Functional Inactivation of the Mouse Nucleolar Protein Bop1 Inhibits Multiple Steps in Pre-rRNA Processing and Blocks Cell Cycle Progression*

Received for publication, May 6, 2002, and in revised form, June 3, 2002
Published, JBC Papers in Press, June 4, 2002, DOI 10.1074/jbc.M204381200

Zˇaklina Strezoska, Dimitri G. Pestov, and Lester F. Lau‡

From the Department of Molecular Genetics, University of Illinois College of Medicine, Chicago, Illinois 60607-7170

Bop1 is a conserved nucleolar protein involved in rRNA processing and ribosome assembly in eukaryotes. Expression of its dominant-negative mutant Bop1Δ in mouse cells blocks rRNA maturation and synthesis of large ribosomal subunits and induces a reversible, p53-dependent cell cycle arrest. In this study, we have conducted a deletion analysis of Bop1 and identified a new mutant, Bop1N2, that also acts as a potent inhibitor of cell cycle progression. Bop1N2 and Bop1Δ are C-terminal and N-terminal deletion mutants, respectively, and share only 72 amino acid residues. Both mutant proteins are localized to the nucleolus and strongly inhibit rRNA processing, suggesting that activation of a cell cycle checkpoint by Bop1 mutants is linked to their inhibitory effects on rRNA and ribosome synthesis. By using these dominant-negative mutants as well as antisense oligonucleotides to interfere with endogenous Bop1, we identified specific rRNA processing steps that require Bop1 function in mammalian cells. Our data demonstrate that Bop1 is required for proper processing at four distinct sites located within the internal transcribed spacers ITS1 and ITS2 and the 3′ external spacer. We propose a model in which Bop1 serves as an essential factor in ribosome formation that coordinates processing of the spacer regions in pre-rRNA.

The biosynthesis of ribosomes is a major expenditure of cellular resources, consuming more than half of the total cellular transcriptional and translational productivity in unicellular organisms such as yeast (1). Accordingly, the rate of ribosome biogenesis is tightly coordinated with cell growth and proliferation. Although the precise mechanisms by which cell growth and cell cycle progression are coordinated are still poorly understood, introns are being made in understanding the connection between ribosome biosynthesis and proliferative signals. For example, growth-promoting signals leading to activation of phosphatidylinositol 3-kinase or target of rapamycin result in the preferential translation of ribosomal proteins, at least in part through phosphorylation of the ribosomal protein S6 (2, 3).

One of the major impediments in understanding the role of ribosome biogenesis in proliferation control lies in the limited knowledge of the ribosome biosynthetic machinery in higher eukaryotes. The synthesis of rRNA and ribosome assembly is a highly complex process that takes place largely in the nucleolus (4). The mammalian 18 S, 5.8 S, and 28 S ribosomal RNAs are derived from a single 47 S precursor (pre-rRNA), which is processed to the mature species through a series of endonucleolytic, exonucleolytic, and modification steps (5). Targeted inhibition of specific components of ribosome biosynthetic machinery, a fruitful approach for elucidating pre-rRNA processing in yeast (6, 7), has not been feasible in mammalian cells until recently. To address questions relating ribosome biogenesis and cell proliferation, we have devised a system to block specific steps of pre-rRNA processing in cultured mouse cells by using a dominant-negative mutant of the nucleolar protein Bop1 (8, 9).

Bop1 is a component of large nuclear ribonucleoprotein complexes that represent ribosome precursors. Expression of a Bop1 deletion mutant that lacks 231 amino acids at the N terminus, Bop1Δ, leads to a specific block in the maturation of the 28 S and 5.8 S rRNAs without affecting maturation of 18 S rRNA (9). Pulse-chase analysis showed that Bop1Δ expression resulted in a partial inhibition of conversion of the 36 S precursor to 32 S pre-rRNA and a complete inhibition of synthesis of mature 28 S and 5.8 S rRNAs from 32 S pre-rRNA. Concomitant with these defects in pre-rRNA processing, expression of Bop1Δ abolished formation of new 60 S ribosomal subunits (9).

Bop1 is conserved throughout eukaryotes and contains five WD motifs (10), implicated in protein-protein interactions (11). Analysis of the Saccharomyces cerevisiae homolog of Bop1, ERB1, has shown that its functions in pre-rRNA and ribosome maturation are also conserved. Depletion of Erb1p in yeast resulted in the inhibition of the synthesis of mature 25 S and 5.8 S rRNA, paralleling effects of interfering with Bop1 in mouse cells (10).

