Positioned Nucleosomes Due to Sequential Remodeling of the Yeast U6 Small Nuclear RNA Chromatin Are Essential for Its Transcriptional Activation*

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Transcription from the yeast SNR6 (U6 small nuclear RNA) chromatin, a gene transcribed by the enzyme RNA polymerase III, depends on its transcription factor IIC (TFIIIC) and the promoter elements (the intragenic box A and box B located downstream to its terminator) to which TFIIIC binds. The genes transcribed by polymerase III generally lack the upstream promoter elements where TFIIIC is known to recruit the transcription initiation factor TFIIIB. The TFIIIC-dependent chromatin remodeling of the gene in vitro that involves translational positioning of a nucleosome between boxes A and B is found to be essential for its transcriptional activation. We show here that the role of TFIIIC is not limited to the recruitment of TFIIIB on chromatin templates. The pre-binding of TFIIIB to the SNR6 TATA box in the upstream gene region does not alleviate TFIIIC requirement for transcriptional activation of the chromatin. Binding of TFIIIC to an array of pre-positioned nucleosomes results in an upward shift of the single nucleosome between boxes A and B. The ~40-bp shift of this nucleosome in the 3’ to 5’ direction leads to increased nuclease sensitivity of the ~40-bp DNA 3’ to the upstream TATA box. Further chromatin remodeling accompanies the binding of TFIIIB in the next step. This two-step remodeling mechanism using the basal factors of the gene yields high transcription levels and generates a chromatin structure similar to that reported for the gene in vivo.

Organization of the eukaryotic genome into chromatin links the process of transcription intimately with the structure of its template. Gene expression in eukaryotes is regulated by several mechanisms that alter the chromatin structure either to allow or disallow an activity using it as the substrate or template. These mechanisms include the covariant histone modifications, exchange with histone variants, and ATP-dependent chromatin remodeling, demonstrated in a large number of studies with the genes transcribed by the enzyme RNA polymerase II (pol II) (1–4). Fewer examples of chromatin transcription by the RNA pol III are available that may have a different relationship with its template structure due to the short gene units and characteristically intragenic promoter elements.

Pol III transcribes genes encoding structural RNAs required for translation (tRNA, 5 S rRNA), tRNA processing (PRP1), and splicing (U6 snRNA). The pol III transcription machinery in yeast is made up of the 17-subunit polymerase and its two complex factors (TFIIB and TFIIIC) that are involved in promoter recognition and/or initiation of all the three classes of the pol III genes (5–8). Transcription factor IIA, which binds to the intragenic box C, is required only for the 5 S ribosomal DNA transcription (9). TFIIIC binds to the intragenic promoter elements, boxes A and B (9), and the role attributed to it in pol III transcription has been to recruit the true transcription initiation factor TFIIIB in the upstream region of the pol III-transcribed genes (10). The TFIIIB-DNA complex is extremely stable (11). It does not dissociate from its upstream site after transcription initiation and is capable of directing multiple rounds of transcription in the absence of TFIIIC on naked DNA templates. Transcription of a pol III-transcribed gene takes place on the template covered by its own factors. Thus, the presence of nucleosomes on these genes may need transcription mechanisms different from those known for the pol II-transcribed genes.

Transcription from the yeast U6 snRNA gene (SNR6, a class III gene transcribed by pol III) in vivo and on chromatin templates assembled in vitro is dependent on the presence of box B and TFIIIC (12–15). This pol III gene is atypical in having a TATA box at the −30 position and an extragenic box B located further downstream to its terminator, making the distance between boxes A and B unusually large (~200 bp) (12). We have previously shown that chromatin remodeling plays a significant role in transcriptional activation of the SNR6 chromatin by TFIIIC in vitro (15), wherein one nucleosome gets translationally positioned between boxes A and B to which TFIIIC binds. Thus, complete repression of SNR6 transcription on the chromatin templates is due to the non-availability of its upstream TATA box for TFIIIB binding, and TFIIIC directed remodeling may be required for the purpose of making a room around the TATA box for TFIIIB binding. If this is the only role for TFIIIC, a pre-bound TFIIIB on chromatin templates should not require TFIIIB binding or chromatin remodeling for transcriptional activation. Similarly, a nucleosome positioned between boxes A and B without TFIIIB addition should give a high level transcription, associated with TFIIIC-dependent positioning of nucleosome reported in our previous study (15). We have explored the above-proposed possibilities in vitro by using a modified template that allows chromatin assembly to give pre-positioned nucleosomes on the U6 gene region in vitro. We find that TFIIIC is indispensable for the activation; neither a pre-positioned nucleosome between boxes A and B nor a pre-bound TFIIIB on the TATA box can give the full-level activation of transcription on the chromatin templates. Thus, the role of TFIIIC is not limited to the recruitment of TFIIIB on the chromatin DNA (10).

