The Budding Yeast Homolog of the Human EBNA1-binding Protein 2 (Ebp2p) Is an Essential Nucleolar Protein Required for Pre-rRNA Processing*

The human EBP2 protein was found by two-hybrid analysis to interact with the Epstein-Barr virus nuclear antigen 1 (EBNA1). Homologs of human EBP2 can be found in Caenorhabditis elegans, Schizosaccharomyces pombe, and in Saccharomyces cerevisiae, and they all share a conserved 200–300-amino acid block of residues at their C termini. To understand the cellular function of EBP2, we have begun to study the protein in S. cerevisiae. The yeast Ebp2p protein contains N-terminal, nucleolar-associated KKE motifs, and deletion analysis reveals that the C-terminal conserved region is required for the activity of the protein. The EBP2 gene codes for an essential protein that localizes to the nucleolus. Temperature-sensitive ebp2-1 mutants become depleted of ribosomes and cease to divide after several generations at the restrictive temperature of 36 °C. This decline in ribosome levels is accompanied by a diminution in the levels of the 35 S-derived recombinant RNAs (rRNAs) (in particular the 25 S and 5.8 S rRNAs). Pulse-chase, Northern, and primer extension analysis of the rRNA biosynthetic pathway indicates that ebp2-1 mutants are defective in processing the 27 SA precursor into the 27 SB pre-rRNA.

One approach for determining how viruses depend on and alter normal host cell functions is to identify and characterize host factors with which the viral proteins interact. Such an approach can potentially identify host cell proteins that are utilized for aspects of the viral life cycle as well as cellular targets of viral oncoproteins. Such a strategy has been applied to the Epstein-Barr virus, a ubiquitous human herpesvirus that is causally associated with infectious mononucleosis and several cancers (1). During latent infection of human host cells, EBV genomes are maintained in the cell nucleus as double-stranded DNA episomes. Like the cellular chromosomes, the EBV genomes are marked by the viral origin of DNA replication, oriP, and the EBNA1 binding to oriP is thought to activate EBV DNA synthesis and govern the segregation of the episomes by mediating interactions with host cell proteins. In mitosis, EBV genomes and the EBNA1 protein have been observed to associate with the host metaphase chromosomes (5, 6), and therefore, EBNA1 may govern the partitioning of EBV genomes by mediating interactions between the EBV episomes and a component of the condensed host chromosomes. Characterizing factors that interact with EBNA1 are also important because EBNA1 may play a direct role in the development of EBV-associated cancers (7).

Previously, we used a yeast two-hybrid system to identify human proteins that specifically interact with EBNA1 (1). We isolated a cDNA molecule encoding a 35-kDa protein, termed EBP2 (EBNA1-binding protein), and verified its interaction with EBNA1 using three independent assays. The sequence of EBP2 is identical to the human nucleolar protein p40 (8). Immunofluorescence studies on this protein previously showed that it is predominantly nucleolar in interphase and is associated with proliferating cells, but its cellular function had not been determined (8). Functional analyses of EBNA1 mutants defective for EBP2 binding showed that the ability to bind EBP2 correlated with the ability of EBNA1 to mediate the stable segregation of oriP plasmids but not with the ability of EBNA1 to activate DNA replication (1). Recent immunofluorescence studies showed that EBP2 colocalizes with EBNA1 on the cellular mitotic chromosomes. These results suggested that the EBNA1-EBP2 interaction is important for EBNA1-mediated DNA segregation; however, the normal cellular function of EBP2 remained to be elucidated.

Data bank searches with the human EBP2 sequence identified homologs of this protein in Schizosaccharomyces pombe, and Caenorhabditis elegans, none of which has been characterized. To better understand the conserved cellular function of EBP2, we investigated EBP2 in budding yeast. Here we show that Ebp2p is an essential protein that, like human EBP2, localizes to the nucleolus. A yeast strain containing a temperature-sensitive allele of EBP2 was generated and found to exhibit defects in pre-rRNA processing. Detailed analysis of the yeast RNA-processing pathway indicates that Ebp2p is required for efficient processing of the 27 SA into the 27 SB precursor rRNA.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Media

The genotypes of the strains used in this study are listed in Table I, and the plasmids used in this study are indicated in Table II. Standard yeast genetic techniques and media were used throughout (10). Sequencing of constructs was performed at the University of Pennsylvania DNA sequencing facility.

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The abbreviations used are: EBV, Epstein-Barr virus; EBNA1, EBV nuclear antigen 1; r-, recombinant; GFP, green fluorescent protein; DAPI, 4',6'-diamidino-2-phenylindole.

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The sequencing facility, and the MOBIX Central facility at McMaster University. Restriction enzymes were purchased from New England Biolabs, and Taq polymerase was purchased from Promega.

