Nhp6 Is a Transcriptional Initiation Fidelity Factor for RNA Polymerase III Transcription in Vitro and in Vivo*

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The binding of the RNA polymerase III (pol III) transcription factor TFIIIC to the box A intragenic promoter element of tRNA genes specifies the placement of TFIIIB on upstream-lying DNA. In turn, TFIIIB recruits pol III to the promoter and specifies transcription initiating 17–19 base pairs upstream of box A. The resolution of the pol III transcription apparatus into recombinant TFIIIB, highly purified TFIIIC, and pol III is accompanied by a loss of precision in specifying where transcription initiation occurs due to heterogeneous placement of TFIIIB. In this paper we show that Nhp6a, an abundant high mobility group B (HMGB) family, non-sequence-specific DNA-binding protein in Saccharomyces cerevisiae restores transcriptional initiation fidelity to this highly purified in vitro system. Restoration of initiation fidelity requires the presence of Nhp6a prior to TFIIIB-DNA complex formation. Chemical nucleo-footprinting of TFIIIC- and TFIIIB-TFIIIC-DNA complexes reveals that Nhp6a markedly alters the TFIIIC footprint over box A and reduces the size of the TFIIIB footprint on upstream DNA sequence. Analyses of unprocessed tRNAs from yeast lacking Nhp6a and its closely related parologue Nhp6b demonstrate that Nhp6 is required for transcriptional initiation fidelity of some but not all tRNA genes, in vivo.

The sites at which RNA polymerase III (pol III)2 initiates tRNA gene transcription are specified by sequence-specific interactions of its two transcription factors, TFIIIC and TFIIIB, and by pol III itself. TFIIIC is a 520-kDa protein, containing six distinct subunits arranged into two globular subdomains, αA (subunits Tfc1, Tfc4, and Tfc7) and β (subunits Tfc3, Tfc6, and Tfc8), which are connected by a flexible linker (Tfc8; see Refs. 1–3 for reviews). TFIIIC binds to two intragenic sequence elements, the start site-proximal box A (binding αA) and the distal box B (binding β), which are optimally separated by 30–60 bp. The β-box B interaction contributes nearly all of the affinity of TFIIIC for tRNA genes (Ka >1 nM) (4). Nevertheless, it is the weak αA-box A interaction that primarily specifies initiation 17–19 bp upstream of box A (5, 6). Specification is indirect; the αA-box A interaction determines the placement of TFIIIB, which, in turn, recruits pol III. TFIIIB placement is mediated through an interaction between Tfc4 and the Brf1 subunit of TFIIIB. Brf1 forms a stable complex with TBP, and the major DNA contacts during TFIIIC-dependent assembly of TFIIIB upstream of the start site of transcription are mediated by TBP. Consistent with this role of TBP, the DNA segments occupied by TFIIIB (−bp −40 to bp −10, relative to predicted start sites of transcription) are uniformly AT-rich (>70% (7–9)).

The notion that preferential sequence selection by TBP can occur during TFIIIC-dependent assembly of TFIIIC comes from in vivo and in vitro studies in which a shift in placement of a TATA box or partial TATA box shifts the start site accordingly but retains dependence on TFIIIC for transcription (10, 11). Pol III also contributes to start site selection in a sequence-dependent manner, since initiation only occurs at sites specifying a purine as the initiating nucleotide preceded by a pyrimidine in the non-transcribed strand (i.e. −1/1 +1 is always YR). In vitro and in vivo studies on SL54 tRNA genes indicate that spacings of 17 to 20 bp are optimal for transcription and that spacings of 16 bp or less and 22 bp or more are severely compromised (5, 6). In this regard, an analysis of all 273 Saccharomyces cerevisiae tRNA genes (9) shows that only 26 genes lack a YR motif for initiation between bp −19 and −17 upstream of box A. Twenty-three of these genes accommodate initiation with a 16-bp spacing; of the remaining three, two have perfect TATA boxes specifying initiation bp upstream of box A (8), and one is known to function poorly for transcription in vitro (12). The combined DNA sequence-specific interactions of TFIIIC, TFIIIB, and pol III are inadequate for specifying the start site in vitro: 1) substantial purification of TFIIIC from yeast increases the proportion of minor initiation events at bp −4 + 4 and +8 on the SL54 tRNA gene in assays with TFIIIC and pol III purified to near homogeneity (cf. Ref. 10); 2) replacement of TFIIIB with recombinant subunits elevates bp +4 +8 +8 initiation events to the level at bp +1 (13, 14). A crude Bdp1 fraction was also found to contain an excess of a factor or factors that re-establishes the fidelity of initiation at bp +1 on the SL54 gene with recombinant TFIIIB. EMSA implicated a protein or proteins that interact with TFIIIC- and TFIIIB-TFIIIC-SL54 DNA complexes (13), although modification of TFIIIB, TFIIIC, and/or pol III (e.g. by a protein kinase) could not be excluded.

