cerevisiae* DNA damage-responsive 48 gene, DDRP 48 (1). Further evidence confirming that YP 75 is DDR 48 gene product DDRP 48 was obtained from amino acid composition analyses. As demonstrated in Table II, the amino acid composition of YP 75 is in good agreement with that of DDRP 48.

As illustrated in Fig. 1, a and b, the apparent molecular mass of DDRP 48 varied from 75 kDa to 65 kDa as the concentration of the acrylamide in SDS-PAGE was increased from 10% to 15%. No further significant changes in the apparent molecular mass of DDRP 48 were observed when the acrylamide concentration was higher than 15%. Such anomalous mobility in SDS-PAGE is considered to be typically characteristic of glycoproteins (30). Given the fact that the abundances of asparagine and serine residues in DDRP 48 are 23% and 26%, respectively, both N-link or O-link glycosylations seemed possible. In order to study the protein-carbohydrate linkages in DDRP 48, it was digested by PGNase

**FIG. 1.** a, SDS-PAGE of DDRP 48 when the acrylamide concentration was 10%. Lane 1, Bio-Rad low range protein molecular mass standards, from top to bottom: phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa); lane 2, 2 μg of ethanolamine affinity column-purified DDRP 48; b, SDS-PAGE of DDRP 48 when the acrylamide concentration was 15%. Lane 1, Sigma high range protein molecular mass standards, from top to bottom: β-galactosidase (116.0 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), fumarase (48.5 kDa), and carbonic anhydrase (31.0 kDa); lane 2, 2 μg of ethanolamine affinity-purified DDRP 48.
Purification and Characterization of DDRP 48

Fig. 2. The sequence of first 30 N-terminal amino acids of YP 75 compared with the N-terminal sequence predicted for DDRP 48.

**TABLE II**

| Amino acid composition of YP 75 compared with that predicted for DDRP 48 |
|-----------------|-----------------|-----------------|
| Amino acid residue | Amino acid composition of YP 75 | Amino acid composition predicted for DDRP 48 |
|-------------------|---------------------|---------------------|
| Ala               | 0.39 ± 0.03         | 0.46                |
| Asn + Asp         | 34.17 ± 1.2         | 36.48               |
| Arg               | 1.56 ± 0.27         | 1.39                |
| Glu + Gln         | 2.04 ± 0.22         | 2.31                |
| Gly               | 10.89 ± 0.33        | 11.32               |
| Ile               | 1.40 ± 0.40         | 0.69                |
| Leu               | 0.619 ± 0.41        | 0.23                |
| Lys               | 7.22 ± 0.31         | 6.93                |
| Met               | 0.41 ± 0.05         | 0.46                |
| Phe               | 1.11 ± 0.14         | 1.15                |
| Ser               | 26.89 ± 0.55        | 26.32               |
| Thr               | 0.43 ± 0.02         | 0.46                |
| Tyr               | 9.00 ± 0.53         | 10.16               |
| Val               | 1.47 ± 0.41         | 0.92                |
| His               | 0.77 ± 0.15         | 0                   |
| Pro               | 1.65 ± 0.36         | 0                   |

*The amino acid composition of YP 75 was the average result of three HPLC amino acid composition analyses.
*The amino acid composition of DDRP 48 was calculated based on the primary sequence deduced from the cDNA sequence (1).

F, POGase, and a mixture of PNGase F and POGase, respectively. The resulting mixtures were analyzed by SDS-PAGE. As shown in Fig. 3, three common protein bands of approximately 38 kDa, 44 kDa, and 55 kDa, respectively, were observed after DDRP 48 was treated with PNGase F and PNGase F/POGase (lanes 3 and 5). The protein band of approximately 38 kDa was the PNGase F which cleaves all types of asparagine-bound N-glycans when both the amino group and the carboxyl group are present in a peptide linkage (31). After DDRP 48 was treated with POGase (160 kDa), which liberates the disaccharide GalP(1-3)GalNAc from serine- or threonine-attached glycans (32), only one common band of approximately 33 kDa was observed (lane 4 and lane 5). As expected, the protein bands shown in lane 5 were the exact sum of those in lane 3 and lane 4. The identities of the fragments resulting from the deglycosidase digestions were not tested. While the possibility that the DDRP 48 breakdown was caused by the contaminating protease activity in the deglycosidases could not be ruled out, the fact that the PNGase F treatment resulted in a fragment of approximately 44 kDa, which coincides with the molecular mass of DDRP 48 protein predicted from the primary sequence, may suggest that DDRP 48 protein was N-linked to carbohydrates. According to the method of Segrest and Jackson (33), 65 kDa, the minimum apparent molecular mass of intact DDRP 48 on SDS-PAGE, is considered to be the real molecular mass of DDRP 48 whole molecule, 30.77% of which is glycan.

