MOLECULAR AND CELLULAR BIOLOGY, May 2001, p. 3096–3104 Vol. 21, No. 9
0270-7306/01/$04.00+0 DOI: 10.1128/MCB.21.9.3096–3104.2001

High-Mobility-Group Proteins NHP6A and NHP6B Participate in Activation of the RNA Polymerase III *SNR6* Gene

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Received 20 December 2000/Accepted 13 February 2001

Transcription of yeast class III genes involves the formation of a transcription initiation complex that comprises RNA polymerase III (Pol III) and the general transcription factors TFIIIB and TFIIIC. Using a genetic screen for positive regulators able to compensate for a deficiency in a promoter element of the *SNR6* gene, we isolated the *NHP6A* and *NHP6B* genes. Here we show that the high-mobility-group proteins NHP6A and NHP6B are required for the efficient transcription of the *SNR6* gene both in vivo and in vitro. The transcripts of wild-type and promoter-defective *SNR6* genes decreased or became undetectable in an *nhp6A*Δ *nhp6B*Δ double-mutant strain, and the protection over the TATA box of the wild-type *SNR6* gene was lost in *nhp6A*Δ *nhp6B*Δ cells at 37°C. In vitro, NHP6B specifically stimulated the transcription of *SNR6* templates up to fivefold in transcription assays using either cell nuclear extracts from *nhp6A*Δ *nhp6B*Δ cells or reconstituted transcription systems. Finally, NHP6B activated *SNR6* transcription in a TFIIIC-independent assay. These results indicate that besides the general transcription factors TFIIIB and TFIIIC, additional auxilliary factors are required for the optimal transcription of at least some specific Pol III genes.

Transcription of small genes by RNA polymerase III (Pol III) in yeast involves a multistep assembly of transcription factors into a preinitiation complex which recruits RNA Pol III (for a review, see reference 35). The A and B blocks found in most Pol III promoters are first recognized by a multisubunit complex called Pol III transcription factor C (TFIIIC). TFIIIC, one of the largest and most complex transcription factors known, has a molecular mass of about 600 kDa and is composed of six subunits. It acts as an assembly factor to direct the binding of the initiation factor TFIIIB to an upstream gene position. Once assembled into a highly stable protein-DNA complex at Pol III promoters, TFIIIB can direct multiple rounds of transcription by Pol III in vitro in the absence of TFIIIC (17, 18). TFIIIB is composed of three loosely associated polypeptides, the TATA-binding protein (19), a general transcription factor used by all eukaryotic and archeal RNA polymerases (14, 27); B″ or TFIIIB90, which displays little homology to other known proteins except for a putative SANT domain (1, 20, 28, 29); and Brf1 or TFIIIB70, which shows 44% similarity to TFIIB in its N-terminal 320 residues (3, 7, 21).

In addition to these basal factors, there are hints that additional components exist which influence transcription efficiency or accuracy. A protein called TFIIIE, which has yet to be characterized, is able to stimulate transcription under certain conditions (9, 29). TFIIIE has been suggested to act by facilitating TFIIIB recruitment, by inducing conformational rearrangements of TFIIIB, or by stabilizing transcription complexes. A partially purified B″ fraction was found to direct a more efficient and more accurate transcription initiation than the recombinant TFIIIB90 protein (6, 29), but the factors postulated to influence start site selection and transcription efficiency remain to be identified. Among the potential candidates, factors belonging to the class of chromatin proteins might play a role in adjusting Pol III transcription to the cell physiology, but this hypothesis has not been explored so far.

In this paper we report the first characterization of yeast Pol III gene-specific activating factors. Using a screen for multicopy suppressors of a mutation affecting an extragenic promoter element of the *SNR6* Pol III gene, we isolated the *NHP6A* and *NHP6B* genes. Both genes encode proteins with DNA-binding domains similar to those of the HMG1 and HMG2 proteins. NHP6A and NHP6B were found to increase specifically the transcription efficiency of wild-type and mutant *SNR6* genes in vivo and in vitro.

MATERIALS AND METHODS

Yeast strains. The *Saccharomyces cerevisiae* strains used for this study are derived from YPH500α (31) and Y865 (8). MCM260 is a derivative of YPH500α. It corresponds to strain FTY115 (22) with the *snr6Δ2* allele at the chromosomal locus, but it is rescued at 30°C by the 2μm plasmid pRS425-*snr6Δ2* instead of the centromeric plasmid pRS314-U6 for FTY115 (the *snr6Δ2* allele has a 2-bp deletion in its B block, which strongly reduces its functionality in vitro and in vivo). YPH500α was used as a tester strain to monitor the effects of *NHP6A* or *NHP6B* overexpression on the transcription of the *SNR6* genes. The wild-type Y865 and the *nhp6A*Δ *nhp6B*Δ double mutant Y869 have been described (8).

Isolation of high-copy-number suppressors of *snr6Δ2*. A yeast genomic DNA library carried in the multicopy, *URA3*-marked vector pFL44 (32) was transformed into MCM260, and transformants were directly selected at 37°C on a medium lacking uracil. Of 60,000 transformants, 16 colonies were identified for growth at 37°C. To ensure that the ability to grow at 37°C was due to the presence of the genomic clone in pFL44, the thermoresistant colonies were streaked on 5-fluoroorotic acid (5-FOA) plates and tested again for growth at 37°C. All colonies tested became thermosensitive. The plasmids were then rescued into *Escherichia coli* and retested for suppressor activity by transformation into MCM260; all plasmids restored thermoresistance. The plasmids were finally

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sequenced and identified by comparison with sequences in the GenBank data base.

Plasmids. pRS425-*snr6Δ2* was obtained by subcloning the *SNR6* sequences of pB6Δ238-239 (4) into the *LEU2*-marked 2μm plasmid pRS425 (31) using the *Bam*HI and *Hin*dIII sites. All the plasmids used for the in vitro transcription assays are derived from the Bluescript SK vector (Stratagene) and contain the region of the *SNR6* gene spanning bp −140 to +314 relative to the *SNR6* transcription start site, except for the Aup-ΔB construct, which harbors a truncated fragment from −120 to +122 lacking the B block (4). These fragments were mutated as described previously (4). To study the transcriptional activity of wild-type and mutated *SNR6* genes in yeast, we used YEp352-derived plasmids (15), harboring *SNR6* genes with a 59-pb DNA fragment inserted in their transcribed sequences and the same mutations as those described above. Their construction has been described previously (22).

The tetO-NHP6A and tetO-NHP6B constructs were generated as follows. The entire coding sequences of the *NHP6A* and *NHP6B* genes were amplified by PCR using as a template the genomic sequences harbored by the pFL44 plasmids isolated by our screen. After digestion with *Bam*HI and *Hpa*I, the PCR products were cloned into the *Bam*HI-*Hpa*I sites of pCM183 (13), behind the tetracycline operator tetO.

