A gene-specific effect of an internal deletion in the Bdp1 subunit of the RNA polymerase III transcription initiation factor TFIIIB

Akira Ishiguro*, George A. Kassavetis

Division of Biological Sciences and Center for Molecular Genetics, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0634, USA

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Abstract **The *Saccharomyces cerevisiae RPR1* gene encodes the RNA subunit of its RNase P, which processes RNA polymerase (pol) III primary transcripts. *RPR1*, which is transcribed by pol III, has been isolated as a multicopy suppressor of a specific small internal deletion (amino acids 253–269) in the Bdp1 subunit of transcription factor TFIIIB, the core pol III transcription factor. The selective effect of this Bdp1 deletion on *RPR1* transcription has been analyzed in vitro. It is shown that TFIIIC-dependent assembly of TFIIIB on the *RPR1* promoter is specifically sensitive to this Bdp1 deletion, leading to gene-specifically defective single-round and multiple-round transcription.**

Key words: Eukaryotic transcription; RNA polymerase III; RNase P; TFIIIB; Bdp1

1. Introduction

Less than 7% of *Saccharomyces cerevisiae* nuclear genes, encoding stable, mostly small RNA, are transcribed by RNA polymerase (pol) III. The most prominent distinguishing features of all these yeast genes are their transcription unit-internal promoter elements, which serve as binding sites for essential pol III transcription initiation factors (TF): the *box* C elements of the ~140 5S rRNA genes bind 5S gene-specific TFIIIA; the *box* B and *box* A elements of all other pol III-transcribed genes bind two loosely linked domains, τ_B and τ_A, of the general pol III transcription factor TFIIIC. The *box* A–*box* B separation varies considerably between genes, but generally falls within a range that is optimal for TFIIIC-DNA binding [1]. The U6 spliceosomal RNA gene with its transcription unit-external, downstream *box* B and very wide *box* A–*box* B separation constitutes a singular exception to this rule, and its non-canonical *box* A–*box* B spacing is associated with specific consequences for the regulation of its transcription [2–4]. However, the placement of *box* A relative to transcriptional start sites is closely constrained (15–18 bp separate the beginning of the *box* A motif from the pyrimidine–purine motif that marks the transcriptional start in all but 7 of the ~270 tRNA genes): *box* A is the DNA-binding site of the τ_A domain of TFIIIC, and the Tfc4 (τ_{131}) subunit, which is part of τ_A, is principally responsible for TFIIIC-dependent recruitment of TFIIIB to its DNA site upstream of the transcriptional start of pol III genes; TFIIIB, in turn, is responsible for recruiting pol III to its promoters [5–10].

All three TFIIIB subunits – TBP, Brf1 and Bdp1 – are essential in vivo, and essential for transcription of pol III genes in vitro. Its TATA binding protein subunit enables TFIIIB to bind independently of TFIIIC to promoters with strong TATA *box*es. A small number of *S. cerevisiae* tRNA genes and its U6 gene have strong TATA *box*es but most *S. cerevisiae* genes do not [11]. In contrast, all *Schizosaccharomyces pombe* genes transcribed by pol III have TATA *box*es as essential promoter elements [12].

Analysis of the *BDP1* gene has defined three segments that are essential for viability. Deletion of the small segment encoding amino acids 253–269, located between two of these essential parts, severely impairs growth at 37°C. This temperature-sensitive phenotype is specifically suppressed by multicopy overexpression of the *RPR1* gene [13]. *RPR1* is transcribed by pol III. Its product is the RNA subunit of RNase P, which is required for 5′-end processing of the primary transcripts of tRNA genes. A preliminary analysis identified defective transcription as a possible contributing cause of the growth defect generated by deleting amino acids 253–269 of Bdp1. It was also found that Bdp1 interacts with RNase P/*RPR1* RNA, implying a possible role for TFIIIB in 5′ end processing of its conjugate transcripts [13]. The experiments that follow extend the analysis of the effect that deleting amino acids 253–269 of Bdp1 exerts on transcription of the *RPR1* gene in vitro. It is shown that TFIIIC-dependent assembly of TFIIIB on the *RPR1* promoter is specifically sensitive to this Bdp1 deletion.