The loss of transcriptional initiation fidelity when recombinant TFIIIB, purified TFIIIC, and pol III are used to form pre-initiation complexes on the SL54 tRNA gene appears to reflect a heterogeneity in TFIIIB placement upstream of the start site of transcription by TFIIIC. This has hampered mapping regions within TFIIIB subunits that are proximal to specific DNA locations upstream of the start site of transcription (15). To alleviate this problem we decided to turn to the start site selectivity factor.

Here we show that Nhp6a, an abundant S. cerevisiae HMGI-related protein and a component of yFACT, a factor that facilitates pol II transcription of chromatin templates (16, 17), is the factor that enforces the fidelity of transcriptional initiation of the SL54 tRNA gene by pol III in vitro and in vivo.

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2 The abbreviations used are: pol, polymerase; EMSA, electrophoretic mobility shift assay; MPE, methylidenepropyl-EDTA.

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EXPERIMENTAL PROCEDURES

DNA—Plasmids pTZ2 (SUP4), pLNG56 (containing a transcriptionally inactive SUP4 gene with a mutated box B), and pPC1 (tRNA-L subunit) have been described (18). The 250-bp SUP4 DNA fragment used for EMSA and footprinting was generated by PCR from pTZ2 with a 5’-32P-labeled upstream primer (5’- bp-77, relative to the start site of transcription +1) and an unlabeled downstream primer (5’- bp +173) and purified by non-denaturing PAGE. Primers for reverse transcription were 5’-32P-labeled; sequences are available upon request.

Proteins—The purification and quantitation of pol III, Bdp1, Brf1, TBP, TFIIIC, and the “crude” Brα fraction have been described (18-20). Nhp6a and Nhp6b were generously provided by R. Johnson (UCLA) and HU from Thermotoga maritima and Helicobacter pylori and S. cerevisiae HMO1 by A. Grove (Louisiana State University). Human HMG1 was purchased.

Assays—Quantities of Brf1, TBP, and Bdp1 are specified as femtomoles of protein. Quantities of TFIIIC and pol III are specified as femtomoles of DNA binding activity and enzyme active for specific transcription, respectively (18). Protein-DNA complexes for the EMSA, and footprinting were formed at 20°C in 18-20 μl of reaction buffer (40 mM Tris-Cl, pH 8.0, 7 mM MgCl2, 3 mM dithiothreitol, 100 μg/ml bovine serum albumin, 6-8% (v/v) glycerol, and 90 mM NaCl). All proteins were diluted prior to use in a buffer solution designed to yield 20 mM NaHepes, pH 7.8, 0.2 mM Na3 EDTA, 7 mM MgCl2, 10 mM 2-mercaptoethanol, 100 mM NaCl, 20% (v/v) glycerol, 0.005% (v/v) Tween 20, 200 μg/ml bovine serum albumin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin and pepstatin (bovine serum albumin diluted (21)).