Remarkably, perturbation of Bop1 activities in murine 3T3 cells by Bop1Δ induced a strong and reversible cell cycle arrest in G1 (8, 12). This cell cycle arrest was associated with down-regulation of Cdk2 and Cdk4 kinase activities and hypophosphorylation of pRb, indicating an inability of cells to progress through G1 into S phase (12). This G1 arrest occurred prior to a significant impediment in the global translation rate that might occur due to depletion of the cytoplasmic ribosome pool.

Most interestingly, inactivation of functional p53 circumvented the Bop1Δ-induced cell cycle block without restoring normal pre-rRNA processing. These results have led to the proposal that ribosome biosynthesis serves as cell cycle checkpoint subject to surveillance by p53 (12).

In this study, we have undertaken a deletion analysis of Bop1 to dissect its structure and function relationships. Deletions of either the N terminus or C terminus of Bop1 create two different dominant-negative mutants (Bop1Δ and Bop1N2, re-
spectively) that inhibit proliferation and cause overlapping but not identical blocks in pre-rRNA processing. The comparison of processing defects caused by the two deletion mutants and the effects of antisense inhibition of endogenous Bop1 demonstrate a role of Bop1 in processing of the ITS1, ITS2, and the 3′-ETS in mouse pre-rRNA. Moreover, this analysis shows that effects of pre-rRNA processing are correlated with effects on pre-rRNA maturation.

MATERIALS AND METHODS

Plasmids—Expression constructs driving expression of Bop1, Bop1Δ, HA-tagged Bop1, and HA-Bop1 in the IPTG-inducible expression vector pX11 (15) have been described previously (9). Deletion mutants of Bop1 were obtained by cloning appropriate restriction fragments of Bop1 into pX11 with an in-frame N-terminal HA tag sequence. Details of the cloning procedures are available upon request.

Cell Culture—Cells were cultured as described previously (8). LAP3 is a clonal cell line derived from NIH 3T3 cells that constitutively expresses the IPTG-inducible transactivator protein LAP267 (14) to support expression from pX vectors (13). For stable transfections and isolation of clonal lines, LAP3 cells were cotransfected with a puromycin resistance marker pPGK-puro and either the empty pX11 vector or HA-tagged deletion constructs using calcium phosphate method (15), followed by selection in medium containing 1 μg/ml puromycin (Sigma). Clonal lines LAP3/A and Bop1/A/6 have been described previously (8). BrdUrd light treatment was performed according to a protocol described previously (15).

RNA Blot Analysis—Total RNA was isolated using Trizol (Invitrogen) following the manufacturer's protocol. RNA was separated on a 1% agarose-formaldehyde gels and analyzed by Northern blot hybridization using standard techniques (16). The following oligonucleotides were used as probes for hybridization: 5.8 S probe (5′-GGGTTCAAGGTTGGGATGATCAATGTGTCCTGCAATTCAC) complementary to nt 65–108 of mouse 5.8 S rRNA; ITS2-2 (5′-ACTGGTGAAGCCGCGTCCGGAGGCCGCGCCGACG) complementary to nt 239–271 of the ITS2 region; ITS1-4 (5′-GTATCGATGATCAATGTGTCCTGCAATTCAC) complementary to nt 972–1001 of ITS1; 3′-ETS (5′-AGAGCGAGCGAGGGAAAGAGAAACGAAC) complementary to nt 158–187 of 3′-ETS. Probes were 5′-labeled using [γ-32P]ATP and T4 polynucleotide kinase.

Western Blot Analysis—Cells were lysed in RIPA buffer (16) and lysates containing equal amounts of protein, determined by the DC protein assay (Bio-Rad), were resolved by SDS-PAGE. Western blot analysis was carried out following a standard protocol (16) using affinity-purified anti-Bop1 antibodies (9), anti-HA monoclonal antibodies (Bio-Rad), or anti-actin antibodies (Roche Molecular Bichemicals) and anti-Cdk4 antibodies C-22 (Santa Cruz Biochemicals).

