Chapter III

HIGH LEVEL TRANSCRIPTIONAL ACTIVATION OF SNR6 GENE IN CHROMATIN BY TFIIC
3.1 Overview

The SNR6 gene is a single copy gene in the yeast *S. cerevisiae* and is essential for the cell survival (Brow and Guthrie, 1988). Because of its presence in the spliceosomal complex and its role in pre-mRNA processing, there is a high demand for the U6 snRNA gene transcript for the cell growth and survival. For this requirement to be met, the rate of transcription on this single copy gene has to be very high.

Transcription of this gene as naked DNA template is TFIIIC independent, as the TATA box situated upstream of the start site can recruit TFIIIB on its own. But inside the cell, DNA is packaged into chromatin. It was observed that the transcription *in vivo* and in crude *Xenopus* oocyte extracts *in vitro* was dependent on the presence of box B and TFIIIC (Burnol et al., 1993). Therefore, it was postulated that the requirement for TFIIIC could be related to chromatin repression rather than to transcription complex assembly. TFIIIC probably helps other transcription factors gain access to the DNA in chromatin, but how TFIIIC might fulfill this function has not been reported till now. This chapter describes the experiments and results that address the first question asked in Section 1.7 of the first chapter.

In order to elucidate the possible role of TFIIIC in de-repression of chromatin transcription, we decided to use S-190 assembly extract from *Drosophila* embryos to assemble nucleosomes over plasmid template containing the SNR6 gene to mimic the *in vivo* situation. We then looked at the ability of the pol III transcription apparatus (reconstituted from purified proteins) to transcribe such templates in the absence and the presence of TFIIIC. This was done to check for the involvement of TFIIIC in de-repression of transcription on chromatin templates in our system.

In order to elucidate whether transcriptional activation was accompanied by a change in the nucleosomal organization in the SNR6 gene region, various techniques were used. Perturbation in the MNase digestion pattern over a large area of DNA was checked to look for chromatin remodeling following TFIIIC binding.

Low and high-resolution footprinting analyses were used as tools to look for structural alteration in the gene region. Indirect end labeling (IEL) is a classical method used to locate positioned nucleosome (Wu, C., 1980) and has been used in my study too. High resolution DNase I footprinting was done to check the ability of TFIIIC to bind to box B on both naked DNA and chromatin templates. Micrococcal
nuclease (MNase) footprinting was used to map the exact position of the nucleosome in and around the gene region at a single base pair resolution.

Based on the results from the above experiments, we have presented a model for the structural alteration of a nucleosome core in the gene region upon TFIIC binding. And, since TFIIC is absolutely required for transcription of SNR6 gene as chromatin, this structural alteration is proposed to have a role in transcriptional activation over chromatin templates, as evidenced in later parts of this thesis.

3.2 TFIIC activates U6 transcription in a chromatin dependent manner

Transcription over pCS6 template was carried out as described under Section 2.9 of Chapter II. Two schemes were followed. Both the schemes were similar except for the time of addition of pol III to the template. Rationale for the differences and consequent results are described in the following sections.

3.2.1 Multiple round specific in vitro transcription on chromatin templates

In order to look at the role of TFIIC on transcription of chromatin templates, transcription was carried out on templates assembled using S-190 extract in the absence or presence of TFIIC. Transcription was carried out according to the scheme in Fig. 3.1A. Our results showed that over naked DNA templates, TFIIC is neither required for U6 transcription with highly purified components nor does it activate transcription (Fig. 3.1B compare lanes 1 and 2). Reconstitution of pCS6 plasmid into chromatin for a period of 4.5 h prior to addition of TFIIB abolished pol III transcription (Fig. 3.1B, lane 3) but transcription was restored when 2-fold molar excess of TFIIC was added along with TFIIB on such templates (lane 4). The transcription was activated about 50-fold over the repressed chromatin level. Interestingly, when TFIIC was added at the start of assembly (lane 5) or at the end of assembly (lane 7) (in 2.5 fold excess), the activation due to TFIIC was about 100-150 fold above repressed chromatin (Fig. 3.1C). Addition of extra TFIIC along with TFIIB did not increase transcription further (Fig. 3.1B compare lanes 6 and 8 with 5 and 7) probably because box B was already saturated with TFIIC, or because TFIIB and pol III were limiting. These results show that TFIIC can stimulate transcription on SNR6 gene template in a chromatin dependent manner. Our result is similar to the
Fig 3.1: Transcription of pCS6 using purified pol III proteins when RNA polymerase III was added along with TFIIIC and TFIIIB:

(A) Scheme of the transcription experiment. TFIIIC was added at a 2.5-fold molar excess at the start and the end of the assembly. TFIIIC in italics shows the addition of 2-fold molar excess of TFIIIC during transcription.

(B) Representative gel showing the primer extension product of the transcript and the recovery marker. The 5' end of the 32P end labeled primer binds to 98th nucleotide from the 5' end of the transcript.

(C) Fold activation due to TFIIIC on chromatin templates calculated against repressed chromatin. Standard deviation from three independent experiments are specified.
previously reported observations by Burnol et al. (1993) in that, chromatin greatly represses TFIIIB-directed naked U6 DNA transcription, and that TFIIIC can partially reverse this repression. However, in the experiments by Burnol et al., the activation by TFIIIC on chromatin templates assembled either using Xenopus egg extracts or by salt dialysis method did not exceed that of the naked DNA templates. To rule out the possibility that some other component of S-190 was bringing about higher activation of transcription on SNR6 template as chromatin, we carried out a few control transcription experiments, as described below.

3.2.2 Control transcription experiments

Although Drosophila S-190 extract may contain some TFIIIB, TFIIIC and pol III, transcription of the SNR6 gene was completely dependent on exogenous yeast proteins (Fig. 3.2A). Transcription was done on naked DNA templates in the absence of pol III (lane 4) or in the absence of both TFIIIC and pol III (lane 3). When compared with control samples that had pol III (lanes 1 and 2), we could see that the transcription was completely dependent on the presence of the yeast transcription factors and pol III.

S-190 extract is the soluble fraction of cells and it also has some endogenous histones. Therefore, we have tested for the presence of NTPs in our S-190 preparation, and the effect of the endogenous histones on chromatin transcription (Fig. 3.2B). This was done either by carrying out assembly in the absence of exogenous histones (lane 1) or conducting transcription by omitting the addition of rNTPs in the transcription mix (lane 4). Control reaction in lane 2 (chromatin assembled in the presence of exogenous histones and transcription carried out by supplementation of rNTPs) gave ~47-fold activation whereas that carried out in the absence of histones and rNTPs gave ~10-fold and ~12-fold activation respectively, over the repressed chromatin, only in the presence of TFIIIC. Therefore, on chromatin templates, when RNA polymerase III is added along with TFIIIB and TFIIIC at the start of 40 min incubation for the TFIIIC-TFIIIB-DNA complex formation, a low
**Fig 3.2: Control transcription experiments:**

(A) 50 fmols of naked DNA was incubated in the presence of TFIIIB with (lanes 2 and 4) or without (lanes 1 and 3) TFIIIC. 100μg of S-190 proteins were added to lanes 3 and 4 to check for TFIIIC and pol III activity, and pol III was added to lanes 1 and 2 as marked above the gel. The 98nt primer extension product and the recovery marker have been marked.

(B) The amounts of DNA and transcription proteins were as described in A. In the chromatin lanes, TFIIIC was added during transcription in a 2 fold molar excess.

(C) 5 fmols of pol III was added either along with TFIIIB and TFIIIC at the start of the 40 min incubation for the pre-initiation complex formation, or at the end of the 40 min incubation along with the rNTP mix.
were compared on naked DNA and chromatin templates (Fig. 3.2C), we found that for reactions in which pol III was added along with TFIIB (lanes 2 and 4), there was a 1.5 fold increase in the level of transcription on naked DNA templates and a 2.1 fold increase in transcription on the chromatin templates. The higher value (2.1 Vs 1.5) in the chromatin templates might be due to the endogenous rNTPs present in S-190.

