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ATR1, a *Saccharomyces cerevisiae* Gene Encoding a Transmembrane Protein Required for Aminotriazole Resistance

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In *Saccharomyces cerevisiae*, 3-amino-1,2,4-triazole (aminotriazole) competitively inhibits the activity of imidazoleglycerolphosphate dehydratase, the product of the *HIS3* gene. Wild-type strains are able to grow in the presence of 10 mM aminotriazole because they induce the level of imidazoleglycerolphosphate dehydratase. However, strains containing *gcn4* mutations are unable to grow in medium containing aminotriazole because they lack the GCN4 transcriptional activator protein necessary for the coordinate induction of *HIS3* and other amino acid biosynthetic genes. Here, we isolated a new gene, designated *ATR1*, which when present in multiple copies per cell allowed *gcn4* mutant strains to grow in the presence of aminotriazole. In wild-type strains, multiple copies of *ATR1* permitted growth at extremely high concentrations of aminotriazole (80 mM), whereas a chromosomal deletion of *ATR1* caused growth inhibition at very low concentrations (5 mM). When radioactive aminotriazole was added exogenously, cells with multiple copies of *ATR1* accumulated less aminotriazole than wild-type cells, whereas cells with the *atr1* deletion mutation retained more aminotriazole. Unlike the mammalian *mdr* or yeast *PDR* genes that confer resistance to many drugs, *ATR1* appears to confer resistance only to aminotriazole. Genetic analysis, mRNA mapping, and DNA sequencing revealed that (i) the primary translation product of *ATR1* contains 547 amino acids, (ii) *ATR1* transcription is induced by aminotriazole, and (iii) the *ATR1* promoter region contains a binding site for the GCN4 activator protein. The deduced amino acid sequence suggests that ATR1 protein is very hydrophobic with many membrane-spanning regions, has several potential glycosylation sites, and may contain an ATP-binding site. We suggest that *ATR1* encodes a membrane-associated component of the machinery responsible for pumping aminotriazole (and possibly other toxic compounds) out of the cell.

In the bakers' yeast *Saccharomyces cerevisiae*, the general control system coordinately regulates the expression of many genes encoding amino acid biosynthetic enzymes from different pathways (reviewed in references 12 and 16). Transcription of these genes is induced coordinately when yeast cells are starved for any one of several different amino acids. The coordinate induction is mediated by the binding of the GCN4 trascriptional activator protein to the promoter regions of amino acid biosynthetic genes (2, 15). GCN4 binds as a dimer (15a) to target sites whose consensus is the 9-base-pair (bp) dyad ATGA(C/G)TCAT (10). GCN4 protein is synthesized only when cells are starved for amino acids even though the mRNA is present under all conditions (11, 36). This regulation of GCN4 protein levels, which occurs by a novel translational control mechanism, accounts for the fact that the amino acid biosynthetic genes are induced only during starvation conditions.

Extensive deletion analysis of the *HIS3* promoter region indicates that although the GCN4-binding site is required for induction, it is insufficient for maximal mRNA levels during amino acid starvation (33). A separate element, which includes the TGCCTC sequence between −142 and −137 with respect to the transcriptional initiation site, is also required for wild-type induction levels. Although this sequence appears to be related to GCN4-binding sites, DNase I footprinting experiments indicate that its affinity for GCN4 is at least 50-fold lower than that of the primary binding site, and they showed no evidence for cooperativity (2, 15). From this and several other observations, we proposed that yeast cells contain a DNA-binding protein that is distinct from GCN4, but which recognizes related but nonidentical sequences (33). This protein, which was suggested from genetic experiments, may or may not be identical to a protein detected in crude extracts that binds near the GCN4-binding site in the *HIS4* promoter (2).

The original purpose of the experiments described in this paper was to clone the gene encoding the hypothetical DNA-binding protein described above. We reasoned that cells containing an excess of this protein might show increased levels of *HIS3* mRNA in the absence of GCN4. In principle, this increased expression could have been due to more efficient binding to the TGCCTC sequence between −137 and −142 and/or to the related GCN4-binding sequence. Thus, we attempted to identify a yeast gene that would cause increased *HIS3* expression when present in multiple copies per cell in a *gcn4* mutant strain.

The genetic selection for such a gene relied on the fact that *gcn4* strains are unable to grow in medium containing 3-amino-1,2,4-triazole (aminotriazole), a competitive inhibitor of the *HIS3* gene product, imidazoleglycerolphosphate (IGP) dehydratase (17, 31). At a concentration of 10 mM, aminotriazole causes histidine starvation, but wild-type strains are able to grow because they induce *HIS3* transcription and hence IGP dehydratase above the basal level. However, strains containing *gcn4* mutations fail to grow under these conditions because they are unable to induce *HIS3* expression in response to starvation (13, 24). In this paper, we identify a new yeast gene (*ATR1*) that when

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present in multiple copies confers increased aminotriazole resistance. However, characterization of this gene strongly suggests that it does not encode the hypothetical transcription factor, but rather a membrane-associated protein that may be involved in the excretion of aminotriazole (and possibly other toxic compounds) out of the cell.

MATERIALS AND METHODS

Yeast strains. The yeast strains used in this study were KY114 (a *ura3-52 ade2-101 trp1-Δ1 lys2-801*) (30), S288C (wild type), KY413 (a *leu2-2,112 gcn4-1*) (derived from a strain obtained from Alan Hinnebusch), and MP40A-8B (a *ura3-52 gcn4-2*). These strains and their derivatives were grown at 30°C either in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or in minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose) with appropriate supplements at 2 μg/ml. Unless otherwise specified, aminotriazole was added to a final concentration of 10 mM to minimal medium lacking histidine. Yeast strains were transformed with hybrid DNA molecules as described previously (30).

Construction of hybrid DNA molecules. The procedures for constructing hybrid DNA molecules have been described previously (29). To make the hybrid pools, we cloned 5- to 15-kilobase (kb) fragments of yeast DNA obtained by partial cleavage with *Sau*3A into the *Bam*HI site of YEp13 (6) or of YEp24 (5). The YEp13 hybrid pool was constructed by and obtained from Kim Nasmyth.