Indirect Immunofluorescence—Cells were grown on slides, incubated with IPTG for 12 h, and fixed with paraformaldehyde, followed by permeabilization with 0.5% Triton X-100 and incubation with monoclonal anti-HA antibodies in phosphate-buffered saline containing 0.5% bovine serum albumin for 1 h. After washing, slides were incubated with anti-mouse Alexa 488-conjugated antibodies (Molecular Probes) and photographed using MicroMAX digital camera mounted on an Axioplan II Zeiss microscope. Pictures were processed using Canvas software (Deneba).

Metabolic Labeling and Analysis of RNA Transcripts—Various cell lines were plated in 6-well plates at 10^5 cells per well. One day after plating, cells were either left untreated or treated with 1 μM IPTG for a further 24 h to induce expression. Pulse-chase experiments were carried out using [3H]methionine due to a rapid turnover of the cellular methionine pool. Cells were preincubated in methionine-free medium for 15 min and then incubated in medium containing 50 μCi/ml [3H]methionine (PerkinElmer Life Sciences) for 30 min. Cells were then chased in medium containing 10-fold excess of nonradioactive methionine, after which RNA was isolated using Trizol, and equal amounts of total RNA were analyzed by Northern blotting and fluorography (9).

Cells to be labeled with radioactive phosphate (for improved detection of small RNAs) were pretreated in phosphate-free medium for 1 h and labeled with 20 μCi/ml [32P]orthophosphate (PerkinElmer Life Sciences) for 30 min followed by a chase in a regular medium for 2 h. Equivalent RNA amounts were separated on a 5% polyacrylamide, 7 M urea gel. The gel was stained with ethidium bromide for photography and then dried for autoradiography.

Deletion Analysis of Bop1—In previous studies (13), a genetic selection was established to identify cDNAs that inhibit cell proliferation when expressed. By using this selection procedure, a cDNA that confers a powerful and reversible cell cycle block was identified, and this cDNA (Bop1Δ) encodes an N-terminal deletion of Bop1 (8). Acting in a dominant-negative manner, expression of Bop1Δ blocks maturation of the 28 S and 5.8 S rRNA, 60 S ribosome subunit biogenesis, and cell cycle progression (9, 12). To understand the structural elements that mediate Bop1 functions, we constructed a series of N-terminal and C-terminal deletions of Bop1 linked to an HA epitope tag (Fig. 1A). IPTG-regulated expression constructs encoding these mutant proteins were transfected into LAP3 cells, and expression of the deletion mutants in stable transfected pools induced with IPTG was verified by immunoblotting (Fig. 1C).

We first determined the subcellular localization of the deletion mutants by indirect immunofluorescence staining with anti-HA antibodies. All N-terminal deletion mutants were present in the nucleus in substantial amounts, but their subnuclear distribution was different (Fig. 2). Bop1C1 (aa 153–732) was primarily localized to the nucleolus, whereas Bop1C4 was almost exclusively nuclear. Interestingly, Bop1C3 (aa 392–732) and Bop1C4 (aa 490–732), although capable of efficient translocation into the nucleus, appeared to be excluded from the nucleolus (Fig. 2).

Among the C-terminal deletion mutants, only Bop1N2 (aa 1–323) was primarily localized to the nucleolus. Two other mutants, Bop1N4 (aa 1–489) and Bop1N5 (aa 1–704), were excluded from the nucleus in the majority of transfected cells and accumulated in the cytoplasm, indicating that either their nuclear import or retention was impaired. In a fraction of cells, however, these proteins also exhibited some degree of nuclear staining, possibly because they still contained a nucleolar targeting signal that was unaffected by the deletions that prevented their efficient nuclear accumulation. A short internal fragment of Bop1, Bop1Δf (aa 231–413), which is likely capable of entering into the nucleus due to small size, was concentrated in the nucleolus (Fig. 2). These results suggest that the nuclear localization signal is located in the center region of Bop1 (see Fig. 1A).