2. Materials and methods

2.1. In vitro transcription

Pol III, TFIIIC, recombinant TBP, Brf1 and Bdp1 were purified as described or referenced [14]. Pre-initiation complexes were formed in 20 μl of transcription buffer (40 mM Tris–HCl, pH 8.0, 70 mM NaCl, 7 mM $MgCl_2$ 3 mM dithiothreitol (DTT), 100 μg/ml bovine serum albumin) with 100 ng (50 fmol) plasmid DNA, 50 fmol TBP, 50 fmol Brf1, 150 fmol Bdp1, 26 fmol TFIIIC and 5 fmol pol III as the standard assembly mix. Transcription was started by adding 5 μl transcription buffer containing ATP, CTP, GTP and [α-^{32}P]UTP at concentrations specified in the figure legends. Pre-initiation complexes for single-round transcription were assembled for 40 min, then incubated with pol III (10 fmol), 200 μM ATP, 25 μM [α-^{32}P]UTP, and either 200 μM CTP (*SUP4*) or GTP (*RPR1*) for the times indicated. Transcript elongation was subsequently re-started by adding either

*Corresponding author. Present address: Laboratory for Developmental Neurobiology, RIKEN Brain Science Institute, Wako-shi, Saitama 351-0198, Japan. Fax: (81)-048-467 9744.
E-mail addresses: akiraishiguro@brain.riken.go.jp (A. Ishiguro), gak@ucsd.edu (G.A. Kassavetis).

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GTP (*SUP4*) or CTP (*RPR1*) to 200 μM and heparin to 200 μg/ml for 10 min. Samples were processed and transcripts were analyzed by 8 M urea/6 or 8% polyacrylamide gel electrophoresis as described [15] and quantified by phosphor image plate analysis.

2.2. Gel shift assay

Plasmids pTZ1 (*SUP4* $tRNA^{tyr}$ gene; [15]), and p*RPR1* (*RPR1* gene; [13]) were digested with *Bam*HI and end-labeled with Klenow fragment DNA polymerase and [α-^{32}P]dCTP. Promoter-containing DNA fragments were gel-purified as described [16]. Protein–DNA complexes were formed in 20 μl of binding buffer (40 mM Tris–HCl, pH 8.0, 7 mM $MgCl_2$, 3 mM DTT, 100 μg/ml bovine serum albumin, 90 mM NaCl and 100 μg/ml vector-plasmid DNA [pGEM1 for *SUP4*, pBluescript KS^+ for *RPR1*]) with, under standard conditions, 50 fmol TBP, 50 fmol Brf1, 150 fmol Bdp1, 52 fmol TFIIIC and 4 fmol labelled probe DNA. DNA–protein complexes were separated on 4% polyacrylamide gel under native conditions, as described [17] and quantified by phosphor image plate analysis.

3. Results and discussion

The *RPR1* gene is much more weakly transcribed in vitro by pol III (in conjunction with TFIIIC and wild type TFIIIB) than is the more commonly examined *SUP4* $tRNA^{Tyr}$ gene (Fig. 1B and [13]). Specific features of *RPR1* that could be responsible for this low rate of transcription (Fig. 1A) include its T_4:A_4 tract at bp 2–5 (U_4 at nt 2–5 of the primary transcript), which might generate abortive initiation and defective promoter clearance at low concentrations of UTP, or reiterative incorporation of UMP, also leading to aborted transcription, at high UTP concentrations (cf. [18,19]). For *S. cerevisiae* pol III, a run of 5 or more U residues (T_n with $n > 4$ in the non-transcribed strand) signals termination [20]. Since a mere three successive steps of UMP addition significantly slow RNA chain elongation [21], specifying UMP addition for the first four steps of *RPR1* RNA synthesis may lead to extremely slow clearance of the *RPR1* promoter by pol III at all concentrations of UTP. Inefficient transcription can also be due to suboptimal *box* A and *box* B promoter elements [22]. The *RPR1* gene contains an A instead of T at position 54 of *box* B (in standard tRNA numbering) which has been shown to decrease TFIIIC binding affinity 40-fold [23] and transcription approximately two-fold [24]. *RPR1* also has C instead of a purine at position 15 of *box* A, which may act like T at this