Additionally, the chromatin structure analysis over the wild type and the modified genes explains the mechanism of the ATP-dependent chromatin remodeling and its relationship to the transcriptional activation of this gene.
**EXPERIMENTAL PROCEDURES**

DNA—Plasmid DNAs carrying either the wild type or a modified form of the yeast U6 snRNA gene were used for transcription as naked DNA or in vitro assembled chromatin. The wild type gene from the plasmid pCS6 (12) was put in a different vector backbone to give the plasmid pU6LNS (16), giving the two high affinity lac repressor binding sites (Lac O1) immediate downstream to its boxes A and B. The terminator sequence centered at +112, and pseudo boxes A or the pseudo initiation site in pCS6 have been deleted in this plasmid construct (Fig. 1, A and B), whereas the sequence of box A was altered. The altered box A sequence (TGGCCAAATGGT) is similar to an Aup mutant (TGGCCAAATGGT) that showed increased TFIIIC-dependent transcription on nucleosomal templates (17). Chromatin assembly over pU6LNS in the presence of a mutant lac repressor protein R3 (18), which does not tetramerize to loop out the intervening DNA, gives an array of five positioned nucleosomes (16) such that one of them gets positioned between boxes A and B.

Chromatin Assembly and Structure Analysis—Chromatin assembly on the plasmid DNA using the S-190 extract of Drosophila embryos and Drosophila core histones, structure analysis by indirect end labeling (IEL) analysis at longer range, and the micrococcal nuclease (MNase) footprinting by the primer extension method for structure probing at closer range were done as described previously (15, 16, 19). When required, TFIIIC/TFIIIB were added in molar excess to saturate the binding sites at different steps of the chromatin assembly (15). Restriction enzymes AlwNI and XmnI were used for the secondary digestion of the MNase-digested samples for IEL. The probe was used close to the AlwNI site, present in opposite orientations on both the plasmids. TFIIIB was added in a 2.5-fold molar excess (active molecules), whereas TFIIIC was added in a 5-fold molar excess (active molecules) over DNA for the IEL experiments. Calibrating the blots with the molecular size markers identified the MNase cut sites in IEL blots. A mutant lac repressor protein R3 (18) was added in a 10-fold molar excess over the operators at the start of the assembly. R3 was dislodged from the DNA by adding excess isopropyl 1-thio-β-D-galactopyranoside (IPTG), and apyrase enzyme was used to stop the ATP-dependent chromatin remodeling (16). For all footprinting analyses, an 8-fold molar excess of TFIIIC over DNA was used. The quality of the chromatin assembly and periodicity of the nucleosomal arrays were confirmed by the formation of MNase-resistant nucleosomal ladders (19). Profiles of all the lanes from phosphorimages of the Southern blots/footprinting gels were generated using the Image Gauge program. Every footprint was confirmed, and boundaries of protections were ascertained by comparing profiles of the lanes with matching levels of digestions. Peaks were assigned numbers that denote cuts by the MNase at bp positions with respect to the transcription initiation site of the gene taken as +1.

**In Vitro Transcription**—Yeast TFIIIC (purified by DNA affinity chromatography) and TFIIIB subunits TBP, Bef1, and Bdp1 (purified from overexpression clones) were prepared, and active molecules were estimated as described previously (Ref. 15 and references therein). RNA pol III was purified from a yeast strain harboring a gene encoding its C128 subunit with double tag of hexahistidine and FLAG (20). Templates were pre-saturated with TFIIIC/B, added at a 2.5-fold molar excess (active molecules). Transcription on the naked DNA and chromatin templates was carried out without or with the addition of a 2.5-fold molar excess of TFIIIC (active molecules) according to the given schemes (referred to as "extra TFIIIC"), and the transcript formation was visualized by a primer extension method, as described previously (15). The products were resolved on 10% gels and quantified by phosphorimage analysis (15). Transcript yield in each lane was normalized with respect to the recovery marker, and -fold activation was calculated as the ratio of the normalized transcript amount to the normalized reference transcript amount.

**RESULTS**

Pre-binding of TFIIIB to Chromatin Templates Does Not Alleviate Transcriptional Repression of the SNR6 Chromatin—Unlike naked DNA, no transcription from the chromatin templates could be seen when only TFIIIB was provided with pol III (15). The requirement of TFIIIC for chromatin transcription (15) suggests that the TFIIIC-directed chromatin remodeling is required for TFIIIB binding. Therefore, we explored whether similar levels of transcriptional activation could be achieved by using pre-bound TFIIIB so that the requirement for TFIIIC addition and chromatin remodeling could be bypassed. Knowing that the complex formed by TFIIIB on pol III gene promoters is highly stable (11), we assembled chromatin over pCS6 having a pre-bound TFIIIB/C to use as a template for transcription according to the scheme given in Fig. 2A. Transcription of naked pCS6 DNA does not require TFIIIC (compare lanes 1 and 5, Fig. 2B). Transcription of the templates saturated with TFIIIB shows the highest levels without the addition of...
TFIIIC (compare lane 2 with the rest of the naked DNA lanes, Fig. 2B). However, the chromatin formation does not allow full activation of transcription from the DNA pre-saturated with TFIIIB (lane 10 versus lanes 2, 8, and 9). The full activation can be seen only when TFIIIC is added without or with prior saturation with TFIIIB (Fig. 2, B, lanes 11–16, and C, quantifications). Once bound, TFIIIB was reported to direct multiple rounds of transcription from the naked transfer DNA even in the absence of TFIIIC (10). However, TFIIIB alone is found insufficient for transcriptional activation from the chromatin templates. These results suggest that blocking the nucleosome formation over the TATA box by pre-bound TFIIIB does not overcome the repression due to chromatin formation on the gene region, and TFIIIC-directed chromatin remodeling is an important component of the transcriptional activation from the yeast U6 gene.