Proteins. Recombinant NHP6B was produced in *E. coli* strain BL21(DE3) (*hupA*::Cm *hupB*::Km) as previously described (36). Crude yeast extracts were prepared from the Y869 yeast strain as previously described (4), except for the DEAE-Sephadex column purification stage, which was omitted. The recombinant TBPm3, TFIIIB70, and TFIIIB90 were a gift from Giorgio Dieci (10). The purified fractions containing Pol III or TFIIIC were obtained as previously described (16).

RNA analysis. The multicopy plasmids YEp352 harboring the various *SNR6* constructs were introduced into YPH500α, Y865, and Y869. Yeast transformation procedures, RNA extraction, and Northern blot analysis were performed as previously described (4), using body-labeled DNA fragments encompassing the *SNR6, SNR31*, and $tDNA^{His\text{-}KL}$ coding sequences. Alternatively, the primers 5′-TGTTGCTATAAGCACGAAGCTCTAACCACT-3′ and 5′-GTCAGGCTC TTACCAGCTTAA-3′ were phosphorylated by T4 polynucleotide kinase and used to detect the $tRNA^{Ile(UAU)}$ and 5S RNA, respectively. Quantifications were performed using PhosphorImager software (Amersham Pharmacia).

Chromatin analysis by MNase. Cultures of strains Y865 (wild type) and Y869 (double mutant *nhp6AΔ nhp6BΔ*) were grown in yeast extract-peptone-dextrose (YPD) at 30°C to 1×10^7 to 2×10^7 cells/ml. Cells were harvested and converted to spheroplasts using Zymolyase. For chromatin analysis at 37°C, cultures were grown in YPD at 30°C to 1×10^7 to 2×10^7 cells/ml, incubated at 37°C for 4 h, and spheroplasted at 37°C. Chromatin and genomic DNA were prepared and digested with micrococcal nuclease (MNase) for 5 min at 37°C, and the cutting sites were mapped by indirect end labeling from the *Pst*I site as previously described (22).

In vitro transcription assays. In vitro transcription reactions were performed using 150 ng of either Bluescript SK-derived plasmids, harboring *SNR6* genes, or the KS-tDNAIle(TAT)199 plasmid (10), the pSIRT plasmid (23), or the pFL44-t(His)K plasmid (24), harboring the I(TAT)LR1, the 5S DNA, and the $tDNA^{His\text{-}KL}$ genes, respectively. The templates were incubated at 25°C for 40 min in 40-μl reaction mixtures (20 mM HEPES buffer [pH 7.5], 0.1 mM EDTA, 5% glycerol, 5 mM $MgCl_2$, 5 mM dithiothreitol, 8 U of RNasin (Promega), 0.6 mM each ATP, CTP and GTP, 0.03 mM UTP, and 10 μCi of [α-^{32}P]UTP) with or without 10 to 200 ng of NHP6B recombinant protein. Transcription reaction mixtures with purified components contained 50 ng of purified Pol III (16) and recombinant TBPm3 (40 ng), TFIIIB70 (80 ng), and TFIIIB90 (80 ng) (10), with or without 100 ng of purified TFIIIC (16). Transcription reactions with cell extracts prepared from the Y869 strain contain 20 μg of proteins.

RESULTS

Identification of high-copy-number suppressors of the temperature-sensitive *snr6Δ2* gene. *SNR6* is a Pol III gene which encodes the yeast U6 RNA, the catalytic part of the spliceosome. This gene has unusual promoter elements. Its A block, at position +21 relative to the transcription start site, is degenerate compared with the consensus tRNA gene element, its B block is located downstream of the transcribed sequence, 202 bp away from the A block, and it has a TATA box at position −30 (2). A mutant *snr6Δ2* strain in which the chromosomal *SNR6* gene has been inactivated by a 2-bp deletion in the B block is not viable but could be rescued by the same *snr6Δ2* allele if the allele is harbored by a multicopy plasmid. This new *snr6Δ2* strain, MCM260, grew slowly at 30°C and failed to form colonies at 37°C (see Fig. 1). We reasoned that by searching multicopy suppressors of the *snr6Δ2* allele, which is impaired only in a gene-distal promoter element, we should select for genes involved in the transcriptional activation of *SNR6*.

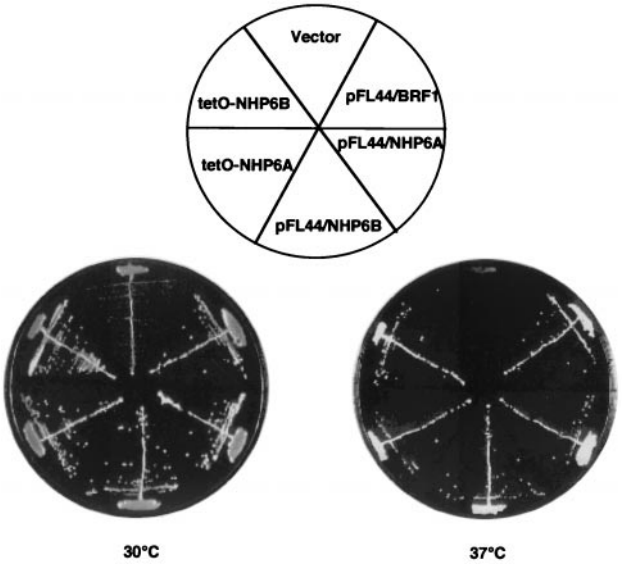

FIG. 1. Overexpression of the *BRF1, NHP6A*, or *NHP6B* genes rescues the thermosensitivity of *snr6Δ2*. MCM260 transformants containing either 2μm plasmids with the *BRF1* (pFL44/BRF1), *NHP6A* (pFL44/NHP6A), or *NHP6B* (pFL44/NHP6B) genes, or the tetO-NHP6A or tetO-NHP6B constructs, or an empty vector (vector), were streaked on YPD plates and grown at 30 or 37°C for 3 days.

A yeast genomic DNA library in a 2μm multicopy plasmid (32) was transformed into MCM260, and transformants were selected for growth at 37°C. Of 60,000 transformants, 16 plasmids that reproducibly allowed growth at 37°C were isolated. The yeast genomic DNA inserts harbored by these plasmids were identified by sequence analysis and fell into seven classes. One of the largest classes of suppressor plasmids had four members and contained the wild-type *SNR6* gene. The *BRF1* gene encoding the TFIIIB70 subunit of TFIIIB was found in three plasmids and defined a second class. Two classes of inserts present in six plasmids contained either the *NHP6A* or the *NHP6B* gene and were selected for further study. The other three classes of plasmids contained uncharacterized yeast genes, whose analysis will be reported elsewhere.