The polypeptide sequence of DDRP 48 protein core does not have any homology with that of human asparagine synthetase (EC 6.3.1.1) (34) which catalyzes the biosynthesis of L-asparagine as follows (35):

\[
\text{L-Aspartate + ATP + L-glutamine (or NH}_3\text{) } \rightarrow \text{L-asparagine + AMP + PP}_i + \text{L-glutamate} \]

Nevertheless, when the nature of DDRP 48 purification was not known, the possibility of DDRP 48 acting as a yeast intrinsic asparagine synthetase was investigated, because it was co-purified with recombinant human asparagine synthetase by immunoaffinity chromatography, and therefore had been suspected to share some structural and functional similarities with that enzyme. DDRP 48 was added into the substrate mixtures optimized for asparagine synthetase (36). Asparagine synthetase activities were determined either by measuring the production of L-asparagine by HPLC on an Applied Biosystems 420 Derivatizer/130A Separation System, or by measuring the production of PP, by a coupled enzyme activity assay. The coupled PP, assay was derived from the method of O’Brien (27). Asparagine synthetase substrates had no effect on the coupling enzymatic reactions. When pure PP, was added as described under “Experimental Procedures,” a linear detection range of 0–125 μM PP, was obtained. The results of numerous HPLC assays indicated that DDRP 48
was unable to catalyze L-asparagine synthesis in vitro. Under the same reaction conditions, however, the results of the coupled enzyme activity assays demonstrated significant stimulations of PPi production by DDRP 48. The effects of each substrate of asparagine synthetase on this DDRP 48-catalyzed PPi formation were carefully examined. It was found that only ATP and Mg\textsuperscript{2+} were required. In the following studies, DDRP 48 activity was always assayed in a reaction mixture containing 50 mM Tris-HCl (pH 7.0), 50 mM NaCl, 10 mM ATP, and 15 mM MgCl\textsubscript{2}. Several nucleotides were tried as ATP substitutes. As reported in Table III, DDRP 48 activity was found only when ATP was replaced by GTP. The $K_m$ values of ATP and GTP in DDRP 48 reactions were 0.29 mM and 0.58 mM, respectively (see Fig. 4). When ATP hydrolysis activities of DDRP 48 were measured by coupled PPi assay with additional 10 mM concentrations of AMP or ADP, the DDRP 48 specific activities were reduced 18% and 20%, respectively. In a parallel experiment, the addition of 10 mM cAMP resulted in a nearly 40% decrease of DDRP 48 specific activity (see Fig. 5).

The DDR 48 gene was so named because its transcripts were elevated more than 10-fold after S. cerevisiae cells were exposed to heat shock or chemical mutagenesis (19). While little is known about the role, if any, of DDRP 48 in yeast cellular responses against DNA-damaging treatments, it is important to find out whether the DDRP 48 protein concentrations were also elevated in response to the above stimuli. When DDRP 48 was first co-purified with recombinant human asparagine synthetase, rabbit polyclonal antisera against a mixture of these two proteins was produced. The polyclonal antisera thus obtained was used in DDRP 48 immunoblotting analysis. The quantities of DDRP 48 were estimated by a secondary antibody-linked alkaline phosphatase reaction (24). As expected, the polyclonal antisera showed cross-reactivities with human asparagine synthetase (see Fig. 6). In addition, the polyclonal antisera bound to a protein band of 55 kDa, which could be a major degradation product of DDRP 48 (see Fig. 3). A detectable DDRP 48 signal was obtained from every untreated S. cerevisiae strain tested. This result is consistent with a previous observation that low abundances of DDR 48 transcripts in untreated yeast cells were always detectable (19). The intensities of DDRP 48 signals in Fig. 6 appeared to vary from strain to strain with no obvious correlations to the yeast genotypes.

