Diazaborine Treatment of Baker’s Yeast Results in Stabilization of Aberrant mRNAs*

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Upon Northern blotting, Saccharomyces cerevisiae that was treated with diazaborine showed aberrant mRNAs that were extended at the 3’-end and terminated at secondary processing sites. These bands were also detected in untreated supf1, Δxrn1, and rat7-1 mutants. This finding demonstrates that the aberrant mRNAs also occur in untreated strains in small quantities and can reach the cytoplasm, where they are normally degraded by Xrn1p. Diazaborine treatment stabilizes these mRNAs. The detection of the aberrant bands in the untreated rat7-1 strain indicates that Rat7 is involved in quality control of RNA. The aberrant mRNAs were not detected after diazaborine treatment of a DRG1-1 mutant. Drg1p, a member of the family of AAA (ATPases associated with a variety of cellular activities) proteins, which are thought to represent specific chaperones, may be involved in the process of unfolding the mRNA-ribonucleoprotein complex or in the recognition of aberrant mRNA molecules in the cytoplasm.

Eukaryotic mRNA 3’-end formation involves an intricate processing event by which the precursors are cleaved at a predetermined position. Specific sequence elements on the pre-mRNA act as signals for the attachment of the processing factors. In a further step, the pre-mRNA generated is polyadenylated, and the downstream fragment is degraded (1). The mRNA processing signals in higher eukaryotes are well defined, but in the yeast Saccharomyces cerevisiae, the identification of these signals is complicated because of their high degree of variability and redundancy (2). One of the best-studied examples in yeast is gene CYC1, encoding iso-1-cytochrome c, where the processing signals are well characterized and necessary for proper 3’-end formation (3, 4). These signals are the AU-rich efficiency element, an A-rich positioning element, and the poly(A) site. Deletion of the efficiency element prevents utilization at this site, but secondary processing sites located downstream are concomitantly recognized and utilized by the processing complex. A deletion in the efficiency element in the cyc1-512 mutant resulted in a 90% decrease in CYC1 mRNA and led to the appearance of a set of eight elongated mRNAs ranging from 850–3500 nucleotides in length (5).

Defects in mRNA 3’-end formation can profoundly alter cell viability, growth, and development. Yeast genes involved in 3’-end formation are essential, which underscores the fundamental nature of this process. After mRNA maturation, which takes place in the nucleus, the processed mRNAs are exported to the cytoplasm by a series of steps that are still far from being fully understood. At one stage during transport, the 5’→3’ exonuclease Rat1, an essential enzyme, is required (6). The precise nature of this enzymatic step is still unclear. The mRNA is not exported in naked form but rather as a ribonucleoprotein particle, which, upon arrival in the cytoplasm, needs to be uncoated and transferred to the translation machinery that attaches it to the initiating ribosomal subunits. During this journey, the ribonucleoprotein particle has to get in contact with proteins of the nuclear pore, like Rat7 (7), and with RNA helicases, which are important for the unwinding of the RNA-protein agglomerate (8–10). A quality control mechanism scans the mRNA for the appearance of premature stop codons and thus prevents the synthesis of incomplete and potentially deleterious protein molecules. This nonsense-mediated decay pathway (11) involves as a key component the ATP-dependent RNA helicase Upf1p, which, in combination with other proteins, detects aberrant mRNAs (12, 13) and renders the mRNA to the exonuclease Xrn1p for immediate 5’→3’ degradation. Xrn1p is homologous to the nuclease Rat1p, but it is nonessential (14). Before Xrn1p can degrade the mRNA, it has to be decapped (15). mRNA degradation can also occur in the 3’→5’ direction. This pathway involves the exosome of which Ski2p, a putative RNA helicase, is a key component (16). The steps that a mRNA molecule has to go through from its appearance in the cytosol to its degradation are only incompletely understood. In particular, it is not entirely clear whether aberrant mRNA molecules are able to pass the nuclear pore or what characterizes them as abnormal.

