Significant Effect of the N-terminal Region of the Mitochondrial ADP/ATP Carrier on Its Efficient Expression in Yeast Mitochondria*

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The low-level expression of the bovine heart mitochondrial ADP/ATP carrier (bovine type 1 ADP/ATP carrier (bAAC1)) in the yeast mitochondrial membrane is significantly improved by replacement of its N-terminal region with corresponding regions of the yeast type 1 and 2 carriers (yAAC1 and yAAC2) (Hashimoto, M., Shinohara, Y., Majima, E., Hatanaka, T., Yamazaki, N., and Terada, H. (1999) Biochim. Biophys. Acta 1409, 113–124). To understand why the bAAC1 chimeras were highly expressed in yeast mitochondria, we examined the effects of the length and sequence of the N-terminal region extending into the cytosol on the expression of bAAC1 and yAAC2 derivatives in yeast mitochondria. For this, their N-terminal regions were replaced with peptide fragments of various lengths and sequences derived from those of bAAC1, yAAC1, and yAAC2. We found that a specific amino acid sequence and a definite length of the N-terminal region of yAAC2 were required for high expression of bAAC1 and yAAC2 in yeast mitochondria. We also examined the steady-state transcript levels and expression of these derivatives in whole yeast cells. Based on our results, we discuss the role of the N-terminal region in efficient expression of bAAC1 and yAAC2 in yeast mitochondria.

The ADP/ATP carrier (AAC or ANT) is a major member of the mitochondrial solute carrier family (1), mediating the exchange transport of ADP and ATP across the inner membrane of mitochondria. The carrier consists of three homologous domains, each of which contains two membrane-spanning regions linked by a hydrophilic loop facing the matrix space, and both the N- and C-terminal regions extend into the cytosol (2–5). Several AAC isoforms are known to be expressed, and those in the mitochondria of bovine heart and Saccharomyces cerevisiae have been studied extensively (2–7). The type I isoform of the bovine carrier known as ANT1, referred to as bAAC1 in this study, is predominantly expressed in heart mitochondria (8, 9). Three isoforms of the yeast carrier (yAAC1, yAAC2, and yAAC3) are expressed in the mitochondria of S. cerevisiae depending on the environmental conditions (10–12). Of these, yAAC2 is the major isoform responsible for growth with a non-fermentable carbon source such as glycerol or lactate (11); yAAC1 is expressed at low levels under aerobic conditions (11); and yAAC3 is expressed only under anaerobic conditions (12). Hence, the structure/function relationships of AACs have mainly been studied with bAAC1 and yAAC2 (2–7).

Most mitochondrial proteins are synthesized as preproteins on cytosolic polyosomes. The N-terminal targeting sequence (presequence) attached to the sequence of the mature form is cleaved after import into mitochondria (13–18). However, the AAC is synthesized without a presequence (15–21) because it contains targeting information within the mature form (19–21). It has been shown that amino acid sequence 72–111 of yAAC1 and two-thirds of the whole sequence containing the C-terminal region of the AAC expressed in Neurospora crassa are of importance for import into mitochondria (19, 20). In addition, each of three homologous domains of the AAC of N. crassa contains information for mitochondrial targeting (21).

Recently, we succeeded in functionally expressing mammalian mitochondrial bAAC1 and human AAC1 (hAAC1) in the yeast mitochondrial inner membrane (22, 23). Although bAAC1 and hAAC1 were expressed slightly in yeast mitochondrial membranes, their expression was significantly improved by replacement of their N-terminal regions, which extend into the cytosol, with the corresponding regions of the yeast carriers yAAC1 and yAAC2. The oxidative phosphorylation ability of the AAC-disrupted yeast strain WB-12, in which the intrinsic AAC genes are disrupted, was complemented well by the enhanced expression of chimeric bAAC1 and hAAC1. Furthermore, the transport activities of the bAAC1 chimeras were very similar to that of bAAC1 in bovine heart mitochondria, indicating that the bAAC1 chimeras functioned like native bAAC1 in bovine heart mitochondria. From these results, the N-terminal regions of yeast carriers are thought to be important for efficient expression of the AAC in the yeast mitochondrial inner membrane, but not for its transport function (22, 23).

As an extension of this study, we examined the effects of N-terminal regions of various lengths and amino acid sequences on the expression of derivatives of bAAC1 and yAAC2 in the yeast mitochondrial membrane. This study should be helpful for understanding the mechanism of expression of the AAC in the yeast mitochondrial membrane.