The *BRF1* gene was found in some genomic inserts in the absence of any other open reading frame, so its identification as a suppressor gene was immediate. To confirm the suppressor activity of the *NHP6A* and *NHP6B* genes, their coding sequences were cloned into a yeast expression vector under the control of the tetracycline operator (tetO). The tetO-NHP6A and tetO-NHP6B constructs improved the growth rate of MCM260 transformants at 30°C and suppressed their thermosensitive phenotype at 37°C, confirming the identification of *NHP6A* and *NHP6B* as suppressors of the *snr6Δ2* allele (Fig. 1).

In vivo transcript levels of mutant *SNR6* genes are increased by overexpression of *NHP6A* or *NHP6B*. To confirm that the

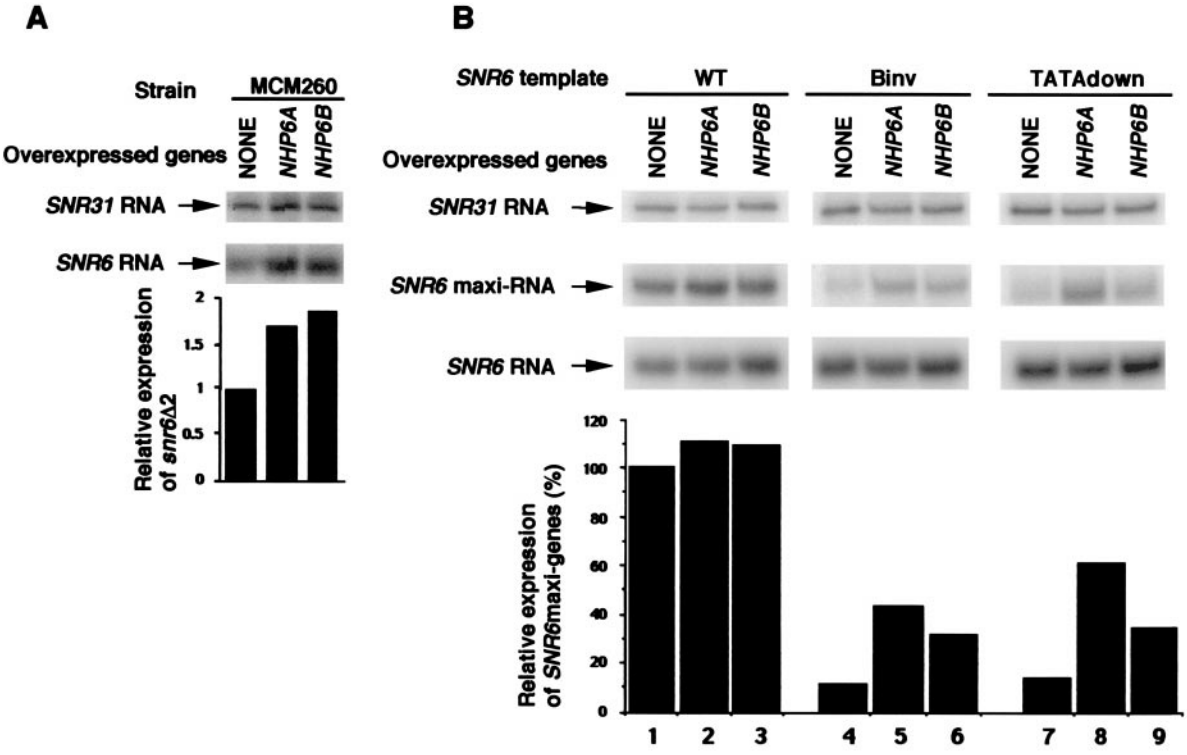

FIG. 2. The transcription of mutant *SNR6* genes is increased by overexpression of *NHP6A* or *NHP6B*. (A) The tetO-NHP6A and tetO-NHP6B constructs were introduced into the MCM260 strain, which contains only the *snr6Δ2* allele. Transcripts derived from the *snr6Δ2* genes were quantified in Northern blots by PhosphorImager analysis, using *SNR31* transcripts as internal controls. (B) The Northern blot illustrates the transcriptional activation of *SNR6* constructs harboring a 59-bp insert in the transcribed region and different mutations. These genes are borne by multicopy plasmids and generate transcripts (*SNR6* maxi-RNA) easily distinguishable from the *SNR6* RNA produced from the wild-type, chromosomal *SNR6* gene. The steady-state levels of transcripts derived from the *SNR6* maxigene constructs were analyzed in the wild-type strain YPH500α, with or without the overexpression of *NHP6A* or *NHP6B*, and quantified using *SNR31* transcripts as internal controls. The transcription level of the wild-type (WT) maxigene construct without the overexpression of *NHP6A* or *NHP6B* (lane 1) was arbitrarily assigned the value 100%.

suppression of *snr6Δ2* thermosensitivity was due to an effect on *SNR6* expression, the steady-state levels of *SNR6* transcripts in the MCM260 strain at 30°C, in the presence or the absence of the tetO-NHP6A and tetO-NHP6B constructs, were analyzed by Northern blotting (Fig. 2A). Equivalent amounts of RNA, as determined by measuring of optical density, were loaded in each lane of the gel, so as to normalize the levels of *SNR6* transcripts in relation to rRNA. We also noticed that the quantities of RNA derived from *SNR31*, a Pol II-transcribed gene, showed no correlation with the levels of NHP6A and NHP6B proteins, and we used them systematically to correct for variations in RNA loading and to quantify more precisely the amounts of *SNR6* transcripts. As shown in Fig. 2A, the overexpression of *NHP6A* or *NHP6B* increased significantly the level of *SNR6* transcripts (derived from both the plasmid and chromosomal *snr6Δ2* genes).