DNA fragments from the original plasmids containing *ATR1*, YEp13-Sc4018 and YEp24-Sc4020, were subcloned as follows. From YEp24-Sc4020, the 2.5-kb *Sph*I fragment (Sc4021) was subcloned back into YEp24. From YEp13-Sc4018, the 1.8-kb *Bam*HI-*Hin*dIII fragment (Sc4022), the 2.6-kb *Bam*HI-*Eco*RI fragment (Sc4023), the 2.4-kb *Sac*I-*Hin*dIII fragment (Sc4024), and the 3.2-kb *Sac*I-*Eco*RI fragment (Sc4025) were inserted into the relevant sites of YRp74. YRp74, a 3.4-kb vector constructed by a complex series of manipulations to be described elsewhere, contains the *Eco*RI-*Hin*dIII polylinker from M13mp18 (22), the 1.1-kb *Hin*dIII fragment encoding the *URA3* structural gene (3), the 350-bp *Hin*dIII-*Hin*cII fragment encoding the *ARS1* chromosomal replicator (37), and the 2.1-kb *Nde*I-*Eco*RI fragment encoding the ampicillin resistance gene from pBR322 (35) in which the origin of DNA replication has been replaced by equivalent sequences from the high-copy vector pUC9 (39).

Mutations in the *ATR1* gene were generated as follows. To make *atr1*-Δ1, which deletes the *Ssp*I fragment from +280 to +775, YRp74-Sc4025 DNA was partially cleaved with *Ssp*I and the desired fragment was purified by agarose gel electrophoresis and circularized. To make *atr1*-Δ2, which deletes the region between the *Bam*HI site at −225 and the *Hin*dIII site at +1569, the plasmid YRp74-Sc4025 DNA was partially cleaved with *Bam*HI and *Hin*dIII (because of the presence of both sites in the YRp74 vector) and treated with the Klenow fragment of DNA polymerase I to fill in the ends, and the desired fragment was purified by gel electrophoresis and circularized. To make *atr1*-L1, the *ATR1* fragment was recloned in YIp5 (34) and a 430-bp *Sph*I fragment of bacteriophage λ DNA was inserted into the *Sph*I site.

For purposes of DNA sequencing and general utility, the *Eco*RI-*Xho*I fragment of YEp13-Sc4018 (Sc4026) was subcloned between the *Eco*RI and *Sal*I sites of the single-stranded vectors M13mp18 and M13mp19 and the plasmid vectors pUC18 and pUC19 (39).

Constructing yeast strains with *atr1* mutations. DNAs containing *atr1* mutations were cleaved with *Spe*I to increase the integration frequency and to direct integration to the *ATR1* locus. DNAs purified by electrophoresis in low-gelling-melting-temperature agarose followed by phenol extraction were introduced into KY114 by selecting for Ura^+ colonies. Transformants resulting from chromosomal integration of the introduced DNA were distinguished from those resulting from autonomous replication by assaying the mitotic stability of the Ura^+ phenotype (34). Colonies showing a stable Ura^+ phenotype were then grown in YPD medium, and Ura^- segregants were selected by virtue of their resistance to 5-fluoro-orotic acid (4).

To determine which of the Ura^- segregants were due to replacement of the *ATR1* locus by the *atr1* deletion mutations, we did genomic hybridization experiments as described previously (34). Yeast DNA was prepared from 2-ml cultures of cells, digested with *Eco*RI, electrophoretically separated in a 0.7% agarose gel, and then transferred to a nitrocellulose filter. The separated fragments were hybridized with ^{32}P-labeled pUC18-Sc4026 DNA (contains the 3.3-kb *ATR1* fragment) prepared by nick translation. The wild-type *ATR1* allele should produce a single band of hybridization corresponding to a fragment of 5.3 kb, whereas *atr1*-Δ1 and *atr1*-Δ2 should generate bands that, respectively, are 4.8 and 3.5 kb.

RNA analysis. Total and poly(A)-containing RNA was isolated from yeast cells as described previously (32). For quantitation of *ATR1* RNA levels in cells grown under various conditions, total RNA was electrophoretically separated in a 1.7% agarose gel containing 6% formaldehyde and then transferred to a nitrocellulose filter. The separated RNAs were hybridized with a mixture of ^{32}P-labeled probes prepared by nick translation. The hybridization probes were YIp5 (contains the *URA3* gene) (34) and the 1.6-kb *Bam*HI-*Sph*I fragment of *ATR1*.

To determine the 5′ ends of *ATR1* RNA, we performed S1 nuclease mapping essentially as described previously (32). The hybridization probes were made by cleaving CsCl-purified YRp74-Sc4025 DNA with *Mlu*I, *Hin*dIII, *Dde*I, or *Sau*3A and labeling the 5′ ends with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. In the *Mlu*I case, the DNA was subsequently cleaved with *Hin*dIII, and in the *Hin*dIII case, the DNA was subsequently cleaved with *Mlu*I. The labeled 1.8-kb *Mlu*I-*Hin*dIII fragments (the *Mlu*I site is located at position +708 with respect to the AUG initiation codon, and the *Hin*dIII site is located in the YRp74 vector), the 379-bp *Dde*I fragment (from positions +306 to −74), and the 495-kb *Sau*3A fragment (from positions +276 to −220) were isolated by agarose gel electrophoresis. Mixtures containing a DNA probe and 5 μg of poly(A)$^+$ RNA were heated to 73°C in 70% formamide–0.3 M NaCl–0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.0)–1 mM EDTA for 15 min to denature the DNA and then immediately transferred to the hybridization temperature that had been determined empirically (50°C for the *Mlu*I and *Hin*dIII probes and 46°C for the *Sau*3A and *Dde*I probes). After hybridization for 18 h, the RNA-DNA hybrids were digested with S1 nuclease and the resulting products were analyzed on a thin 4% acrylamide gel containing 7 M urea.

DNA sequencing. The DNA sequence of the 3.3-kb *Xho*I-*Eco*RI fragment of *ATR1*, Sc4026, was determined by the dideoxy chain termination method (26). Templates for DNA sequencing were generated in two ways. First, double-stranded M13mp19-Sc4026 DNA was digested with DNase I in the presence of Mn^{2+} to generate double-strand breaks at random positions in the DNA (14). The resulting linear molecules were purified by electrophoresis in 0.7% agarose,

cleaved with *Eco*RI, treated with the large fragment of DNA polymerase I, and circularized with T4 DNA ligase. Second, to sequence the opposite strand, we cleaved double-stranded M13mp18-Sc4026 DNA with *Sac*I or *Mlu*I. The linear molecules, purified by electrophoresis in a 0.7% agarose gel, were then treated with *Bal* 31 nuclease for various incubation times, filled in with the large fragment of DNA polymerase I, cleaved with *Eco*RI, and subjected to gel electrophoresis to purify DNA fragments of different lengths. The resulting fragments were ligated into M13mp18 between the *Sma*I and *Eco*RI sites.