Creation of EBP2 Strains

Knock-out Analysis—To generate an EBP2 deletion strain, a 2-kilobase pair 

Deletion Analysis—Plasmids pMM112 (EBP2 LEU2) and pMM113 (EBP2 URA3) were created by cloning a 2-kilobase pair 

EBP2 is Required for Pre-rRNA Processing

TABLE I

| Strain | Alias | Relevant genotype | Reference or source |
|--------|-------|-------------------|---------------------|
| yMM13  | CH1584| MAT a leu2Δ1 trp1Δ3 ura3-52 | Connie Holm         |
| yMM41  | FY1123 × FY104 | MAT a ura3-52 LYS2 LEU2 TRP1 his3 | Fred Winston       |
| yMM42  | MAT a EBP2 ura3-52 LYS2 LEU2 TRP1 his3 | This study          |
| yMM48  | MAT a EBP2 ura3-52 LYS2 LEU2 TRP1 his3 | This study          |
| yMM49  | MAT a ebp2::HIS3 his3 ura3-52 leu2Δ2 trp1Δ63 lys2-1285 | This study          |
| yMM57  | MAT a ebp2::HIS3 his3 ura3-52 leu2Δ2 trp1Δ63 lys2-1285 | This study          |
| yMM58a | MAT a ebp2::HIS3 his3 ura3-52 leu2Δ2 trp1Δ63 lys2-1285 | This study          |
| yMM60  | CH325 | MAT a leu2-801 ura3-52 his4-539 top2-4 | Ref. 20            |
| yMM64  | Y168  | MAT a his7 ura1 edc28 | Connie Holm         |
| yMM138 | MAT a ebp2::HIS3 his3 ura3-52 leu2Δ2 trp1Δ63 lys2-1285 | This study          |
| yMM144 | MAT a ebp2::HIS3 his3 ura3-52 leu2Δ2 trp1Δ63 lys2-1285 | This study          |
| yMM148 | MAT a ebp2::HIS3 his3 ura3-52 leu2Δ2 trp1Δ63 lys2-1285 | This study          |
| yMM151 | MAT a ebp2::HIS3 his3 ura3-52 leu2Δ2 trp1Δ63 lys2-1285 | This study          |
| yMM178 | MAT a EBP2.Kan leu2Δ1 trp1Δ63 ura3-52 | This study          |
| yMM179 | MAT a ebp2-1 Kan leu2Δ1 trp1Δ63 ura3-52 | This study          |
| yMM196 | MAT a EBP2 Kan ura3-52 hom3 can-1 leu2Δ2 trp1 | This study          |
| yMM199 | MAT a ebp2-1 Kan ura3-52 hom3 can-1 leu2Δ2 trp1 | This study          |
| yMM239 | MAT a EBP2 Kan leu2Δ1 trp1Δ63 ura3-52 | This study          |
| yMM240 | MAT a EBP2.Kan leu2Δ1 trp1Δ63 ura3-52 | This study          |

* Variants of this strain were used to express the different ebp2 deletion alleles, including ebp2ΔN62 (yMM143), ebp2ΔN90 (yMM140), ebp2ΔN127 (yMM145), ebp2ΔN178 (yMM147), ebp2ΔN231 (yMM174), ebp2ΔC50 (yMM135), ebp2ΔC105 (yMM136), ebp2ΔC151 (yMM131), and ebp2ΔN178ΔC50 (yMM180).

TABLE II

| Plasmid        | Relevant genotype | Reference or source |
|----------------|-------------------|---------------------|
| PCF15          | RDN1 Ampβ ori     | Scott Holmes        |
| pFA6-GPP-kanMx | GFP(S65T) Kan’ CEN ARS | New England Biolabs |
| pLitmus38      | Ampβ ori          | This study          |
| pMM88          | EBP2 Ampβ ori     | This study          |
| pMM89          | ebp2::HIS3 Ampβ ori | This study          |
| pMM112         | EBP2 LEU2 CEN ARS | This study          |
| pMM113         | EBP2 URA3 CEN ARS | This study          |
| pMM131         | ebp2ΔC50 LEU2 CEN ARS | This study          |
| pMM152         | ebp2ΔC105 LEU2 CEN ARS | This study          |
| pMM153         | ebp2ΔC151 LEU2 CEN ARS | This study          |
| pMM144         | ebp2-1 LEU2 CEN ARS | This study          |
| pMM146         | ebp2ΔN90 LEU2 CEN ARS | This study          |
| pMM147         | ebp2ΔN62 LEU2 CEN ARS | This study          |
| pMM156         | ebp2ΔN127 LEU2 CEN ARS | This study          |
| pMM160         | EBP2.Kan’ LEU2 CEN ARS | This study          |
| pMM161         | ebp2-1 Kan’ LEU2 CEN ARS | This study          |
| pMM163         | ebp2ΔN178 LEU2 CEN ARS | This study          |
| pMM169         | GFP-ebp2ΔN62 LEU2 CEN ARS | This study          |
| pMM200         | ebp2ΔN231 LEU2 CEN ARS | This study          |
| pMM203         | ebp2ΔN178ΔC50 LEU2 CEN ARS | This study          |
| pNOY353        | GAL7-35 S rDNA 5 S rDNA TRP1 2μm Ampβ | Ref. 24 |
| pRS415         | LEU2 CEN ARS      | Ref. 9             |
| pRS416         | URA3 CEN ARS      | This study          |
| pYST122        | ACT1 Ampβ ori     | Scott Holmes        |
These deletion fragments were created by polymerase chain reactions employing oligonucleotides engineered to contain the appropriate restriction sites, start, and stop codons. The EBP2 deletion plasmid constructs were tested for activity in yeast by introducing them into an eby2::HIS3 disruption strain by the plasmid shuffle technique. The LEU2-containing EBP2 constructs were transformed into strain yMM49, and the Leu+ transformants were recovered (yMM58 and its siblings). The transformants were then transferred onto plates containing 5-fluoorotic acid to select for loss of the URA3 EBP2 plasmid pMM113.