To test whether the effect of NHP6A or NHP6B overexpression was restricted to the *snr6Δ2* mutation or could also affect other promoter-defective alleles of *SNR6*, we analyzed the expression of two other mutant *SNR6* genes at 30°C in wild-type cells overexpressing *NHP6A* or *NHP6B*. We used another *SNR6* mutant affected in the B-block element, the Binv mutant, whose B block and surrounding sequences have been inverted, and an *SNR6* gene whose TFIIIC-binding sequences have been left intact but whose TATA box (5′-TATAAAT-3′) has been replaced by an unrelated sequence (5′-GTGCACG-3′), the TATAdown mutant, and a wild-type *SNR6* gene as a control. A 59-bp DNA fragment was introduced into the transcribed regions of these genes, at position +73, to distinguish the corresponding transcripts from the endogenous, wild-type *SNR6* RNA (22). These *SNR6* maxigene constructs were inserted into the multicopy YEp352 plasmid and introduced into the wild-type strain YPH500α. As previously described (22), in the absence of *NHP6A* and *NHP6B* overexpression, the level of transcripts derived from the Binv maxigene represented about 15% of the level produced from the wild-type construct, whereas the TATAdown construct generated fewer RNA molecules than the wild-type maxigene and produced transcripts which were slightly shorter, in agreement with the proposed role of the TATA box on the selection of the transcription start site (Fig. 2B, lanes 1, 4, and 7). The overexpression of *NHP6A* or *NHP6B* was found to increase the steady-state levels of the transcripts derived from the TATAdown and Binv constructs (lanes 4 to 9), whereas it had no effect on the transcript level of the wild-type construct (lanes 1 to 3). Similarly, the overexpression of *NHP6A* or *NHP6B* had no effect on the abundance of the transcripts derived from the wild-type, chromosomal *SNR6* gene. In conclusion, it seems that the overexpression of *NHP6A* or *NHP6B* increased the steady-state levels only of transcripts derived from *SNR6* genes that are impaired in their promoter elements, either TFIIIC- or TFIIIB-binding sequences: the *snr6Δ2* allele, the TATAdown construct, and the

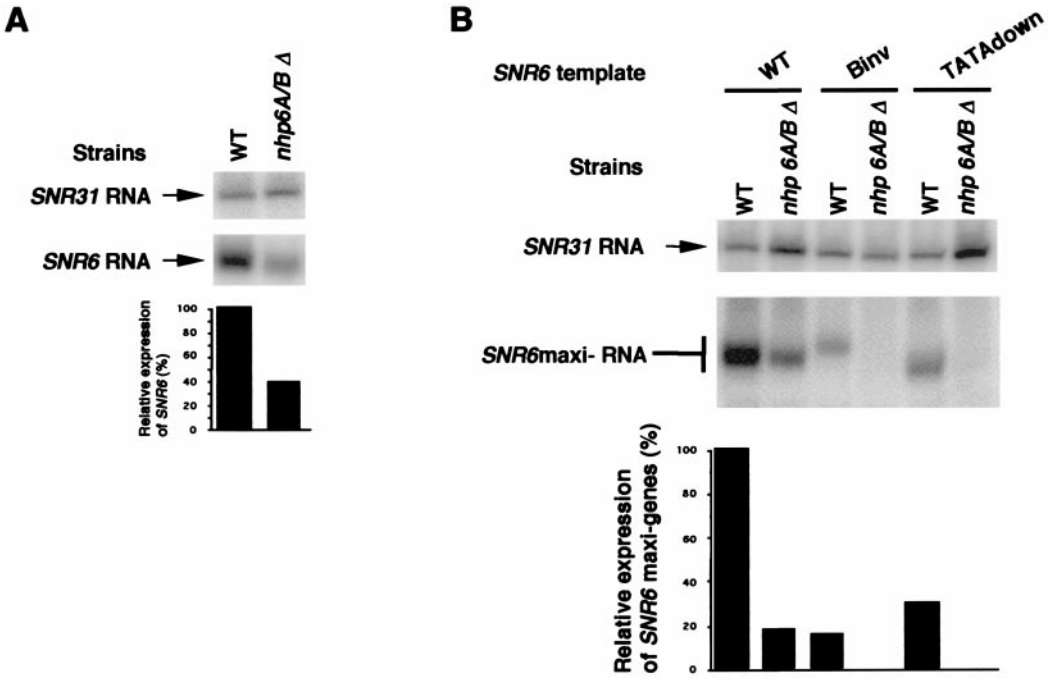

FIG. 3. The transcriptional activity of wild-type and mutant *SNR6* genes is reduced in the absence of *NHP6A* and *NHP6B* at 30°C. The transcription of the wild-type (WT), chromosomal *SNR6* gene (A) and of *SNR6* maxigene constructs either wild type or harboring different mutations (B) was monitored by Northern blotting in wild-type (WT, Y865 [8]) and *nhp6A*Δ *nhp6B*Δ mutant (Y869 [8]) cells. The steady-state levels of *SNR6* RNA or maxi-RNA were quantified by PhosphorImager analysis, with *SNR31* transcripts as internal controls. The transcription level of the wild-type maxigene construct in the wild-type strain was assigned the value 100%.

Binv construct. These observations confirmed that the suppressor activity of *NHP6A* and *NHP6B* was due to a direct effect on *snr6Δ2* expression and implied that wild-type levels of NHP6A and NHP6B were not a limiting factor at 30°C for transcription of the wild-type *SNR6* gene.

The expression of wild-type and mutant *SNR6* genes is reduced in the absence of *NHP6A* and *NHP6B*. To assess the contribution of NHP6A and NHP6B to the expression of the wild-type *SNR6* gene, the abundance of *SNR6* transcripts was analyzed in wild-type and in double-mutant *nhp6A*Δ *nhp6B*Δ cells grown at 30°C. As shown in Fig. 3A, the transcript level of the wild-type *SNR6* gene was specifically reduced 2.6-fold in the *nhp6A*Δ *nhp6B*Δ cells compared to that of other genes, such as *SNR31*. Likewise, the RNA levels derived from the *SNR6* maxigene constructs were systematically reduced in the mutant *nhp6A*Δ *nhp6B*Δ strain, whether these *SNR6* constructs were impaired in their promoter elements or not (Fig. 3B).