Measuring intracellular levels of aminotriazole. Yeast strains were grown in glucose minimal medium lacking histidine to A_{600} = 1 and then concentrated 20-fold in the same medium by centrifugation. In a typical experiment, 2 μl of 0.1 M [^{14}C]aminotriazole (2.75 mCi/mmol; ICN Radiochemicals) was added to 18 μl of cells that had been prewarmed to 30°C. After 15 min of incubation at 30°C, 5 μl of cells was diluted into 1 ml of ice-cold medium, immediately filtered onto a pre-wet Whatman GF-A filter, and washed three times with 1.5 ml of ice-cold medium. The filters were dried under a heat lamp for 5 min, soaked in Liquiscint (National Diagnostics), and then analyzed in a Beckman LS 3801 scintillation counter. For comparing aminotriazole incorporation in different strains, the amount of radioactivity retained on the filter was normalized to the number of cells in the assay. Kinetic experiments indicated that incorporation of aminotriazole into cells proceeded linearly for approximately 10 min, at which time it remained constant for at least one more hour. Essentially no aminotriazole was incorporated when cells were incubated on ice.

RESULTS

Cloning of *ATR1*. Two independent hybrid pools were prepared, each consisting of genomic yeast DNA segments cloned into vectors containing the 2μm origin of replication. In one case, yeast DNA segments were cloned in YEp13, a vector containing the *LEU2* gene (6), and then introduced into KY413 (a *leu2-2,112 gcn4-1*). In the other case, yeast DNA was cloned in YEp24, a vector containing the *URA3* gene (5), and then introduced into MP40A-8B (a *ura3-52 gcn4-2*). Leu$^+$ or Ura$^+$ transformants were selected and then screened for their ability to grow on medium containing 10 mM aminotriazole. Plasmid DNAs from several aminotriazole-resistant colonies were recovered and then reintroduced back into the appropriate strain. From each original hybrid pool, one cloned DNA segment allowed cells to grow at wild-type rates in medium containing 10 mM aminotriazole. Restriction mapping indicated that these two DNA molecules, YEp13-Sc4018 and YEp24-Sc4020, contained approximately 3.3 kb of DNA in common (Fig. 1). For reasons to be discussed below, we propose the gene designation *ATR1* (aminotriazole resistance) to refer to the segment of DNA that confers aminotriazole resistance. Hybridization of ^{32}P-labeled Sc4023 (a *Bam*HI-*Eco*RI fragment; Fig. 1) to a filter containing electrophoretically separated yeast chromosomes (kindly provided by Anne Happel and Fred Winston) revealed that *ATR1* was located on either chromosome XIII or XVI.

Functional mapping of *ATR1*. To determine the location of *ATR1*, we subcloned various regions of the original DNA molecules into YEp24 or into YRp74, a multicopy vector that contains the *ARS1* chromosomal replicator and the *URA3* gene. The resulting molecules were introduced into MP40A-8B, and the Ura$^+$ transformants were tested for growth on 10 mM aminotriazole (Fig. 1). Plasmids containing Sc4025 permitted cells to grow at wild-type growth rates, thus localizing the *ATR1* gene to a 3.2-kb region between the *Sac*I and *Eco*RI sites. A plasmid containing the *Sph*I fragment (Sc4021) did not confer aminotriazole resistance, indicating that the *Sph*I site lies within the *ATR1* gene. On the other end of the gene, wild-type expression of *ATR1* appears to involve sequences beyond the *Bam*HI site because strains containing Sc4023 (the *Bam*HI-*Eco*RI fragment) grow slowly in medium containing 10 mM aminotriazole. Strains containing the *Bam*HI-*Hin*dIII fragment (Sc4022) are unable to grow under these conditions, suggesting that sequences beyond the *Hin*dIII site can affect *ATR1* expression. Although this suggestion appears to be inconsistent with the observation that strains containing the *Sac*I-*Hin*dIII fragment (Sc4024) grow at wild-type rates, such strains may have reduced *ATR1* function which cannot be detected by the phenotypic assay.

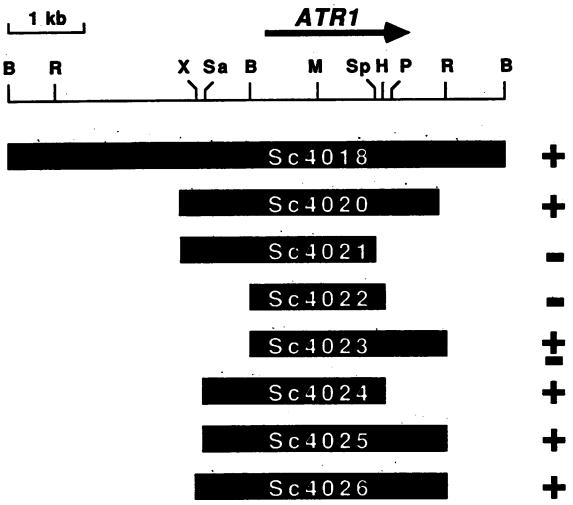

FIG. 1. Restriction map and cloned DNA fragments from the *ATR1* gene region. The locations of restriction endonuclease sites on the original plasmid YEp13-Sc4018 are indicated as vertical bars (abbreviations are B, *Bam*HI; H, *Hin*dIII; M, *Mlu*I; P, *Pst*I; R, *Eco*RI; Sa, *Sac*I; Sp, *Sph*I; X, *Xho*I), and the position of the *ATR1* coding region is shown as a thick arrow (drawing is to scale). The extents of cloned DNA fragments are diagrammed as black bars, and their *ATR1* phenotypes are indicated by their ability to permit growth on 10 mM aminotriazole: +, wild-type growth; ±, slow growth; −, no growth.

Aminotriazole sensitivity of strains containing *atr* mutations. To gain further insight into the role of *ATR1*, we generated strains containing *atr* mutations by replacing the wild-type locus with mutant DNA. Two deleted derivatives of YRp74-Sc4025 (contains the 3.2-kb *Eco*RI-*Sac*I *ATR1* fragment cloned in YRp74) were constructed (Fig. 2). One deletion, *atr1*-Δ1, lacks the 495-bp *Ssp*I fragment within the *ATR1* coding region. The other mutation, *atr1*-Δ2, lacks 1.8 kb of DNA between the *Bam*HI and *Hin*dIII sites and hence represents almost a complete deletion of the *ATR1* gene. The mutant DNAs were cleaved with *Spe*I and introduced into KY114 (relevant genotype, *ura3-52 HIS3 ATR1*) by selecting for Ura$^+$ colonies. Transformants resulting from chromosomal integration of the introduced DNA were grown in nonselective medium, and Ura$^-$ segregants were selected by growth on medium containing 5-fluoro-orotic acid (4). Genomic hybridization experiments indicated that 5 of 15 Ura$^-$ segregants contained *atr1*-Δ1, whereas only 1 of 8 Ura$^-$ segregants contained *atr1*-Δ2 (Fig. 2). All six *atr1* deletion