For Figs. 1 and 3B, transcription complexes were formed by adding TFIIIB (100 fmol of Bdp1, 100 fmol of TBP, and 80 fmol of Brf1) pre-mixed with or without Nhp6a, HU, and HMO1 (as specified in the figures) in 4-μl volume to 14 μl of the above reaction buffer containing 100 ng of pTZ2 or pPC1 (50 fmol) and 50 fmol of TFIIIC and incubated for 40 min; 2 μl of pol III (5 or 10 fmol) was then added with an additional 20-min incubation. Single-round transcription was initiated by addition of 2.5 μl of reaction buffer containing a 2 mM concentration each of ATP and CTP and 250 μM [α-32P]UTP (20 cpm/μmol) for 10 min followed by addition of 2.5 μl of reaction buffer containing 2 mM GTP and 2 mg/ml heparin for 5 min. Transcription reactions for primer extension analysis used unlabeled UTP. Primer extension with avian myeloblastosis virus reverse transcriptase was performed as described (19) using intron-specific primers. Reaction mixtures were processed for denaturing gel electrophoresis as described (18). For reaction mixtures that contained the crude Brα fraction, 3.7 μl of this fraction in the requisite volume of bovine serum albumin diluted was used in place of TFIIIB and bovine serum albumin diluted in place of TFIIIC and pol III in the protocol described above. The order of addition transcription analysis shown in Fig. 2 used the following variations of the above protocol: 1) the reaction mixture containing TFIIIC and pTZ2 was incubated for 5 min followed by the addition of Nhp6a and TFIIIB for 40 min; 2) the reaction mixture with pTZ2, TFIIIC, and TFIIIB (16 μl in this case) was incubated for 40 min followed by the addition of Nhp6a in 2 μl volume for 5 min; and 3) the reaction mixture with pTZ2 and Nhp6a (14 μl in this case) was incubated for 5 min followed by the addition of TFIIIC and TFIIIB in 4 μl for 40 min.

Protein-DNA complexes for the EMSA analysis shown in Fig. 3A were formed under the conditions used for transcription, except that 100 ng of pLNG56 (which differs at 2 bp within box B to knock out its function) replaced pTZ2 and 3 fmol of a labeled 250-bp pTZ2-derived probe was included. Following 60 min of incubation, EMSA was performed on a 4% polyacrylamide gel as described (22). Two-dimensional methidiumpropyl-EDTA (MPE)-Fe(II) footprinting was performed as follows. Protein-DNA complexes were formed for 60 min in 20 μl of reaction buffer containing 100 ng of pLNG56 as nonspecific competitor, 200 fmol of TBP, 160 fmol of Brf1, 100 fmol of Bdp1, 200 ng of Nhp6a, and 75 fmol of TFIIIC (as indicated in Fig. 5) with 6 fmol of the 250-bp SUP4 probe (12 fmol for naked DNA and Nhp6a only). Partial DNA cleavage was achieved by sequential addition of 1.2 μl of 23 mM sodium ascorbate and 1.2 μl of 46 μM MPE-Fe(II) (34 μM for naked DNA and Nhp6a alone) for 2 min and quenched by adding 2 μl of 60 μM bathophenanthroline. OH-treated complexes were separated by EMSA and footprints of individual complexes were resolved by denaturing gel electrophoresis of the extracted DNA as described previously (20).

5’-End Mapping of Start Sites in Vivo—RNA was isolated from exponentially growing S. cerevisiae strains DLY150 (reference type) and DY2381 (nhp6ΔΔ) (kindly provided by D. Stillman, University of Utah (23)) using the guanidinium thiocyanate-hot phenol extraction method (24) and an SDS-phenol extraction method (25). Primer extension, with 3 or 6 μg of total RNA, was performed as described (19).

RESULTS

Because the crude Bdp1 fraction (26, 27) containing the start site selectivity factor also contains significant levels of DNA-binding proteins (13), we first examined whether a nonspecific DNA-binding protein might impose greater stringency on TFIIIC-dependent placement of TFIIIB. The HMG1-related Nhp6a was chosen to mimic the effect of nonspecific DNA-binding proteins present in crude Bdp1 fractions. Nhp6a and the closely related Nhp6b play a positive role in pol III transcription of SNR6 (U6 snRNA gene) by a proposed compacting of DNA between the grossly suboptimally spaced box A and box B TFIIIC binding sites of that gene. Nhp6a and Nhp6b were not found to play a positive role in the transcription of any tRNA gene tested (28-30). However, under conditions of limiting pol III with recombinant TFIIIB and highly purified TFIIIC, Nhp6a and Nhp6b were seen to stimulate tRNA gene transcription by shielding partial TATA boxes that otherwise lead to TFIIIC-independent, TFIIIB-DNA complex formation, depleting the availability of pol III for specific transcription and increasing nonspecific transcription (31). A bacterial HU protein, which like Nhp6 binds to DNA non-specifically and bends DNA sharply, also stimulates tRNA gene transcription under these conditions.4