From these observations (summarized in Fig. 1B), we conclude that efficient nuclear import and/or retention of Bop1 is largely dependent on the C-terminal portion, whereas the nucleolar localization requires the region of Bop1 located between aa 251 and 323. Based on these data, three large functional regions in Bop1 can be defined as follows: the N-terminal domain (aa 1–250), the central domain (aa 251–323) required for nucleolar localization, and the C-terminal domain containing WD repeats (aa 324–732), which plays a role in nuclear localization. Both the N-terminal and central regions of Bop1 contain stretches of polar amino acids termed PEST motifs (Fig. 1A) (9).
Effects of Bop1 Deletion Mutants on Cell Proliferation—To determine whether the Bop1 mutants affect cell proliferation, we induced their expression in stable pools of transfected cells and subjected these cells to BrdUrd light selection that kills proliferating cells (13). When the N-terminally truncated mutant Bop1\(N\)/H9004 is expressed in cells, activation of a p53-dependent checkpoint reversibly blocks the cell cycle in G1 phase (8, 12). Cells that are transiently arrested by Bop1\(N\)/H9004 survive the BrdUrd light treatment and form numerous colonies following the transfer to IPTG-free medium in which Bop1\(N\)/H9004 expression is repressed (Fig. 3). By using the BrdUrd light assay to analyze cell cycle inhibitory properties of Bop1 mutants, we found that the C-terminally truncated mutant Bop1N2 induced a reversible cell cycle arrest in a large fraction of transfected cells as efficiently as Bop1\(N\)/H9004 (Fig. 3). This result was surprising because Bop1N2 (aa 1–323) and Bop1\(N\)/H9004 (aa 251–732) are C-terminal and N-terminal deletions, respectively, and overlap in only a short stretch of 72 amino acids (Fig. 1A).

A modest increase in cell survival in the BrdUrd light assay was also observed with the nucleolar mutant Bop1C1 (Fig. 3), indicating that this mutant protein can also inhibit cell cycle progression in a fraction of transfected cells. The inhibitory effects of Bop1C1, however, were much less pronounced than those of Bop1\(N\)/H9004 and Bop1N2. In addition, we did not observe cell cycle inhibition in several individually isolated clonal lines with low to moderate expression of Bop1C1, suggesting that its effect may require high expression levels (data not shown). The remaining Bop1 deletion mutants did not promote cell survival in the BrdUrd light assay, indicating that these mutants are not capable of inducing a reversible cell cycle arrest (Fig. 3).

**Fig. 1.** Deletion analysis of Bop1. A, schematic representation of Bop1 and its deletion mutants. PEST sequences 1 and 2 are shown as hatched boxes and WD repeats 1–5 as solid black boxes. B, summary of the subcellular localization of Bop1 deletion mutants and their effects on cell proliferation and pre-rRNA processing. Predominant localization of HA-tagged proteins in the nucleolus (No) and nucleoplasm (Np) is indicated when it occurs in the majority of transfected cells. The asterisk indicates that the localization is detected only in a fraction of cells. ND, not determined. The ability of the deletion mutants to evoke a reversible cell cycle arrest in LAP3 cells was assessed by the BrdUrd light assay (see text for details). C, expression of deletion mutant proteins. Total cell lysates were prepared from cell populations stably transfected with the IPTG-inducible constructs indicated after induction with IPTG for 20 h. Lysates were normalized by protein content, separated by SDS-PAGE, and analyzed by Western blotting using antibodies against the HA tag.
Both Bop1N2 and Bop1Δ Block Synthesis of 28 S and 5.8 S rRNAs—Both Bop1Δ and Bop1N2 are localized predominantly to the nucleolus, suggesting that these mutants affect nucleolar functions (Fig. 2). We have shown previously that Bop1Δ inhibits processing of pre-rRNA into mature 28 S and 5.8 S rRNAs (9). Maturation of rRNA in mammalian cells is a complex process that involves a series of endonucleolytic, exonucleolytic, and modification steps to generate 18 S, 5.8 S, and 28 S rRNAs from a primary 47 S transcript (Fig. 4). Bop1Δ interferes with several steps in pre-rRNA maturation, causing a loss of 12 S pre-rRNA as well as 28 S and 5.8 S rRNAs and partially inhibiting the conversion of 36 S pre-rRNA to 32 S pre-rRNA, which results in the accumulation of the 36 S precursor (9).