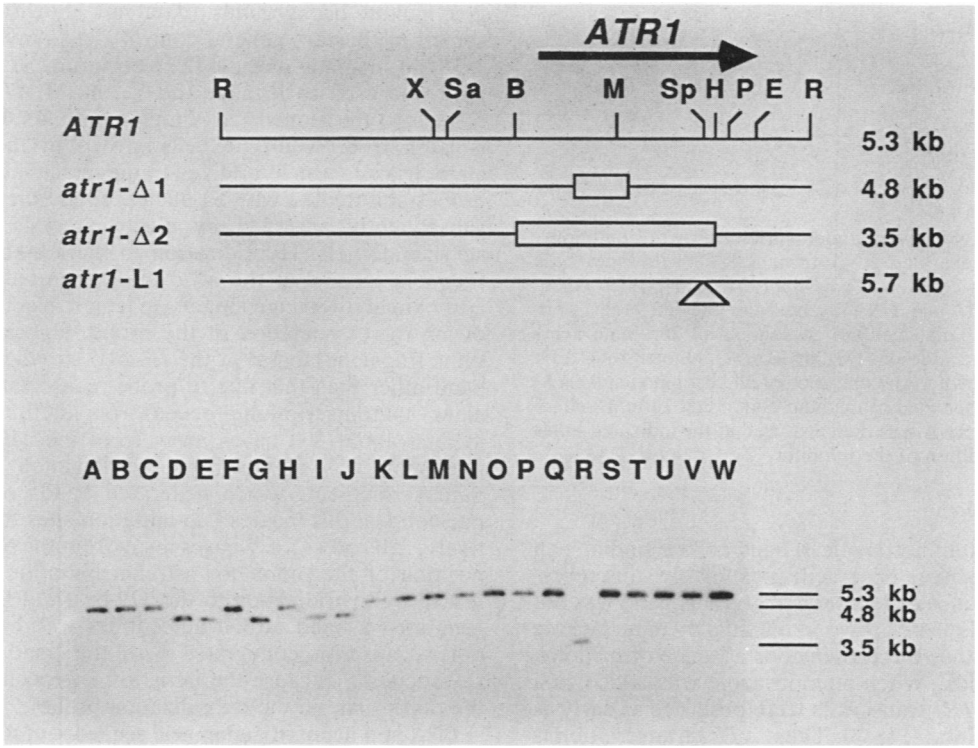

FIG. 2. Generation of strains containing *atr1* mutations. The top part of the figure diagrams the physical structures of the *atr1* mutant DNAs with deletions indicated by open boxes and the insertion of the λ DNA fragment indicated as a triangle (drawing is to scale). Abbreviations for restriction sites are as defined in the legend to Fig. 1 with the addition of E for *Spe*I. Shown below is an autoradiogram that represents genomic hybridization analysis from Ura⁻ segregants from transformants originally containing *atr1*-Δ1 (lanes A to O) and *atr1*-Δ2 (lanes P to W). The sizes of the DNA fragments were calibrated with respect to standards produced by cleavage of bacteriophage λ DNA with *Hin*dIII (data not shown).

strains were unable to grow in medium containing 10 mM aminotriazole, although they grew at wild-type rates in the absence of this inhibitor. A similar phenotype was observed when the *ATR1* gene was replaced by *atr1*-L1, a derivative in which a 430-bp *Sph*I fragment from bacteriophage λ DNA was inserted into the *Sph*I site (Fig. 2). Thus, although *ATR1* is not essential for cell viability, loss of *ATR1* function causes strains to be hypersensitive to aminotriazole.

Effect of *ATR1* on resistance to other toxic compounds. The mammalian *mdr* and yeast *PDR* genes are involved in resistance to a number of apparently unrelated drugs (1, 7, 9, 27). To determine whether *ATR1* is specific for aminotriazole resistance, we diluted saturated cultures of wild-type, *atr1*-Δ1, *atr1*-Δ2, and YRp74-*ATR1* strains to a concentration of approximately 50 cells per μl. For each condition to be examined, 1-μl samples from each culture were spotted on a plate containing a toxic compound at a defined concentration. In this way, growth of the strains could be compared directly on the same plate by determining the size of the colonies after a given amount of time; artifacts due to cell density and reversion were avoided.

As expected, the strains behaved very differently in the presence of aminotriazole. Strains containing *atr1* deletions were almost completely inhibited at 10 mM and were partially inhibited even at 5 mM; this level of inhibition appeared somewhat less pronounced than that seen in *gcn4* strains. Strains overproducing *ATR1* formed colonies in 2 to 3 days even in the presence of 80 mM aminotriazole. The growth of wild-type strains was completely inhibited at this concentration, strongly inhibited in 40 mM aminotriazole, and barely if at all inhibited at 20 mM (or lower). Thus, excess *ATR1* gene product confers super-resistance to aminotriazole.

In contrast, growth of these strains could not be distinguished in the presence of a variety of other toxic compounds over a range of different concentrations. The compounds tested included triazolealanine (resistant below 2 μg/ml and sensitive about 10 μg/ml), ethionine (resistant below 0.2 μg/ml and sensitive above 0.75 μg/ml), canavanine (resistant below 0.2 μg/ml and sensitive above 1 μg/ml), cycloheximide (resistant below 0.2 μg/ml and sensitive above 1 μg/ml), chloramphenicol (resistant below 4 μg/ml and sensitive above 15 μg/ml), antimycin (resistant below 0.4 μg/ml and sensitive above 2 μg/ml), oligomycin (resistant below 0.5 μg/ml and sensitive above 3 μg/ml), *p*-fluorophenylalanine (resistant below 2 μg/ml and sensitive above 8 μg/ml), vinblastine (slighlty sensitive at 40 μg/ml), and daunomycin (slightly sensitive at 7 μg/ml). Triazolealanine, ethionine, canavanine, and *p*-fluorophenylalanine respectively inhibit histidine, methionine, arginine, and phenylalanine biosynthesis; vinblastine inhibits mitosis; daunomycin, cycloheximide, and chloramphenicol inhibit protein synthesis; and antimycin and oligomycin block electron transport. Thus, the *ATR1* gene seems to be involved specifically in aminotriazole resistance and hence is unlikely to be allelic to yeast *pdr* mutations.