Nhp6 Promotes Initiation Fidelity—Fig. 1 examines the effect of Nhp6a on start site selection on the SUP4 tRNA gene. In A, TFIIIB, with or without Nhp6a, was added to TFIIIC-DNA complexes to form TFIIIB-TFIIIC-DNA complexes; pol III and NTPs were subsequently added under conditions that limit transcription to a single round (see “Experimental Procedures”). Lanes 1 and 3 compare SUP4 transcription with a crude pol III transcription fraction (Brα (22)) and with purified TFIIIC, pol III, and recombinant TFIIIB, respectively. Brα generated a relatively sharp SUP4 transcript band upon denaturing gel electrophoresis, reflecting initiation occurring almost exclusively at bp +1 with a trace of initiation at bp +4 (primer extension 5’-end mapping in B, lane 1; profile of this lane in C). In contrast, the SUP4 transcript generated with purified components was diffuse (A, lane 3), reflecting high levels of initiation at bp +1, +4, and +8 and weaker initiation at bp +12 (B, lane 2; profile in C). This pattern reflects initiation at every YR motif preceding the mature tRNA 5’-end—specifying sequence of SUP4: CAA-CAAATTGATGA (bp -1 to +12). The diffuse nature of the SUP4 transcript band in lane 3 of A arises from heterogeneity of 5’-ends and

4 G. A. Kassavetis and David F. Steiner, unpublished results.
A heterogeneous termination within the run of 7 Ts that comprises the SUP4 terminator. Addition of Nhp6a together with TFIIIB to TFIIIC-DNA complexes substantially sharpened the band of SUP4 transcripts (A, compare lanes 4–6 with lane 3), resulting from suppression of initiation at bp +4, +8, and +12 (B, lanes 3 and 4; C profile). The degree of fidelity of initiation with 50 ng of Nhp6a was comparable with that of BRα for bp +4 initiation with only a trace residuum of bp +8 and bp +12 initiation retained (C, compare left and right profiles). Suppression of downstream initiation also occurred under conditions of multiple round transcription (data not shown), and the closely related Nhp6b likewise suppressed downstream initiation (D). Nhp6a and Nhp6b also reduced nonspecific transcription as noted previously (31).

Although transcriptional initiation downstream of bp +1 with purified components probably results from TFIIIB complexes that are misplaced downstream of their canonical site, it remains conceivable that Nhp6a suppresses downstream initiation at a step subsequent to TFIIIB-DNA complex formation (e.g. by shading the more confined space between downstream-shifted TFIIIB and the box A-bound ρ domain of TFIIIC from access by pol III). This possibility was addressed in the experiment shown in Fig. 2. When TFIIIC was allowed to bind to DNA first, followed by the simultaneous addition of TFIIIB and Nhp6a, suppression of downstream initiation was observed (compare lanes 2–4 with lane 1). When Nhp6a was allowed to bind to DNA first, followed by the simultaneous addition of TFIIIC and TFIIIB, downstream initiation was also suppressed (compare lanes 10–12 with lane 9), but this did not happen when Nhp6a was added to preformed TFIIIB-TFIIIC-DNA complexes (compare lanes 6–8 with lane 5). We conclude that Nhp6a must be present as the TFIIIB-DNA complex forms to suppress downstream initiation.