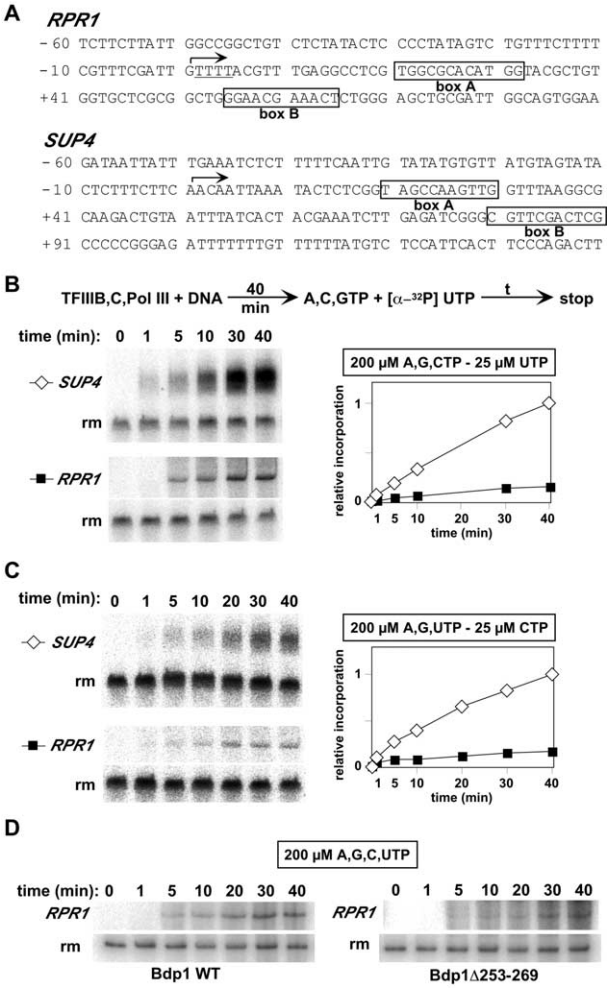

Fig. 1. Transcription of the yeast *SUP4* and *RPR1* genes by RNA pol III in vitro. A: Promoter sequences of *SUP4* and *RPR1*. Bent arrows indicate transcriptional start sites. The start-proximal TTTT of *RPR1* is underlined. B: After assembling pre-initiation complexes for 40 min, transcription for the times indicated was started by addition of 200 μM ATP, CTP, GTP and 25 μM [α-^{32}P]UTP. C: As B, but transcription was with limiting CTP (here at 25 μM) instead of UTP (here at 200 μM). rm: recovery marker. D: As B but with equal concentrations of all 4 nt (200 μM).