Regulation of *ATR1* transcription. Total RNA was prepared from wild-type cells grown under a variety of environmental conditions, electrophoretically separated in a denaturing agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled probes corresponding to the *ATR1* and *URA3* genes (Fig. 3). When cells were grown in the

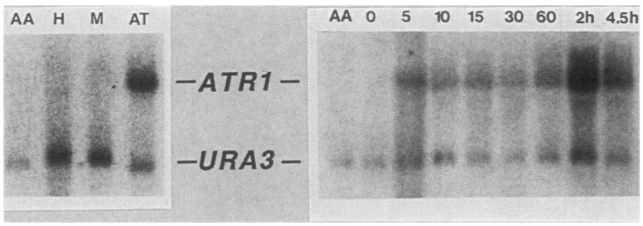

FIG. 3. *ATR1* mRNA levels under various growth conditions. Electrophoretically separated RNA from S288C (wild-type) cells grown under various conditions was hybridized to probes corresponding to the *ATR1* and *URA3* genes. On the left, cells were grown in glucose minimal medium containing all 20 amino acids (AA), histidine (H), no additions (M), or 10 mM aminotriazole (AT). On the right, cells grown in the presence of all 20 amino acids (AA) were pelleted and suspended in medium containing 10 mM aminotriazole at time zero. RNA was then analyzed at the indicated times (in minutes) after addition of the inhibitor.

presence of 10 mM aminotriazole, a band corresponding to a 1.9-kb RNA species was observed; presumably, this represents transcription of *ATR1*. Interestingly, this band was not observed when cells were grown in minimal medium lacking aminotriazole in either the presence or absence of histidine or all 20 amino acids. When aminotriazole was added to a growing culture, *ATR1* transcripts were observed as early as 5 min after the addition (Fig. 3). Thus, *ATR1* transcription is regulated as a function of aminotriazole concentration.

This regulation of *ATR1* transcription could reflect a direct response to aminotriazole or a response by the GCN4-mediated general control system to the histidine limitation caused by aminotriazole. At least part of the response appears to involve the general control pathway because when leucine is added to 10 mM, a condition that inhibits valine and isoleucine biosynthesis (23), *ATR1* transcription is also induced (data not shown). However, the observation that elevated levels of *ATR1* transcripts occur even in certain *gcn* mutant backgrounds (data not shown) suggests that something besides general control is also involved.

5′-end mapping of the *ATR1* transcript. S1 nuclease mapping was used to localize the 5′ end of *ATR1* RNA with respect to the cloned DNA region (Fig. 4). Poly(A)$^+$ RNA isolated from wild-type cells grown in the presence of aminotriazole was hybridized to the indicated 5′-end-labeled probes and treated with S1 nuclease, and the products were subsequently analyzed by electrophoresis in denaturing acrylamide gels. Hybridization to the 1.8-kb *Mlu*I-*Hin*dIII fragment labeled at the *Mlu*I site generated a broad band approximately 800 nucleotides in length in addition to a band owing to reassociation of the probe. Hybridization to the same fragment labeled at the *Hin*dIII site did not produce a band other than that due to probe reassociation. This indicates that transcription proceeds from left to right from a site located about 800 bases away from the *Mlu*I site. Better resolution was obtained by hybridization to the 495-bp *Sau*3A fragment, which generated bands of 330 and 445 nucleotides; this locates two initiation sites that are, respectively, 330 and 445 ± 5 bases away from the *Sau*3A site. The position of the more downstream initiation site was confirmed by hybridization to the 379-bp *Dde*I fragment which generated a band of 360 nucleotides (the band due to the other transcript comigrates with the band due to probe reassociation because the initiation site occurs upstream of the *Sau*3A site in the hybridization probe).

DNA and deduced amino acid sequence of *ATR1*. To define the structure of *ATR1* more precisely, we determined the DNA sequence of both strands of the 3.3-kb *Xho*I-*Eco*RI fragment of YEp13-Sc4018 by the dideoxy chain termination method. The clones that were sequenced are shown in Fig. 5, and the DNA sequence is shown in Fig. 6.

Analysis of both strands of the DNA sequence revealed a single long open reading frame of 547 amino acids, and several lines of evidence strongly suggest that this is the primary *ATR1* translation product. First, the length and direction of this reading frame are consistent with the length

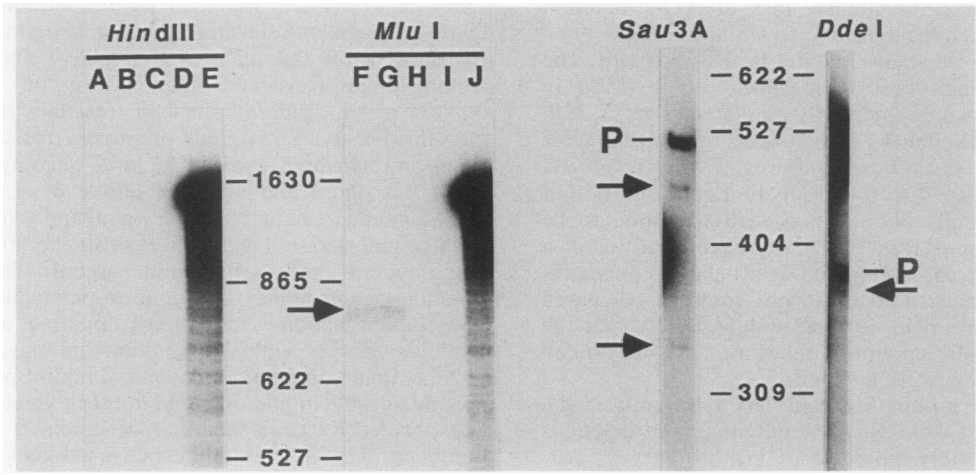

FIG. 4. Mapping the 5′ ends of *ATR1* mRNA by S1 nuclease analysis. For the experiment with the probe labeled at the *Mlu*I site (position +708 with respect to the AUG initiation codon) or at the *Hin*dIII site (located in the YRp74 vector), 5 μg of poly(A)$^+$ RNA from strain KY114 (lanes A, B, F, and G) or 30 μg of *Escherichia coli* tRNA (lanes C to E and H to J) was hybridized and then treated with S1 nuclease for 30 min (lanes A, C, F, and H), 90 min (lanes B, D, G, and I), or 0 min (lanes E and J). For the experiment with the probe labeled at the *Dde*I site (position +306) or the *Sau*3A site (position +276), 5 μg of the same poly(A)$^+$ RNA preparation was hybridized and treated with S1 nuclease for 30 min. Bands owing to RNA-DNA hybrids (arrows) and to probe reassociation (P) are shown with respect to the positions of DNA fragments of known length (determined on neighboring lanes on the same gel).