Therefore, we modified the scheme of our transcription experiment described in Section 3.2.1 to the one described below and the following transcriptions were used to calculate more accurate levels of activation.

3.2.3 A modified multiple round specific in vitro transcription

In the modified scheme (Fig. 3.3A), transcription conditions were identical to that in Fig 3.1A except that RNA polymerase III (5 fmol) was added along with 500 µM rNTPs at the end of TFIIB-TFIIC-DNA complex formation. This was done to ensure that no transcription happens on chromatin templates during the incubation for pre-initiation complex assembly, because of the rNTPs that might be present in the S-190 extract. The representative gel (Fig. 3.3B) shows that there was a 43-fold activation of transcription due to TFIIC on chromatin templates (lane 4 vs. lane 1) and 90 to 100-fold activation when TFIIC was added at the onset of assembly (lanes 5 and 6) or at the end of assembly (lanes 7 and 8). From the quantitations shown in Fig. 3.3C, we can see that transcription of chromatin in the presence of TFIIC exceeded that of naked DNA (4.5-fold, compare lanes 3 and 4). Addition of extra TFIIC either before or at the end of 4.5 h assembly generated an additional approximately two-fold increase in transcription over that obtained when TFIIB and TFIIC were added together. Since the residual transcription in lane 1 is barely distinguishable from the gel background, we also calculated the fold activation relative to naked DNA. The results suggested that TFIIC and the chromatin structure together resulted in an activation of approximately 5 to 15-fold over that of naked U6 template (data from three individual experiments are summarized in Fig. 3.3C), and the activation of transcription mediated by TFIIC relative to chromatin repressed TFIIB only reaction was found to be at least 50-100 fold.
Fig 3.3: Transcription of pCS6 using purified pol III proteins when RNA polymerase III was added along with rNTPs:

(A) Scheme of the transcription experiment. TFIIIC was added at a 2.5-fold molar excess at the start, the end of the assembly and during transcription along with TFIIIB.

(B) Representative gel showing the 98nt primer extension product.

(C) Fold activation due to TFIIIC on chromatin templates calculated against naked DNA in the without TFIIIC.
3.2.4 Conclusions

The restoration of transcription of the U6 gene in chromatin by TFIIIC addition suggests a TFIIIC-dependent alteration of chromatin structure that allows accessibility for both TFIIIC and TFIIIB. Alternatively, one can conclude that a TFIIIB-dependent rearrangement of chromatin structure may occur, that is insufficient for pol III binding and transcription but is sufficient for TFIIIC binding, which, in turn allows pol III access. From our results we see that, with the addition of TFIIIC prior to TFIIIB at 4.5 hrs of assembly, or at the outset of nucleosome deposition, there is a further increase in transcription (compare lanes 5-8 with 4 in Fig.3.3B). Therefore, the above transcription experiments suggest that TFIIIC is capable of binding to its high-affinity box B binding site in the context of dynamic chromatin, and in turn alters the chromatin structure so as to promote initiation complex assembly. This leads us directly to the second question; can TFIIIC access its sites in chromatin and does it cause some structural rearrangements in and around its binding sites.