EXPERIMENTAL PROCEDURES

Materials and Reagents—The haploid strain of S. cerevisiae W303-1B (MATa ade2-1 leu2-3,112 his3-112 trp1-1 ura3-1 can1-100) (11) was a gift from Dr. B. Shimizu (Osaka University). The AAC-disrupted yeast strain of WB-12 (MATa ade2-1 trp1-1 ura3-1 can1-100 aux1::LEU2 aux2::HIS3) and the single-copy type yeast shuttle vector pRS314-YA2P containing TRP1 were prepared as described (22). Other materials and reagents were of the highest grade commercially available.

Preparation of DNA Fragments Encoding Various AACs—DNA frag-
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Western and Northern Blotting—SDS-PAGE on 10% polyacrylamide gel was performed following the method of Laemmli (25). The amounts of various AACs were determined by Western blotting using antiserum against either a synthetic peptide of the bAAC1-specific sequence (His$_2$-lle$_6$) or that of yAAC2 (Asn$_5$-Lys$_{15}$) as reported previously (4, 22).

Preparation of Yeast Mitochondria and Extraction of Total Yeast Cellular Proteins—Yeast mitochondria were prepared as described (22).

All the DNA fragments prepared were subcloned into the NdeI site of pRS314-YA2P for transformation of the AAC-disrupted yeast strain WB-12 (22). Yeast cells were grown as described (22). From the immunostaining band intensities, we determined the effect of the length and sequence of the N-terminal region on the expression of bAAC1 and yAAC2 in yeast mitochondria. We prepared various derivatives of these AACs in which the N-terminal regions were replaced with peptides of various lengths and sequences derived essentially from those of bAAC1, yAAC1, and yAAC2 (Fig. 1). For simplicity, we abbreviated these AACs as B, Y1, and Y2, respectively, and their structural features are shown by their N-terminal region/trunk AAC. Namely, Y2/B represents a class of bAAC1 (B/B) derivatives with an N-terminal region derived from that of yAAC2. In addition, the sequence numbers of the N-terminal regions of the derivatives and those with a deletion (Δ) or reversion (R) are shown in parentheses. Namely, Y2-(1–16)/B is a derivative of bAAC1, Y2-(3–17)/B is a derivative of the N-terminal region of which consists of sequence 1–17 of yAAC2 connected to sequence 8–16 of yAAC1; and Y2Δ(3–6)/B is a Y2/B derivative, the N-terminal region of which consists of sequences 1–2 and 7–26 of yAAC2, but is devoid of sequence 3–6. Y2R-(3–20)/Y2 is a yAAC2 (Y2/Y2) derivative in which sequence 3–20 of the N-terminal region of yAAC2 is reversed (Y2-(1,2,20–3,21–26)/Y2). The length of the N-terminal region is shown by the number of amino acid residues (n). The n values of bAAC1, yAAC1, and yAAC2 are 11, 16, and 26, respectively. The prepared expression vectors encoding these AAC derivatives were introduced into the AAC-disrupted yeast strain WB-12. These yeast transformants were grown, and the amounts of these AAC derivatives expressed in yeast mitochondrial membranes and their total amounts in the yeast cells were examined by Western blotting.

Effect of the Length of the N-terminal Region on the Expression of bAAC1 Derivatives in Yeast Mitochondria—Recently, we found that the slight expression of bAAC1 in the yeast mitochondrial inner membrane was significantly improved by replacement of its N-terminal region with the corresponding region of yAAC1 or yAAC2 (22). The expression of the Y2-(1–26)/B chimera (n = 26) in the Y2/B class was greater than that of the Y1-(1–16)/B chimera (n = 16) in the Y1/B class (for their N-terminal sequences, see Fig. 1A). These bAAC1 chimeras correspond to y2NhAAC and y1NhAAC, respectively, in our previous report (22). To understand the mechanism of the remarkable expression of the bAAC1 chimeras in the yeast mitochondrial membrane, we examined the effect of the length of the N-terminal region of Y2-(1–26)/B on its expression in yeast mitochondria by deleting stepwise the amino acid residues in its N-terminal region. The sequences of the N-terminal regions of the Y2/B derivatives are summarized in Fig. 1A.