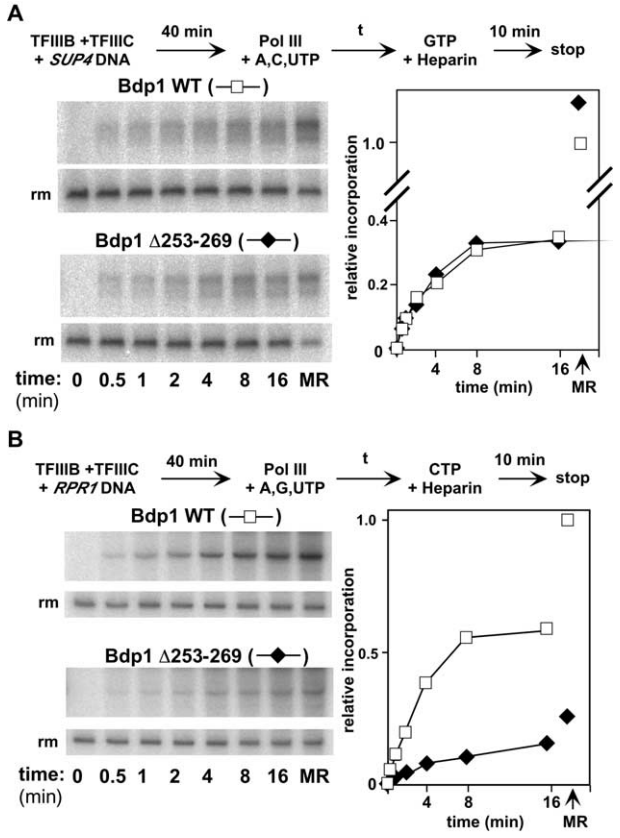

Fig. 2. Single-round transcription: comparing the activity of TFIIIB assembled with Bdp1Δ253-269 and wild type (WT) Bdp1. After TFIIIC–TFIIIB–DNA complex assembly for 40 min, pol III and 3 nt were added, and incubated for the times shown. A single round of transcription was re-started by adding the fourth nucleotide and heparin for 10 min. As a control, the same reactions without heparin show multiple rounds of transcription in 10 min following 16 min of initiation in the presence of 3 nt (lanes marked MR). A: *SUP4* transcription; B: *RPR1* transcription. The inset graphs quantify UMP incorporation in single-round transcription normalized to multiple round transcription in 10 min after initiation for 16 min with wild type Bdp1.

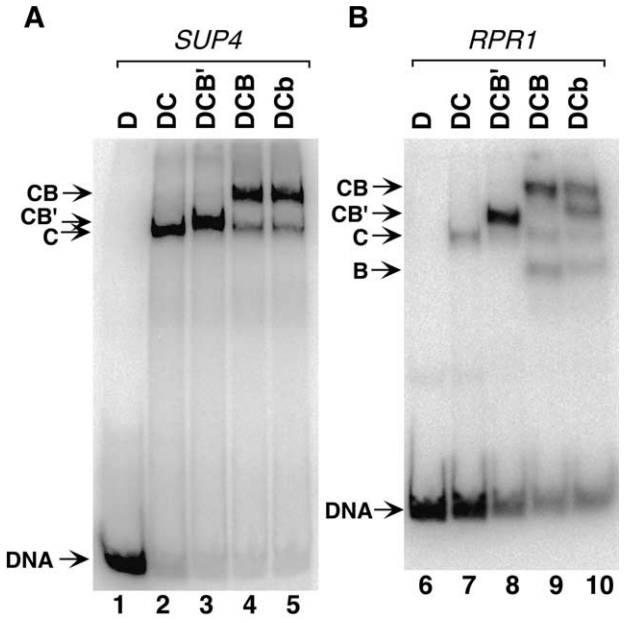

Fig. 3. Promoter complexes. Protein-DNA complexes were formed on the *SUP4* (A) and *RPR1* (B) genes and analyzed by polyacrylamide gel electrophoresis under native conditions (electrophoretic mobility shift analysis). Components are identified above each lane. Lanes 1 and 6: DNA only; lanes 2 and 7: DNA+TFIIIC; lanes 3 and 8: DNA, TFIIIC, TBP and Brf1; lanes 4 and 9: DNA, TFIIIC, TBP, Brf1 and Bdp1; lanes 5 and 10: DNA, TFIIIC, TBP, Brf1 and Bdp1Δ253-269. Arrows at the side indicate the mobilities of protein-DNA complexes and free DNA. B′: TFIIIB without Bdp1 (i.e. TBP+Brf1); b: TFIIIB assembled with Bdp1Δ253-269.

position to impair the *box* A element [24]. The 22 bp spacing between *box* A and *box* B is also suboptimal [1]. The combined effect of all three non-consensual elements is not known.

Fig. 1B–D shows that *RPR1* is weakly transcribed regardless of whether UTP is present at low or high concentration and regardless of whether its concentration relative to CTP is low (1:8), high (8:1) or unity. Evidently, the peculiar start site-proximal U tract of the *RPR1* transcript does not function as a control element for adjusting production of RNase P to pyrimidine nucleotide metabolism [18].

Transcription of the *RPR1* gene (as supercoiled DNA) is also specifically sensitive to substituting Bdp1Δ253-269 for wild type Bdp1 in TFIIIB, as shown previously [13] and confirmed under the conditions of Fig. 1D: transcription with the deletion-mutant TFIIIB was approximately one-fifth of transcription with wild type TFIIIB (analysis not shown, but see Fig. 2B). The possibility that the *RPR1*-specific inactivity of Bdp1Δ253-269 might be due to a defect of pol III recycling was examined by comparing single-round transcription (Fig. 2) with the previously analyzed multiple-round transcription (Fig. 1D; [13]). The *RPR1*-specific defect of Bdp1Δ253-269 persisted in single-round transcription (Fig. 2B), eliminating recycling as a major source of this relative inactivity.