The expression of DDRP 48 could be regulated at both the transcriptional and translational levels. The DDR 48 gene has two overlapping open reading frames each encoding a protein of approximately 45 kDa. The DDRP 48 characterized in this study is encoded by the second open reading frame. No translational regulation studies on DDRP 48 have been performed. Yet, some evidence suggests that the transcription of the DDR 48 gene was indirectly regulated by yeast RAD genes. In order to investigate the roles of RAD genes in DDRP 48 expression, a wild type S. cerevisiae strain, BJ2168, and a rad 52 mutant strain, JN934, were chosen for use in a DDRP 48 induction experiment. After the cells were either heat-shocked or treated with EMS, equal amounts of cell-extractable protein from each culture were subjected to Western blotting with polyclonal antisera. As shown in Fig. 7, untreated S. cerevisiae BJ2168 (lane 2) produced less DDRP 48 than untreated S. cerevisiae JN934 (lane 5). DDRP 48 expression was stimulated to approximately the same extent by heat shock and EMS in BJ2168 cells (lane 3 and lane 4). No significant induction of DDRP 48 occurred in JN934 cells upon the same treatments (lanes 5–7). The difference in the DDRP 48 induction pattern between BJ2168 and JN934 may imply that RAD 52 gene was somehow involved in the regulation of DDRP 48 expression. Since the high abundance of DDRP 48 was also found in other untreated yeast S. cerevisiae strains, e.g. AB116/pBS24GAS1 (lane 8 in Fig. 6) carrying the wild type RAD 52 gene, the role of RAD genes in DDRP 48 expression may not be as important as previously proposed.

**DISCUSSION**

Since 1984, when it was first found that the transcripts of S. cerevisiae DNA damage-responsive gene DDR 48 and DDR

---

**TABLE III**

| Nucleotides | DDRP 48 specific activity* (nmol PPi/min/mg) |
|------------|------------------------------------------|
| ATP        | 25.6                                     |
| GTP        | 15.7                                     |
| CTP        | ND\textsuperscript{a}                   |
| ITP        | ND\textsuperscript{a}                   |
| XTP        | ND\textsuperscript{a}                   |

\textsuperscript{a}DDRP 48 activities were measured by the PPi-coupled assay at 37 °C for 30 min. The reaction mixtures contained reconstituted PPi assay reagent, 10 mM nucleotide, 15 mM MgCl\textsubscript{2}, 50 mM Tris-HCl (pH 7.0), 50 mM NaCl. Each specific activity is the average result of two duplicate experiments.

\textsuperscript{b}No detectable PPi production was observed under the assay condition as described in a.
A2 were specifically induced when the cells were exposed to UV irradiation or DNA-alkylating reagent N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) (10), studies of the DDRP 48 gene have been focused on its transcription regulation. The elevation of DDRP 48 transcripts is found to be a result of increased mRNA synthesis which is dually regulated by DNA-damaging reagent treatments and thermal stress (10). It is worth noting that the DDRP 48 sequence has no homology with any known yeast heat shock genes. The possibility that the DDRP 48 gene and its product, DDRP 48, may be involved in a yeast SOS-like pathway (10) has been investigated. It seems unlikely that DDRP 48 is directly associated with DNA-repair activity. Some evidence suggests that the wild type DDRP 48 gene is required for maintaining the rate of spontaneous mutagenesis in yeast (19). Current understanding of

FIG. 5. Inhibition of DDRP 48-ATP hydrolysis activity by nucleotides. DDRP 48 activities were determined by the coupled PP, assay as described under "Experimental Procedures." Cyclic AMP, AMP, and ADP were added separately to the reaction mixtures containing 50 mM Tris-Cl (pH 7.0), 50 mM NaCl, 5 µg of purified DDRP 48, 10 mM ATP, 15 mM MgCl₂, and 20 µl of reconstituted PP, assay reagent. The final concentrations of cyclic AMP, AMP, and ADP were 10, 10, and 10 mM, respectively.