Restoration of Transcriptional Initiation Fidelity Is Specific to Nhp6a and Nhp6b—We originally speculated that any nonspecific DNA-binding protein might promote start site fidelity and, accordingly, analyzed the effects of four other nonspecific DNA-binding proteins: human HM1G1 and yeast HMO1, which are HMG B class proteins like Nhp6a (32), and bacterial HU proteins from *T. maritima* and *H. pylori*, which, like HMG B class proteins, are non-sequence specific and bind DNA. Nhp6a can, in fact, partially substitute for the function of HU in *Escherichia coli* nucleoid compaction and chromosome segregation (33). Of these proteins, only HMO1 and *T. maritima* HU could be verified by EMSA to associate stably with the SUP4 gene under the conditions used for transcription (Fig. 3A). HMO1 did not substitute for Nhp6a in restoring start site fidelity (compare lanes 6 and 7 with lanes 3 and 4 in the primer extension analysis shown in Fig. 3B). HU from *T. maritima* also had no effect on start site selection (Fig. 1B, lane 5); doubling the concentration of HU greatly inhibited transcription but the residuum retained the same relative abundance of downstream initiation events (data not shown).
Nhp6a and Nhp6b Are Required for Initiation Site Fidelity in Vivo—
The inability of nonspecific DNA-binding proteins similar to Nhp6a to promote initiation fidelity prompted us to examine whether Nhp6a has a role in start site selection in vivo. For this purpose, RNA was purified from logarithmically growing yeast deleted for the genes encoding Nhp6a and Nhp6b and from the otherwise isogenic strain. RNA 5′-ends were mapped by primer extension with reverse transcriptase, using a primer complementary to the intron of the wild type allele of SLIP4 (tRNA-Y JR). The non-coding 5′-leaders of tRNA gene transcripts are rapidly processed, but unspliced intron-containing tRNAs are greatly enriched for pre-tRNAs retaining the 5′-leader. This intron-specific primer is complementary to two additional tyrosyl-tRNA genes (tRNA-Y DR2 and tRNA-Y OL; Fig. 4A, bottom). Relative to the position of the SLIP4 bp +1 reverse transcription product, YR motifs that place initiation sites within the optimal 17–20-bp spacing from the start site of transcription are implicated in determining the need for Nhp6 to reinforce initiation fidelity.

Nhp6a Alters TFIIIC-Box A Interaction—The order-of-Nhp6a-addition experiment in Fig. 2 implied that Nhp6a must act prior to TFIIIB-DNA complex formation to prevent initiation of transcription downstream of bp +1. Two-dimensional hydroxyl radical footprinting with MPE-Fe(II) was employed to gain insight into the process by which Nhp6a specifies stringency in TFIIIB placement (Fig. 5). Naked DNA or protein-DNA complexes containing combinations of Nhp6a, TFIIIC, and TFIIIB were treated with MPE-Fe(II), and the complex of interest was isolated by EMSA (A, inset), followed by denaturing gel electrophoresis. A compares the MPE-Fe(II) digestion profiles of a 250-bp SLIP4 tRNA gene probe, 5′-end-labeled on the non-transcribed strand, as free DNA and as the Nhp6a-DNA complex formed with 200 ng of Nhp6a (0.9 μM). This concentration of Nhp6a is substantially less than required to completely cover the DNA probe (≥7 μM Nhp6a, data not shown; Fig. 3). Weaker Nhp6a-dependent protection was observed near the label-distal end of the probe (at the left in the profile shown in A) and directly upstream of the start site of transcription (−bp −7 to −32) in a region of alternating TA and TG residues believed to be somewhat preferred for Nhp6a binding due to inherently greater local flexibility (34). Most yeast tRNA genes have upstream flanking sequences that are rich in these motifs ((8, 12).1 Nevertheless, we were surprised to detect little protection in gel-isolated Nhp6a-DNA complexes under single-hit conditions and no requirement for additional MPE-Fe(II) to achieve digestion levels comparable with naked DNA. It is possible that the equilibrium between bound and unbound DNA states favors the latter
under transcription and footprinting conditions but the former at the low salt concentrations of EMSA. Weak protection by Nhp6a was also observed upstream of the start site when DNase I was used as the cutting agent (data not shown).

Nhp6a generated two effects on the interaction of the TFIIIC τA domain with box A (Fig. 5B). First, it stabilized the interaction of TFIIIC with box A as indicated by increased protection from MPE-Fe(II) cleavage. Second, it generated an upstream extension of the footprint from bp −15 to +10. This extended protection may reflect a conformational change in the τA domain of TFIIIC or may reflect a TFIIIC-dependent and site-specific interaction of Nhp6a with DNA upstream of box A. A single Nhp6a molecule is expected to shield ∼10 bp of the DNA minor groove from MPE-Fe(II) access (34, 35).