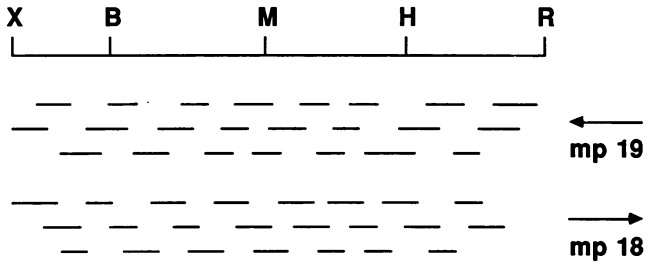

FIG. 5. Strategy for DNA sequencing. The lines indicate the lengths and positions of the DNA sequences determined by the dideoxy chain termination method. The directions of sequencing for the fragments cloned in M13mp18 and M13mp19 are rightward and leftward, respectively. Abbreviations for restriction sites are as defined in the legend to Fig. 1, and the drawing is to scale.

and direction of *ATR1* RNA. Second, the two mRNA initiation sites map approximately at nucleotides −55 and −165 with respect to the presumptive AUG initiation codon, and there is a consensus TATA sequence (TATAAA) at nucleotides −102 to −97. The −55 transcript is likely to be translated into *ATR1* protein because the presumptive initiation codon would be the first AUG in the RNA. However, translation of the −160 transcript is unlikely as there are several AUG codons upstream of the presumptive initiation codon, each defining an incorrect reading frame. With regard to potential promoter elements, the TGACTCA sequence at nucleotides −284 to −278 is identical to the internal 7 bp of the optimal GCN4-binding site (10). Third, deletion of the C-terminal 34 amino acids (Sc4021) abolishes *ATR1* activity, and deletion of promoter sequences upstream of −225 (Sc4023) reduces activity. Interestingly, however, deletion of the C-terminal 23 amino acids (derivatives Sc4024 and Sc4022) appears to have a relatively minor affect on *ATR1* function.

The most obvious feature of the presumptive *ATR1* protein is its hydrophobicity. The diagram in Fig. 7 with the algorithm and hydropathy values of Kyte and Doolittle (19) with a window of 19 amino acids indicates that there are 12 very hydrophobic regions (all with values greater than 1.2 and nine with values greater than 1.5) and one extraordinarily hydrophobic region (value of 3.0). Essentially the entire *ATR1* protein is hydrophobic with the exception of the 70 N-terminal amino acids and two short regions near the C terminus which are hydrophilic. Given the extremely hydrophobic nature of ATR1, we also examined the amino acid sequence for other features that are often associated with membrane proteins. Potential N-linked glycosylation sites (Asn-X-Ser/Thr, as defined in reference 18) are found at amino acid residues 3, 18, 394, and 471. A possible ATP-binding site may be defined by the pair of sequences at positions 426 to 440 and 528 to 536. From an analysis of ATPases, it has been proposed that G-$(X)_4$-GK(T)-$(X)_6$-I/N and R/K$(X)_3$-G-$(X)_3$-L-(hydrophobic)$_4$-D represent a pair of sequences that constitute an ATP-binding fold (41). Amino acids 426 to 440 fit the latter motif except that there are four residues between the glycine and leucine instead of three, and amino acids 528 to 536 fit the former motif except for the first glycine. Otherwise, a computer search of all known protein sequences failed to reveal significant homology between ATR1 and any other protein.

Intracellular concentration of aminotriazole. To determine the relative intracelleular concentration of aminotriazole, cells were equilibrated in 10 mM [^{14}C]aminotriazole and then separated from the free inhibitor by filtration. Incorporation of aminotriazole into cells reached a steady-state value after 10 min at 30°C, did not occur at 0°C, and was competed by an excess of unlabeled aminotriazole (Table 1). When compared with the relative level of aminotriazole in a wild-type strain, the *atr1* deletion strain contained approximately twice as much aminotriazole, whereas the *ATR1* multiple-copy strain had only about half as much. Thus, it appears that increased aminotriazole resistance was inversely correlated with the intracellular concentration of aminotriazole. However, owing to the relatively small differences between strains, it was not possible to determine whether the differences in the steady-state level reflected alterations of transport into or out of the cell.

DISCUSSION

This paper describes the isolation and characterization of a new gene, *ATR1*, which is involved in the resistance to aminotriazole in the yeast *S. cerevisiae*. When compared with wild-type strains, cells with multiple copies of *ATR1* grow in very high concentrations of aminotriazole, whereas elimination of the gene causes cell growth to be inhibited at lower concentrations of the inhibitor. Thus, it appears that the level of aminotriazole resistance is determined by the amount of *ATR1* gene product. In this sense, *ATR1* is analogous to dominant drug resistance genes of bacteria such as those involved in resistance to ampicillin, tetracycline, or chloramphenicol or to a mammalian gene involved in multidrug resistance. Perhaps *ATR1* will prove to be useful as a general selectable marker for transformation of *S. cerevisiae* (and possibly other eucaryotic) cells.

Structure of ATR1 protein. From the sequence of the *ATR1* gene, we suggest that ATR1 is a transmembrane protein and propose a tentative structure (Fig. 8). The hydropathy analysis shown in Fig. 7 utilizes stringent criteria for hydrophobic regions in that it was carried out with a window of 19 amino acids, a stretch that is large enough to span a membrane (19). By this analysis, there are 12 separate regions of ATR1 protein with hydropathic values greater than +1.2, a number strongly indicative of a membrane-spanning region (8, 19), and most of these regions are considerably more hydrophobic (nine are greater than +1.5). The N terminus of ATR1 is very highly charged and hence does not resemble a signal sequence that is typically used for passage through the membrane (40). Thus, we propose that the N-terminal region is oriented on the cytoplasmic side of the membrane. If the 12 hydrophobic regions represent six transmembrane loops, this would mean that the C terminus of ATR1 would also be oriented on the cytoplasmic side of the membrane.

In the proposed structure, two of the putative glycosylation sites (at positions 394 and 471) are predicted to occur on the extracellular side of the membrane, the expected

TABLE 1. Aminotriazole incorporation[a]

ATR1 allele	Treatment	Aminotriazole (cpm)
Wild type	Normal	2,523
Wild type	0°C	103
Wild type	+ 0.1 M	447
atr1-Δ1	Normal	3,843
YRp74-*ATR1*	Normal	988

[a] The amount of [^{14}C]aminotriazole incorporated into various strains after 15 min of incubation at 30°C (normal), 0°C, or in the presence of 0.1 M unlabeled aminotriazole. The entries represent the average of four determinations.