We have a long-standing interest in the antibacterial drug diazaborine. In bacteria, diazaborine inhibits fatty acid biosynthesis (17, 18). We demonstrated recently that the drug also inhibits the growth of the yeast S. cerevisiae and that two distinct mechanisms lead to resistance (19). One mechanism is connected with allelic forms of the gene DRG1, and the other involves overexpression of drug efflux pumps that are activated by mutated forms of transcription factors or by overexpression of the transcription factor Yap1 (20). We have investigated the mode of action of diazaborine in yeast and observed that treatment of yeast cells with subinhibitory concentrations of this inhibitor results in a characteristic change in the length of certain mRNAs. These changes, which we describe in the present communication, are the result of a drug-induced disturbance of the cells’ ability to handle aberrant mRNA molecules.

### EXPERIMENTAL PROCEDURES

**Yeast Strains and Media**—The S. cerevisiae strains used in this study are listed in Table I. Cells were grown in rich medium (YPD) or synthetic medium (SD), supplemented with appropriate nutrients for maintenance of plasmids, as described by Sherman et al. (21). Yeast was grown routinely at 30 °C.
Diazaborine and Aberrant mRNAs

**TABLE I**
Genotypes of strains used in this study

| Strain | Genotype | Source |
|--------|----------|--------|
| W303   | MATa ura3 leu2 his3 trp1 ade2 can1          | S. D. Kohlwein |
| ESY212 | MATa ura3 leu2 his3 trp1 ade2 can1DRG1-1 | This laboratory |
| D311-3A| MATa CYC1+ his1 lys2 trp2                  | F. Sherman    |
| B-4060 | MATa cyc1-512 his1 lys2 trp2               | F. Sherman    |
| B-9637 | MATa cyc1-512 trp2 ura3                   | F. Sherman    |
| B-988  | MATa cyc1-512 trp2 ura3 upf1-1 URA3       | F. Sherman    |
| RW2889 | MATa, ura3, his3                          | A. W. Johnson |
| RKY1981| MATa, ura3, his3, xrn1-URA3               | A. W. Johnson |
| RW2966 | MATa, trp1, leu2, pho3, pho5             | A. W. Johnson |
| RKY1982| MATa, trp1, leu2, pho3, pho5, ski2-LEU2  | A. W. Johnson |
| FY22   | MATa, ura3, leu2, trp1                    | C. N. Cole    |
| DAY1-1 | MATa, ura3, leu2, trp1, rat1-1            | C. N. Cole    |
| LGY101 | MATa, ura3, leu2, trp1, rat7-1            | C. N. Cole    |

**Plasmids**—The plasmids used in this study were YEp351 and pFW300 (19). The plasmids were transformed into *S. cerevisiae* by the method of Ito et al. (22).

**Northern Blot Analysis**—The yeast strains were grown overnight in YPD medium or, when plasmid-carrying strains were used, in SD medium lacking leucine. The cells were diluted to an *A*_{600} of 0.05 and cultured in YPD medium to an *A*_{600} of 0.3–0.4. From this cell suspension, aliquots were taken and cultured with and without diazaborine for an additional 30 min. Alternatively, other drugs such as cycloheximide, ketoconazole, or terbinafine were used. Samples of total RNA were prepared from each aliquot as described in the Qiagen RNeasy Handbook. RNA samples (10–20 μg) were denatured and separated on a 1.2% agarose gel containing 1.2 M formaldehyde. The amount of 26 S and 18 S rRNA was used as a control to monitor RNA loading and quantitated on a densitometer. To measure the length of the characteristic RNA bands, RNA molecular weight markers (Roche Diagnostics) were used in some of the gels. The gels were transferred to Hybond N (Amersham Pharmacia Biotech) filters and hybridized at 65 °C in 0.5 M NaOH, pH 7.2, 1 mM EDTA, 7% SDS, 1% bovine serum albumin, and 100 μg/ml salmon sperm DNA with 32P-labeled DNA probes for 12–16 h. The filters were washed four times in 40 mM NaOH, pH 7.2, 1% SDS at 65 °C for 25 min, and the radioactivity was made visible by autoradiography on an x-ray film at ~70 °C and quantitated on a laser densitometer.