To investigate whether Bop1N2 might also inhibit pre-rRNA maturation, we isolated single cell clonal lines that inducibly express Bop1N2. Indeed, expression of Bop1N2 resulted in a blockade of 28 S rRNA synthesis as judged by [3H]uridine labeling (data not shown). To examine the specific pre-rRNA processing steps affected by Bop1N2, we performed pulse-chase labeling with [methyl-3H]methionine. A Bop1N2-inducible clonal line (Bop1N2/7) was either left untreated or treated with IPTG, pulse-labeled for 30 min, followed by chase with nonradioactive methionine for various times (Fig. 5). The short-lived, primary 47 S rRNA transcript in mouse cells is rapidly converted to 46 S and then to the relatively stable 45 S pre-rRNA (see Fig. 4). In the absence of IPTG, the major 45 S rRNA precursor, which comigrates with the less abundant 47 S and 46 S pre-rRNAs, was processed to 41 S and 36 S pre-rRNAs and converted by 30 min to 32 S pre-rRNA and mature 18 S rRNA (Fig. 5). Formation of the mature 28 S rRNA was almost completed by 1 h after the pulse.

After 15 min of chase, Bop1N2 expression led to the appearance of novel 41 S* and 36 S* precursors that migrated slightly slower than the 41 S and 36 S pre-rRNAs (Fig. 5). Strikingly, no 32 S rRNA was formed by this time. After 30 min of chase, Bop1N2 expression resulted in a decreased amount of mature 18 S rRNA and the appearance of small amounts of 36 S and 32 S pre-rRNAs, in addition to the aberrant 41 S* and 36 S* species. Processing of the 47/45 S precursor was significantly delayed, and this pre-rRNA could be found even after 1 h of chase, in contrast to uninduced cells. New 28 S rRNA in Bop1N2/7 cells was virtually absent, whereas the mature 18 S rRNA was produced with only a slightly reduced efficiency. To determine whether the synthesis of 5.8 S rRNA was affected, we separated small RNA species labeled with radioactive orthophosphate on a polyacrylamide gel. Induction of either Bop1Δ or Bop1N2 led to a nearly complete inhibition of the 5.8 S rRNA synthesis (Fig. 6).

Comparison of the effects of Bop1Δ and Bop1N2 showed that both mutant proteins abolished the synthesis of mature large ribosome subunit rRNAs, 28 S and 5.8 S rRNAs, without diminishing the generation of 18 S rRNA significantly (9) (Figs. 5 and 6). However, these two mutants exerted different effects of the levels of rRNA precursors. In Bop1Δ-expressing cells, the label from the 32 S precursor disappears after ~1 h (9), indicating that either 32 S pre-rRNA or its derivatives are rapidly degraded. In Bop1N2-expressing cells, the 32 S precursor is stabilized and remains detectable even after 2 h of chase (Fig. 5). Bop1Δ also delayed the processing of 36 S pre-rRNA, leading to its accumulation (9). In contrast, Bop1N2 expression did not cause significant accumulation of 36 S pre-rRNA but appeared to delay early processing steps and also resulted in the formation of novel RNA species, 41 S* and 36 S* (Fig. 5).

Role of Bop1 in Processing of ITS1, ITS2, and 3′-ETS in Pre-rRNA—In addition to the major pre-rRNA processing pathway in mouse cells (Fig. 4), several alternative processing pathways have been observed (17) in which an altered sequence of cleavages may give rise to pre-rRNA species different from the precursors depicted in Fig. 4. To investigate the nature of the novel rRNAs (41 S* and 36 S*) observed in pulse-chase labeling of Bop1N2-expressing cells (Fig. 5), we performed hybridizations of RNA isolated from clonal cell lines Bop1Δ/6, Bop1N2/7, and LAP3/1 (vector-transfected) with oligonucleotide probes complementary to different regions of the primary rRNA transcript (Fig. 7).