3.3 Structural analysis of the chromatin template in the presence of TFIIIC

3.3.1 TFIIIC can bind box B over chromatin templates

Results from our transcription experiments suggested that TFIIIC could bind to box B site in the context of chromatin. Therefore, we examined the ability of TFIIIC to bind box B site in chromatin assembled on pCS6 plasmid by DNase I footprinting (Fig. 3.4 A and B). The amount of TFIIIC used in this experiment was a 10-fold molar excess over the DNA amounts (in saturating levels). The DNase I digested fragments from naked DNA and chromatin in the absence or in the presence of TFIIIC were probed using primers located upstream to TATA box (Fig. 3.4A, probe A), and downstream to box B (Fig. 3.4B, probe B). The gel in Fig. 3.4 B shows that TFIIIC added to U6 template assembled into chromatin results in a substantial protection (lanes 12-14 vs. lanes 9-11) comparable to that over naked DNA (lanes 5-8 vs. lanes 1-4) indicating that TFIIIC can bind to box B in chromatin. As box A is not a high affinity binding site for TFIIIC, as expected, the region did not show protection due to TFIIIC (Fig. 3.4A) both on naked DNA and chromatin.
Fig 3.4: DNase I footprinting of pCS6 as naked DNA and chromatin:

125ng of naked DNA and chromatin were incubated with a 10-fold molar excess of TFIIIC followed by digestion with 0.42-2.5 ng of DNase1 in case of naked DNA and 0.625-1.25 μg of DNase1 in the case of chromatin. All digestions were carried out for 1 min at room temperature in the presence of 3mM CaCl$_2$.

(A) The 5'end of the primer extension probe was 170nt away from +1, complementary to the bottom strand.

(B) The 5'end of the primer extension probe was 135bp away from the 3' end of box B, complementary to the top strand (non-transcribed strand). Boxes A and B and TATA box are marked at the side of the gel.
3.3.2 Gross structural analysis of chromatin by MNase digestion: TFIIIC does not disrupt the periodicity of nucleosomal array

Binding of many proteins to their DNA binding sites over chromatin is known to disrupt the periodicity of the nucleosomal array. Therefore, MNase cleavage pattern of assembled chromatin in the presence or absence of TFIIIC was inspected. 250 ng of DNA was assembled into chromatin under conditions described in Section 2.5 in Chapter II with or without the addition of 2.5-fold molar excess of TFIIIC at the start of assembly or at the end of a 4.5 hr assembly. Fig. 3.5 shows that addition of TFIIIC either before or after chromatin assembly did not disrupt the periodicity of nucleosomal arrays. Fig. 3.5A shows the blot probed with promoter proximal probe to look at the region close to the U6 gene and Fig. 3.5B shows the same blot probed with a primer located distal to the gene (894 bp upstream to TATA box). The results indicated that binding of TFIIIC to box B does not disrupt nucleosome periodicity at a gross structural level.

3.3.3 Indirect end labeling analysis of the chromatin structure on the U6 snRNA gene

IEL of DNA displays protection from double stranded MNase cleavage that can be interpreted to show changes in the positions of several nucleosomes at the same time. Fig. 3.6A shows a representative gel of one such experiment. Upon TFIIIC binding to naked DNA, except for the slight protection seen in the box B region due to TFIIIC binding (lane 2 vs. lane 1; Fig. 3.6B), no change in the MNase digestion pattern was seen. The digestion pattern of chromatin in the absence of TFIIIC was similar to that of naked DNA in and around the gene region (compare lanes 1 and 3) indicating that there is no preference for positioning of nucleosomes on this plasmid template. Interestingly, in the presence of TFIIIC added either 30 min before the start of assembly (lane 4), at the start of assembly along with histones (lane 5), or near the end of assembly (lane 6), a protected segment between box A and box B was seen. This is marked as a gray ellipse on the right of the gel and in the aligned profiles of lane 3 vs. lanes 4, 5 and 6 (Fig. 3.6C). Molecular weight marker shown in the gel was used to calibrate and estimate the MNase cut positions on the gene (Fig. 3.7). The protection generated by TFIIIC was found to be ~180 bp long (between ~+20 and ~+200) and
Fig 3.5: Effect of TFIIIC on the periodicity of nucleosomal array:

250ng of chromatin assembled in the absence or presence of 2.5-fold molar excess of TFIIIC added at the start or at of 4.5 hrs of assembly was digested with 0.1U to 0.004U of MNase. The fragments were electrophoretically resolved, blotted and probed either with primer proximal to the gene (53bp upstream to the TATA box) or gene distal probe that hybridizes 894bp upstream to TATA box as shown schematically at the bottom of the panel.
Fig 3.6: IEL analysis of the chromatin structure on the U6 snRNA gene:

(A) 125ng of naked DNA and chromatin samples in the presence or absence of 10-fold molar excess of TFIIIC were digested with MNase and probed with a primer that hybridizes 894bp upstream of the TATA box. Positions of boxes A and B are marked on the left. M represents the DNA marker generated from the same plasmid and used to calibrate the sizes of the bands on the blot.

