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A Nucleosome Positioned in the Distal Promoter Region Activates Transcription of the Human U6 Gene

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To investigate the consequences of chromatin reconstitution for transcription of the human U6 gene, we assembled nucleosomes on both plasmids and linear DNA fragments containing the U6 gene. Initial experiments with DNA fragments revealed that U6 sequences located between the distal sequence element (DSE) and the proximal sequence element (PSE) lead to the positioning of a nucleosome partially encompassing these promoter elements. Furthermore, indirect end-labelling analyses of the reconstituted U6 wild-type plasmids showed strong micrococcal nuclease cuts near the DSE and PSE, indicating that a nucleosome is located between these elements. To investigate the influence that nucleosomes exert on U6 transcription, we used two different experimental approaches for chromatin reconstitution, both of which resulted in the observation that transcription of the U6 wild-type gene was enhanced after chromatin assembly. To ensure that the facilitated transcription of the nucleosomal templates is in fact due to a positioned nucleosome, we constructed mutants of the U6 gene in which the sequences between the DSE and PSE were progressively deleted. In contrast to what was observed with the wild-type genes, transcription of these deletion mutants was significantly inhibited when they were packaged into nucleosomes.

The expression of eukaryotic genes in vivo occurs in a chromatin environment which in many cases is thought to regulate initiation of gene transcription in a negative sense (11, 18, 23). In order to overcome such a repressive influence of nucleosomal organization, gene-specific mechanisms which render the chromatin accessible for transcription factors exist. The SWI-SNF complex of different species has been shown to rearrange nucleosomal structures, thereby leading to enhanced binding of transcription factors (5, 7, 15, 20, 28, 47). Recently, the SWI-SNF complex was found to interact with activators, which resulted in a relief of a transcriptional elongation block caused by nucleosomes (3). It has been postulated that the products of the SWI-SNF genes form a complex with other members of the transcription apparatus, thereby facilitating gene expression in vivo (27, 49). Moreover, it is well established that transcription factors like GAGA and heat shock factor can disrupt chromatin structure in conjunction with cofactors like nucleosomal rearrangement factor (NURF) (43–46). Interestingly, one of the subunits of NURF has been shown to be a member of the SWI-SNF family of proteins (44).

However, chromatin structure must not necessarily be considered a negative regulator of gene expression, and in some examples it is known to facilitate transcription of a gene. For instance, expression of the *Xenopus laevis* vitellogenin B1 gene is enhanced by a positioned nucleosome which brings two regulatory elements into juxtaposition, thereby enabling transcription factors bound to the respective elements to interact (36). The *Drosophila* Adh gene was found to position a nucleosome juxtaposing enhancer and promoter elements (16). Recently, the efficient transcription of the *Drosophila melanogaster* hsp26 gene has been shown to depend on specific nucleosomal positioning between two heat shock elements (24). The *D. melanogaster* hsp27 promoter is a further example of a positioned nucleosome, which shortens the distance between the TATA box and two heat shock elements (32).

Most experiments concerning the effect of chromatin structure on transcription by RNA polymerase III (Pol III) have been conducted on genes encoding ribosomal 5S RNA (2, 12, 13). An additional example of a Pol III gene analyzed in the context of chromatin is the yeast U6 small nuclear RNA (snRNA) gene. It was shown that transcription could be rescued by the transcription factor IIIC (TFIIIC) once this gene had been reconstituted into chromatin (4). In the absence of chromatin, there was no need of TFIIIC for efficient U6 gene transcription in vitro. However, recent data presented for the yeast U6 gene suggested an additional role for TFIIIC in determining polymerase specificity in vivo (35). It remains to be clarified whether TFIIIC alone could gain access to a B-box element downstream of the yeast U6 gene or whether TFIIIC binding would lead to a local disruption of chromatin structure.

The human and other vertebrate U6 snRNA genes have a different promoter structure, lacking a B-box element in the downstream region (19), and nothing is known about their transcription in the context of chromatin structure. We hence investigated this question for the case of the human U6 gene.

Two different experimental strategies were used to reconstitute chromatin on either plasmid or linear fragment DNA containing the human U6 gene. Both methods showed increased transcription of nucleosomal templates compared to naked DNA. Structural analyses of the nucleosomes revealed strong nucleosome-positioning sequences in the region of the gene between the distal sequence element (DSE) and the proximal sequence element (PSE). These data support the conclusion that expression of the human U6 gene by Pol III is positively influenced upon chromatin reconstitution.