**Generation of eby2-1 Strains**—The eby2-1 allele was created by using a combination of mutagenic polymerase chain reaction, followed by a gap-repair method of yeast transformation. An EBP2 DNA fragment was amplified under conditions of low Mg²⁺ concentration (1.5 mM), and it was transformed into strain yMM49 along with linearized plasmid pMM112 (EBP2 LEU2) that had been digested with EcoRI and StuI to remove the EBP2-coding sequences. Leu+ transformants were recovered and plated to 5-fluoorotic acid plates at temperatures from 16 °C to 36 °C. A single temperature-sensitive colony was isolated out of more than 600 transformants that could not grow at temperatures above 34 °C. The plasmid containing the eby2-1 allele (pMM144) was recovered and sequenced. EBP2 KAN and eby2-1 KAN integration alleles were created by cloning a 1.2-kilobase pair FspI KAN fragment from plasmid pFA6-GFP-kamMX6 into the EcoRI site of plasmids pMM112 and yMM49 respectively, yielding plasmids pMM160 and pMM161. Integrated EBP2.KAN or eby2-1 KAN yeast strains yMM178 and yMM179 were created by transforming strain yMM13 with pMM160 and pMM161 and selecting transformants with G418. The integrity of the integrated alleles was confirmed by sequence analysis.

**Construction of GFP-EBP2 Strains**—The GFP-eby2AN62 fusion construct pMM169 was created by cloning a DNA fragment containing the S65T mutant of the green fluorescent protein from plasmid pFA6-GFP-kamMX6 into the EcoRI site of plasmids pMM112 and yMM149, respectively, yielding plasmids pMM160 and pMM161. Plasmid yMM179 was created by transforming strain yMM49 along with linearized plasmid pMM160 and selecting transformants with G418. The integrity of the integrated alleles was confirmed by sequence analysis.

**Fluorescent Microscopy**

To visualize cellular and nuclear morphologies, 1 ml of early log cultures grown in liquid yeast extract/petone/x-dextrose medium at 28 °C was collected, sonicated, and fixed in 3.7% formaldehyde. Cells were then washed twice in phosphate-buffered saline (150 mM NaCl, 10 mM sodium phosphate, pH 7.4) and resuspended in 50 μl of phosphate-buffered saline containing 10% dimethyl sulfoxide and 1% Triton X-100. Five μl of cells were added to 5 μl of mounting medium containing 1 mg/ml p-lysine and 0.2% methanol. Cells were incubated for 2.5 min. Cold methionine was then added to a final concentration of 50 mM. One-ml samples were collected at the times indicated, and total RNA was prepared as described above. Equal number of counts per sample were resolved on 1.25% denaturing agarose gels and transferred onto Zeta-membranes. The blots were cross-linked, soaked in Amplify solution (Amersham Pharmacia Biotech), and exposed to phosphorimaging analysis.

**RESULTS**

**The EBP2 Protein Is Conserved among Eukaryotes**—To identify human proteins that interact with the Epstein-Barr virus-encoded EBNA1 protein, a two-hybrid screen was performed using the EBNA1 protein as a bait to screen an expression library derived from human B lymphocytes (1). This screen identified a human protein that interacts specifically with EBNA1, and it was named EBP2. The full-length cDNA encoding the EBP2 protein was cloned and sequenced, revealing that EBP2 encodes for a 306-amino acid protein. Data bank searches revealed an exact match between the human EBP2 sequence and nucleolar protein p40 (GenBank™ accession number U86060). Nucleolar protein p40 was previously shown to localize to the nucleoli of proliferating cells, but it had not been functionally characterized (8). Significant matches were also found between the human EBP2 protein sequence and predicted proteins encoded by C. elegans (C18A3.3), S. cerevisiae (YKL172w), and S. pombe (SPAC17H2) genes, each of which were identified as a result of genomic sequencing projects (17, 18). Each of these proteins contains a C-terminal block of 200–300 amino acids that is at least 38% identical to sequences from human EBP2 (Fig. 1A), but as yet there is no information on their respective functions. The 427-amino acid sequence of budding yeast Ebp2p was scanned for recognizable protein motifs, and it was found to contain two putative nuclear localization signals near its C terminus and a basic domain (lysine rich) followed by an acidic domain (glutamic acid rich) at its N terminus (Fig. 1B). Within the N-terminal sequences there are five copies of the KRE motif, which is associated with yeast nucleolar proteins (19). Other than these limited features, Ebp2p contained no obvious matches to previously identified protein consensus sequences that would provide insight into its function.

