Characterization of the Export of Bulk Poly(A)⁺ mRNA in *Saccharomyces cerevisiae* during the Wine-Making Process

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Ethanol stress affects the nuclear export of mRNA similarly to heat shock in *Saccharomyces cerevisiae*. However, we have little information about mRNA transport in actual alcoholic fermentation. Here we characterized the transport of mRNA during wine making and found that bulk poly(A)⁺ mRNA accumulated in the nucleus as fermentation progressed.

Adverse effects of ethanol on *Saccharomyces cerevisiae* cells are key problems facing wine growers and brewers. Therefore, the response to ethanol stress of yeast has been investigated with great interest. It has been reported that ethanol stress blocks the export of bulk poly(A)⁺ mRNA similarly to heat shock (42°C) (6, 9).

In eukaryotic cells, including *S. cerevisiae* cells, mRNA is synthesized and processed in the nucleus and exported to the cytoplasm through nuclear pore complexes (NPCs). In *S. cerevisiae*, matured mRNA is exported as messenger ribonucleoprotein complexes by mRNA export factors (2, 7, 20). It is well known that mRNA export is a target in the regulation of stress. Yeast cells alter not only their transcriptional patterns but also the types of mRNA to be exported from the nucleus to adapt rapidly to stress. Under conditions of heat shock, stress-induced transcripts such as *SSA4* encoding Hsp70 are efficiently exported, whereas bulk poly(A)⁺ mRNA accumulates in the nucleus (6, 9, 16). The mechanisms of such a selective mRNA export caused by the stress seem to be quite complex and remain controversial (4, 10, 14, 18). Additionally, it still remains to be clarified whether ethanol and heat shock affect mRNA export in the same way. Furthermore, we have almost no information about the transport of yeast mRNA in actual alcoholic fermentation such as wine making.

We and others have recently reported that rapid changes in the localization of Rat8p, an mRNA export factor, contribute to the nuclear accumulation of bulk poly(A)⁺ mRNA under ethanol stress conditions but not under heat shock conditions (8, 15). Rat8p is one of the DEAD-box proteins and shuttles between the nucleus and the cytoplasm (3, 11, 12, 17). Since it is usually concentrated at the cytoplasmic fibrils of NPCs, Rat8p has been suggested to participate in a terminal step of mRNA export through the removal of proteins that accompany mRNA through NPCs (2, 3, 13). We found that the intracellular localization of Rat8p changes rapidly and reversibly in response to ethanol stress (15). Here we investigated changes in the transport of mRNA and the intracellular localization of Rat8p during wine making.

The strain of wine yeast used in this study was *S. cerevisiae* OC-2, which was obtained from the Institute for Fermentation, Osaka, Japan. Grape must was obtained from Chardonnay grapes grown in Yamanashi prefecture, Japan. Grapes were crushed in a stainless steel tub after being destemmed and compressed to yield grape must. The must was stored at –20°C prior to use. Sulfur dioxide and glucose were added to the must at final concentrations of 50 mg/liter and 64.8 g/liter, respectively. The indices of must were pH 3.3, 26.0 Brix, and 7.5 g of total acid/liter. The must (500 ml) was filtered through a 0.22-μm-pore-size membrane filter and inoculated with a 3% starter culture of wine yeast OC-2 cells (ca. 10⁶ cells/ml) and then fermented for 30 days at 15°C. No effect of the filtration was observed on the increase in the concentration of ethanol, the synthesis of protein, and the localization of Rat8p and bulk poly(A)⁺ mRNA. The ethanol concentration was measured by gas chromatography (AL-2; Riken Keiki Co., Tokyo, Japan). To determine the pattern of protein synthesis during wine making, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was carried out as follows: 10 μl of [³⁵S]methionine-cysteine solution (Pro-mix [³⁵S] in vitro cell labeling mix; Amersham Bioscience, Piscataway, N.J.) was added per 10 ml of the must, and OC-2 cells were incubated for 60 min at 15°C. Cell extracts were subsequently prepared and subjected to 2D-PAGE analysis as previously described (5). Dry strips of immobilized polyacrylamide gel with pH 3 to 10 gradients (Bio-Rad, Richmond, Calif.) were used in this study.