MATERIALS AND METHODS

Plasmid DNA and DNA fragments of the human U6 snRNA gene. A fragment of the human wild-type U6 gene harboring sequences from −232 to +289 was cloned blunt end into the *Sma*I site of pUC18. For DNase I footprinting and transcription analyses, this fragment was prepared by *Eco*RI/*Hin*dIII restriction and labelled at the *Hin*dIII site (corresponding to the 5′ end of the gene) with the

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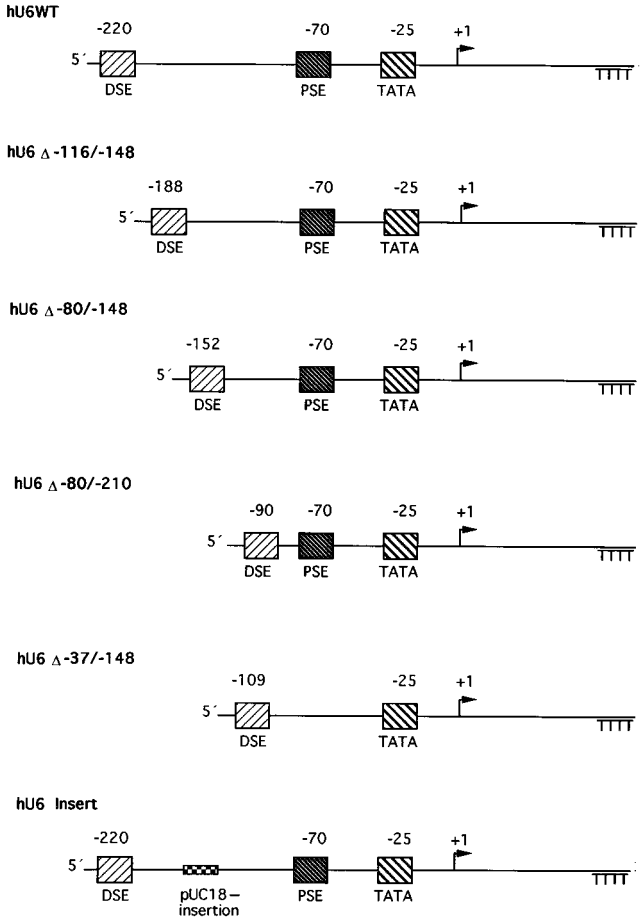

FIG. 1. U6 wild-type (WT) gene and mutated constructs used for chromatin assembly. The mutants shown were constructed by following an ExoIII experimental strategy (see Materials and Methods for details). The designations of the mutants reflect the DNA sequences deleted by ExoIII.

Klenow-Fill-In reaction with [^{32}P]dCTP. The construct from which the DSE was deleted (hU6ΔDSE) contains sequences of the U6 gene from −149 to +189 and was also subcloned into the *Sma*I site of pUC18. For Exonuclease III (ExoIII) footprinting, a fragment of the U6 gene harboring sequences from −243 to −44 (DSE to PSE) was subcloned into the *Sma*I site of pUC18. To construct the reinsertion mutant of the U6 gene, the wild-type sequence between −116 and −148 was replaced by pUC18 sequences harboring 32 bp of the multiple cloning region between the *Sma*I and *Hin*dIII sites.

Construction of U6 deletion mutants. Deletion mutants of the U6 gene were generated by an ExoIII treatment of the U6 wild-type gene. The U6 mutants used in this study were constructed with the Stratagene ExoIII deletion kit according to the manufacturer's instructions. The vector harboring the U6 sequences from −232 to +289 was cut with *Bam*HI and *Pst*I. The latter enzyme generates 3′ protruding ends. By variations of time and temperature, we obtained U6 mutants which were deleted at their 5′ ends to different degrees. After religation and amplification of the deleted constructs, they were linearized by *Hin*dIII restriction and subsequently ligated to the U6 wild-type sequence from −232 to −148. Figure 1 outlines the mutants of the U6 gene used in the present study. The mutant hU6 Δ−80/−210 was created by a combined ExoIII-PCR method by amplification of the DSE and subsequent cloning of the PCR product into a *Hin*dIII-restricted pUC18 vector harboring the U6 gene with deleted sequences to −80.

Reconstitution of chromatin on plasmid DNA. For chromatin reconstitution on plasmid DNA harboring the human U6 snRNA gene, the plasmids were assembled into chromatin by using cytoplasmic extracts derived from *X. laevis* (oocyte S150) (21, 37). The plasmids were packaged into minichromosomes as described previously (41). The nucleosomes generated with this system are regularly spaced.