The effect of Nhp6a on TFIIIB assembly is shown in Fig. 5C. As observed previously (18), addition of TFIIIB to the TFIIIC-DNA complex increased protection of box A (C, compare with B) and generated protection upstream of the start site of transcription attributable to bound TFIIIB. This footprint extends from approximately bp −7 to bp −38. The addition of Nhp6a reproducibly generated a decrease in TFIIIB protection between bp −7 and approximately bp −15 with increased protection between approximately bp −24 and bp −38 (C). (In two repetitions of this footprinting analysis, interpretation of the inside borders of these two regions of decreased/increased protection varied by ±2–3 bp, and the degree in the loss of protection between bp −7 and −15 also varied, but the result as just stated was uniformly observed.) For D, TFIIIC-TFIIIB-DNA complexes formed in the presence or the absence of Nhp6a were treated with heparin prior to the addition of MPE-Fe(II). Heparin strips TFIIIC from DNA leaving only TFIIIB (36, 37). Heparin also strips the bulk of Nhp6a from DNA (data not shown). Protection by TFIIIB was decreased between bp −4 and approximately bp −22 and was increased between approximately bp −30 and bp −38 in complexes formed in the presence of Nhp6a. Overall, Nhp6a generated a compaction of the TFIIIB footprint by decreasing the already weak protection at the start site-proximal end of the footprint without introducing additional protected DNA residues upstream of bp −38. This is consistent with the following interpretation: the TFIIIB footprint of the promoter complex formed in the absence of Nhp6a represents an ensemble of heterogeneously positioned TFIIIB complexes; Nhp6a limits TFIIIB to its most upstream site that specifies initiation at bp +1.

DISCUSSION

The impetus for examining whether Nhp6a might suppress incorrect initiation downstream of bp +1 on the SLP4 tRNA gene came from the
observation that Nhp6a suppresses TFIIIC-independent formation of TFIIIB-DNA complexes at partial TATA boxes (31). We reasoned that the initiation events downstream of bp +1 with recombinant TFIIIB and highly purified TFIIIC and pol III might result from interaction of the τ subdomain with overlapping box A variants with residence times sufficient to nucleate TFIIIB assembly at correspondingly shifted positions. It was supposed that, if a nonspecific DNA-binding protein like Nhp6a suppresses TFIIIB binding at partial TATA boxes, it might also suppress TFIIIC interaction with partial box A sequences. In this regard, 17 of the 46 distinct mature tRNA sequences encoded in yeast contain AT-rich upstream sequences, similar to the limited number of 2–3-bp substitutions within box A that have been analyzed in vitro, nondecreased transcription by more than 60% (38–40).

We have shown that Nhp6a does restore fidelity of initiation site selection to transcription with highly purified TFIIIC and pol III and recombinant TFIIIB (Fig. 1) and that this effect requires the intervention of Nhp6a prior to the formation of TFIIIB-DNA complexes (Fig. 2). Nhp6b also restores initiation fidelity (Fig. 1D). However, our original notion that Nhp6a might function solely as a nonspecific DNA-binding protein in suppressing TFIIIC interaction with variant, overlapping, downstream box A sequences appears to be invalid. 1) Enforcement of initiation fidelity is not strictly due to nonspecific binding to and bending of DNA by Nhp6, since two bacterial HU proteins and two other HMG B class proteins that bind DNA and bend it similarly (reviewed in Ref. 32) fail to restore initiation fidelity (Figs. 1B and 3 and data not shown). Instead, Nhp6 is a specific pol III initiation fidelity factor, since cells that lack both Nhp6a and Nhp6b lose initiation fidelity at some tRNA genes (Fig. 4). 2) Some, but not all, tRNA genes (Box 4) have a variant box A that deviates at two positions. Among the limited number of 2–3-bp substitutions within box A that have been analyzed in vitro, none decreased transcription by more than 60% (38–40).

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