TFIIIC-directed Nucleosome Positioning between Boxes A and B Makes the Upstream Region Sensitive to MNase—To allow the assembly of transcription initiation complex ~30 bp upstream to the start site, TFIIIC-directed chromatin remodeling should make the TATA box accessible to TFIIIB. The nucleosome positioned between boxes A and B due to TFIIIC binding protects the DNA from bp +50 to +190 in pCS6 (15) and bp +40 to +180 in pU6LNS (not shown). An increased MNase sensitivity (vertical bars) of this nucleosome upstream to this nucleosome was observed after binding of TFIIIC to both the wild type (Fig. 3A) and the modified genes (Fig. 3B). On the wild type gene (profiles not shown), this hypersensitivity is found spread from bp +48 to −10, whereas the peaks falling between the region +46 and −24 show higher intensities in the presence of TFIIIC for pU6LNS chromatin samples (Fig. 3C). The profile alignment in Fig. 3C also revealed a hypersensitivity from −110 to −140 bp and an −85-bp protection (between bp −25 and −110) immediately upstream of the TATA box in pU6LNS. The protection in the corresponding region of pCS6 was observed between −10 to −90 (pronounced) and −100 to −150 (weak). These weakly protected regions on both the genes (the ellipses) are probably due to a positioned nucleosome flanked by the hypersensitive regions. This may be the reason that no positioned nucleosome was seen upstream to box A in the structure analysis on the wild type gene in our previous study (15). However, on both of the plasmids, the MNase hypersensitivity of the region upstream to box A (Fig. 3, A–C) upon TFIIIC binding suggests an increased accessibility of the DNA to TFIIIB.

Changes in the Upstream Chromatin Structure with TFIIIB Binding—Hypersensitivity of box B-flanking regions associated with binding of TFIIIC has been observed previously (15), and the hypersensitivity of the region around box A observed here may also be due to the binding of TFIIIC to box A. Poor cutting of box A by the MNase (single cut at bp +28) and the hypersensitivity of the region around box A make the TFIIIC footprint on box A difficult to see. A TFIIIC footprint over box A or a TFIIIB footprint over the TATA box could not be detected on the chromatin templates in high resolution gels (not shown), probably due to hypersensitivity of the region observed in Fig. 3, A–C.

We used IEL analysis (Fig. 4) to follow the changes, if any, due to the binding of TFIIIB to the chromatin after the TFIIIC-dependent chromatin remodeling. Comparison of the chromatin and naked DNA samples from pCS6 in Fig. 4A shows positioned nucleosomes (marked with ellipses) in the presence of TFIIIC (compare lane 2 with lanes 1, 5, and 6), one between boxes A and B and probably two more with less pronounced protections in the flanking regions. The addition of TFIIIB alone at the end of the chromatin assembly did not give any positioned nucleosomes (lane 3 versus lanes 1 and 7 in Fig. 4A and the profiles in Fig. 4B), suggesting that TFIIIB either cannot bind a chromatinn structure on its own, or its binding does not cause any structural change in the chromatin. However, the addition of TFIIIC along with TFIIIB (lanes 4) shows better protection both between the boxes and upstream to box A (marked with an ellipse in the lower part of the gel) than that in lane 2, suggesting the presence of the positioned nucleosomes. This result shows that in the presence of TFIIIC, as suggested by the analysis in Fig. 3, TFIIIB binding of the TATA box leads to the further stabilization/remodeling of the immediate upstream chromatin structure of the gene, as confirmed by the results of the experiments described in the following sections.

The structural analysis by IEL of the pU6LNS chromatin in the presence of TFIIIC with/without TFIIIB (Fig. 4C) shows positioning of three nucleosomes in the presence of TFIIIC (lanes 3 and 5, marked with the dark gray ellipses), although the nucleosome marked with a question mark (?) is difficult to ascertain in lane 3 due to a weaker protection. Binding of a mutant lac repressor R3 to the two lac operator sites in pU6LNS has been reported to give five positioned nucleosomes in a single register (lane 2, Fig. 4C; Ref. 16). Numbering the nucleosome between the two operators as 0, the flanking two nucleosomes on both...
Chromatin Remodeling on Yeast SNR6

FIGURE 3. Binding of TFIIIC to the chromatin increases the MNase sensitivity of the DNA upstream. Panels A and B, high resolution MNase footprinting analysis by primer extension method in the upstream region of the wild type gene in pC56 (A) and the modified gene in pU6LNS (B). In each set of the four lanes, the first three lanes have samples subjected to three levels of MNase, whereas the last lane shows primer extension over the undigested sample. Primer was located 65 (A) or 68 (B) bp downstream to box A, hybridizing to the top strand of the gene. Vertical bars mark the chromatin region with increased sensitivity to MNase in lanes 13–16 downstream of the TATA box, and the ellipse spans the protection observed in profile comparisons. C, comparison of the profiles of lanes 11 (black) and 15 (gray) from the gel in panel B shows the nuclease hypersensitivity from −24 to +53 and a protection from −110 to −25 on the pU6LNS chromatin with TFIIIC.

the sides were numbered as −1, −2 and +1, +2 (the light gray ellipses). Mapping of the approximate locations of these nucleosomes on the IEL blot (analysis not shown) showed positions of −2, +1, and +2 nucleosomes as similar to those reported for positioned nucleosomes flanking the gene region in vivo (21). Although the reported chromatin structure does not confirm the presence of positioned nucleosomes on the gene region in vivo (14, 21), we assembled a chromatin over pU6LNS using R3 that gave positioned nucleosomes (−1 and 0) on the gene region along with a structure similar to that reported for the gene flanking regions in vivo. We refer to the positioned nucleosomes on this template as “pre-positioned.”