(B) Aligned profiles of naked DNA (lane1) and naked DNA+ TFIIIC (lane2).

(C) Aligned profiles of chromatin (lane3) with that of lanes 4, 5, and 6. The gray ellipse marks the nucleosomal protection between boxes A and B.
Fig 3.7: Summary of the MNase cuts before and after TFIIIC-induced chromatin positioning over the SNR6 gene:

The numbers indicated are with reference to the transcription start sites. Black arrows represent the MNase cut sites which are present both in the absence and the presence of TFIIIC. Gray arrows represent the MNase cut sites on chromatin that get protected in the presence of TFIIIC. The protection generated due to TFIIIC is ~180 bp (between ~+20 and ~+200).
indicated that it could be due to a positioned nucleosome between boxes A and B. A weaker protection was also seen upstream to box A, but further analysis could not confirm the presence of a nucleosome in that region. Thus, from the IEL experiments, we can conclude that, irrespective of the time of addition of TFIIC (pre-bound to DNA templates, added at the start of assembly or near the end of assembly), the chromatin structure in the gene region gets altered to position a nucleosome on the SNR6 gene.

3.3.4 High resolution analysis of the TFIIC dependent chromatin remodeling

Based on the IEL analysis, we could conclude that when TFIIC was bound to chromatin templates, a positioned nucleosome was present in the gene region between boxes A and B. Since the resolution of IEL blots is not good enough to map the exact position of the nucleosome; MNase footprinting was done to see the protection at a single base resolution and was used to define the exact boundaries of the nucleosome. Fig. 3.8A shows a gel in which four sets of MNase titrations were used to compare the partial digestion products of naked DNA and chromatin in the absence and the presence of TFIIC. The primer used for the extension of digested samples in Fig. 3.8A was located 31 bp downstream of the 3' end of box B. The TFIIC footprint on box B sequence in both naked DNA (lanes 5-8 vs. lanes 1-4) and in chromatin (lanes 13-16 vs. lanes 9-12) was readily apparent on the pCS6 DNA digested with MNase.

Comparison of MNase digestion profiles of chromatin and naked DNA (Fig. 3.8B) shows chromatin-mediated protection upstream to box B (bp +178 to +219) and hypersensitivity to MNase cleavage (+157 and +227). Comparison of the partial digestion products of chromatin in the absence (lanes 9-12) and presence of TFIIC (lanes 13 to 16) showed a ~140 bp protection between ~ bp +50 and +190 (depicted as gray ellipse in Fig. 3.8C). This ~140 bp protection corresponds to approximately one nucleosome core DNA size. The difference in the size of protection due to the nucleosome between IEL and high-resolution footprinting (180 bp vs. 140 bp) is due to the requirement of double stranded cleavage in the former case and single stranded cleavage in the latter case. Interestingly, from the above analysis we find that the transcriptional terminator (bp 109 to 118) is placed near the dyad of the nucleosome, which is at bp +120.
Fig 3.8: Analysis of chromatin structure by higher-resolution MNase footprinting of the gene region:

(A) Gel showing MNase digestion pattern of naked DNA and chromatin in the presence and absence of 11 fold molar excess of TFIIIC. The amount of MNase used was $1.5 \times 10^{-4}$ to $9.3 \times 10^{-4}$ U for the naked DNA digestions and $7.4 \times 10^{-3}$ to $6.7 \times 10^{-2}$ U for chromatin digestions. The primer used for extension anneals 31bp downstream of box B.