For construction of DNAs encoding these derivatives, the first N-terminal residue was replaced with Met as a start codon for translation. Taking into consideration the fact that the Ser$_2$ residues of mature bAAC1 and yAAC2 are N$^\text{a}$-acetylated (8, 26), the second residue was substituted with Ser. We prepared DNA fragments encoding various Y2/B derivatives and inserted them into the single-copy plasmid pRS314-YA2P with the promoter region of the yAAC2 gene. The expression vectors thus prepared were introduced into the yeast strain WB-12, in which the intrinsic yAAC1 and yAAC2 genes are disrupted (22, 23). We also prepared transformants of bAAC1, Y2-(1–26)/B, and Y1-(1–16)/B.

First, we examined the growth of these WB-12 transformants on plates containing 1% yeast extract, 2% bactopeptone, and 3% glycerol (YPGly) as a non-fermentable carbon source. As shown in Fig. 2, the Y2-(1–26)/B and Y1-(1–16)/B transformants grew well in contrast to WB-12 and the transformant of bAAC1 (B-(1–11)/B), indicating that the oxidative phosphorylation ability of WB-12 was well complemented by introduction of these bAAC1 chimeras, but not by native bAAC1, as we observed previously (22). The growth rates of the transformants of Y2Δ/B derivatives were determined to be less than those of the Y2-(1–26)/B transformants, with the growth rate depending on the length of the N-terminal region (n) (Fig. 2). The growth doubling times of the Y2Δ(3–17)/B, Y2Δ(3–11)/B, Y2Δ(3–6)/B, and Y2-(1–26)/B transformants in liquid YPGly medium were >50, 24, 15, and 8 h, respectively. The Y2Δ(3–22)/B transformant essentially did not grow like WB-12 and the bAAC1 transformant. In addition, the growth doubling time of Y1-(1–16)/B was ~24 h.

To determine the amounts of these derivatives expressed in yeast mitochondrial membranes, we next carried out SDS-PAGE and Western blotting of the mitochondrial proteins using antiserum against a synthetic peptide of the bAAC1-specific sequence His$_2$-Ile$_6$, which reacted well with 34-kDa Y2-(1–26)/B and 32.5-kDa Y1-(1–16)/B and to the same degree as that of 30-kDa native bAAC1 (22). This antiserum did not cross-react with yAAC2 (Fig. 3, W7 and yAAC2 lanes), and immunostained bands were detected for Y2Δ(3–6)/B, Y2Δ(3–11)/B, and Y2Δ(3–17)/B, but not for Y2Δ(3–22)/B, as shown in Fig. 3.