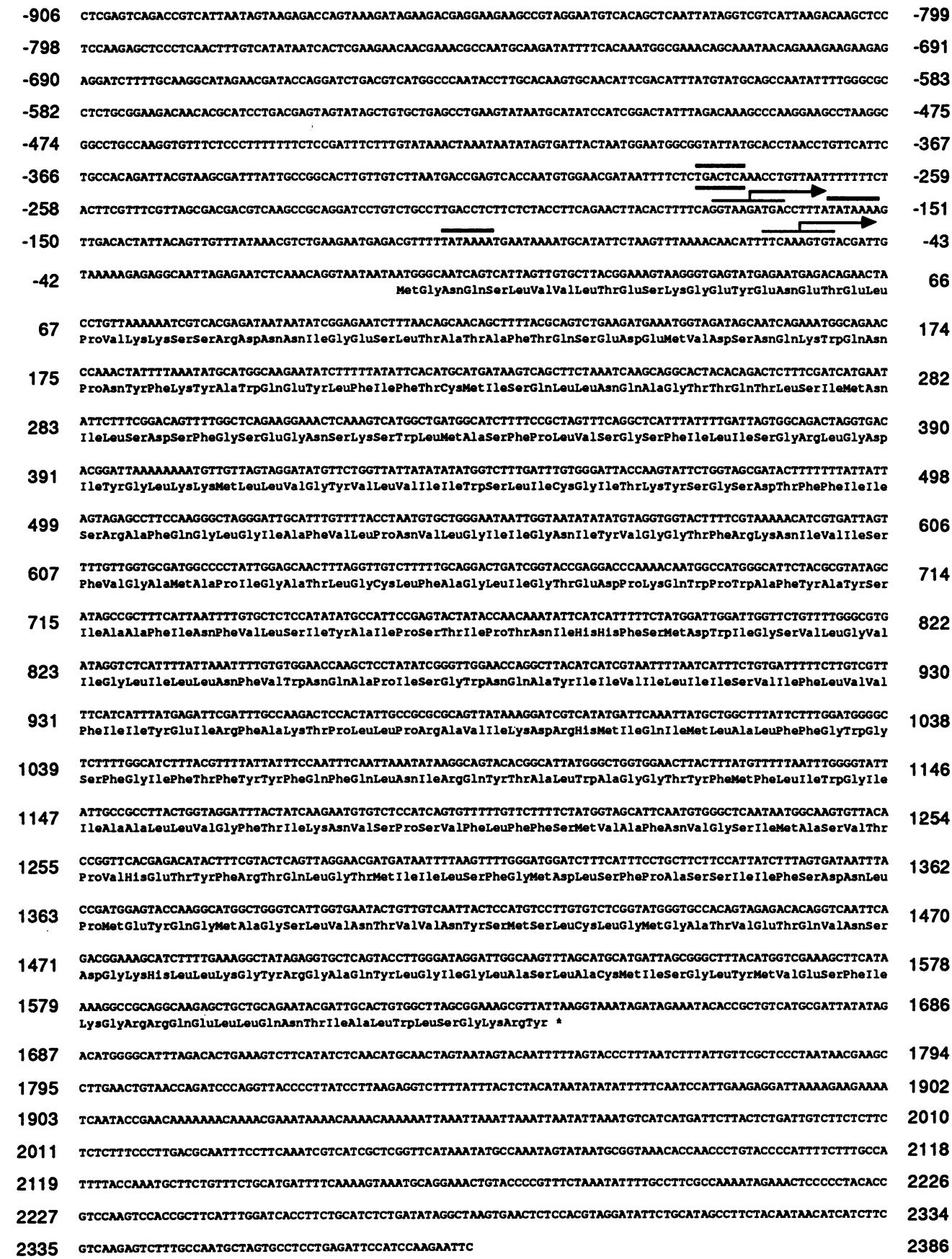

FIG. 6. Nucleotide sequence of the *ATR1* gene region. The nucleotide sequence of the *ATR1* structural gene and upstream and downstream flanking regions is shown for the mRNA-coding strand. Shown below the DNA sequence is the deduced amino acid sequence of the *ATR1* gene product. The nucleotide coordinates are defined such that +1 represents the presumptive AUG initiation codon. The positions of RNA initiation sites (rightward arrows; the indicated positions are accurate to ±5 nucleotides), prospective TATA promoter elements (thick overline), and GCN4-binding site (thick over- and underline) are also indicated. *, Stop codon.

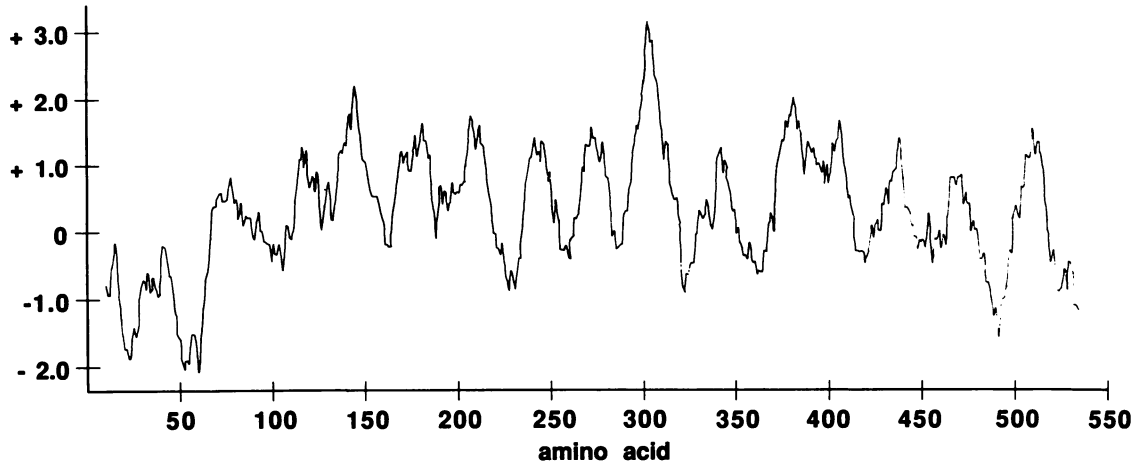

FIG. 7. Hydropathy analysis of ATR1 protein. Plotted at each amino acid position of ATR1 is the average hydropathic value (19) for the indicated amino acid and the nine flanking amino acids on each side, i.e., a window of 19 amino acids. Positive numbers indcate hydrophobic regions with values greater than 1.2 suggestive of a transmembrane region, and negative numbers indicate hydrophilic regions.

position. The other two sequences lie within the highly charged and intracellularly located N terminus and would not be expected to be glycosylated. Both sequences that make up the putative ATP-binding site would be located on the intracellular side as expected, although they would be separated by one transmembrane loop. With respect to the *atr1* deletion mutants, removal of theC-terminal 23 amino acids has a minor effect on function and does not disrupt the basic structure, whereas deletion of the C-terminal 34 amino acids destroys the last transmembrane region and abolishes *ATR1* function. The *atr1*-Δ1 mutation, which abolishes function, would remove five transmembrane domains and hence would lead to a faulty orientation of the protein.