Protection over the TATA box of the wild-type *SNR6* gene is dramatically altered in *nhp6A*Δ *nhp6B*Δ cells. To investigate whether the absence of NHP6A and NHP6B would affect *SNR6* chromatin structure, chromatin of the *SNR6* locus in the wild-type and *nhp6A*Δ *nhp6B*Δ strains was analyzed by MNase digestions. MNase preferentially attacks linker DNA between the nucleosomes and leads to double-strand cut, but it may also introduce single-strand nicks on the nucleosome surface. Genomic chromatin and deproteinized DNA were digested with different amounts of MNase, and double-strand-cutting sites were displayed by indirect end labeling (Fig. 4). The bands in the DNA lanes represent the preferential cutting sites for MNase in deproteinized DNA. Some of these sites were protected in chromatin, whereas others remained or became accessible. Protected regions of 140 to 200 bp were interpreted as positioned nucleosomes (open boxes) (33). As previously described (22), the *SNR6* chromatin structure in a wild-type strain at 30°C was characterized by the organization of the upstream and downstream regions into series of positioned nucleosomes, by a protection of the TATA box, and by hypersensitive sites around the A and B blocks (Fig. 4, lanes 1 to 4). Only minor changes were observed when the wild-type strain was grown at 37°C: the protection over nucleosome 1 was stronger, and the relative accessibility of the two sites between nucleosome 1 and the B block was slightly altered (lanes 3 to 6). In the *nhp6A*Δ *nhp6B*Δ cells, the nucleosomal organization in the upstream and downstream regions of the *SNR6* locus was maintained at 30 and 37°C (lanes 7 to 10) and was similar to that of the wild-type cells. The relative sensitivities of the two sites around the A block and of the two sites between nucleosome 1 and the B block were similar to those of the wild-type strain grown at 37°C. The most dramatic change, however, which was induced by the absence of NHP6A and NHP6B, was observed at the TATA box: while the TATA box was completely protected in the wild-type cells at both temperatures (lanes 3 to 6), it was slightly accessible in the mutant at 30°C, as revealed by a weak band (lanes 7 and 8), and the protection appeared to be completely lost at 37°C, as indicated by a strong band (lanes 9 and 10). Loss of the footprint on the TATA box has been previously observed for the *snr6Δ2* allele, whose transcriptional activity is crippled by a 2-bp deletion in the B block (22). These observations hinted that the occupancy of the A and B blocks by TFIIIC could be modified in the

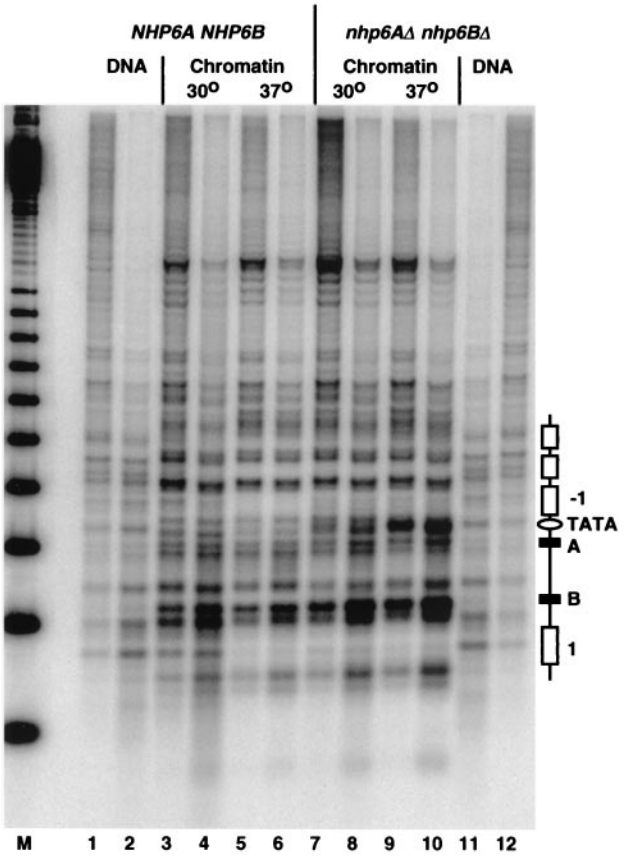

FIG. 4. Protection over the TATA box of the *SNR6* gene is dramatically altered in *nhp6A*Δ *nhp6B*Δ cells. Cultures of Y865 (*NHP6A NHP6B*) and Y869 (*nhp6A*Δ *nhp6B*Δ) were grown at 30°C (lanes 3 and 4 and lanes 7 and 8, respectively) and shifted to 37°C for 4 h (lanes 5 and 6 and lanes 9 and 10, respectively). Chromatin and genomic DNA were prepared and digested with different amounts of MNase. To display the cutting sites, the DNA was digested with *Pst*I, fractionated on a 1% agarose gel, blotted to a nylon membrane, and hybridized to a probe close to the *Pst*I site. Indicated are the nucleosome positions (white boxes), A and B blocks (black boxes), and TATA box (white oval) of the *SNR6* gene. The marker (M) represents multiples of 256 bp and was hybridized separately. The protection of the TATA box (lanes 3 to 8) was lost when Y869 was shifted to 37°C (lanes 9 and 10).

*nhp6A*Δ *nhp6B*Δ cells and strongly suggested that TFIIIB positioning on the TATA-box region was destabilized and lost in the absence of NHP6A and NHP6B at 37°C. The effects of *NHP6A* and *NHP6B* deletion on the chromosomal structure of the *SNR6* locus indicate that NHP6A and NHP6B are acting at the level of *SNR6* transcription complex formation and rule out the possibility that NHP6A and NHP6B are simply stabilizing *SNR6* RNA.

In vitro transcription of *SNR6* genes is stimulated by NHP6B. We next analyzed the effect of NHP6B on the in vitro transcription of several mutant *SNR6* genes. The mutant template called Binv has been described above. The constructs Aup and Aup-ΔB have the *SNR6* degenerate A block replaced by a consensus A block derived from the sequences of tRNA genes (4), and their B blocks have been either left intact (Aup) or deleted (Aup-ΔB). We used crude nuclear extracts so as to mimic as closely as possible the complexity of transcription processes occurring in the cell nucleus. The wild-type and mutant *SNR6* templates were thus transcribed in a cell extract prepared from *nhp6A*Δ *nhp6B*Δ cells, with or without the addition of NHP6B (Fig. 5). NHP6B significantly stimulated the transcription of the wild-type and Binv templates. Interestingly, NHP6B strongly activated the transcription of the Aup-ΔB template but had no effect on the Aup construct, which remained the only *SNR6* template insensitive to NHP6B activity. In all cases, in vitro as well as in vivo, the size of the transcripts was the same in the presence and in the absence of NHP6B, suggesting that the activation of the *SNR6* genes by NHP6B did not alter the transcription start site.

NHP6B activates the transcription of *SNR6* in a reconstituted transcription system. To get some insight into the molecular mechanisms underlying *SNR6* transcriptional activation by NHP6A and NHP6B, we performed in vitro transcription reactions using purified fractions (TFIIIC and polymerase III) and recombinant proteins (TFIIIB70, TFIII90, and TBP). In contrast to many tRNA genes, naked DNA templates of *SNR6* can be transcribed in vitro using only purified fractions of TFIIIB and Pol III. TFIIIC is not required for this reaction, although its presence increases the transcription efficiency. We first tested the effect of NHP6B on a transcription reaction involving the complete system with purified TFIIIC and recombinant TFIIIB. As shown in Fig. 6, a fivefold increase in *SNR6* transcription was observed at optimal concentrations of NHP6B. This stimulation level was comparable to that observed, using the same concentration range, in yeast cell extracts. This result strongly suggested a direct stimulatory effect of NHP6B on the transcription system. Remarkably, as seen in Fig. 6, NHP6B was able to give a ca. threefold stimulation of *SNR6* transcription directed by TFIIIB alone. This lower stimulation level might be due to the repression of transcription observed at the highest concentration of NHP6B, in the absence of TFIIIC.