The position of the nucleosome between boxes A and B in lane 3 is found to be between pre-positioned nucleosomes 0 and −1 (lane 2), whereas the flanking nucleosomes in Fig. 4C, lane 3, are in the same location as the R3-directed nucleosomes −2, +1, and +2 in lane 2. The −2 nucleosome in lane 3 (marked with a ?) is found upstream of box A, touching the TATA box on its 3’ side and could be the same as that seen in Fig. 3, B and C. Its position in lane 6 does not change after TFIIIC binding but appears to have moved upward or been disturbed with TFIIIB binding in lanes 5 and 7 (black ellipse). Similarly, when TFIIIC is added to the chromatin with pre-positioned nucleosomes (lanes 6 and 7), all the R3-directed, gene-flanking nucleosomes remain in their respective places, but nucleosomes 0 and −1 on the gene region appear disturbed. This structure appears to be an intermediate between the R3-dependent and TFIIIC-dependent structures due to either loss of nucleosomes or loss of positioning.

The presence of pre-bound TFIIIB (Fig. 4C, lane 4) does not change the chromatin structure anywhere (compare with lanes 1, 8, and 10) except for a protection upstream of the TATA box (black ellipse), which is seen even in the presence of TFIIIC (lane 5). Profile comparisons of lanes 1, 4, and 5 in Fig. 4D confirm this result. Because TFIIIB can bind to the assembled chromatin only after TFIIIC binding and the addition of TFIIIC before or after the chromatin assembly leads to the same structure (15), these changes in the gene-upstream region (lanes 4 and 5) suggest that the binding of TFIIIC to chromatin leads to a further shift of the TFIIIC-directed nucleosome positioned in the region upstream of the TATA box.

A Template with Pre-positioned Nucleosomes on and around the U6 Gene Requires TFIIIC for Full Transcriptional Activation—TFIIIC binding was reported as essential for the positioning of a nucleosome between boxes A and B, which is important for transcriptional activation of the yeast SNR6 chromatin in vitro (15). Therefore, we hypothesized that a template having a pre-positioned nucleosome between boxes A and B may not require TFIIIC for transcriptional activation. We compared the transcription (Fig. 5) over naked DNA as well as chromatin-assembled DNA according to scheme A in the presence or absence of the R3 protein. TFIIIC was either added (e) or not added (d) during transcription of the templates without or with pre-bound TFIIIB/TFIIIC. As evident from Fig. 5, B–F, full activation from chromatin templates is seen only in the presence of TFIIIC. Under every condition of the experiment, R3 gives a 4–5-fold inhibition of naked DNA transcription (compare the corresponding naked DNA lanes in Fig. 5, panels B and D). Chromatin formation represses transcription completely, and TFIIIC can activate it ~50-fold with or without R3 (lane 13 versus lane 9 in Fig. 5, B and D; quantifications in Fig. 5, C and E). This result suggests that the pre-positioned nucleosomes help overcome the tran-
Chromatin Remodeling on Yeast SNR6

FIGURE 4. Indirect end-labeling analysis of the chromatin structure on SNR6 with added TFIIIC and TFIIIB. Ellipses on the left-hand side mark the positioned nucleosomes in the corresponding lanes. Sizes of marker DNA in lane M of the gel are 1995, 1348, 1047, 955, 871, and 725 bp, from top to bottom. A, lanes 1–8 show digestion products of the pCS6 chromatin, whereas lanes 1–8 show that of naked DNA. At the end of the 4.5-h chromatin assembly, TFIIIB was added in lanes 1, 4, 7, and 8, whereas lanes 2, 4, 6, and 8 received TFIIIC. Positions of boxes A and B, the TATA box, and nucleosomal protections (marked as ellipses) are shown. B, comparison of the digestion profile of lane 1 with lanes 3 and 4 from the panel A. The schematic at the bottom of the figure shows the position of the probe with respect to boxes A and B. C, indirect end-labeling analysis of the chromatin structure on the modified U6 gene. Chromatin was assembled on the pU6LNS DNA with the proteins added as shown. Digestion products of the naked DNA (lanes 8–13) and chromatin (lanes 1–7) were probed with a probe that hybridizes ~900 bp downstream to box B. Saturating amounts of TFIIIC/TFIIIB were added 15 min before the start of the chromatin assembly (lanes 3–5) or at the end of the 4-h assembly (lanes 6 and 7). Positions of boxes A and B and the TATA box are marked. The t represents the uncertainty of this protection in lane 3. Lighter gray ellipses and the numbers −2, −1, 0, +1, and +2 mark the nucleosomes positioned due to pre-bound R3 in lanes 2, 6, and 7, whereas the black ellipse marks the protection due to TFIIIB in lanes 4 and 5. D, comparison of the digestion profile of lane 1 with lanes 4 and 5 from the panel C. The schematic at the bottom of the figure shows the position of the probe with respect to boxes A and B.

transcription inhibition due to the R3 on chromatin templates in the presence of TFIIIC. When TFIIIC is not added for transcription, pre-bound TFIIIB in the presence of R3 gives lower transcription levels from the chromatin as compared with that in the absence of R3 (lane 10 versus lane 9 in Fig. 5, B and D; quantifications in Fig. 5, C and E). However, with pre-bound TFIIIC (TFIIIC additions (b); lanes 11, 12, 15, and 16 in Fig. 5, B and D), transcriptional activation (versus lane 9) is 2–3 times lower in the presence of R3 than in its absence (Fig. 5E compared with Fig. 5C). The result suggests that TFIIIC binding does not exclude R3 binding to its sites, and the pre-bound TFIIIC cannot give full transcriptional activation in the presence of R3.