(B) Aligned profiles of naked DNA (lane 2) and chromatin (lane 10). The numbers represent the positions of the bases with respect to the start site.

(C) Aligned profiles of chromatin without (lane 9) and with TFIIIC (lane 14). Gray box denotes the protection due to TFIIIC and the gray oval denotes protection due to a positioned nucleosome.
Since the primer used for extension in Fig. 3.8A was located very close to box B we used the same MNase digested samples for primer extension using a primer located 135 bp downstream of box B to see box B protection due to TFIIIC (Fig. 3.9). Fig. 3.9A shows that the protection of box B due to TFIIIC binding on naked DNA extended from +199 to +267, and on chromatin from +219 to +260 (as shown in Fig. 3.9 B and C). This gel also confirmed the protection due to a nucleosome between +50 and +190.

We also explored whether TFIIIC generated a positioned nucleosome downstream of box B and upstream of TATA box. Fig. 3.10 shows a gel in which the primer extension probe anneals ~200 bp downstream of box B. The footprint of TFIIIC at box B and the TFIIIC dependent nucleosomal protection between boxes A and B were seen again but no positioned nucleosome could be seen downstream of box B (Fig 3.10A, compare lanes 9-11 with 13 to 15, and Fig. 3.10 B). The asterisks in Fig. 3.9A and Fig. 3.10A denote hypersensitive sites flanking box B due to TFIIIC binding to box B in chromatin.

Fig. 3.10C is a gel showing regions upstream to TATA box. The primer used for extension anneals ~230 bp upstream of start site and probes the bottom strand. From the gel, we can see that TFIIIC does not position a nucleosome upstream to the TATA box and the chromatin structure in this region does not change much in the absence (lanes 9-12) or presence (lanes 13 to 16) of TFIIIC.

Taken together, the high-resolution footprinting analyses showed that the TFIIIC positioned nucleosome lies between two sites of enhanced MNase cleavage (bp +47 and bp +192) giving an ~140 bp protection. The DNase I digestion pattern in this region does not display a 10 bp periodicity of nuclease accessibility, thus ruling out the possibility of a rotationally positioned nucleosome. Therefore, binding of TFIIIC results in a translationally positioned nucleosome between boxes A and B.

3.4 Discussion

Contrary to the general belief that the chromatin structure is repressive for transcription, it has been observed that the folding of DNA over a single positioned nucleosome or an array of positioned nucleosomes can activate certain genes by bringing regulatory elements that are widely separated closer together in space (Schild et al., 1993; Thomas and Elgin, 1988; Urmov and Wolfe, 2001; Zhao et al., 2001).
**Fig 3.9**: Analysis of chromatin structure by higher-resolution MNase footprinting using a downstream primer:

(A) Gel showing MNase digestion pattern of naked DNA and chromatin in the presence and absence of 11 fold molar excess of TFIIIC. The primer used for extension anneals 135 bp downstream of box B. The asterisk denotes the hypersensitivity on either side of the box B footprint by TFIIIC.

(B) Aligned profiles of naked DNA in the absence (lane2) and presence of TFIIIC (lane6). Gray box denotes the protection due to TFIIIC.

(C) Aligned profiles of chromatin without (lane9) and with TFIIIC (lane13). Gray box denotes the protection due to TFIIIC and the gray oval denotes protection due to a positioned nucleosome.
Fig 3.10: Structure of SNR6 gene downstream of box B and upstream of TATA box:

(A) Gel showing MNase digestion pattern of naked DNA and chromatin in the presence and absence of 11 fold molar excess of TFIIIC. The primer used for extension anneals 174bp downstream of box B. The asterisk denotes the hypersensitivity on either side of the box B footprint by TFIIIC.

(B) Aligned profiles of chromatin without (lane9) and with TFIIIC (lane13). Gray box denotes the protection due to TFIIIC and the gray oval denotes the positioned nucleosome.