**EBP2 Is an Essential Gene in Yeast**—To further investigate the function of Ebp2p, we determined whether or not the EBP2 gene is essential in yeast. We isolated the EBP2 gene from a λ clone (ATCC 3657) that contained yeast genomic sequences from the left arm of chromosome XI, and this clone was used to create an eby2::HIS3 disruption allele (see “Experimental Procedures”). A heterozygous disruption strain was generated, and it was transformed into strain yMM49 along with linearized plasmid pMM160 and selecting transformants with G418. The integrity of the integrated alleles was confirmed by sequence analysis.
sporulated, and dissected, yielding only two viable spores from each tetrad (Fig. 2A). The viable colonies were His<sup>+</sup>, indicating that the inviable spores had inherited the disrupted ebp2::HIS3 allele. The inviable colonies were found to contain microcolonies of 1 to 4 cell bodies, indicating that the deletion strains were able to undergo at most two rounds of cell division before they ceased to divide. To confirm that the inviability associated with the ebp2::HIS3 disruption was due to loss of EBP2 function, the heterozygous diploid strain was transformed with an EBP2-expressing plasmid. This extra plasmid-borne, wild type copy of EBP2 was sufficient to restore viability in the strains that inherited the disrupted ebp2::HIS3 allele (data not shown). These experiments indicate that EBP2 is an essential gene in yeast.

Deletion Analysis of Ebp2p—In the effort to identify the regions of the Ebp2 protein that contribute to its essential function, we undertook a deletion analysis of the EBP2 gene (Fig. 2B). A series of plasmid-borne N- and C-terminal EBP2 deletion alleles was constructed and tested for the ability to complement an ebp2::HIS3 disruption allele by the plasmid shuffle method (see “Experimental Procedures”). For the N-terminal deletions, we created constructs that encoded for...
Ebp2 proteins containing deletions of 62, 90, 127, 178, and 231 amino acids. We found that, with the exception of the 231-amino acid deletion, all of these alleles were functional, suggesting that the N terminus of the protein is dispensable for the essential Ebp2p activity. For the C-terminal deletions, we created alleles removing 50, 105, and 151 amino acids from the end of the protein. Unlike the N terminus, the amino acids at the C terminus of the Ebp2p are critical for its activity. The strain bearing the 50-amino acid deletion construct grew slowly, and the 105- and 151-amino acid deletion alleles were non-functional. We also constructed an allele that combined the 178-amino acid N-terminal deletion with the 50-amino acid C-terminal deletion. The strain containing this construct was nearly completely dependent on the wild type EBP2 plasmid for viability, as only a few cells from the culture grew up on the 5-fluoroorotic acid plate. Therefore, the regions of yeast Ebp2p that are critical for its essential activity correspond to the C-terminal two-thirds of the protein and include the amino acids that are most highly conserved among the other EBP2 homologs as well as the putative nuclear localization signals.

The Ebp2 Protein Colocalizes with the Nucleolar Protein Nop1p—To investigate whether yeast Ebp2p was in fact a nucleolar protein, we constructed a fusion construct containing the sequences from the A. victoria GFP fused to the N-terminal region of Ebp2p. The resulting GFP-ebp2ΔN62 construct was tested for Ebp2p activity, and it was found to complement an ebp2::HIS3 disruption allele. Cells expressing the GFP-Ebp2 fusion protein alone from the native EBP2 promoter were collected, stained with DAPI, and observed by fluorescence microscopy (Fig. 3). The GFP-Ebp2p signal was found to localize to the nuclear region of the cells, but the staining pattern did not always exactly overlap with the DAPI signal. Since the nucleolus often does not stain well with DAPI, we investigated whether the GFP-Ebp2p signal corresponded to the nucleolus. Anti-Nop1p antibodies were used as a nucleolar marker, and it was found that the Nop1p staining pattern coincided with the GFP-Ebp2p signal. Thus, like the reported location of human EBP2, yeast Ebp2p also appears to be a nucleolar protein.

Phenotypes of Temperature-sensitive ebp2-1 Mutants—To further elucidate the function of the Ebp2 protein, we created and characterized a conditional ebp2 mutant. Random mutations were introduced into the EBP2 gene by mutagenic polymerase chain reaction, and the mutated fragments were introduced into yeast cells by the gap-repair method (see “Experimental Procedures”). Colonies were screened for their ability to grow at temperatures ranging from 16 °C to 36 °C, and a temperature-sensitive mutant (ebp2-1) was recovered that could not grow at temperatures above 34 °C (see below). The ebp2-1 allele was recovered from yeast, sequenced, and found to encode for a protein that differs from wild type Ebp2p at four amino acids (Q92P, S140P, F217S, and E243G). We further investigated the contribution of these four amino acids to Ebp2p function by creating additional EBP2 alleles that contained either the Q92P and S140P or the F217S and E243G substitutions. We observed that the allele containing the Q92P and S140P or the F217S and E243G substitutions. Interestingly, the E243G substitution corresponds to a glutamic acid residue that is conserved among all four of the EBP2 homologs.