**DNase I and Protease Treatment of RNA**—The Qiagen RNase-free DNase I Set was used for DNase I treatment of total RNA as described in the Qiagen RNeasy Handbook. 50–100 μg of prepared RNA was treated with 50 μg of Qiagen protease in 30 mM Tris-HCl buffer (pH 8.0) and incubated for 30 min at 50 °C. RNeasy Mini Kits were used to clean up the treated RNA as described in the Qiagen RNeasy Handbook.

**RESULTS**

Diazaborine Affects the mRNA Stable-state Levels of Several Genes—When we tested the expression of the gene FLR1 in Northern blotting experiments by growing yeast in the presence of subinhibitory concentrations of diazaborine, a marked decrease in mRNA content was observed. The decrease was accompanied by the appearance of slower-migrating mRNAs of the gene FLR1. The result is also shown in Fig. 1. In none of these cases could we observe larger mRNA species and a concomitant decrease of the main band. Cycloheximide showed an increase of the mRNA band, which is probably due to the inhibition of mRNA degradation as a result of blocked protein synthesis (23). Treatment with terbinafine and ketoconazole showed no change at all.

The Slow-moving Bands Are Not Due to Bandshifting—To exclude the possibility that the high molecular weight RNAs are artifacts resulting from protein-RNA aggregation, DNA-RNA aggregation, or contamination by DNA, we incubated the RNA preparations from diazaborine-treated and untreated cells with protease and DNase I and probed them by Northern blotting with either the FLR1- or CYC1-specific probe (Fig. 2).

The High Molecular Weight mRNA Species Are Extended at the 3′ End—To estimate the approximate length of the slow-migrating mRNAs, we hybridized the RNA preparation from diazaborine-treated and untreated yeast cells with radioactive probes designed to anneal at various regions downstream of the
Gene FLR1. The locations of the probes are indicated in Fig. 3. The most distant probe, S4, is located 1400 nucleotides downstream from the stop codon of FLR1 and within the neighboring reading frame. Regardless of which probe was used, we always observed a signal at the same position in the Northern blot of RNA from dazaborine-treated yeast. This finding indicates that in the presence of dazaborine, the transcript extends into the next reading frame. The transcript of gene YBR007C starting at its own promoter was also detected when probe S4 was used for hybridization.

The influence of dazaborine on the appearance of extra long mRNAs was investigated in detail using the gene CYC1. When the efficiency element is deleted, as in the case of the cyc1-512 mutant, only 10% of normal length CYC1 mRNA is formed. Higher molecular weight RNAs appear instead, which arise from alternative downstream 3’-end processing sites (5). We decided to use this model system to investigate the action of dazaborine in detail. As seen in Fig. 4, the cyc1-512 mutant showed a strong decrease in CYC1 mRNA accompanied by the appearance of the characteristic high molecular weight species (5). When we treated the wild type strain with dazaborine, we observed a low mRNA identical in size to the band of 1.8 kb seen in the untreated cyc1-512 mutant strain. Treatment of the cyc1-512 mutant with dazaborine resulted in an increase in the amount of the high molecular weight band and the appearance of additional signals with even slower electrophoretic mobility. Das et al. (5) showed recently that the nonphysiologically elongated cyc1-512 mRNAs are stabilized in a Δupf1 background. Using this mutant, we observed the described stabilized bands, which occurred at the same positions in the Northern blot as bands from the cyc1-512 mutant treated with dazaborine. In the dazaborine-treated RNA sample, one very long species of 5 kb was present that could not be seen in the Δupf1 mutant. This experiment shows that some of these longer RNA species are formed even in the absence of dazaborine but are undetectable in wild type cells due to their fast degradation. Dazaborine treatment of the Δupf1 mutant gave the same electrophoretic pattern except for the appearance of this additional very slow-migrating band.