From the immunostaining band intensities, we determined
### Table I

**PCR primers for preparation of DNA fragments encoding various AACs**

| DNA fragment | Primer | Sequence (5' → 3') | Location<sup>a</sup> | Origin<sup>b</sup> |
|--------------|--------|--------------------|----------------------|-------------------|
| **A. Open reading frames** | | | | |
| hAAC1 | D<sup>d</sup>,<sup>e</sup> HT431 | CCGGTGTCGcatATGAGCGATCA | −11 to −11 | hAAC1 |
| | U,<sup>c</sup> HT432 | CAAAGGATCTGGAACCAATG | 936 to 917 | hAAC1 |
| yAAC2 | D, HT499 | ATACATATGGCTTTACACGCC | −6 to −16 | yAAC2 |
| | U, HT501 | AGGATCCAGGACGATTGAC | 978 to 958 | yAAC2 |
| **B. N-terminal regions** | | | | |
| Y1(1–16) | D, HT809 | ACAGCAcatATGCTCACACAGA | −9 to −14 | yAAC1 |
| | U, HT811 | AGAAAGCGCAGAGCAAGAAGT | 69 to 47 | yAAC1 |
| Y2(1–26) | D, HT999 | ATACATATGGCTTTACACGCC | −6 to −16 | yAAC2 |
| | U, HT544 | GCCTGACCACCGCAGTAAAGA | 101 to 79 | yAAC2 |
| Y2A(3–6) | D, HT686 | cAtatgtctGTCAAACCCACACTACC | 10 to −36 | yAAC2 |
| Y2A(3–11) | D, HT686 | cAtatgtctCTCCACCCACACTCCA | 25 to −51 | yAAC2 |
| Y2A(3–17) | D, HT687 | cAtatgtctAAGAGGAAATCTAATTT | 43 to −69 | yAAC2 |
| Y2A(3–22) | D, HT688 | cAtatgtctTTTTGTATGATTCTTAA | 58 to −84 | yAAC2 |
| Y1(1–10) | D, HT889 | catatgtctCACACAGACAG | 101 to 79 | yAAC2 |
| | U, HT891 | GCCAGTCGCAGAGGAGTTTC | −3 to −21 | yAAC1 |
| Y1(2–16) | D, HT888 | gCTGACAGAGGGAGCTTCTCAG | 36 to 13 | yAAC1 |
| | U, HT811 | AGAAAGCGCAGAGCAAGAAGT | 69 to 47 | yAAC1 |
| Y2R(3–20) | D, HT895 | catATGCTGaaAaagGctcAGGCCCCACCCATCaCCACCAGGGAAGAaC | −3 to −57 | yAAC2<sup>f</sup> |
| | U, HT896 | GCCAGGTAAGAATCACTAAAAGTTAGAagagATgagcTcGacCtGCTGG | 90 to 37 | yAAC2<sup>f</sup> |
| Y2R(12–20) | D, MB22 | GCCAGGTAAGAATCACTAAAAGTTAGAagagATgagcTcGacCtGCTGG | −3 to −57 | yAAC2<sup>f</sup> |
| | U, MB23 | GCCAGGTAAGAATCACTAAAAGTTAGAagagATgagcTcGacCtGCTGG | 90 to 34 | yAAC2<sup>f</sup> |
| Y2(1–11) Y1(2–16) | D, MB19 | catATGCTGCTGAGAGAAGAAGT | −3 to −33 | yAAC2<sup>f</sup> |
| | U, MB20 | GCCAGGTAAGAATCACTAAAAGTTAGAagagATgagcTcGacCtGCTGG | 42 to −27 | yAAC1<sup>f</sup> |
| Y2(1–17) Y1(8–16) | D, MB21 | GCCAGGTAAGAATCACTAAAAGTTAGAagagATgagcTcGacCtGCTGG | 60 to 4 | yAAC1<sup>f</sup> |
| | U, MB24 | GCCAGGTAAGAATCACTAAAAGTTAGAagagATgagcTcGacCtGCTGG | 22 to −27 | yAAC1<sup>f</sup> |
| | U, MB24 | GCCAGGTAAGAATCACTAAAAGTTAGAagagATgagcTcGacCtGCTGG | 60 to 22 | yAAC1<sup>f</sup> |
| | U, MB24 | GCCAGGTAAGAATCACTAAAAGTTAGAagagATgagcTcGacCtGCTGG | 51 to 34 | yAAC1<sup>f</sup> |
| **C. C-terminal region of yAAC2** | | | | |
| yAAC2 | D, HT685 | TTAGGCGGCGGTTGTCAGTGC | 82 to −105 | yAAC2 |
| | U, HT501 | AGGATCCAGGACGATTGAC | 978 to 958 | yAAC2 |

<sup>a</sup> Underlined and lowercase letters represent the created restriction site of *NdeI*, *BamHI*, *NcoI*, or *NheI* and altered nucleotide, respectively.

<sup>b</sup> Nucleotide numbers of the primer.

<sup>c</sup> Origin of the primer.

<sup>d</sup> Downstream primer.

<sup>e</sup> Upstream primer.

<sup>f</sup> Without template.
the amounts of the derivatives expressed in yeast mitochondria, and the results are summarized in Fig. 4 as a function of the length of the N-terminal region (n). Although expression of Y2D(3–22)/B (n = 6) was not clear, other derivatives were distinctly expressed. The expression of Y2-(1–26)/B was the highest, and that of Y2Δ(3–22)/B was the least. The expression of Y2Δ(3–17)/B was almost the same as that of Y2Δ(3–11)/B (n = 17), although the amino acid sequences of their N-terminal regions were quite different. These results show that the length of the N-terminal region of yAAC2 is
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Effect of the Amino Acid Sequence of the N-terminal Region on the Expression of bAAC1 Derivatives in Yeast Mitochondria—We next examined the effect of the N-terminal sequence on the expression of bAAC1 derivatives in the yeast mitochondrial membrane by changing the sequences of the N-terminal regions of the derivatives, keeping the length the same as that of Y2-(1–26)/B \((n = 26)\). The N-terminal regions of the bAAC1 derivatives examined were as follows: a repeat of the N-terminal region of yAAC1 \((Y1Y1/B)\), reversed sequences of the N-terminal region of yAAC2 \((Y2R/B)\), and composite sequences of the N-terminal regions of yAAC2 and yAAC1 \((Y2Y1/B)\), as shown in Fig. 1A.