Having isolated the conditional ebp2-1 allele, we then sought to characterize the phenotypes that were associated with this mutation. The plasmid-borne ebp2-1 gene was used to create an integration allele (ebp2-1.KAN), and this construct was used to replace the wild type chromosomal EBP2 gene by homologous recombination. Like the plasmid-bearing strain, the integrated ebp2-1 strain (YMM179) exhibited a temperature sensitivity that could be complemented by a plasmid containing a wild type EBP2 gene (Fig. 4A and data not shown). In addition, we created a EBP2.KAN strain (YMM178), and these two strains were used in temperature shift experiments to determine how loss of Ebp2p activity affects yeast cell growth. In liquid culture, the ebp2-1 strain exhibited a longer doubling time than the wild type strain at 28 °C (2.4 h versus 1.7 h). After shifting...
to 36 °C, the EBP2 strain continued to divide, with a doubling time of 1.5 h (Fig. 4B). In contrast, the ebp2-1 culture divided for several generations at 36 °C, but it did so with increasingly longer generation times, such that by 14 h at 36 °C, cell division had nearly ceased. To confirm that the ebp2-1 cells were able to cycle for a few generations at 36 °C, we performed a similar temperature shift experiment on plates. When single ebp2-1 cells were incubated on yeast extract/peptone/dextrose plates at the restrictive temperature of 36 °C, they formed microcolonies of up to 10–20 cells before cell division terminated (data not shown). Furthermore, temperature shift experiments revealed that ebp2-1 cells could recover from incubations at 36 °C for up to 16 h. These results suggest that the loss of Ebp2p activity that is associated with the ebp2-1 mutation does not rapidly or irreversibly terminate cell division. The ebp2-1 mutation is, however, a strong allele, since we observed a complete, albeit delayed, block of cell division at the restrictive temperature.

**ebp2-1 Mutants Are Defective in rRNA Biosynthesis**—Since the human EBP2 protein was originally identified as a nucleolar antigen that was abundant in proliferating cells, we assayed ebp2-1 mutants for defects associated with ribosome biosynthesis. Cell extracts from EBP2 and ebp2-1 strains grown at 28 and 36 °C were prepared and fractionated on sucrose gradients to separate free proteins from monosomes and polysomes (Fig. 5.). For the EBP2 strain at both 28 and 36 °C, we could detect an 80 S monosome peak and up to 7 peaks within the larger polysome portion of the profile. We could also visualize two minor peaks of sizes smaller than 80 S, consistent with the expected sedimentation properties of the free 40 S and 60 S ribosomal subunits. For the ebp2-1 strain at 28 °C, the monosome and polysome peaks were smaller than those of the EBP2 strain, and there was an increase in the size of a peak at the expected position for a 40 S particle. These changes in the ribosome profiles were more pronounced in the samples that were prepared from the ebp2-1 strain after it was incubated for 4 h at 36 °C. This overall drop in ribosome levels in ebp2-1 mutants at the restrictive temperature indicates that Ebp2p likely plays a role in either ribosome synthesis or ribosome stability.

The production of the ribosomal subunits requires rRNAs that are transcribed from the many rDNA repeats located within the nucleolus. Each rDNA repeat contains a gene encoding for the large 35 S precursor rRNA transcript that is processed into the mature 25 S, 18 S, and 5.8 S rRNAs as well as a gene that encodes for the 5 S rRNA (reviewed in Kressler et al. (21)). These genes are transcribed in opposite directions by two different RNA polymerases (RNA polymerase I and III, respectively), and the transcriptional status of these genes is coordinated to achieve appropriate levels of expression (22). To test whether the observed drop in ribosome content was related to changes in rRNA metabolism, we prepared total RNA from equal numbers of EBP2 and ebp2-1 cells growing at 28 °C and after shifting to 36 °C for up to 8 h (Fig. 6A). Because of the abundance of the larger 25 S and 18 S rRNAs, they could be detected directly by ethidium bromide staining. The levels of these rRNAs were found to be comparable in the EBP2 samples from 28 °C and 36 °C as well as from the ebp2-1 strain at 28 °C. However, upon shifting to the restrictive temperature, the amounts of the 25 S and 18 S rRNA bands in the ebp2-1 strain were found to drop significantly. This drop could be detected even after 2 h at the restrictive temperature, and by 8 h the levels of these rRNAs were reduced by severalfold.