NHP6A and NHP6B exert pleiotropic effects on the expression of Pol III genes. NHP6A and NHP6B are required for the induction of a subset of genes transcribed by Pol II (25). Since NHP6A and NHP6B are involved in the transcription of *SNR6*,

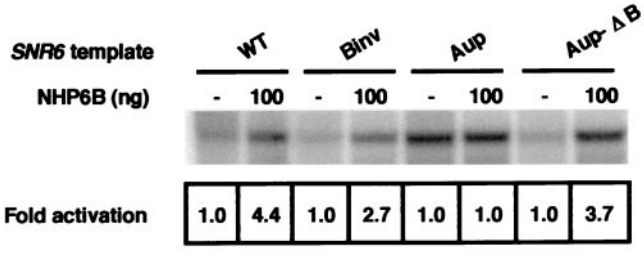

FIG. 5. NHP6B stimulates the transcription of wild-type and mutant *SNR6* genes in vitro. NHP6B was added to in vitro transcription reaction mixtures containing cell extracts prepared from *nhp6A*Δ *nhp6B*Δ mutant cells (Y869 [8]) and different *SNR6* templates, either wild type (WT) or mutated in the A or B block. The templates used were Bluescript derivatives harboring *SNR6* wild-type or mutant genes. Reaction mixtures were incubated for 40 min at 25°C, and the transcription products were electrophoresed in a 6% polyacrylamide gel. The *SNR6* transcripts were quantified by PhosphorImager analysis, and for each template, the basal level of *SNR6* transcription in the absence of NHP6B was arbitrarily assigned the value of 1 unit.

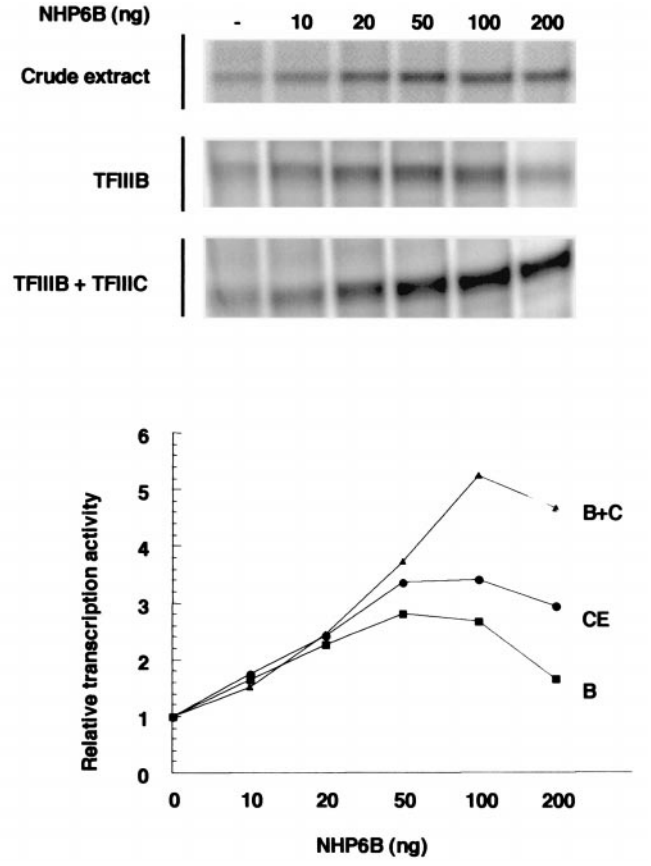

FIG. 6. NHP6B activates *SNR6* transcription in a reconstituted system. NHP6B protein was added as indicated to in vitro transcription reaction mixtures containing either cell extracts (CE) prepared from *nhp6A*Δ *nhp6B*Δ mutant cells (Y869 [8]) or purified Pol III and recombinant TFIIIB (B) or Pol III, TFIIIB, and TFIIIC (B+C). A Bluescript-derived plasmid harboring the wild-type *SNR6* gene was used as a template. Reaction mixtures were incubated for 40 min at 25°C, and the transcription products were electrophoresed in a 6% polyacrylamide gel. The *SNR6* transcripts were quantified by PhosphorImager analysis, and the basal level of *SNR6* transcription in the absence of NHP6B was arbitrarily assigned the value of 1 unit in each case.

we investigated whether these proteins could also influence the transcript levels of other Pol III genes. We analyzed by Northern blotting the levels of the 5S rRNA, $tRNA^{Ile(UAU)}$, and $tRNA^{His}$ in wild-type cells grown at 30°C, with or without the overexpression of *NHP6A* or *NHP6B*, and in mutant *nhp6A*Δ *nhp6B*Δ cells. The $tRNA^{Ile(UAU)}$ genes were selected because they contain a canonical TATA sequence around position −30, like the *SNR6* gene. Also, like *SNR6*, these $tRNA^{Ile(UAU)}$ genes can be transcribed in vitro in the absence of TFIIIC (10). As shown in Fig. 7A, the levels of the 5S rRNA and of the $tRNA^{His}$ were similar in all the strains analyzed whereas, unexpectedly, the abundance of the $tRNA^{Ile(UAU)}$ strongly increased in the absence of NHP6A and NHP6B, in contrast to *SNR6* transcript level that decreased (Fig. 7A, lane 5). $tRNA^{Ile(UAU)}$ is encoded by two genes, I(TAT)LR1 and I(TAT)DR2, with identical transcribed sequences. To confirm these in vivo results, we investigated the effect of NHP6B on the in vitro transcription of 5S RNA, $tRNA^{His}$, and $tRNA^{Ile(UAU)}$ genes using cell extracts prepared from *nhp6A*Δ *nhp6B*Δ cells, with or without the addition of NHP6B. As shown in Fig. 7B, NHP6B stimulated only the in vitro transcription of the *SNR6* gene. Remarkably, under the same condition, the transcription of both $tRNA^{Ile(UAU)}$ genes remained unaffected by the addition of NHP6B (Fig. 7B and data not shown).

DISCUSSION

We present genetic and biochemical evidence that the nonhistone chromatin proteins NHP6A and NHP6B are required for optimal transcription efficiency of wild-type and mutant *SNR6* genes in vivo and in vitro. These observations suggest the potential of chromatin-associated proteins to act as positive or negative cofactors in Pol III transcription.