The WB-12 transformants of Y1Y1/B and Y2Y1/B derivatives such as Y1-(1–10)Y1-(2–16)/B, Y2-(1–11)Y1-(2–16)/B, and Y2-(1–17)Y1-(8–16)/B grew on YPGly plates with doubling times of \(\sim 23\), 13, and 8 h, respectively (Fig. 2). After Western blotting (Fig. 3), we determined their amounts expressed in the yeast mitochondrial membrane. Their expression was not the same, being \(-30, 75\), and 95% of that of Y2-(1–26)/B, respectively (Fig. 4). Hence, the sequence of the N-terminal region, as well as its length, affects the expression of bAAC1 derivatives. We further examined the expression of Y2R/B derivatives. For this, we prepared the Y2R-(3–20)/B and Y2R-(12–20)/B transformants with \(n = 26\) (Fig. 1A) and examined their growth on YPGly plates and in liquid YPGly medium. They all grew (Fig. 2), and their doubling times were 24 and 9 h, respectively. The amounts of their expression in mitochondrial membranes were determined from the immunostained band intensities by Western blotting (Fig. 3). The amounts of Y2R-(3–20)/B and Y2R-(12–20)/B were determined to be \(-22\) and 92%, respectively, of that of Y2-(1–26)/B (Fig. 4). Here again, expression was dependent on the sequence of the N-terminal region.

It is noteworthy in Fig. 4 that the expression of Y1-(1–10)Y1-(2–16)/B and Y2R-(3–20)/B was similar to that of Y1-(1–16)/B \((n = 16)\) and Y2A-(3–11)/B \((n = 17)\), being 20–30% of that of Y2-(1–26)/B. Therefore, it is possible that bAAC1 derivatives are expressed up to this level depending solely on the length of the N-terminal region, irrespective of its sequence. As the sequence Ser\(^3\)-Leu\(^{11}\) was common to the N-terminal regions of the well expressed derivatives Y2-(1–17)Y1-(8–16)/B, Y2R-(12–20)/B, and Y2-(1–26)/B, this sequence was suggested to be effective for efficient expression of bAAC1 derivatives in yeast mitochondria. Although Y2-(1–11)Y1-(2–16)/B contained this effective sequence, its expression was lower than that of the well expressed derivatives. This might be due to the lack of the sequence Pro\(^{15}\)-Ala\(^{16}\)-Pro\(^{17}\), which is commonly observed in the well expressed derivatives.

Effect of the N-terminal Region on the Expression of yAAC2 Derivatives in Yeast Mitochondria—To further understand the role of the N-terminal region, we next studied its role in the expression of yAAC2. For this, we examined the expression of yAAC2 by changing the length and sequence of its N-terminal region, as we did with bAAC1 derivatives. For examination of the effect of the length of the N-terminal region, we constructed DNA fragments encoding various Y2ΔY2 derivatives such as Y2A-(3–6)/Y2 \((n = 22)\), Y2A-(3–11)/Y2 \((n = 17)\), Y2A-(3–17)/Y2 \((n = 11)\), and Y2A-(3–22)/Y2 \((n = 6)\) (see Fig. 1B); inserted them into pRS314-YA2P; and then introduced these vectors into WT, wild-type yeast strain W303-1B.