A mutation in box A was reported to cause the upstream initiation of transcription from the positions −2, −4, −8, and −10 of the SNR6 gene in vivo (14). Generation of the same upstream-initiated transcripts in the presence of R3 (lanes 11–16, Fig. 5D) suggests an impaired interaction of TFIIIC with box A due to steric hindrance by the R3 in its vicinity, which probably results in improper placement of TFIIIB at the TATA box upstream.

Quantifications of -fold activations against the corresponding naked DNA levels in Fig. 5F confirm that in the absence of TFIIIC, transcription remains repressed from the template with pre-positioned nucleosomes (lane 9, Fig. 5, D and E). The higher -fold activation for chromatin in the presence of R3 (Fig. 5F) may be due to the pre-positioned nucleosomes or the lower transcription levels obtained from the naked DNA in the presence of R3. Different levels of transcriptional inhibition by R3 on naked DNA and chromatin templates can be attributed to a possible change in the chromatin structure with TFIIIC binding adjacent to the lac operators without dislodging R3. High resolution footprinting was carried out to resolve the issue.

Binding of TFIIIC to Box B Moves a Pre-positioned Nucleosome Upward—Footprint analysis in Fig. 6 shows the outcome of TFIIIC binding to the chromatin with R3-positioned nucleosomes. R3 remains bound to its site after TFIIIC binds to the adjacent boxes A and B (Fig. 6A) both in the chromatin (lanes 20–22) and naked DNA (lanes 8–10). Alignment of profiles (Fig. 6B) of the chromatin lanes from panel 6A reveals that the R3-directed nucleosome 0 gets positioned from bp +75 to +215 (red ellipse) without blocking access of box B (5’-end at bp +217) to TFIIIC. This nucleosome shifts ~40 bp upward, to bp +35 to +175 (blue ellipse) after TFIIIC binding, resulting in protection of bps +72 and +195 and generation of hypersensitivity 5’ to box B. The new position is similar to that observed for the TFIIIC-directed positioned nucleosome (~40 to +180, not shown) on the pU6LNS, as suggested by IEL analysis in Fig. 4C.

Footprint analysis in the upstream region of the gene (Fig. 6C) shows the R3-positioned nucleosome −1, protecting base pairs +10 to −125 (compare lanes 16–18 to lanes 12–14; red ellipse). The protection due to this R3-directed nucleosome −1 overlaps the protection seen in Fig. 3C due to TFIIIC binding to the chromatin (−25 to −110). However, when TFIIIC binds this chromatin (lanes 20–22), the regions from bp +29 to −22 between box A and the TATA box as well as −100 to −140,
on both the sides of the pre-positioned nucleosome −1 due to R3, show a hypersensitivity to MNase. The 3’ hypersensitive region that runs ~15 bp into the nucleosome is the same as that seen in Fig. 3, B–C, suggesting that binding of TFIIIC to box A probably destabilizes this nucleosome. The profiles of chromatin digests in the presence of R3 without and with TFIIIC (Fig. 6D) overlap perfectly except in the hypersensitive regions, confirming that TFIIIC binding does not result in either loss or a change in the position of the pre-positioned nucleosome −1. The matched profiles in this panel also show protection close to box A (blue box) due to TFIIIC, which was difficult to see in other analyses. Thus, subsequent binding of TFIIIB to the SNR6 TATA box probably results in encroachment of the downstream edge of a nucleosome and pushes it further upstream, as indicated by the far upstream protection in lanes 4, 5, and 7 of Fig. 4, C and D.