Stepwise assembly of pre-initiation complexes was examined by electrophoretic mobility shift analysis (Fig. 3). On the *SUP4* promoter, assembly of TFIIIC–TFIIIB–DNA complexes with wild type Bdp1 and Bdp1Δ253-269 was essentially indistinguishable and relatively efficient (Fig. 3, lanes 4 and 5) as observed previously [16]. TFIIIC bound less efficiently to *RPR1* than to *SUP4* (lanes 7 and 2, respectively) as expected in view of its weakened *box* B element, but DNA-binding was aided by addition of TBP and Brf1 (B′; compare lane 8 with lane 7), which is known to stabilize TFIIIC *box* A and *box* B interaction and generate additional TFIIIC-DNA interactions near the start site of transcription [25]. Assembly of the TFIIIB+TFIIIC pre-initiation complex with wild type TFIIIB was less efficient at the *RPR1* promoter than at the *SUP4* promoter (compare lane 9 with lane 4) and addition of Bdp1 (along with TBP and Brf1) led to some shedding of TFIIIC from DNA (compare lane 9 with lane 8). TFIIIC shedding may reflect the Bdp1-mediated displacement of TFIIIC from the start site of transcription (and possibly from Brf1; [25]) in the absence of a strong *box* B site. Bdp1Δ253-269 was additionally defective in converting the TFIIIC-B′ complex to a TFIIIC–TFIIIB complex (compare lane 10 with lane 9). Varying the relative concentrations of protein components (Fig. 4) did not substantially mitigate this *RPR1*-specific defect of Bdp1Δ253-269: the principal differ-

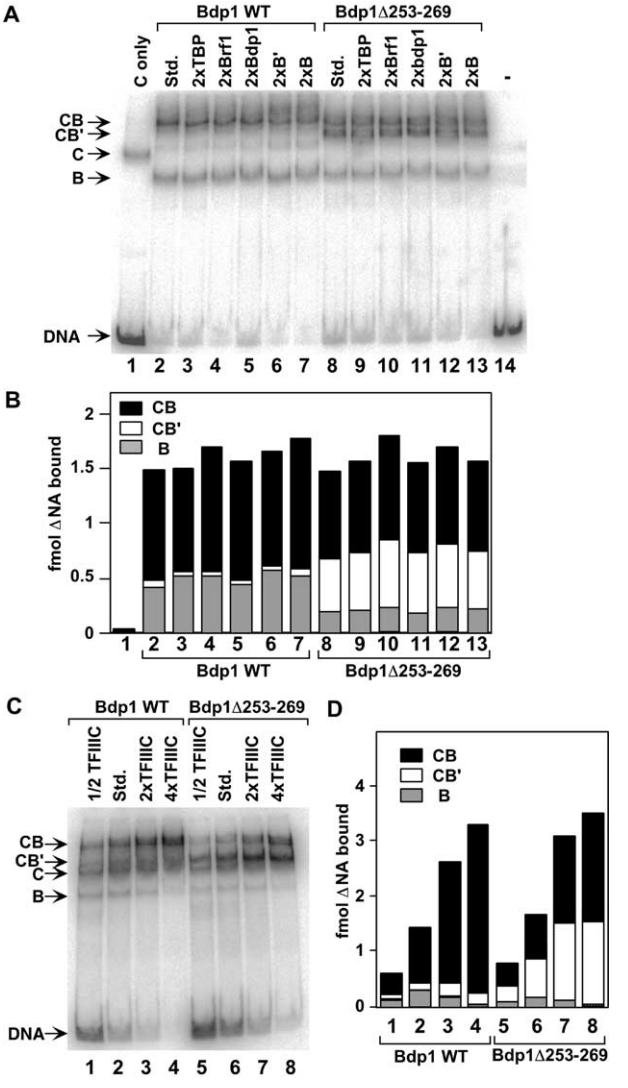

Fig. 4. Effects of varying protein concentration on the conversion of CB′ complexes at the *RPR1* promoter to CB complexes with Bdp1Δ253-269. A: Lack of apparent effect of doubling concentrations of factors. B: Quantified averages of three experiments like A. The numbers below the columns corresponds to lanes in A. C: TFIIIC does not drive Bdp1Δ253-269 into TFIIIB-containing DNA complexes. D: Quantified averages of three experiments like C.