The Exonuclease Xrn1 Degradates the Aberrant mRNA—The experiment with the untreated Δupf1 mutant demonstrated that the aberrant, high molecular weight mRNA species failed to be degraded. To identify the degradation pathway involved, we tested the effect of dazaborine in two mutants defective in the genes coding for Xrn1p and Ski2p. The 5’→3’ exonuclease Xrn1p degrades mRNA in the cytoplasm after its release from

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1 The abbreviation used is: kb, kilobase(s).
Cyc1 and Flr1 was drastically increased as compared with that in the wild type. This effect is probably caused by post-translationally accumulated mRNA that failed to be degraded because of the absence of the exonuclease. Protein Flr1 was previously shown to mediate resistance to diazaborine when overexpressed (20). We found that the Δxrn1 mutant has the same sensitivity to diazaborine as the wild type. Hence, the highly abundant mRNA is not translated. The Δski2 mutant, which is defective in a 3′→5′ exonuclease activity, showed no stabilization of the longer mRNA species, indicating that Ski2p is not involved in removal of these nonphysiologically long transcripts (Fig. 6).

A rat7-1 Mutant Shows the 5.5-kb FLR1 RNA Band—To study the export of aberrant RNAs from the nucleus, we tested two mutants defective in RNA transport. A wild type strain, a rat1-1 mutant, and a rat7-1 mutant were grown with and without diazaborine at 25 °C. The extracted RNA was analyzed in a Northern blot for FLR1-specific mRNA. In the untreated rat7-1 cells, a weak band of the aberrant 5.5-kb FLR1-containing RNA was observed (Fig. 7). This band was absent in the RNA from the wild type and the rat1-1 mutant. In the samples from the diazaborine-treated cells, the 5.5-kb FLR1-containing RNA was present in each of the strains investigated.

The Aberrant RNA Species Do Not Occur in the DRG1-1 Mutant—We tested DNA-aberrant-resistant strains for the appearance of aberrant RNA molecules in the presence of diazaborine. In previous work, we have isolated different types of resistant strains, one of which detoxifies the cell by activating efflux pumps via overexpression of the transcription factor Yap1p, and another that mediates resistance in an unknown way through an allelic form of the gene DRG1. When a Yap1p-overexpressing strain was tested, we still detected aberrant RNAs. This can be seen in Fig. 8, where mRNAs isolated from yeast grown with subinhibitory concentrations of the drug was hybridized with a FLR1-specific probe. Northern blots with Cyc1 probes gave essentially the same result (data not shown). In no case did we see aberrant mRNAs when we isolated RNA from a diazaborine-resistant strain with the allele DRG1-1. The cellular function of Drg1p was hitherto unknown, but the results of Fig. 8 demonstrate that DRG1-1 is intrinsically involved in the generation of correct mRNAs in the presence of diazaborine.

**DISCUSSION**

Treatment of yeast with diazaborine affected both the amount and the correct length of mRNA molecules of various genes in a specific way. The steady-state level of longer transcripts increased with higher drug concentrations, and we could show that these RNAs are extended at the 3′-end and terminate at clearly defined, secondary termination sites. We demonstrated this by using the cyc1-512 mutant, which is a well-characterized system for studying transcript termination in yeast (5). The main band of 1.8 kb in the cyc1-512 mutant,
which results from pre-mRNA cleavage at an alternative termination site, showed the same electrophoretic mobility as the band that arose from diazaborine treatment of wild type cells. In a cyc1-512 Δupf1 double mutant, additional bands were detectable as compared with the cyc1-512 single mutant that again correlated in size with bands detected in the treated cyc1-512 mutant, with the exception of an additional, very high molecular weight band of ~5 kb in the treated strain that was not detectable in the untreated cyc1-512 Δupf1 double mutant. Upf1p is an ATP-dependent RNA helicase and the main component of the nonsense-mediated decay pathway, which recognizes aberrant mRNAs. The appearance of additional bands in the cyc1-512 Δupf1 double mutant as compared with the cyc1-512 mutant means that these RNA species are also formed in the single mutant but are not detectable because they are efficiently removed by the nonsense-mediated decay pathway.