**ATP Is Required for Upward Shift of the Positioned Nucleosome after TFIIIC Binding**—The structural analyses in Fig. 6 have shown that R3 does not dissociate from its site after TFIIIC addition, and the observed shift of the nucleosome by ~30–40 bp takes place only after TFIIIC binding. This shift could be a result of nucleosome sliding due to mechanical push by TFIIIC, independent of the ATP-dependent chromatin remodelers or due to an active remodeling by these complexes known to be present in the S-190 extract. We have previously reported the positioning of a nucleosome between boxes A and B after TFIIIC binding to wild type U6 snRNA chromatin in vitro to be ATP-dependent (15). Using a pre-positioned nucleosome, we could now see that the chromatin remodeling follows the TFIIIC binding and results in the re-arrangement of the nucleosomes. The experimental scheme depicted in Fig. 7A allows abolition of the ATP-dependent remodeling activities of S-190 before TFIIIC binding. In the absence of ATP, although TFIIIC can bind box B (lanes 22–24, blue bar; Fig. 7A; profiles in Fig. 7, B–D, protection at the bp +195), the R3positioned nucleosome does not shift upward. The addition of apyrase at the end of the chromatin assembly allows R3-positioned nucleosome to stay in its place (cf. lanes 13–15 and 19–21; Ref. 16). However, in the presence of...
ATP and TFIIIC this nucleosome shifts upward, showing changes in its boundaries. Therefore, overlapping regions of the nucleosomal protections due to both proteins do not show any change, but the intense peaks at the bps +72 and +195 in the presence of R3 (lanes 13–15 and 19–21 in panel A and red profiles in panels B–D) remain unprotected in the absence of ATP when TFIIIC binds to box B (green profiles, panels C and D). Unlike the difference in profiles in the absence of apyrase (red and blue profiles, Fig. 7D), the profile of chromatin with R3 and TFIIIC in the presence of apyrase (green profile) overlaps that of chromatin with R3 (red profile), except at box B and the region around it (Fig. 7, C and D), where TFIIIC binds. Thus, on the 3' side, the hypersensitivity 5' to box B, which denotes the boundary of the TFIIIC-directed positioned

FIGURE 6. Binding of TFIIIC to the pU6LNS chromatin with pre-positioned nucleosomes. TFIIIC related protections are shown in blue, whereas R3-related protections are shown in red. Rectangles mark protection at the binding sites, whereas ellipses mark the nucleosomal protections. A, high resolution MNase footprinting between boxes A and B. MNase digestions were done as described for Fig. 3. Primer extension products of MNase digest using α-32P-end-labeled primer located 161 bp downstream of box B were resolved on a sequencing gel. GAT shows sequencing lanes, 1–11 show naked DNA products, 12–23 show chromatin samples. Positions of box B and the lac operator next to it are marked with vertical, black bars, whereas the black dot indicates the position of box A immediately upstream to the upper lac operator. Red rectangles and the ellipse show protection due to R3 bound on both the operators (in lanes 4–10 and 16–22) and positioned nucleosomes due to R3 binding (lanes 16–18). Blue rectangles and the ellipse show the protection due to TFIIIC at boxes A and B (lanes 8–10 and 20–22) and the positioned nucleosome between them (lanes 20–22). B, profile comparison of MNase digestion products from lanes 14, 18, and 22 of the gel in panel A. Red and blue boxes/ellipses mark the protections due to R3 and TFIIIC, respectively. Numbers mark the peaks corresponding to bp positions cut by MNase. C, high resolution MNase footprinting upstream to box A. MNase digestions were as described for the panel A and primer hybridized to the top strand of the U6 gene with its 5' end at the bp +78. The red bar shows R3 protection on the lac operator in lanes 4–10 (naked DNA) and 16–22 (chromatin), whereas the ellipse shows the positioned nucleosome –1 upstream of box A in lanes 16–18. The vertical black bars mark the hypersensitive regions in lanes 20–22. D, profile comparison (lanes 12, 16, and 20 from the panel C) shows the hypersensitivity on both the ends (black bars) of the positioned nucleosome (red ellipse). Hypersensitivity at the 3' side spans from the bp –22 to +29. The blue box marks the additional protection seen around box A when TFIIIC binds, whereas the red bar marks the protection due to R3.
nucleosome when TFIIIC binds (lanes 20–22, Fig. 6A, lanes 16–18, Fig. 7A), is not generated. It is evident that in the absence of ATP, no upward shift of the pre-positioned nucleosome takes place.

Footprinting analysis in Figs. 6–7 shows that a pre-positioned nucleosome shifts 30–40 bp upstream due to TFIIIC binding in an ATP-dependent manner. However, it is possible that the presence of R3 at its site immediately downstream of box A acts as a physical barrier for the upward shift of nucleosome in the absence of ATP-dependent remodeling. The experiment in Fig. 8 was done to check whether the removal of R3 can allow an ATP-independent sliding of the nucleosome due to TFIIIC binding. According to the experimental schemes given in Fig. 8, A and B, isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added either before or after TFIIIC addition (but after apyrase addition) to dislodge R3 from its site (16). Boundaries of the R3-directed positioned nucleosome between the two lac operators (bp +36 to +180) were better defined in the absence of ATP (lanes 13–15 in Fig. 8, A and B, and the profile, Fig. 8C, upper panel). A nucleosome was found positioned between boxes A and B in the presence of TFIIIC (lanes 10–12, both gels; blue ellipse, profiles in Fig. 8, A and B) in the same place (bp +36 to +180) as that found in Fig. 6B. With TFIIIC addition, the same

FIGURE 7. ATP dependence of TFIIIC-directed nucleosomal re-positioning. The scheme of the experiment is shown at the top. Positions of box B, lac operators, R3 protections (red boxes), and TFIIIC protections (blue boxes) are indicated. R3-dependent nucleosomal protections (lanes 13–15 and lanes 19–24) are shown as a red ellipse, whereas TFIIIC-directed nucleosome (lanes 16–18) is marked with a blue ellipse. A, the high resolution MNase footprinting is as described for Fig. 6A. The primer hybridized to the top strand of the U6 gene with its 5’-end at bp +288. B, the profiles of lanes 11, 14, and 17 from the gel in panel A reveal the bases are protected due to the positioned nucleosome in the presence of R3 or R3 with TFIIIC on proper alignment. C, alignment of the profiles of lanes 11, 20, and 23 shows a nucleosomal size protection with perfect overlap for lanes 20 and 23 (red and green) after apyrase treatment, with both TFIIIC and R3 still bound (blue and red bars) to their target sites. D, comparison of the profile of lanes 17, 14, and 23 shows that after the apyrase treatment, the profile of lane 23 (green) in the region upstream to box B matches that of lane 14 (red) rather than lane 17 (blue). However, around box B, blue and green profiles match, as both TFIIIC and R3 are still bound (blue and red bars) to their target sites.