Previously, Mizuta and Warner (23) reported that ribosome biosynthesis is moderated by a regulatory network that controls the transcriptional levels of both rRNAs and ribosomal proteins. They demonstrated that when temperature-sensitive secretory pathway mutants (i.e. sly1, sec1) are shifted to the restrictive temperature, the rRNA transcript levels as well as

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**Fig. 4. Temperature sensitivity of ebp2-1 mutants.** A, temperature-sensitive ebp2-1 mutants do not grow at 36 °C. B, wild type and mutant ebp2 strains (yMM178 and yMM179) were grown overnight in rich medium at 28 °C. The cultures were then split and incubated at 28 °C or 36 °C, and the increase in cell density was monitored for 14 h.

**Fig. 5. ebp2-1 mutants have decreased ribosome levels.** EBP2 and ebp2-1 strains were grown at either 28 °C or shifted to 36 °C for 4 h and then prepared for polysome gradient analysis as described under “Experimental Procedures.” The positions of the 80 S monosome peaks and the polysome peaks are indicated.
total RNA was prepared from equal numbers of EBP2 and ebp2-1 cultures growing at 28 or 36 °C. The samples were separated on an agarose gel and stained with ethidium bromide. The positions of the 25 S, 18 S, and 5.8 S rRNAs are indicated. B, RNA samples as in A were blotted and hybridized with probes specific for the 25 S, 18 S, 5.8 S, and 5 S rRNAs, the U3 small nuclear RNA, as well as the indicated mRNAs. C, quantitation of the average transcript levels from the 25 S, 18 S, 5.8 S, 5 S, and U3 probes. For each probe, the transcript level corresponding to the 28 °C culture was defined as 100%, and values represent the averages of at least three independent experiments.

The lowering of rRNA levels in ebp2-1 cells could potentially be due to 1) increased degradation of the rRNAs at the restrictive temperature, 2) decreased rRNA synthesis coupled with the dilution of the ribosomes as cell division continues, or 3) defects in processing of the pre-rRNA transcripts. To investigate these possibilities, EBP2 and ebp2-1 cells were first arrested with a factor before shifting to the restrictive temperature. It was observed that after 4 h at 36 °C, α factor-treated ebp2-1 cultures did not exhibit the same pronounced decline in rRNA levels that was observed in cycling cells (data not shown). Thus, it is unlikely that the existing rRNAs become degraded upon shifting to higher temperatures, and also, the observed decline in rRNA content per cell may reflect a dilution effect that occurs as ebp2-1 cells divide.

To investigate whether the decline in the 35 S-derived rRNAs could be related to defects in rDNA transcription, we employed plasmid pNOY353, which contains a copy of the full 35 S rDNA repeat under the control of the RNA polymerase I-directed GAL7 promoter (24). Previously, it has been demonstrated that this plasmid can compensate for numerous defects in components of the RNA polymerase I machinery (including rpa190 mutations) by bypassing the normal RNA polymerase I-mediated rDNA transcription (25). Plasmid pNOY353 was transformed into both EBP2 and ebp2-1 strains (yMM239 and yMM240), and the transformants were tested for viability in the presence and absence of galactose at 28 and 36 °C. The pGAL7 rDNA plasmid was unable to compensate for the temperature-sensitive phenotype of ebp2-1 mutants. Furthermore when ebp2-1 cells bearing this plasmid were shifted to the restrictive temperature, we could still observe a decline in 25 S and 18 S rRNA levels (data not shown). These data suggest that the ebp2-1-mediated defect was not limited to disruptions in rDNA transcription.

To follow rRNA biosynthesis more directly, EBP2 and ebp2-1 cultures were subjected to pulse-chase analysis (Fig. 7). Cultures were grown at either 22 or 36 °C, pulsed with [methyl-3H]methionine for 2.5 min, and then chased with cold methionine. RNAs were prepared from each sample, and equal numbers of counts per lane were analyzed by agarose gel electrophoresis.

Fig. 7. Pulse-chase experiments with [methyl-3H]methionine. EBP2 and ebp2-1 cultures were grown at 22 °C and then shifted where indicated to 36 °C for 2 h. Cultures were pulsed with [3H-methyl]methionine for 2.5 min and then chased for up to 18 min with cold methionine. RNAs were prepared from each sample, and equal numbers of counts per lane were analyzed by agarose gel electrophoresis.
i.e. the 35 S precursor rRNA is indicated. The positions of the externally transcribed spacer (ETS) and the internally transcribed spacers (ITS) are indicated below the transcript. The positions of the oligonucleotides to the mature 18 S, 5.8 S, and 25 S rRNAs are indicated. The locations of the top band correspond to the 25 S and 5.8 S rRNAs, this result is consistent with the polysome gradient analysis.

rRNA processing in yeast involves a complex series of specific endonucleotylic and exonucleotylic digestions that produce defined intermediate species (reviewed in Ref. 21). Given that the pulse-chase experiments revealed that the ebp2-1 strain exhibited defects in the pre-25 S rRNA processing at 36 °C, we probed Northern blots with labeled oligonucleotides that hybridized to specific rRNA-processing intermediates (Fig. 8). To detect these intermediates, we chose probes that have previously been used to characterize mutants defective in the rRNA-processing machinery. They include probes that are directed toward sequences within ITS1 and ITS2 that are cleaved off during the processing of the 25 S and 18 S rRNAs (probes b, c, and e) as well as probes that recognize the mature 25 S and 18 S rRNA species (probes 25 and 18) (Fig. 8A) (15).