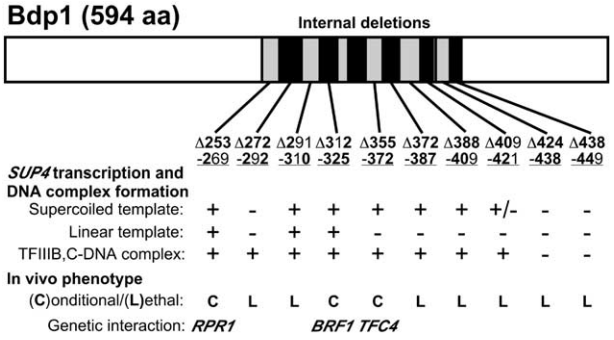

Fig. 5. Properties of Bdp1 internal deletion mutants. The ability of individual deletion mutants to function for TFIIIC-dependent transcription and TFIIIB–DNA complex formation [16,26] in vitro are shown. The resulting in vivo phenotype [13] is also shown along with multicopy gene suppressors (*RPR1*, *BRF1*) or synthetic lethals (*TFC4* mutant *PCF1*-1) of individual Bdp1 deletion mutants. Bdp1Δ327-338 and Bdp1Δ340-353, not shown, behave normally both in vitro and in vivo. All of the Bdp1 internal deletion mutants function for TFIIIC-independent TFIIIB–DNA complex formation and transcription of supercoiled *SNR6* gene templates [16].

ence relative to wild type Bdp1 was the incomplete conversion of TFIIIC-B′ complexes to TFIIIC–TFIIIB complexes (or TFIIIB complexes) upon addition of Bdp1Δ253-269 (panels B and D). Assays comparable to Fig. 4A were performed to measure the formation of heparin-resistant TFIIIB–DNA complex formation: increasing the concentration of TFIIIB subunits had no effect on the level of TFIIIB–DNA complex formation with Bdp1Δ253-269, generating 40–50% of the heparin resistant TFIIIB–DNA complexes formed with wild type Bdp1 (data not shown). Increasing the concentration of TFIIIC favored the formation of both CB′-DNA and CB–DNA complexes, but did not mitigate incomplete conversion of B′-containing DNA complexes to TFIIIB-containing complexes (panels C and D).

These results imply that the decrease in transcription of the *RPR1* gene as a result of deleting amino acids 253–269 of Bdp1 stems from a defect in the assembly of Bdp1 onto the B′-TFIIIC–DNA complex. This template-specific effect may be related to the TFIIIC-mediated interference of certain Bdp1 deletion mutant proteins with assembly of TFIIIB on the *SUP4* tRNA gene [16] summarized in Fig. 5: Bdp1Δ272-292, Bdp1Δ409-421, Bdp1Δ424-438 and Bdp1Δ438-449 function for TFIIIC-independent transcription of the SNR6 gene but not TFIIIC-dependent transcription of *SUP4* or *SNR6*. Bdp1Δ424-438 and Bdp1Δ438-449 fail to assemble into the B′-TFIIIC–DNA complex; Bdp1Δ272-292 and Bdp1Δ409-421 do assemble but fail to displace TFIIIC from the start site of transcription. Although the effect of Bdp1Δ253-269 on *RPR1* transcription is similar to the effect of the above deletions on *SUP4* transcription, it is also distinctive in that Bdp1Δ253-269 allows formation of heparin-resistant TFIIIB–DNA complexes, whereas the above mutants affecting *SUP4* transcription do not form heparin-resistant complexes and are completely defective in *SUP4* transcription. It is conceivable that the abnormally close spacing between *box* A and *box* B in *RPR1* (and therefore an abnormally close juxtaposition of the τ_A and τ_b domains of TFIIIC) makes displacement of TFIIIC by Bdp1 less favorable. The inability to alleviate the defect of Bdp1(Δ253-269) by increasing its concentration suggests that perhaps the close spacing between *box* A and *box* B results in two non-equilibrating forms of the B′-TFIIIC–DNA complex, one amenable to the assembly of Bdp1Δ253-269 and one not.

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