Reciprocal genetic interactions between *SNR6* and the *NHP6A* and *NHP6B* genes. Nonhistone proteins 6A and 6B (NHP6A and NHP6B) belong to a family of proteins characterized by the presence of one HMG box, a conserved domain of about 80 amino acids which mediates DNA binding (for a review, see reference 5). NHP6A and NHP6B are 96% similar and appear to be functionally redundant, as indicated by the absence of phenotype for the single *nhp6A*Δ or *nhp6B*Δ mutants (8). However, the double *nhp6A*Δ *nhp6B*Δ mutant grows slowly at 30°C and is nonviable at 38°C (8). The *nhp6A*Δ *nhp6B*Δ mutant shares many phenotypes with the *pkc1*Δ, *slk1*Δ, and *slt2*Δ mutants, and the *NHP6A* gene has been identified as a multicopy suppressor of the synthetic lethality of the *slk1*Δ and *spa2*Δ mutations, which has led to the suggestion that *NHP6A* and *NHP6B* function downstream of *SLT2* to mediate its function in cell growth and morphogenesis (8). However, we have demonstrated here that the transcription of the *SNR6* gene is strongly reduced in *nhp6A*Δ *nhp6B*Δ mutants and have found that the thermosensitivity of *nhp6A*Δ *nhp6B*Δ cells can be suppressed by the overexpression of either wild-type *SNR6* or *BRF1* (data not shown), which encodes the TFIIIB70 component of TFIIIB and whose overexpression was also found in our screen to suppress the thermosensitivity of *snr6*Δ*2*. Our data thus suggest that the thermosensitivity of the *nhp6A*Δ *nhp6B*Δ mutant could be primarily due to a defect in *SNR6* transcription, which could in turn affect, via splicing defects, the expression of genes belonging to the *SLT2* pathway. The chromatin analysis directly supports this hypothesis: the *nhp6A*Δ *nhp6B*Δ mutant revealed a loss of protection of the TATA box when the cells were shifted from 30°C to the nonpermissive temperature of 37°C.

Are NHP6A and NHP6B general regulators of Pol III transcription? While NHP6 proteins increase the transcription efficiency of *SNR6*, the overexpression or deletion of the *NHP6A* and *NHP6B* genes did not affect the in vivo levels of 5S RNA or $tRNA^{His}$. Remarkably, the level of another tRNA species, $tRNA^{Ile(UAU)}$, was found to be markedly increased in cells lacking NHP6 proteins. This observation raises the possibility that NHP6A and NHP6B could positively or negatively modulate the expression of a subset of Pol III genes. It should be noted, however, that we did not observe any effect of NHP6B on the transcription of $tRNA^{Ile(UAU)}$ genes in vitro. NHP6 proteins might participate in the repression of the $tRNA^{Ile(UAU)}$ genes only in the in vivo chromatin context or affect $tRNA^{Ile(UAU)}$ levels in an indirect fashion. At this point, *SNR6* remains the only Pol III gene whose transcription is unambiguously and directly influenced by NHP6A and NHP6B. It will