From the intensities of the immunostaining bands on Western blots using antiserum against a synthetic peptide of the yAAC2-specific sequence Asn\(^{52}\)-Lys\(^{75}\) \((4, 22, 23)\), we determined their amounts of the YAAC2 derivatives expressed in yeast mitochondrial membranes (Fig. 6). Fig. 7 summarizes the amounts of B-(1–11)/Y2, Y1-(1–16)/Y2, and derivatives of Y2ΔY2 expressed in mitochondrial membranes as a function of the length of the N-terminal region. The amounts expressed increased with increases in \(n\), as observed with derivatives of bAAC1 (see Fig. 4). The amounts of Y2ΔY2 derivatives with \(n = 6, 11, 17,\) and 22 were \(-23, 41, 58,\) and 79%, respectively, of that of Y2-(1–16)/Y2 (53%). It is noteworthy in Fig. 4 that the expression of Y1-(1–10)Y1-(2–16)/Y2, Y2-(1–17)Y1-(8–16)/Y2, and Y2R-(12–20)/Y2 transformants with the same N-terminal length \((26\) amino acids\) as yAAC2 (Fig. 1B). These transformants grew well on YPGly plates, like those of other yAAC2 derivatives (Fig. 5), and their growth doubling times were all \(-4\) h in liquid YPGly medium. The results of Western blotting and expression in yeast mitochondria are shown in Figs. 6 and 7, respectively. In mitochondria, the amounts of Y2-(1–11)Y1-(2–16)/Y2, Y2-(1–17)Y1-(8–16)/Y2, Y2R-(3–20)/Y2, and Y2R-(12–20)/Y2 transformants with the same N-terminal length \((26\) amino acids\) as yAAC2 (Fig. 1B). These transformants grew well on YPGly plates, like those of other yAAC2 derivatives (Fig. 5), and their growth doubling times were all \(-4\) h in liquid YPGly medium. The results of Western blotting and expression in yeast mitochondria are shown in Figs. 6 and 7, respectively. In mitochondria, the amounts of Y2-(1–11)Y1-(2–16)/Y2, Y2-(1–17)Y1-(8–16)/Y2, and Y2R-(12–20)/Y2 were \(-106, 97,\) and 101%, respectively, of that of yAAC2, whereas that of Y2R-(3–20)/Y2 was \(-53\)%. These results were essentially the same as those observed with the corresponding derivatives of bAAC1. The finding that the level of expression of Y2R-(3–20)/Y2 \((53\%)\) was similar to those of Y1-(1–16)/B \((53\%, n = 16)\) and Y2A-(3–11)/Y2 \((58\%, n = 17)\) suggests that yAAC2 derivatives are expressed up to a level of 50–60% depending solely on the length of the N-terminal region. It is noteworthy that the effective sequence Ser\(^{5}\)-Leu\(^{11}\) was commonly observed in all the well expressed yAAC2 derivatives, as observed with bAAC1 derivatives. In contrast to bAAC1 derivatives, the effect of the sequence Pro\(^{15}\)-Ala\(^{16}\)-Pro\(^{17}\) was not clearly observed with yAAC2 derivatives, possibly due to their intrinsically higher expression compared with bAAC1.
derivatives. In addition, the expression of yAAC2 derivatives was always more than that of bAAC1 derivatives with the corresponding N-terminal region (Figs. 4 and 7).

Expression of bAAC1 and yAAC2 Derivatives in Whole Yeast Cells—To understand the role of the N-terminal region of the AAC in its expression in mitochondrial membranes, we determined the expression levels of bAAC1 and yAAC2 and their derivatives in whole yeast cells consisting of those inserted into mitochondrial membranes and those in the cytosol. The bAAC1 derivatives examined were Y2Δ/B, Y2-(1–26)/B, Y1-(1–16)/B, Y1-(1–16)Y1-(2–16)/B, and Y2R-(3–20)/B; and the yAAC2 derivatives were Y2Δ/Y2, Y1-(1–16)/Y2, B-(1–11)/Y2, and Y2R/Y2. After growing the yeast transformants of these AAC derivatives in liquid glycerol medium, the total proteins in yeast cells were extracted by the NaOH/β-mercaptoethanol method (24) and subjected to Western blotting. As shown in Fig. 8A, all the bAAC1 derivatives reacted with the specific anti-peptide antibody against bAAC1, and their expression was determined from the intensities of their immunostaining bands as shown in Fig. 9. The amounts of bAAC1 derivatives tended to increase with increases in n, and no sequence-dependent expression could be observed. Interestingly, the amounts of Y1-(1–10)Y1-(2–16)/B and Y2R-(3–20)/B, which were not high in the mitochondrial membrane (Fig. 4), were almost the same as that of Y2-(1–26)/B in whole cells.