Equal amounts of total RNA were prepared from EBP2 and ebp2-1 strains at 28 °C and 36 °C, and the samples were subjected to Northern analysis. To ensure that the assignment of the detected bands was consistent between experiments, the samples were stripped and reprobed with each oligonucleotide.

We could detect the mature 25 S and 18 S rRNAs in both EBP2 and ebp2-1 samples with the 25 and 18 probes, and these served as internal size markers. One of the early steps in rRNA processing involves cleavage of the large precursor rRNA at the A2 site. This cleavage produces a 20 S species that will be further processed to the 18 S rRNA, and a 27 SA2 species that will be processed into the 5.8 S and 25 S rRNAs. The 20 S intermediate could be readily detected in both strains by the b probe, although the levels of this species was diminished in ebp2-1 samples derived from the 36 °C cultures (Fig. 8B). Similarly, probing with oligonucleotide c revealed the expected 27 SA3 species in all of the EBP2 and ebp2-1 samples. Normally, after cleavage at position A2, there is a cleavage at the adjacent A3 position, yielding the 27 SA3 species. The 5' end of the 27 SA3 species is then subjected to 5'-3' exonucleotylic digestion, which produces the 27 SB species. Two prominent intermediates could be detected in the EBP2 samples with the e probe. Since this probe hybridizes to a sequence 3' of the 5.8 S rRNA, it could potentially detect the 27 SA2, 27 SA3, and 27 SB precursors. The position of the top band corresponded to the position of the 27 SA3 band detected by the c probe. However, due to the small size differences between the 27 SA2 and 27 SA3 pre-rRNAs, these species may not be well resolved under these conditions. We were able to detect a lower band, which corresponds to the 27 SB precursor. This band was prominent throughout the EBP2 samples and present in the ebp2-1 samples derived from the culture at 28 °C. This band was greatly reduced, however, in the ebp2-1 samples derived from cultures at 36 °C. The decrease in the level of the 27 SB species was significant, and it occurred within the first hour of shifting to 36 °C. We also observed that the ebp2-1 samples exhibited an increase in the level of the 27 SA species at 36 °C. These results indicate that ebp2-1 mutants are defective in processing the 27 SA pre-rRNA into the 27 SB species at the restrictive temperature.

Probes b, c, and e could also detect the accumulation of the 35 S precursor at the later time points in ebp2-1 samples at 36 °C. The accumulation of this precursor is suggestive of a disruption to the early steps of the rRNA-processing pathway. Such an effect has been observed for other rRNA-processing mutants (15) and may reflect coordination between processing events. Furthermore, probes c and b detected a 23 S species in ebp2-1 samples derived from cultures at 36 °C. A 23 S intermediate
Ebp2p Is Required for Pre-rRNA Processing

has been detected previously (15, 27, 28), and it represents rRNAs that are cleaved at the A3 site without being cleaved at positions A0, A1, and A2. The appearance of this intermediate argues that cleavage at position A3 can occur in ebp2-1 mutants at the restrictive temperature. Therefore, the observed loss of the 27 SB species may reflect a defect not on the cleavage at A3 but in the subsequent exonucleolytic digestion that processes the 27 SA3 pre-rRNA into the 27 SB species.

To further investigate which rRNA-processing step requires Ebp2p activity, we performed primer extension analysis on RNAs derived from EBP2 and ebp2-1 cultures grown at either 22 or 36 °C (Fig. 9). Oligonucleotide e was chosen for this analysis because it can be used to detect rRNAs derived from processing events that occur within ITS1 (see Fig. 8A). For the EBP2 samples, we could detect strong bands corresponding to the expected cleavage events at B1S, B1L, and A2. We could also detect weaker signals that corresponded to cleavages at site A3 or at sites of known rRNA stem-loop structures. Similar bands were apparent in the ebp2-1 sample derived from cells growing at 22 °C. Upon shifting to 36 °C, however, we could detect a relative decrease in the signal corresponding rRNAs derived from cleavages at B1S and B1L and an increase in the levels of the A2 signal. We could also detect an increase in the intensity of multiple bands that migrated to positions below that expected for cleavages at the A3 site. The accumulation of these rRNA species in ebp2-1 mutants is consistent with the hypothesis that Ebp2p is required for efficient processing of the 27 SA3 rRNA (Fig. 10).