We have recently reported that the localization of Rat8p and bulk poly(A)⁺ mRNA changed in response to 6% ethanol in synthetic dextrose (SD) minimal medium (2% glucose, 0.67% yeast nitrogen base, pH 5.5) when W303-1A, a representative laboratory strain (15, 19), was used. In the present study, we first characterized the localization of Rat8p and mRNA export in OC-2 in SD medium with ethanol. Construction of the plasmid pAUR-GFP-RAT8 and the method of in situ hybridization of bulk poly(A)⁺ mRNA were previously described (15). Nuclear DNA was stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). OC-2 cells in an exponential phase of growth in SD medium with reciprocal shaking (120 strokes/min) at 28°C were treated with ethanol (5 to 9% [vol/vol]) for 15 min, and then the localization of GFP-Rat8p was monitored. The localization of GFP-Rat8p in OC-2 cells

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FIG. 1. Concentration-dependent effects of ethanol on the localization of Rat8p and export of bulk poly(A)⁺ mRNA in wine yeast OC-2. Wine yeast *S. cerevisiae* OC-2 cells in an exponential phase of growth in SD medium at 28°C were treated with ethanol (6 or 9% [vol/vol]) for 15 min, and then the localization of GFP-Rat8p and bulk poly(A)⁺ mRNA was monitored. The localization of GFP-Rat8p was monitored in living OC-2 cells carrying pAUR-GFP-R478. The intracellular localization of bulk poly(A)⁺ mRNA was analyzed by in situ hybridization using a digoxigenin-labeled oligo(dT)₅₀ probe. BF, bright field.

changed partly in response to 6% ethanol and completely in response to 9% ethanol (Fig. 1). Furthermore, the localization of GFP-Rat8p in response to ethanol stress changed quickly and reversibly in OC-2 cells as well as in W303-1A cells (data not shown) (15). In SD medium, no distinct difference was observed between these strains in terms of the response of Rat8p to ethanol. Corresponding to the nuclear accumulation of Rat8p, a partial accumulation of bulk poly(A)⁺ mRNA in the nucleus was observed with 6% ethanol and a complete accumulation was observed with 9% ethanol in OC-2 cells in SD medium (Fig. 1). These results indicate that in SD medium, ethanol stress has almost identical effects on the localization of Rat8p and the export of bulk poly(A)⁺ mRNA in wine yeast OC-2 cells as well as in W303-1A cells.

We next investigated the localization of Rat8p and mRNA export during wine making. A change in the ethanol concentration of the fermenting must is shown in Fig. 2. At the early stage of wine making (days 1 to 7), GFP-Rat8p showed a normal localization around the nuclear envelope (leftmost panels in Fig. 3). However, GFP-Rat8p began to accumulate into the nucleus when the ethanol concentration of the must reached around 6% (days 8 to 12) (Fig. 3, panels in second column from left), and finally showed a complete accumulation in the nucleus when the ethanol concentration reached 9% (after day 13) (Fig. 3, panels in third column from left). GFP-

FIG. 2. Changes in the ethanol concentration, intracellular localization of Rat8p and bulk poly(A)⁺ mRNA, and protein synthesis during the wine-making process. Changes in the ethanol concentration of the must during wine making are indicated. Wine making was carried out using *S. cerevisiae* OC-2 cells. Results are representative of 12 independent wine-making procedures.

Rat8p remained in the nucleus until the end of fermentation. Corresponding to the nuclear accumulation of GFP-Rat8p, a partial accumulation of bulk poly(A)⁺ mRNA in the nucleus was observed with 6% ethanol and an almost complete accumulation was observed with 9% ethanol (Fig. 3). We further investigated whether such a nuclear accumulation of Rat8p and bulk poly(A)⁺ mRNA was caused by the increase in the concentration of ethanol in the must or by other factors such as a lack of essential nutrients. The wine must of day 15 (average ethanol concentration and sugar content were approximately 11% and 12.5 Brix) was divided in two, and one-half was centrifuged to eliminate yeast cells. The centrifuged must was subsequently bubbled with nitrogen gas to remove the ethanol. The ethanol concentration of the must was reduced to approximately 2.5% by nitrogen bubbling for 4 h (ethanol-removed must). Yeast cells were prepared from the other half by centrifugation and added to the ethanol-removed must. After incubation at 15°C for 6 h, the localization of GFP-Rat8p and bulk poly(A)⁺ mRNA was monitored. As shown in the rightmost panels in Fig. 3, cells in the ethanol-removed must showed normal localization of GFP-Rat8p around the nuclear envelope and the export of bulk poly(A)⁺ mRNA. This result indicates that the increase in the concentration of ethanol in the must at the late stage of wine making is probably the primary factor causing the nuclear accumulation of Rat8p and bulk poly(A)⁺ mRNA and that factors other than ethanol in the must have little effect on the blocking of mRNA export.