As with the bAAC1 derivatives, the amounts of yAAC2 derivatives were determined by immunoblotting and measuring band intensities of total cellular proteins treated with anti-peptide antibody against yAAC2 (Fig. 8B). The results are shown as a function of n in Fig. 9. In contrast to the expression of bAAC1 derivatives, all the yAAC2 derivatives were well expressed in yeast cells, and their amounts were similar, being independent of the length and sequence of their N-terminal regions. The amount of Y2Δ(3–22)/Y2, whose expression in the mitochondrial membrane was the lowest of all the yAAC2 derivatives examined (~23% of that of yAAC2), was similar to that of yAAC2 in whole cells. In addition, the amounts of yAAC2 derivatives in yeast cells were essentially more than those of bAAC1 derivatives.

Steady-state Transcript Levels of bAAC1 and yAAC2 Derivatives—Finally, we examined the steady-state transcript levels of various bAAC1 and yAAC2 derivatives in yeast cells. To determine the transcript levels, we prepared two probes of Nael-BamHI fragments encoding bAAC1 and yAAC2 and performed Northern blotting with RNA samples of various yeast transformants using these probes. As shown in Fig. 10, the probes of bAAC1 and yAAC2 hybridized with RNA samples of transformants of the respective AAC derivatives. In addition, the probe of yAAC2, but not that of bAAC1, reacted with the RNA sample of the wild-type strain.

As the transcript level of Y2-(1–26)/B was found to be almost the same as that of yAAC2 in yeast cells, as we observed previously (22, 23), we could compare the transcript levels of all the derivatives of bAAC1 and yAAC2 from the intensities of their hybridized bands using those of Y2-(1–26)/B and yAAC2 as controls. We found that the transcript levels of bAAC1 and yAAC2 derivatives were in a range of ~50–150% of those of Y2-(1–26)/B and yAAC2. As the steady-state transcript levels of these derivatives were not greatly different, we concluded that the transcription of bAAC1 and yAAC2 derivatives is not associated with their N-terminal region-dependent expression.

DISCUSSION

In this study, we prepared expression vectors encoding various bAAC1 and yAAC2 derivatives in which peptide segments derived from these AACS as well as yAAC1 were introduced instead of their N-terminal regions, which extend into the cytosol, and introduced them into AAC-disrupted yeast cells. After growing the yeast transformants of these AAC derivatives in glycerol medium, we determined the levels of AAC derivatives expressed in mitochondrial membranes and whole yeast cells. We found that the expression of both types of derivatives in yeast mitochondria increased with increases in the length of the N-terminal region (n). However, the amounts of the derivatives Y1-(1–10)Y1-(2–16)/B and Y2R-(3–20)/B (both n = 26) in the mitochondrial membrane were about the same as those of Y1-(1–16)/B (n = 16) and Y2Δ(3–11)/B (n = 17), and that of Y2R-(3–20)/Y2 (n = 26) was very similar to those of Y1-(1–16)/Y2 (n = 16) and Y2Δ(3–11)/Y2 (n = 17). Therefore, the N-terminal length-dependent expression could be effective up to n = 16 regardless of sequence. The low expression of bAAC1 (n = 11) in yeast mitochondria could be due at least in part to its shorter N-terminal length compared with yAAC2 (n = 26).

As AAC derivatives well expressed in the mitochondrial membrane commonly contained the sequence Ser3–Leu11...
of yAAC2, this sequence is expected to be important for further enhanced expression. In addition, the sequence Pro15–Ala16–Pro17 of yAAC2 is likely to play an auxiliary role in efficient expression. The lower expression of Y2D(3–6)/B, Y1-(1–10)Y1-(2–16)/B, and Y2R-(3–20)/B compared with that of Y2-(1–26)/B and the lower expression of Y2D(3–6)/Y2 and Y2R/Y2 compared with that of yAAC2(Y2-(1–26)/Y2) could be due to lack of the sequence Ser3–Leu11, and the slightly lower expression of Y2-(1–11)Y1-(2–16)/B compared with that of Y2-(1–26)/B could be due to lack of the sequence Pro15–Pro17. It is noteworthy that the expression of yAAC2 derivatives in yeast mitochondria was greater than that of the corresponding bAAC1 derivatives, showing that the structure of the trunk region of intrinsic yAAC2 is more favorable for expression than that of exogenous bAAC1.