**DISCUSSION**

The data presented here suggest that the EBP2 gene from *S. cerevisiae* encodes a novel, essential protein that is required for rRNA biosynthesis. The observation that genes predicted to encode EBP2 homologs can be identified among the organisms that are the most highly sequenced (i.e. *S. cerevisiae*, *S. pombe*, *C. elegans*, and humans) indicates that yeast Ebp2p represents a member of a family of proteins that are widely conserved among eukaryotes. Each of these EBP2 genes encode a conserved block of 200 to 300 amino acids that is located toward the C terminus of the respective proteins. The importance of this conserved domain for Ebp2p activity is supported by both deletion analysis and the observation that mutations that disrupt yeast Ebp2p function map to conserved residues with this region of the protein. Since database searches did not identify homologs in either bacteria or archeabacteria, the EBP2 family of proteins may be specific to eukaryotes. The observed nuclear localization of both human (8) and yeast Ebp2p is consistent with that hypothesis. Although yeast Ebp2p contains N-terminal KKE motifs that are found in other nucleolar proteins (19, 30), as is the case for Nop56p, Nop58p, Cbf5p, and Dbp3p, these motifs are not required for its essential activity.

The analysis of ebp2-1 mutants indicates that they cease to divide at the restrictive temperature, but that they do so gradually and only after several generations. Even after incubation at the restrictive temperature for the equivalent of several generations, ebp2-1 mutants can recover if returned to permissive conditions. One possibility is that this “slow stop” phenotype reflects a gradual dilution of the ribosome population in ebp2-1 cells due to defects in rRNA production. If ribosome synthesis became interrupted upon shifting to 36 °C, then one would expect that as the cells continued to divide, the existing ribosome population would become limiting, and the generation time would be expected to increase. Eventually, protein synthesis levels would fall below a threshold required to maintain cell division, and the cells would stop dividing. The cells may be expected to recover from this arrest if they were shifted back to the permissive temperature and resumed production of rRNAs and ribosomes.

Investigations into the rRNA metabolism pathways indicates that the initial decline in rRNA levels that is observed when ebp2-1 mutants are shifted to the restrictive temperature is unlikely to be due to either elevated rRNA degradation nor to a block in rDNA transcription. The strongest and most immediate phenotype that we observed was a block in the processing of the 27 SA intermediate into the 27 SB pre-rRNA (probe e in Fig. 8D). There are two steps involved in this conversion: an initial endonucleolytic cleavage at A3 followed by a 5′-3′ exonucleolytic digestion to site B1S (29). The appearance of a 23 S intermediate within the ebp2-1 samples at 36 °C suggests that cleavage at A3 does occur in ebp2-1 mutants. To verify that the 23 S species that we observed did not contain sequences 3′ of the A3 site, we probed the blots with an oligonucleotide complementary to sequences between the A3 and B1L sites. This probe did not detect a 23 S band but did detect the 35 S and 27 SA intermediates (data not shown). The observed 23 S intermediate is therefore not due to cleavage at site B1L. The suggestion that ebp2-1 mutants are proficient in the cleavage at A3 is supported by the observation that rpp2 mutants (which are defective in the A3 cleavage) do not accumulate a 23 S precursor (31).

An accumulation of the 35 S pre-rRNA could be detected in samples derived from ebp2-1 cultures at 36 °C with several of the probes. This species is processed early in the rRNA-processing pathway, and its accumulation suggests that either Ebp2p activity may be required for multiple processing steps or that there is coordination between different processing events (28). Since defects in later processing steps have been shown to lead to an accumulation of the 35 S precursor (21, 32), we prefer the interpretation that the major defect in ebp2-1 mutants is in the processing of the 27 SA pre-rRNA into the 27 SB pre-rRNA. A defect at this stage in the rRNA-processing pathway is consistent with the observation that the 5.8 S and 25 S rRNAs are the earliest RNA species to decline after ebp2-1 mutants are
shifted to the restrictive temperature. Similarly, the depletion of the 25 S and 5.8 S rRNAs would be expected to limit the assembly of the 60 S ribosome subunit. This defect is consistent with the observed increase in the relative proportions of the 40 S-sized peak apparent in the polysome profiles prepared from ebp-2 mutants.

Two homologous proteins (the Rat1p and Xrn1p exonucleases) have previously been implicated in the 27 S to 27 SB step of the rRNA-processing pathway (29). The RAT1 gene encodes for an essential nucleolar protein, mutations in which lead to an accumulation of 5.8 S rRNAs with a 5′-extension. Rat1p has been purified, and it exhibits 5′-3′ exonuclease activity in vitro (31). Similarly, xrn1 mutations are defective in this rRNA-processing step, and Xrn1p also demonstrates 5′-3′ exonuclease activity (31). Since both of these proteins have been implicated in various aspects of RNA metabolism (Ref. 21 and references therein), it will be of interest to determine whether Ebp2p contributes to either of their activities with respect to the rRNA-processing pathway.

Based on the strong homology between the human and yeast EBP2 proteins as well as the observation that both proteins are nucleolar, we presume that human EBP2 also functions in rRNA processing. We initially identified human EBP2 as a protein that interacts with the EBNA1 protein of EBV. Our data strongly suggest that the EBNA1-EBP2 interaction is important during mitosis for the efficient partitioning of the EBV episomes (1),2 but the functional significance of this interaction in interphase remains to be investigated. Since EBNA1 is present in the nucleolus (in addition to other part of the nucleus), it will be interesting to determine whether the interaction of EBNA1 with EBP2 affects rRNA processing and, hence, cell growth.

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