FIG. 6. Western blotting of DDRP 48 from untreated S. cerevisiae strains with polyclonal antisera. The Bio-Rad protein molecular mass standards are indicated by →, from top to bottom: phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), and carbonic anhydrase (31.0 kDa). The recombinant human asparagine synthetase band is indicated by —. The DDRP 48 band is indicated by . . . Lanes 1–8 are 40 µg of cell extract from each of the following S. cerevisiae strains: lane 1, MB4; lane 2, MB4/ YAS; lane 3, SEY; lane 4, AVY105; lane 5, AVY; lane 6, BJ2168/pVTXAS1; lane 7, AB116/pBS24GAS1; lane 8, AB116. Lane 9 is 0.5 µg of purified DDRP 48.

FIG. 7. Western blotting of DDRP 48 from untreated S. cerevisiae strains and DDRP 48 from heat shock or EMS-treated S. cerevisiae strains. The Bio-Rad protein molecular mass standards are indicated by →, from top to bottom: phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), and carbonic anhydrase (31.0 kDa). DDRP 48 bands are indicated by . . . Lane 1 is 0.5 µg of purified DDRP 48; lanes 2–7 are 40 µg of cell extract of the following S. cerevisiae strains: lane 2, untreated BJ2168; lane 3, BJ2168 treated with heat shock; lane 4, BJ2168 exposed to EMS; lane 5, untreated JN934; lane 6, JN934 treated with heat shock; lane 7, JN934 exposed to EMS.

The significance of DDRP 48, as well as the regulation of DDRP 48 expression, in the yeast cellular response to environmental stresses is greatly limited because no characterization of this protein has been reported. The structural and enzymatic characterization of DDRP 48 was made possible by the fact that native DDRP 48 was purified to near homogeneity. As described under "Results," DDRP 48 is a negatively charged glycoprotein with a saccharide component being approximately 30% of the total molecular mass. Since polysaccharides were found to lack the DDRP 48 protein core mainly through N-glycan bonds, the heavy glycosylation seemed to be dictated by the high abundance of asparagine residues in DDRP 48. For future studies, it will be necessary to determine the subcellular distribution of DDRP 48 in S. cerevisiae, because generally the subcellular location of a glycoprotein is related to its physiological function. Considering the unique aspartate/asparagine/serine-rich repetitive sequence, DDRP 48 may represent a new class of eukaryotic stress-inducible proteins.

Our results showed that DDRP 48 had low Mg²⁺-dependent ATP (or GTP) hydrolysis activity. Since one product of ATP (or GTP) hydrolysis by DDRP 48 turned out to be PP, the other product could be either AMP or cAMP (or GMP or cGMP), provided that under the assay conditions, DDRP 48 catalyzed only one reaction. The significant decrease of DDRP 48 specific activity in the presence of cAMP favored the possibility of cAMP being the second product. In an attempt to identify the nucleotide product in the DDRP 48 reaction, [3,3-³²P]ATP and [α-³²P]GTP were used as substrates, respectively, in the DDRP 48 reactions, and the nucleotide species in the reaction mixtures were separated by DEAE-anion exchange paper chromatography. Our preliminary results suggested that small amounts of radioactive cAMP and cGMP were produced (data not shown). Further investigation will be necessary to determine whether the production of cAMP and cGMP was caused by the contaminating cyclase activity in the DDRP 48 preparation.

ATP hydrolysis activity has been related to several stress-induced proteins in both prokaryotes and eukaryotes. The E. coli DnaK, which is involved in the initiation of DNA replication, has been found to bind tightly to ATP and catalyze a weak DNA-independent ATP hydrolysis in vitro, producing ADP and inorganic phosphate (37, 38). SSAI, a member of
the S. cerevisiae hsp 70 family, is involved in the post-translational transport of proteins across mitochondrial membranes (39). It is known that an early step of such protein import into mitochondria is ATP-dependent. In fact, almost all hsp 70 and related proteins in eukaryotes have high affinities for ATP and are often found in association with other proteins (40). Interestingly, our experiments with purified DDRP 48 demonstrated that DDRP 48 could use both ATP and GTP in vitro. ATP appears to bind to DDRP 48 more tightly than GTP. It is not clear whether ATP/GTP hydrolysis is the main activity or only a side reaction of DDRP 48, i.e. DDRP 48 may primarily catalyze an ATP (or GTP)-dependent reaction and, when other substrates are absent in the assay mixture, it could slowly hydrolyze ATP (or GTP). If ATP/GTP hydrolysis is the main function of DDRP 48, a thorough understanding of the mechanism of this enzyme activity would provide more insight into the pathways of yeast-inducible responses to environmental stresses. It would be also important to find out whether some possible in vivo association of DDRP 48 with other proteins could affect the selectivity of nucleotide bindings and whether such different bindings are important for regulating the biological activities of DDRP 48 in yeast-inducible responses to environmental stresses.