(C) Gel showing region upstream to TATA box. The primer used for extension anneals ~230bp upstream of start site.
Even in the case of RNA polymerase III genes, a correlation between positioned nucleosome and transcription has been seen, for example, in the context of 5S rRNA genes (reviewed in White, R.J. 1998; Wolffe, A.P., 1999). A positioned nucleosome has also been observed between the distal (enhancer) and proximal sequence elements in the case of the human U6 snRNA gene (Stunkel et al., 1997). This positioned nucleosome mediates cooperative binding between Oct 1 and SNAPc by bringing their respective binding regions closer to each other in space (Zhao et al., 2001).

The results presented in this Chapter have shown that upon binding of TFIIIC to box B, a nucleosome gets positioned downstream of the transcriptional initiation site between boxes A and B. In the prototype pol III genes i.e. the tRNA genes, the distance between the box A and box B promoter elements is ~50 bp and this is optimal for the binding of the τA and τB domains of TFIIIC to their respective binding sites simultaneously and accommodate the ~10nm linker between the τA and τB domains of TFIIIC. In the case of the SNR6 gene, reducing the distance between the widely separated promoter elements resulted in diminished transcription in vivo and in crude transcription extracts; a deletion of 84-bp decreased transcription two-fold, and this corresponds to approximately one superhelical turn of DNA in the nucleosome core (Eschenlauer et. al., 1993). These results support the fact that the positioning of a nucleosome between boxes A and B as seen in our system is required for transcriptional activation of the SNR6 gene.

Fig. 3.11 represents a model proposed by us based on the results in this Chapter. The translational positioning of a nucleosome due to TFIIIC between bp ~+50 to ~+190 results in alignment of the transcriptional terminator along with box A and box B in a linear fashion at the dyad axis of the nucleosome. Since the terminator is a stretch of 10-bp T:A residues, it is relatively stiff and occupies the unbent stretches of DNA on the nucleosome core particle. This alignment of the terminator and promoter elements may be required for stabilization of the TFIIIC-DNA complex. Whether the presence of terminator in that position has any role in determining the exact position of the nucleosome between the two boxes in the presence of TFIIIC is an interesting question and has been addressed in the coming chapters. Terminator has also been implicated in recycling of transcription factors that results in increased transcription rates.
Fig 3.11: A model for TFIIC dependent nucleosome positioning:

Binding of TFIIC to box B results in the positioning of a nucleosome between the boxes A and B, thus reducing the gap between the two boxes.
In our experiments, we could not see a protection on box A due to TFIIIC because of the reduced susceptibility of the box A DNA sequence to MNase cuts. But, indications that TFIIIC does interact with box A in chromatin is seen by the levels of transcription well above that of the naked DNA, since it is the TFIIIC binding to box A element that specifies the placement of TFIIIB at the TATA box. TFIIIC dependent positioning of nucleosome probably makes the region upstream to the start site (including the TATA box) more accessible to TFIIIB. As TFIIIB binding is a pre-requisite for the binding of RNA polymerase III, the positioning of nucleosome by TFIIIC might be the first step in transcriptional activation over this gene. Experiments have been done to address this question further in the subsequent chapters.

*In vivo* chromatin footprinting of this gene has either shown a sub-nucleosomal size zone of protection between the terminator and box B (Gerlach et al., 1995) or could not detect the presence of a nucleosome between boxes A and B due to decreased susceptibility to MNase cleavage in this region (Marsolier et al., 1995). Gerlach and coworkers reported a region of continuous protection from MNase that was ~90 to 100bp in length rather than the 146bp expected for a nucleosome. This could be explained by partial displacement or disruption of the nucleosome in the U6 coding region during the process of transcription. Our results suggest the presence of a nucleosome in the intragenic region of pCS6 (from ~bp +50 to +190); and this region overlaps with the reported *in vivo* protection (bp +94 to +198) on the gene. Therefore, the positioning of a nucleosome in a TFIIIC - dependent manner may be the first step in the transcription complex assembly.