The correlation in size of the mRNA species that we detected after diazaborine treatment of the single cyc1-512 mutant and the cyc1-512 Δupf1 mutant demonstrates that these bands also occur in the untreated single mutant but are stabilized in the treated strain. We conclude from this experiment that the 1.8-kb CYC1 band we observed in the diazaborine-treated wild type strain is also formed in a wild type strain in small quantities but is degraded in the untreated strain by the UPF system. We should therefore also be able to detect the same bands that we find in various transcripts in treated wild type strains in an untreated Δxrn1 mutant. We could demonstrate that this is indeed the case. Both the 1.8-kb band from the CYC1 message and the 5.5-kb band from the FLR1 message, which are very characteristic for diazaborine treatment, could be detected in the untreated Δxrn1 mutant. In contrast, these bands were not detected in a Δski2 strain that is deficient for the 3’−5’ exosome, indicating that this activity is not involved in the removal of these aberrant RNAs.

Another important conclusion could be drawn from our experiment with the Δupf1 and Δxrn1 mutants. Both enzymes are localized in the cytoplasm. The accumulation of the aberrant RNAs in these mutants therefore indicates that these RNAs can reach the cytoplasm, where they are usually degraded in an Xrn1- and Xrn1-dependent way. Because diazaborine treatment produces the same types of aberrant RNAs that arise from defects in the XRN1 or UPF1 genes, it seems that the drug affects the recognition or the elimination of aberrant mRNA molecules in the cytoplasm. Xrn1p is also responsible for normal mRNA decay. Does diazaborine interfere directly with the function of Xrn1? Our Northern blot experiments with the FLR1 and CYC1 probes in the Δxrn1 mutant indicate that this is not the case. Whereas diazaborine treatment of a wild type strain resulted in a reduction of the main mRNA bands, a strong accumulation of the main bands of both mRNAs was observed in the Δxrn1 mutant due to the block in mRNA decay. Because we did not find an accumulation of major FLR1 and CYC1 mRNAs after diazaborine treatment, we conclude that diazaborine treatment can act at a step earlier than Xrn1p in the degradation pathway and more specifically affects the degradation of aberrant mRNAs.

The 5.5-kb FLR1 RNA band, which is very characteristic for both the diazaborine treatment and the Δxrn1 mutant, also appeared in the RNA from a rat7-1 mutant, but not in the isogenic wild type. Rat7p is a component of the nuclear pore and is known to be involved in mRNA export (7). This protein is located at the cytoplasmic side of the pore structure (25). We propose that Rat7p is involved in checking the quality of mRNA molecules that leave the nucleus and transfers aberrant RNA molecules to the exonuclease Xrn1p for degradation.

The high molecular weight RNA species were absent when a diazaborine-resistant mutant that carries a DRG1 allele was treated with the drug. When a strain that became diazaborine resistant due to overexpression of YAP1 was tested, the longer RNA species were still present, albeit at a reduced quantity. From this observation, we conclude that it is not possible to completely neutralize the effect of the drug on the appearance of abnormal mRNAs by removing diazaborine from yeast cells using efflux pumps.

Instead, Drg1p appears to be inherently involved in the abolition of the effect of the drug. We found that the protein Drg1p is located exclusively in the cytoplasm. Hence, the mutant variant of Drg1p has to be active in the cytoplasm and perform its metabolic activity in this cellular location. This suggests that Drg1p is involved in the recognition of the destruction process of aberrant mRNA molecules in the cytoplasm. Because Drg1p is a member of the AAA (ATPases associated with a variety of cellular activities) family of proteins, which are believed to act as specific chaperones (26), we hypothesize that Drg1p acts in the process of unfolding the mRNA-ribonucleoprotein complex or in the recognition of aberrant mRNA molecules in the cytoplasm and that this reaction is inhibited by diazaborine. The diazaborine-resistant variant of Drg1p catalyzes this reaction undisturbed by the inhibitor.

Why do the intensities of the main CYC1 and FLR1 mRNA bands decrease as a result of diazaborine treatment? At the moment, we cannot answer this question. This decrease of mRNA could be caused by some sort of signal transduction into the nucleus that tells the cell that something is wrong with the handling of aberrant mRNAs. Another possibility is that the inhibition of mRNA unfolding and recognition in the cytoplasm might be accompanied by the failure of a component of the mRNA-ribonucleoprotein to return to the nucleus, thereby depleting the nuclear reservoir of this component. This could affect the rate of pre-mRNA synthesis or its stability or transport to the cytoplasm.

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