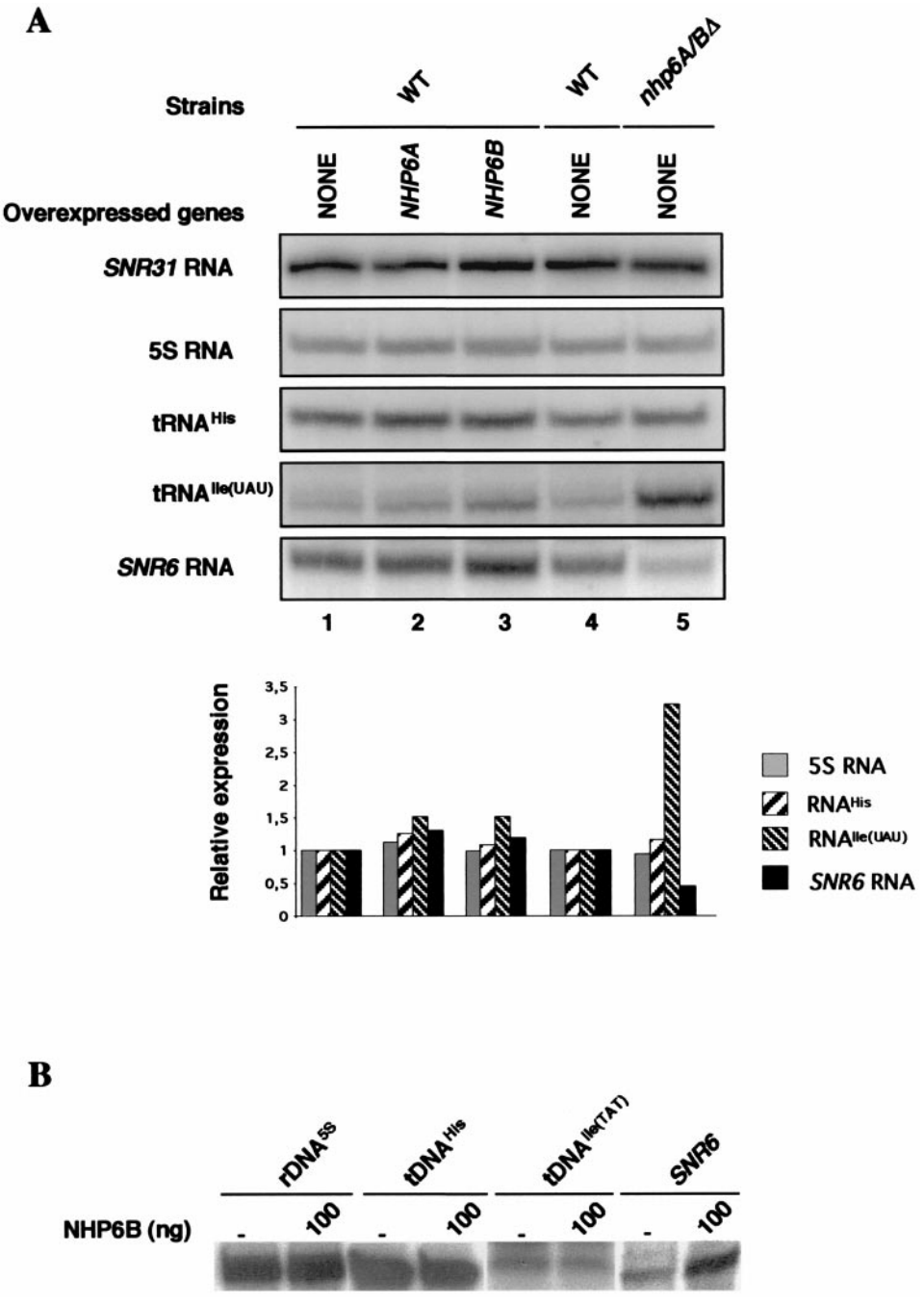

FIG. 7. NHP6A and NHP6B affect the transcript levels of several Pol III genes in vivo. (A) The steady-state levels of 5S RNA, $tRNA^{His}$, $tRNA^{Ile(UAU)}$, and the transcripts derived from the *SNR31* and *SNR6* genes were analyzed by Northern blotting in the wild-type strain YPH500α (WT), with or without the overexpression of *NHP6A* or *NHP6B* (lanes 1 to 3), and in wild-type (lane 4, Y865 [8]) and *nhp6A*Δ *nhp6B*Δ mutant (lane 5, Y869 [8]) cells. The steady-state levels of the RNA were quantified by PhosphorImager analysis, and the quantity of each transcript was normalized with *SNR31* transcripts as internal control. The relative RNA levels in the control wild-type strains (lanes 1 and 4) were arbitrarily assigned the value of 1 unit. (B) NHP6B was added to in vitro transcription reaction mixtures containing cell extracts prepared from *nhp6A*Δ *nhp6B*Δ mutant cells (Y869 [8]) and different templates containing either 5S rDNA, $tDNA^{His}$, $tDNA^{Ile(TAT)}$, or the wild-type *SNR6* gene as a control. The plasmids used are described in Materials and Methods. Reaction mixtures were incubated for 40 min at 25°C, and the transcription products were electrophoresed in a 6% polyacrylamide gel.

be interesting, when the specific DNA microarrays are available, to test the influence of *NHP6* gene deletion on the expression of all the Pol III genes. From the small set of genes examined, it appears that the presence of a canonical TATA sequence (TATAAATA) around position −30 is not a determinant for the stimulatory effect of NHP6. Both *SNR6* and the $tRNA^{Ile(UAU)}$ genes have this core promoter element. Furthermore, the TATAdown mutant *SNR6* gene required NHP6A and NHP6B for detectable expression in vivo (Fig. 3). The only *SNR6* mutant gene that was insensitive to the presence of

NHP6B in vitro was the Aup template, which harbors an intact B block and a canonical A block (instead of the *SNR6* degenerate A block). This mutant *SNR6* gene was efficiently transcribed in the presence or absence of NHP6B. Therefore, the selective effect of NHP6A and NHP6B on the transcription of Pol III genes in vitro appears to be related to the strength of their A block, suggesting a role for NHP6A and NHP6B in transcription complex assembly.

Mechanisms of NHP6A and NHP6B transcriptional activation of *SNR6*. The fact that NHP6B stimulated the transcription of *SNR6* gene in a purified reconstituted system suggested a direct effect on transcription complex formation. In strong support of this conclusion, chromatin analysis with MNase revealed a dramatic loss of protection in the TATA region of the *SNR6* gene in cells lacking NHP6 proteins. The protection of the TATA box is strictly linked to the transcriptional activity of the gene, and the extent of protection was found to correspond to TFIIIB footprinting on naked *SNR6* DNA (22). NHP6A and NHP6B could favor TFIIIB assembly over the TATA region indirectly, by facilitating a TFIIIC-DNA interaction. In gel shift assays, we found that NHP6B interacted with TFIIIC-*SNR6* DNA and TFIIIC-TFIIIB-DNA complexes to generate an upshifted complex. The specificity of this interaction, however, is uncertain because NHP6B by itself caused the formation of a ladder of protein-DNA complexes with *SNR6* DNA (results not shown). The possibility that NHP6 proteins act at the level both of TFIIIC and TFIIIB DNA binding remains open inasmuch as NHP6 stimulated the TFIIIC-independent transcription of *SNR6*.

The mode of action of NHP6 proteins is probably related to their DNA-binding properties. NHP6A and NHP6B belong to the subfamily of non-sequence-specific HMG box proteins. NHP6A was shown to bind linear DNA with little sequence specificity and to induce a large bend (26, 36). The abundance of NHP6A has been estimated to be ~50,000 to ~70,000 molecules per haploid cell, which would correspond to ~1 molecule of NHP6A for every 1 to 2 nucleosomes (25). NHP6A and NHP6B also bind the TATA-box regions of Pol II genes and DNA molecules of random sequences with equivalent affinity in vitro (25, 26). On the other hand, a sequence-dependent binding of the NHP6A- and NHP6B-related HMG1 protein has recently been demonstrated on the BHLF-1 promoter (11). Therefore, NHP6A and NHP6B may contribute to stabilize bent DNA conformations within the preinitiation complexes in a specific or non-sequence-specific manner. This does not exclude the possibility that NHP6A/B could influence *SNR6* transcription by also interacting with components of the preinitiation complex. Gal4(1–147)-NHP6A and Gal4(1–147)-NHP6B fusions were tested in the two-hybrid system (12) with fusions comprising the Gal4 activating domain and all the subunits of TFIIIB or TFIIIC. No interaction between NHP6A or NHP6B and any components of TFIIIC or TFIIIB could be detected in this way (data not shown). These negative results instead suggested that the major role of NHP6 proteins may reside in their DNA-binding and -bending properties. Paull et al. (25) previously reported that NHP6A promotes the formation of a Pol II preinitiation complex and suggested that NHP6A-induced structural changes in the TBP-TFIIA-DNA complex may facilitate TFIIB-DNA binding, which requires considerable DNA distortion. Similarly, TFIID-TFIIA-DNA complex formation was found to be enhanced by HMG2 (30). NHP6A and NHP6B could play a similar role for some Pol III genes. Interestingly, the C-terminal half of human TFIIB90 was found to contain an HMG1- and HMG2-related domain which is required for Pol III transcription (34). The presence of this domain, which is absent in yeast TFIIIB70, suggests that HMG boxes, here embedded in a Pol III common factor, could play a general role in the transcription of vertebrate Pol III genes.

ACKNOWLEDGMENTS

We are very grateful to Reid Johnson for his generous gift of the Y865 and Y869 yeast strains, of the RJ1963 and RJ1964 *E. coli* strains, and of the antibodies against NHP6A and NHP6B. We warmly thank Giorgio Dieci for providing us with the recombinant TBP, TFIIIB70, and TFIIIB90 purified proteins and with the KS-tDNAIle(TAT)199 and KS-tDNAIle(TAT)36 plasmids harboring the I(TAT)LR1 and I(TAT)DR2 genes; Emmanuel Favry for excellent technical assistance; and Olivier Lefebvre and Christine Conesa for stimulating discussions.

S.L. was supported by a Commissariat à l'Energie Atomique postdoctoral fellowship. F.T. and M.L. were supported by the Swiss National Science Foundation.

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