The ITS1–4 probe hybridizes with the region immediately upstream of the 5′ end of 5.8 S rRNA and reveals the steady-state levels of the major precursors 47 S to 45 S, 41S, and 36 S (see Fig. 4). Induction of Bop1 mutants changed the relative abundance of rRNA precursors as compared with control vector-transfected cells and non-induced cells (Fig. 7). The most significant effect of Bop1Δ expression was a strong increase in
the 36 S pre-rRNA level, in agreement with previous data (9). After Bop1N2 induction, 36 S pre-rRNA was only modestly increased, but two additional bands of a slightly larger size than 36 S and 41 S rRNAs were detected, similar to the 36 S* and 41 S* RNAs observed in pulse-chase labeling (Fig. 5). Probe ITS2-2 hybridizes with the 47–45 S, 41 S, 36 S, 32 S, and 12 S pre-rRNAs in all control cells (Fig. 7). Induction of Bop1Δ resulted in a strong increase of the 36 S pre-rRNA level, consistent with the ITS1-4 hybridization, and a decrease in 12 S pre-rRNA (Fig. 7). In Bop1N2 lines, the most striking effect is a large increase in the 32 S pre-rRNA steady-state level, consistent with its stabilization observed in pulse labeling experiments (see above), and the appearance of two additional bands, 41 S* and 36 S* (in Fig. 7, 41 S* is not clearly visible due to a short exposure but is readily detectable after longer exposure times). Interestingly, there is no significant decrease of the steady-state level of 12 S pre-rRNA, although the strong accumulation of 32 S pre-rRNA implies that the cleavage at site 4b, which generates 12 S (Fig. 4), is inhibited. Consistent with inhibition of this cleavage, analysis of 32P-labeled RNA on an agarose gel showed decreased label incorporation into 12 S pre-rRNA after Bop1N2 induction (data not shown). These results suggest that Bop1N2 expression has an additional inhibitory effect on the 12 S pre-rRNA turnover, which may lead to undiminished steady-state levels of this precursor despite reduced formation rates. As shown below, a similar stabilizing effect on 12 S pre-rRNA is also observed after down-regulation of endogenous Bop1 expression.

Probe that hybridizes with the 3'-ETS region downstream of site 6 (Fig. 7) reveals steady-state levels of the 47 S pre-rRNA and major processing sites (labeled 0–6) are shown at the top. The 47 S pre-rRNA is processed through intermediates of different sizes designated according to their relative sedimentation coefficients (S) to eventually yield the mature 18 S, 5.8 S, and 28 S rRNAs. Numbers next to arrows indicate sites processed at the corresponding conversion steps. The steps affected by Bop1Δ and Bop1N2 deletion mutants and down-regulation of endogenous Bop1 through ASO are indicated.
expression of the C-terminal deletion mutant Bop1N2 inhibits formation of 28S rRNA. Cells of an inducible clonal line that expresses HA-tagged Bop1N2 mutant (Bop1N2/7) were either left untreated or treated with 1 mM IPTG for 24 h, pulse labeled with L-[methyl-3H]methionine for 30 min, and chased in non-radioactive medium containing excess methionine for the indicated times. Equal amounts of total RNA were resolved on a formaldehyde-agarose gel, transferred to a nylon membrane, and visualized by fluorography. Positions of major precursors are indicated on the left.

Together, the hybridization data show that expression of Bop1N2 results in slowed cleavage at site 6, giving rise to novel 3'-ETS and N2 mutants, acting as dominant-negative interference with Bop1 function. These data indicate that Bop1N2 inhibits the early processing step at site 6, which removes the 3'-ETS from 46S pre-rRNA (Fig. 4). This inhibition does not completely block subsequent cleavages at sites 1–5, although the increased steady-state levels of 47/46S pre-rRNA detected with the 3'-ETS probe (Fig. 7) and a slowed conversion of the 47/45S precursors in pulse-chase labeling (Fig. 5) suggest that their efficiency is reduced. Notably, very low levels of the 41S* and 36 S* bands are also detectable by hybridization with the 3'-ETS probe after Bop1 induction (Fig. 7), although, as compared with Bop1N2, this is only a minor defect in these cells.

The effects of down-regulation of Bop1 by the ASOs were analyzed by hybridization of total RNA using ITS2-2 and 5.8S probes (see Fig. 7). Cells subjected to mock transfection, as well as cells transfected with an ASO that did not decrease Bop1 protein level (3-3, Fig. 8A), exhibited essentially similar steady-state levels of the 45S, 41S, 36S, 32S, and 12S pre-rRNAs (Fig. 8B). Down-regulation of Bop1 with ASOS 2-1 and 3-1 caused an increase in 36S, 32S, and 12S pre-rRNAs (Fig. 8B), indicating that insufficient levels of Bop1 in the cell can reduce the efficiency of processing in both ITS1 and ITS2. Importantly, the ASO-mediated down-regulation of endogenous Bop1 resulted in pre-rRNA processing defects overlapping with those caused by deletion mutants: inhibited processing of 36S pre-rRNA (an effect shared with Bop1Δ) and 32S and 12S rRNA precursors (similar to Bop1N2), validating the effects of these mutants as a dominant-negative interference with Bop1 function.