Chromatin Remodeling on Yeast SNR6

10468 JOURNAL OF BIOLOGICAL CHEMISTRY

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FIGURE 8. ATP is required for TFIIIC-directed chromatin remodeling even when R3 is dislodged from its site either before or after TFIIIC binding. Comparisons of only chromatin samples are shown. Blue rectangles mark the protections on box B, whereas ellipses mark positioned nucleosomes. Excess isopropyl 1-thio-β-D-galactopyranoside (IPTG) addition was made to all the lanes that had R3, resulting in loss of protection at both the lac operators. A, experiment was carried out according to the scheme shown. The primer used for this high resolution MNase footprinting was the same as that in Fig. 6A. Profile comparison in the left-hand side panel (lanes 8 and 11) shows the TFIIIC-directed nucleosome after R3 is removed, whereas the overlapping profiles in right-hand side panel (lanes 14 and 17) confirm that after apyrase treatment R3-directed nucleosome stays in its place even after R3 is removed and TFIIIC is bound. B, chromatin assemblies and additions of the proteins were made according to the scheme shown. The primer used for the footprinting was the same as that in the gel of Fig. 7. The profile alignment (lanes 8–14) reveals the TFIIIC-dependent positioned nucleosome between boxes A and B in the presence of ATP when R3-directed nucleosome as well as R3 footprint could not be seen due to isopropyl 1-thio-β-D-galactopyranoside addition. C, profile comparisons of chromatin after apyrase treatment. Upper panel (lanes 8–14) shows the R3-dependent nucleosome even after R3 was removed. The lower panel (lanes 14 and 17) shows that the profile remains unchanged with additional protection at box B after TFIIIC addition.
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Chromatin remodeling is observed in the presence of ATP in both the gels (lanes 10–12 versus lanes 7–9). TFIIIC binding to the ATP-depleted chromatin after or before R3 removal does not result in change of the pre-positioned nucleosome (right profile in Fig. 8A and lower panel in Fig. 8C), although its downstream end was protected by TFIIIC bound to box B (lanes 16–18, gels in Fig. 8, A and B). These results show that R3 does not present any physical block to the TFIIIC-directed chromatin remodeling and that the shift of the pre-positioned nucleosome 0 is an important consequence of TFIIIC binding to the chromatin.

Requirement of ATP-dependent Chromatin Remodeling for Full Transcriptional Activation after TFIIIC Binding—Results from Figs. 6–8 show that binding of TFIIIC to box B in chromatin and ATP-dependent chromatin remodeling are two separable activities. We have shown previously that while TFIIIC binding can activate the chromatin transcription, ATP-dependent remodeling is required for full transcriptional activation of the SNR6 gene (15). Therefore, the transcription level from the intermediate state when TFIIIC is bound to the chromatin templates should increase to the full activation level after the pre-positioned nucleosome between boxes A and B is shifted to a precise location due to the TFIIIC-directed ATP-dependent remodeling. For checking the template activity of both the states, ATP was removed by passing the assembled chromatin through a gel-filtration column, and transcription was followed with or without ATP addition according to the scheme given in Fig. 9. Transcription shows absolute dependence on TFIIIC (lanes 5–8 compared with lanes 1–4) and inhibition in the presence of R3 (lanes 6 and 8 compared with 5 and 7). In the absence of R3, the binding of TFIIIC can activate transcription by 34-fold (lane 5), which could be increased to 51-fold (lane 7) after rephosphorylation ATP. However, transcriptional activation over a pre-positioned nucleosome after TFIIIC addition increases from 29-fold (lane 6) to only 36-fold (lane 8) by rephosphorylation ATP. In the presence of ATP and R3 bound to the template, chromatin transcription is inhibited, and the upstream initiated transcripts are produced (Fig. 5). The upstream-initiated transcripts seen in the presence of R3 (lanes 6 and 8, Fig. 9) suggest that R3 remains bound to the chromatin passed through the gel-filtration column. This may be the reason for less transcription recovery in lane 8 compared with lane 7.

We had previously reported the ATP dependence of transcriptional activation on the wild type gene (15). TFIIIC alone could help recover a substantial transcription level, whereas ATP could increase it to ~3-fold higher level. The contribution of ATP to the complete derepression of pU6LNS chromatin transcription by TFIIIC appears to be lower in Fig. 9. The modified gene in pU6LNS shows lower chromatin transcription and activation as compared with the wild type gene in pCS6, probably due to the absence of the gene terminator. It has an altered box A sequence that is reported to increase TFIIIC-dependent transcription in the crude extracts and from the nucleosomal templates (17). This could lead to significant recovery of transcription in lanes 5 and 7 after TFIIIC addition. The alignment of the gene terminator (absent in the pU6LNS) with boxes A and B after the TFIIIC-directed chromatin remodeling on the wild type gene enables the terminator-directed pol III recycling, which further increases the transcription (22). Therefore, on pU6LNS, which lacks the terminator, comparatively less of an increase in transcription was observed even after ATP repopulation, and the observed little increase may be due to the remodeling directed by TFIIIB. This result shows how the chromatin remodeling can contribute to the transcriptional activation of the wild type gene, subsequent to TFIIIC binding.