Such an accumulation of bulk poly(A)⁺ mRNA likely affected the synthesis of proteins at the late stage of wine making. The pattern of protein synthesis during wine making was analyzed by 2D-PAGE. As shown in the left panel of Fig. 4, yeast cells newly synthesized a variety of proteins when cells

FIG. 3. Changes in the localization of GFP-Rat8p and bulk poly(A)⁺ mRNA during wine making. The ethanol concentrations of the must are indicated under the panels. The rightmost panels show the localization of GFP-Rat8p and bulk poly(A)⁺ mRNA in the ethanol-removed must of day 15.

were exporting bulk poly(A)⁺ mRNA at the early stage of wine making (days 1 to 7). However, the number of spots of newly synthesized proteins decreased in parallel with the nuclear accumulation of Rat8p and bulk poly(A)⁺ mRNA, and we detected almost no spot from the must after day 15 (center panel of Fig. 4). This indicates that cells barely synthesized any proteins at the late stage of wine making. Furthermore, as shown in the right panel of Fig. 4, we detected the spots of newly synthesized proteins from the ethanol-removed must of day 15 (described above). This result indicates that the synthesis of protein as well as the export of bulk poly(A)⁺ mRNA resumed on removal of the ethanol (rightmost panels in Fig. 3 and 4). Based on these results, it is likely that the blocking of

mRNA export led, at least in part, to the repression of protein synthesis at the late stage of wine making. Of course, other factors caused by the progress of fermentation, such as depletion of nutrients, also presumably affected the protein synthesis. Actually, the efficiency of protein synthesis was reduced in the ethanol-removed must, despite the resumption of the export of bulk poly(A)⁺ mRNA (right panel in Fig. 4). The number and signal intensity of spots from the ethanol-removed must were less than those from the must at the early stage of wine making (compare right panel with left panel in Fig. 4). However, it is likely that the nuclear accumulation of bulk poly(A)⁺ mRNA is one of the main reasons for the blocking of protein synthesis at the late stage of wine making.

FIG. 4. The pattern of synthesis of newly synthesized proteins during wine making was analyzed by 2D-PAGE with [³⁵S]methionine-cysteine. The ethanol concentrations of the must are indicated over the panels. The right panel shows the pattern of protein synthesis in the ethanol-removed must of day 15.

Here we demonstrated changes in the transport of bulk poly(A)⁺ mRNA during fermentation in wine making. As the ethanol concentration of the wine must increased, so too did the nuclear accumulation of Rat8p and bulk poly(A)⁺ mRNA in yeast cells. In wine making, the nuclear accumulation of Rat8p and blocking of yeast mRNA export seem to be mainly influenced by the concentration of ethanol in the must. Considering the results obtained in SD medium and in the must (Fig. 1 and 3), it is likely that the ingredients of the must, low temperature (15°C), and static culture of wine making have little effect on the localization of Rat8p and bulk poly(A)⁺ mRNA. Additionally, a normal localization of GFP-Rat8p around the nuclear envelope and the export of bulk poly(A)⁺ mRNA were observed in the ethanol-removed must (rightmost panels of Fig. 3). In the case of beer brewing, neither nuclear accumulation of Rat8p nor blocking of the mRNA export was observed (data not shown). This seems quite reasonable, since the ethanol concentration of beer did not rise to more than 5 to 6%.

We also found that the synthesis of new proteins was arrested in yeast cells at the late stage of wine making (Fig. 4). Along with the depletion of nutrients, the nuclear accumulation of Rat8p and mRNA is presumably one of the reasons for the repression of protein synthesis at the late stage of wine making. Ansanay-Galeote et al. have reported that the fermentation rate of wine yeasts is reduced by 6% ethanol, which causes the beginning of the nuclear accumulation of bulk poly(A)⁺ mRNA (Fig. 3) (1). The nuclear accumulation of mRNA may correlate closely with the reduction of alcoholic fermentation.

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