**DISCUSSION**

Much insight into the functions of trans-acting protein factors in ribosome biogenesis has been made possible through genetic approaches in yeast (6, 7). In this study, we demonstrate that transient expression of dominantly acting mutants provides a viable experimental approach to study the pre-ribosome maturation machinery in genetically less malleable systems such as mammalian cells. Exploration of Bop1 functions in pre-rRNA processing using this approach revealed that Bop1 is involved in processing events in the 3'-ETS and intragenic spacers ITS1 and ITS2. These findings suggest a functional link that exists between several specific processing steps...
in pre-rRNA maturation. Moreover, these results establish a set of useful reagents for future studies of pre-rRNA processing in mammalian cells. Furthermore, we show that the effects of Bop1 dominant-negative mutants on pre-rRNA processing are correlated with those on cell cycle progression, providing support to the idea that surveillance of ribosome biogenesis may serve as a mammalian cell cycle checkpoint (12).

Bop1 Is Involved in Processing of Intragenic Spacers and the 3′-ETS—Potent dominant mutants interfering with Bop1 functions can be created by deletions of either N- or C-terminal domains of Bop1 (Fig. 1). Remarkably, the processing steps affected by the dominant-negative forms of Bop1 are not identical, suggesting that Bop1 is a multifunctional protein that harbors domains involved in different aspects of ribosome formation. Expression of the Bop1 mutants most significantly affects late processing steps in ITS1 and ITS2 and, unexpectedly, also interferes with the early processing of the 3′-ETS at site 6 (Fig. 4). Notably, the presence of 32 S* to 41 S* species in the Bop1N2/7 cell line (Figs. 5 and 7) implies that cleavage at site 6 is not an obligatory step for subsequent processing of mouse pre-rRNA at sites 1–3. Consistent with effects of dominant-negative mutants, a reduced efficiency of processing in the ITS1 and ITS2 is also observed after antisense-mediated down-regulation of endogenous Bop1 (Fig. 8). Taken together, these findings demonstrate that Bop1 is involved in processing of three transcribed spacer regions in mouse pre-rRNA as follows: ITS1, ITS2, and 3′-ETS (Fig. 9).

Interdependence of distant cleavage reactions in eukaryotic pre-rRNA has been observed in other organisms. In Schizosaccharomyces pombe, deletions in the 3′-ETS inhibit both the removal of the 3′-ETS region and processing in the ITS1 (21, 22). Likewise, the integrity of a stem loop within 3′-ETS is required for internal cleavages within ITS1 in S. cerevisiae (23), in which efficient production of 25 S rRNA also requires ITS1 sequences (24, 25). Recent studies (26) show that proper secondary structures in ITS2 are important for 5.8 S and 25/28 S rRNA synthesis. In addition to cis-acting elements, one known trans-acting factor, U8 small nucleolar RNA, was shown previously (27) to be involved in processing of both the ITS regions and 3′-ETS in Xenopus oocytes. The present study demonstrates that linkage between processing of the ITS regions and 3′-ETS also exists in mammalian cells and identifies Bop1 as a protein factor required for the proper execution of these processing events.

Multiple effects of Bop1 mutants on pre-rRNA maturation may be explained by a model in which Bop1 functions by coordinating activities of various processing factors within preribosomal complexes. Deletions in parts of the Bop1 molecule that affect different subsets of its interactions in these complexes may thus impair processing of pre-rRNA at discrete sites (Fig. 9). This model is corroborated by biochemical evidence showing that Bop1 is tightly associated with a series of intermediates in the assembly of large ribosome subunits. The yeast homolog of Bop1, Erb1p, has been identified recently (28, 29) as a component of 60–66 S preribosomes. Bop1 cofractionates with preribosomal particles in mouse cells (9), and the nucleolar mutants Bop1Δ and Bop1N2 are present in the same fractions in sucrose gradients, 2 suggesting that their dominant-negative effects result from the incorporation of these defective Bop1 forms into preribosomes.