DISCUSSION

Cells require most of the transcripts generated by pol III in high amounts. For the cell to produce enough pol III transcripts to cater to its needs, the transcription initiation, elongation, termination, and recycling phases must be accurate and efficient. The required accuracy and efficiency can be achieved by co-operation between various factors that compose the pol III transcription machinery and their cognate binding sites in the DNA. Our studies on transcription of SNR6 chromatin show that binding of its basal factors TFIIIC and TFIIIB leads to precisely coordinated structural changes in the chromatin, which are essential for the high transcriptional activation of this single copy gene. Pol II-transcribed genes, in contrast, depend on the activators and factors other than the basal factors to achieve the same results.

Recruitment of Remodeling Activity by TFIIIB on the SNR6 Chromatin—The mechanistic details of the transcriptional activation from SNR6 chromatin in this study show that sequential and concerted remodeling on the gene region takes place after TFIIIC binding (Fig. 10). The chromatin remodeling due to binding of TFIIIC to boxes A and B involves positioning of a nucleosome between them and MNase hypersensitivity of the DNA upstream to box A (Fig. 10, B and C). The binding of TFIIIC in the next step can lead to further nucleosomal re-arrangements upstream of the TATA box and give a template that represents the activated chromatin structure ready for transcription (Fig. 10D). Results from the pU6LNS chromatin with R3 (Fig. 10B) that resembles the in vivo structure of the U6 chromatin (21) better, are more revealing. The TFIIIC-directed re-positioning of nucleosome 0 may remove steric interference of R3 to TFIIIC binding, making transcription possible. The inability of TFIIIC alone to shift the −1 nucleosome over the TATA box of pU6LNS in the presence of R3 (its both ends show MNase hypersensitivity upon TFIIIC binding, Fig. 10, B and C) sug-
gests that the remodeling activity recruited by TFIIIC can mobilize only nucleosome 0 in the gene region and prepare nucleosome −1 for further changes in the structure upstream. Evidently, these changes need recruitment of another remodeler, probably by TFIIIB.

In a global analysis (23), tRNA genes in yeast were identified as the targets of the chromatin remodeler, Isw2. The Bdp1 subunit of TFIIIB was shown to be involved in the recruitment of the Isw2 as well as integration of the Ty1 transposon upstream to the yeast transfer DNA (24). Although this may not be true for the SNR6 gene and none of the templates used in this study had the upstream solo δ elements (remnants of Ty1) of SNR6 found in vivo, the possibility of recruitment of an Isw2 remodeler due to TFIIIB cannot be ruled out with the present data. According to several reports, transcription factors can access their target sites in the nucleosomes by various mechanisms ranging from spontaneous access to ATP-dependent chromatin remodeling (25–28). Recently, the mechanism of the ATP-dependent chromatin remodeling by yeast RSC is shown to involve a directional translocation of the nucleosomal DNA pulling ~20-bp of DNA from one side and releasing it on the other end (29). The observed ~40-bp shift with simultaneous exposure of ~40-bp upstream DNA in this study suggests that TFIIIC recruits a remodeler with a similar mechanism of action from the S-190 extract. Further studies to identify the remodeler from the extract as well as yeast cells are required to confirm the complete mechanism of transcriptional activation of SNR6 chromatin.

**Positioned Nucleosomes in the Transcriptional Activation of the Yeast SNR6**—We have shown that chromatin remodeling of the template with pre-positioned nucleosomes is essential for its transcription. It is possible that a positioned nucleosome is present on the gene in vivo. This nucleosome shifts upward when TFIIIC binds, allowing readjustments in the upstream region. The possibility of recruitment of two remodeling complexes by the two factors of the gene looks promising. This would account for the high rate of chromatin remodeling and a very high transcription rate (15) as both processes on the gene are found closely associated. However, during transcription, with the rapid shuffling of nucleosomes on the gene from the TATA box to box B, the nucleosome between boxes A and B does not get lost and does not generate a completely naked DNA region. This may be the reason only a sub-nucleosomal size (~100 bp) protection of the gene between A and B boxes was observed in vivo independent of TFIIIB or -C binding (14). Similarly, the positioning of nucleosomes in the flanking gene regions observed only after the binding of TFIIIC or/and TFIIIB (Fig. 3, D and E) explains why the chromatin structure with positioned nucleosomes in the flanking region of the gene in vivo depends on the expression of the gene (21).

Several examples of yeast genes are known that use nucleosome positioning as an effective mechanism of regulating their expression (30–33). Yeast genes transcribed by pol II are reported to have positioned nucleosomes flanking the ~200-bp nucleosome-free regions upstream of their start codons (34). Low nucleosome density at the yeast promoters is also found as a common feature in the genome-wide analysis (35). We have shown here that for a small, pol III-transcribed gene like SNR6, the precise positioning of the nucleosomes on the whole gene and its flanking regions is essential for its active transcription.

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Chromatin Remodeling on Yeast SNR6

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