The differences between DDRP 48 and some better characterized yeast heat shock proteins that have ATPase activities are obvious. Firstly, the DDRP 48 protein sequence is not homologous to any known sequences of yeast heat shock proteins. Secondly, as demonstrated in this paper, DDRP 48 is abundant in yeast cells under normal growth conditions and can be induced by more than one kind of DNA-damaging treatments. The DDRP 48 induction patterns in the RAD 52 wild type strain and the rad 52 mutant strain (shown in Fig. 7) were found to be consistent with corresponding DDR 48 gene transcription induction patterns (18). The fact that the DDRP 48 basal concentration varies among RAD 52 wild type S. cerevisiae strains (Fig. 6) suggested that RAD 52 may not be the only gene involved in the regulation of DDRP 48 expression. The substantial amounts of DDRP 48 found in untreated S. cerevisiae, as well as the fact that DDRP 48 transcripts can be elevated by many kinds of stresses, also imply that DDRP 48 expression is subject to a more complicated regulation. In order that yeast could better adapt to frequent environmental changes, it is possible that not only the expression but also the biological activities of DDRP 48 are subject to delicate regulation. As an example of the possibilities, it should be noted that the DDRP 48 characterized in this study was purified from wild type S. cerevisiae cells that had not been treated with any DNA-damaging agents. It would be interesting to find out whether the DDRP 48 from different mutant strains or from cells treated with DNA-damaging agents have the same enzymatic activity and sub-cellular distribution.

Acknowledgment—We thank Dr. Thomas Row for generosity and kind advice.

REFERENCES
1. Witten, E. (1979) Bacteriol. Rev. 43, 869-907
2. Cairns, J., Robbins, P., Sedgwick, B., and Talmud, P. (1981) Proc. Nucleic Acids Res. 26, 237-240
3. Samson, L. and Cairns, J. (1977) Nature 267, 281-282
4. Demple, B., and Holbrook, J. (1983) Nature 304, 466-468
5. Fogliano, P., and Schendel, P. (1981) Nature 289, 196-198
6. Krywun, C. J., and Anderson, N. (1981) Nature 289, 680-681
7. Elledge, S. J., and Walker, G. C. (1988) J. Mol. Biol. 184, 175-192
8. Hinman, O., and George, J. (1986) Mol. Gen. Genet. 177, 629-636
9. Siedler, D., and Moyer, R. (1988) Genetics 98, 763-774
10. McLellan, T., and McIntee, E. (1984) Mol. Cell. Biol. 4, 2356-2363
11. Ruby, S. W., and Szostak, J. W. (1986) Mol. Cell. Biol. 6, 95-100
12. Ruby, S. W., and Scheraga, H. A. (1987) J. Mol. Biol. 201, 20-24
13. Elledge, S. J., and Davis, R. W. (1987) Mol. Cell. Biol. 7, 2785-2792
14. Hurd, H. K., and Rave, C. W., and Robert, J. W. (1987) Mol. Cell. Biol. 7, 3673-3677
15. Ruby, S., Szostak, J. W., and Murray, A. W. (1983) Methods Enzymol. 129, 1-29
16. Ruby, S. W., and Szostak, J. W. (1985) Mol. Cell. Biol. 5, 75-84
17. Ruby, S. W., Szostak, J. W., and Scheraga, H. A. (1987) J. Mol. Biol. 201, 785-792
18. Ruby, S., Szostak, J. W., and Murray, A. W. (1983) Methods Enzymol. 129, 1-29
19. Ruby, S. W., Szostak, J. W., and Scheraga, H. A. (1987) J. Mol. Biol. 201, 785-792
20. Ruby, S. W., Szostak, J. W., and Scheraga, H. A. (1987) J. Mol. Biol. 201, 785-792
21. Ruby, S. W., Szostak, J. W., and Scheraga, H. A. (1987) J. Mol. Biol. 201, 785-792
22. Ruby, S. W., Szostak, J. W., and Scheraga, H. A. (1987) J. Mol. Biol. 201, 785-792