\*Ž. Strezoska, D. G. Pestov, and L. F. Lau, unpublished data.
Depletion of some proteins involved in 5.8 S/25 S rRNA maturation in yeast leads to blocked processing and accumulation of 27 S pre-rRNA, whereas depletion of others results in its loss apparently due to increased degradation (6). Depletion of the yeast homolog of Bop1, Erb1p, results in a decrease in the steady-state level of 27SB pre-rRNA and the disappearance of 7 S pre-rRNA (10). An interesting feature of Bop1 is that deletions in different parts of this protein can lead to opposite effects on the levels of the mouse 32 S and 12 S pre-rRNAs, which are structurally related to the yeast 27SB and 7 S pre-rRNAs. These data suggest that two different types of non-productive complexes may be formed at late stages of ribosome maturation. Complexes formed as a result of Bop1 deletion are open to processing and/or degrading enzymes, as reflected by the rapid 32 S pre-rRNA turnover. In contrast, Bop1N2 may promote formation of complexes trapped in a closed conformation in which the access of RNA-processing enzymes to ITS2 is impaired, thereby causing an accumulation of 32 S pre-rRNA and stabilization of 12 S pre-rRNA. Some of the nucleases involved in the removal of the ITS1 and ITS2 have been characterized in yeast (30, 31); however, the mechanisms that regulate their activities are not yet known. Dominant-negative mutants of Bop1 may thus present an interesting experimental system in which to dissect the interactions between nucleases and other components of preribosomal complexes.

Pre-rRNA Processing and Cell Cycle Progression—Blocking the function of Bop1 in a fast and reversible manner not only provides a novel experimental system for the analysis of the mechanisms of pre-rRNA processing but also has revealed an intriguing connection between preribosome assembly and cell cycle progression in mammalian cells. Induction of Bop1 by LAP3 cells causes a rapid G1 arrest, which occurs sooner than a measurable repression of global protein synthesis due to depletion of the translating ribosome pool (9, 12). Most interestingly, inactivation of functional p53 alleviated the Bop1 depletion-induced cell cycle arrest (12), leading us to propose the hypothesis that perturbations in the ribosome assembly machinery in mammalian cells trigger a nucleolar stress signal that is subject to surveillance by p53 as a cell cycle checkpoint (12).

In this study, we show that two dominant-negative mutants, Bop1Δ and Bop1N2, which cause strong but non-identical rRNA processing blocks, arrest proliferation in transfected cell populations in a potent and reversible fashion (Fig. 3). Like Bop1Δ, Bop1N2 does not significantly affect cell viability but rather induces a transient cell cycle arrest (Fig. 3), which also requires functional p53 (data not shown). Analysis of cell lines expressing various Bop1 deletion mutants to date has revealed none in which the effects on rRNA synthesis and proliferation could be dissociated. The association of effects of Bop1 mutants on pre-rRNA processing and cell proliferation lends further support to the model in which inhibition of pre-rRNA processing can lead to a reversible cell cycle arrest. Further analysis of transdominant mutants of Bop1 as well as other proteins that function in ribosome biogenesis should provide a useful approach to elucidate the molecular mechanisms that link ribosome formation to cell cycle regulation in mammalian cells.

REFERENCES
1. Warner, J. R. (1999) Trends Biochem. Sci. 24, 437–440
2. Thomas, G., and Hall, M. N. (1997) Curr. Opin. Cell Biol. 9, 782–787
3. Gingras, A. C., Raught, B., and Sonenberg, N. (2001) Genes Dev. 15, 807–826
4. Warner, J. R. (1990) Curr. Opin. Cell Biol. 2, 521–527

Fig. 8. Antisense inhibition of endogenous Bop1. A, the effect of antisense oligonucleotide inhibition on the Bop1 protein level. Cells were transfected with either a no-oligo mixture (control sample, lane C), or with oligonucleotides 2-1, 3-1, or 3-3 for 24 h. Bop1 levels were examined by immunoblotting using antibodies against Bop1 (9) in equal amounts of protein lysates. A lower portion of the same blot was probed with anti-Cdk4 antibodies as a loading control. B, effects of down-regulation of endogenous Bop1 on pre-rRNA processing. RNA was isolated from cells transfected with oligonucleotides as in A, and equal amounts of total RNA were separated on 1% agarose gels and subjected to Northern blot analysis. Hybridizations were performed with oligonucleotides complementary to 5.8 S rRNA and ITS2 region (see Fig. 7).

Fig. 9. Bop1 links processing of ITS1 and ITS2 with 3′-ETS processing. Bop1 is envisaged as part of a multiprotein complex in which processing of ITS1, ITS2, and 3′-ETS takes place. Processing at sites 3, 4b, 5, and 6 is inhibited by either deletions of terminal domains of Bop1 or lowering its levels through antisense oligonucleotides.