A problem with the model in Fig. 8 is that one of the sequence motifs proposed to be part of the putative ATP-binding site is located at the extreme C terminus, a region that can be eliminated with only a minor loss of function. This could suggest that the ATR1 protein does not contain an ATP-binding site, that some other region besides the extreme C terminus acts with the conserved sequence between residues 426 to 440 to form an ATP-binding site, or that binding of ATP is not essential for *ATR1* function.

Models for *ATR1* function. In *S. cerevisiae*, it is believed that aminotriazole inhibits cell growth solely because it is a competitive inhibitor of IGP dehydratase, the *HIS3* gene product (17, 31). This belief stems primarily from the observation that aminotriazole does not inhibit growth when histidine is present in the medium. Given this assumption, there are three major factors that influence aminotriazole resistance: the level of the *HIS3* gene product, the amount of IGP (the substrate that is competed by aminotriazole), and the amount of aminotriazole that enters the cell.

On the basis of these considerations, several models for ATR1 function can be proposed. First, ATR1 could positively regulate *HIS3* expression, possibly by encoding the hypothetical DNA-binding protein mentioned in the Introduction. By this model, aminotriazole sensitivity caused by *atr1* mutations would be explained by postulating that ATR1 plays a role in basal-level expression of *HIS3* (and possibly other amino acid biosynthetic genes). Second, *ATR1* could directly or indirectly increase the flux through the histidine biosynthetic pathway, possibly by altering the amino acid biosynthesis in a *gcn4*-independent manner. In this case, inhibition of IGP dehydratase would result in higher levels of IGP, the immediate precursor. Since aminotriazole is a competitive inhibitor, cells containing increased IGP levels would require higher levels of aminotriazole to cause the equivalent degree of inhibition. Third, *ATR1* could indirectly affect the inhibition of IGP dehydratase by aminotriazole, possibly by altering the metabolism of compounds such as phosphate or sulfate which act synergistically with aminotriazole. Fourth, *ATR1* could reduce the effective concentration of aminotriazole by degradation, inhibition of uptake, intracellular compartmentalization, or export.

Although direct proof for any of these potential mechanisms is lacking, the observation that incorporation of [^{14}C]aminotriazole is relatively low in *ATR1* multicopy cells but relatively high in *atr1* deletion cells strongly suggests that *ATR1* confers aminotriazole resistance by reducing the intracellular concentration of this drug. This suggestion is supported by the fact that the *ATR1* gene product has all the features of a transmembrane protein. It seems very unlikely that such a transmembrane protein would be a transcription factor that increases *HIS3* expression. Moreover, we doubt that *ATR1* causes increased flux through the histidine pathway or causes major alterations in metabolism because strains that have increased or decreased *ATR1* activity grow normally and behave indistinguishably in the presence of other inhibitors including triazolealanine, an inhibitor of

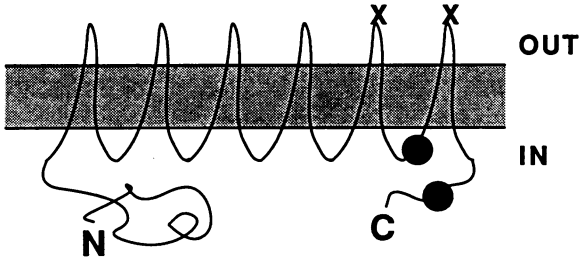

FIG. 8. Structural model of ATR1 protein. A proposed orientation of ATR1 (N and C termini indicated) with respect to the membrane (shaded) and the intracellular and extracellular compartments is indicated along with potential glycosylation sites (X) and a nucleotide-binding site (closed circles). See text.

histidine biosynthesis. Although the differences in [^{14}C] aminotriazole incorporation are fairly small, it seems possible that they account for the observed phenotypic differences between the strains.

There are several ways in which the *ATR1* gene product could reduce intracellular levels of aminotriazole. First, by analogy with the ampicillin and chloramphenicol resistance genes, which respectively encode β-lactamase and chloramphenicol transacetylase, *ATR1* could encode an enzyme that degrades aminotriazole. We do not favor this explanation because hydrolytic enzymes are usually secreted rather than transmembrane proteins; in this regard, the hydrophilic and acidic nature of the N terminus of ATR1 argues against secretion of the protein (40). Second, ATR1 could inhibit the uptake of aminotriazole. This seems unlikely because there are at least two routes for entry of aminotriazole into the cell (43). Moreover, unlike *CAN1* which encodes an arginine permease and is involved in resistance to canavanine (42), *ATR1* cannot encode a permease for aminotriazole because drug resistance is the dominant trait. Third, ATR1 could be involved in the passage of aminotriazole between the vacuole, the normal storage compartment for amino acids (21), and the cytoplasm, where it would inhibit IGP dehydratase. Fourth, by analogy with the tetracycline resistance gene or with plasmid-encoded genes involved in resistance to cadmium or arsenate (20, 25, 28, 38), *ATR1* could encode a protein that is involved in actively pumping aminotriazole out of the cell. Such an active export mechanism, which we favor, has also been proposed for the *mdr* gene product that confers multidrug resistance in mammalian cells (1, 7, 9). Perhaps, as has been suggested for the *mdr* gene product, active export of aminotriazole by ATR1 protein requires the hydrolysis of ATP. However, *ATR1* appears to differ from *mdr* in that resistance does not occur to a variety of drugs but rather appears specific to aminotriazole.

Comments on the physiological role of *ATR1*. Although drug resistance conferred by *ATR1* seems to be specific for aminotriazole, it is difficult to believe that yeast cells have evolved a gene whose normal physiological role is to export a compound not found in nature. A clue to the normal function may come from the observation that *ATR1* appears to be a gene that is subject to general control; i.e., expression is induced coordinately with many genes involved in amino acid biosynthesis, and the promoter region contains a GCN4-binding site. It seems likely that cells containing high levels of amino acid biosynthetic enzymes will accumulate precursors to amino acids, some of which may be toxic. Perhaps *S. cerevisiae* has maintained the *ATR1* gene to excrete such toxic compounds that may arise upon severe metabolic stress. Alternatively, *ATR1* may play an important role in pumping out toxins that cells may encounter in the natural environment. Another possible physiological role for *ATR1* could be to regulate the transport of amino acids between the vacuole and the cytoplasm; such a process would certainly be sensitive to changes in the amino acid biosynthetic capacity of the cell such as those controlled by GCN4. However, for any of these potential roles, the natural substrates for ATR1 are unknown.

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