The expression of derivatives of bAAC1 and yAAC2 in the mitochondrial membrane is the result of various sequential steps. Of these, transcription and translation of the AAC, transfer of the translated AAC to mitochondria, and its translocation across the outer mitochondrial membrane and insertion into the inner mitochondrial membrane constitute major events. Consistent with our previous finding that the steady-state transcript level of hAAC1 in the transformed WB-12 cells was similar to those of chimeric Y1-(1–16)/B, Y2-(1–26)/B, and yAAC2 as well as that of yAAC2 in the wild-type strain (22, 23), the transcript levels of all the derivatives of bAAC1 and yAAC2 examined in this study did not differ greatly from one other. Therefore, we concluded that the N-terminal region is associated with the post-transcriptional process.

The total amounts of bAAC1 derivatives in yeast cells were essentially dependent on their N-terminal length rather than their N-terminal sequence. In contrast, all the yAAC2 derivatives were expressed in yeast cells to similar extents, irrespective of their N-terminal region, and their amounts in yeast cells were essentially higher than those of the corresponding derivatives of bAAC1. In view of the N-terminal region-dependent expression of bAAC1 derivatives in yeast cells, the expression of yAAC2 derivatives in yeast cells could also be dependent on their N-terminal length. However, this could not be clearly observed, possibly due to their high expression in yeast cells. The moderate expression of bAAC1 derivatives should be more favorable than the high expression of yAAC2 derivatives for...
distinguishing between the role of the N-terminal sequence and that of the N-terminal length.

The amount of bAAC1 expressed in yeast cells was very low, being <10% of that of Y2-(1–26)/B. As its steady-state transcript level was as high as that of the well expressed yAAC2, its low expression in yeast cells could be due to its inefficient translation and/or stability of the translated bAAC1 in the cytosol. As both Y2-(1–26)/B and B-(1–11)/Y2 were expressed well, in similar amounts compared with yAAC2 in yeast cells, the translation of bAAC1 should be efficient, and its low stability, such as high susceptibility to protease, could be a reason why its expression in yeast cells was low. The N-terminal region seems to be related to stability of the translated bAAC1 and yAAC2.

Previous studies showed that the N-terminal region of the AAC is not necessary for its import into the mitochondrial membrane (19–21). In fact, a distinct amount of the yAAC2 derivative Y2Δ(3–22)/Y2 with a short N-terminal region was consistently expressed in mitochondria, although the amount expressed was not high. In general, the AAC consists of three homologous domains (6), and each domain is reported to be associated with translocation across the mitochondrial membrane (21). The first and second domains of the AAC of *N. crassa* were translocated across the outer mitochondrial membrane irrespective of the membrane potential (Δψ), but they were not inserted into the inner membrane. Therefore, they are thought to contain the signal for translocation across the outer membrane. The third domain was translocated across the outer membrane and inserted into the inner mitochondrial membrane in a strictly Δψ-dependent manner. Therefore, the third domain contains signals for the Δψ-dependent translocation across the outer membrane and insertion into the inner mitochondrial membrane (21).

Although the levels of expression of Y1-(1–10)/Y1-(2–16)/B and Y2R-(3–20)/B in whole yeast cells were as high as that of Y2-(1–26)/B, their expression in yeast mitochondria was much lower than that of the latter. Furthermore, the total amounts of yAAC2 derivatives in yeast cells were essentially similar to that of yAAC2, whereas their amounts in yeast mitochondria were dependent on the length and sequence of their N-terminal regions. In view of these results, together with the efficient role of the trunk region of the *N. crassa* AAC in expression in mitochondria (19–21), the N-terminal region of yAAC2 with a specific sequence and definite length should be effective in transfer of AACs to yeast mitochondria, rather than in their translocation across the outer membrane and insertion into the inner membrane. Possibly, interaction of the translated AAC with cytosolic cofactors such as chaperones stabilizes the translated AAC derivatives and enhances transfer to mitochondria.

In this study, we first showed that the N-terminal region is quite effective in the expression of bAAC1 and yAAC2 derivatives in the yeast mitochondrial membrane. Although the N-terminal region of yAAC2 is not associated with its transport activity (22, 23), this region was found to be important for efficient expression in the yeast mitochondrial membrane. The highest expression of yAAC2 of the three yeast AAC isoforms is considered to be due to its high transcript level (12). However, the results of this study suggest that N-terminal region-dependent expression is also responsible for its high expression in the mitochondrial membrane. It is noteworthy that the amino acid sequences of the trunk regions of these yeast AAC isoforms are similar, whereas those of their N-terminal regions are quite different (6). Further study to determine the role of the N-terminal region in the expression of the AAC in the mitochondrial membrane is under way.

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