Reconstitution of mononucleosomes on labelled U6 gene fragments. To obtain mononucleosomes, either nucleosomal arrays from avian erythrocytes depleted in linker histones (22) or purified HeLa core histones were prepared. Both methods were shown to provide the same results. For the second method, purified histones were used for the assembly of chromatin following a protocol described earlier, with several modifications (40). First, histones from HeLa cell nuclei were bound to hydroxyapatite and linker histones were subsequently removed by several washing steps with a buffer containing 0.7 M NaCl and 50 mM sodium phosphate. Finally, the core histone fraction was eluted with a 2.5 M NaCl–50 mM sodium phosphate buffer. If necessary, the core histones were concentrated in a Filtron macrosep concentrator by centrifugation at 5,000 × *g* for about 4 h in a Sorvall HB 4 rotor. After the purification steps, the histone integrity and purity were checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. For transcription experiments as well as DNase I and ExoIII footprinting assays, isolated core histones were mixed with 3 to 5 μg of the U6 restriction fragments with a histone/DNA ratio of 0.9 under high salt conditions (2 M NaCl buffer) and then dialyzed against 250 mM NaCl buffer containing 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA (pH 8.0). In contrast to former published protocols, urea was omitted from these reactions (6). It is important to note that a small reaction volume of approximately 30 μl is recommended for optimal efficiency of the reconstitution process.

Investigation of DNA topology. Changes in DNA topology were analyzed after reconstitution of U6 plasmid DNA with increasing amounts of S150 extract. After reconstitution, the samples were treated with RNase A for 30 min and finally digested with 500 μg of proteinase K at 37°C overnight. The DNA was purified according to standard procedures and subsequently loaded onto 1.2% agarose gels. DNA topoisomers were separated by electrophoresis at 50 V for about 20 h and stained with ethidium bromide.

Micrococcal nuclease cleavage and indirect end labelling. Prior to nuclease digestion, 500 ng of plasmid DNA was reconstituted into chromatin with the *X. laevis* S150 extract. After the reconstitution reaction, $CaCl_2$ was added to a final concentration of 3 mM and the samples were subsequently digested with 1 to 5 U of micrococcal nuclease. The fragments generated were separated by electrophoresis on 1.2% agarose gels at 50 V running overnight. To investigate nucleosomal positioning on U6 plasmids by indirect end-labelling analyses (50), the DNA fragments generated after micrococcal nuclease digestion were purified and then recut with *Eco*RI. After purification, the DNA was separated by electrophoresis on 1.2% agarose gels as described above and blotted onto a positively charged nylon membrane (Qiagen). Finally, the blotted fragments were hybridyzed against a ^{32}P-labelled *Eco*RI/*Apa*LI probe derived from the U6 gene carrying 120 nucleotides (nt) from the *Apa*LI site to the *Eco*RI site of the pUC18 polylinker. After hybridization according to standard protocols, the membrane was washed in 0.1× SSC–10% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 20 min at 65°C and autoradiographed with an intensifying screen for 1 to 3 days at −80°C. Size analyses of the detected fragments were performed with an Epson GT-8000 scanner using Scanpack software from Biometra.

DNase I footprint analyses. To determine the rotational and translational phasing of reconstituted nucleosomes, the samples were treated with up to 70 ng of DNase I dissolved in 25 mM $MgCl_2$ and 0.5 mM $CaCl_2$. The reaction proceeded for 1 min at room temperature. To stop the reaction, 100 μl of stop solution including 450 mM sodium acetate, 0.1% SDS (wt/vol), and 10 mM EDTA was added to the samples. Finally, the DNA was processed as described previously (41). Prior to loading onto a 6% (wt/vol) sequencing gel, the DNA pellets were dissolved in 95% formamide loading buffer and denatured at 95°C.

ExoIII digestion. Prior to nucleosome reconstitution, a *Sac*I/*Hin*dIII U6 fragment from −243 to −44 was dephosphorylated and labelled at both ends with polynucleotide kinase and [γ-^{32}P]ATP. To maintain the radioactive 3′ protruding end, the fragment was recut with *Xba*I within the polylinker. The fragment was then reconstituted into a mononucleosome and subjected to digestion with ExoIII (Promega). The samples were incubated with 40 U of ExoIII for 5 to 60 min in the case of free DNA and with 200 U of ExoIII for 5 to 120 min in the case of nucleosomal DNA in a total volume of 200 μl at room temperature. Aliquots were taken at several time points, and reactions were stopped as described for the DNase I experiments. DNA was purified and finally electrophoresed on 6% (wt/vol) denaturing sequencing gels.

In vitro transcription. All transcription reactions were performed essentially as described previously (17). The reaction buffer used in the presented assays contained 20 mM Tris (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, and 3 mM dithiotreitol. For transcription of the U6 fragments as either nucleosomal or naked DNA, we employed 50 to 250 ng of DNA. It should be noted that efficient transcription of linear DNA fragments of the U6 gene in vitro requires a reconstituted system containing fraction A precipitated with 20 to 40% ammonium sulfate (150 μg of protein), as well as 75 μg of fraction B and 25 μg of fraction C, obtained after chromatography of a HeLa S100 extract through phosphocellulose. Additionally, the assays contained 50 ng of bacterially expressed recombinant human TATA-binding protein (TBP).

RESULTS

Nucleosomal organization of the U6 gene stimulates transcription, and the DSE is required for this effect. To investi-

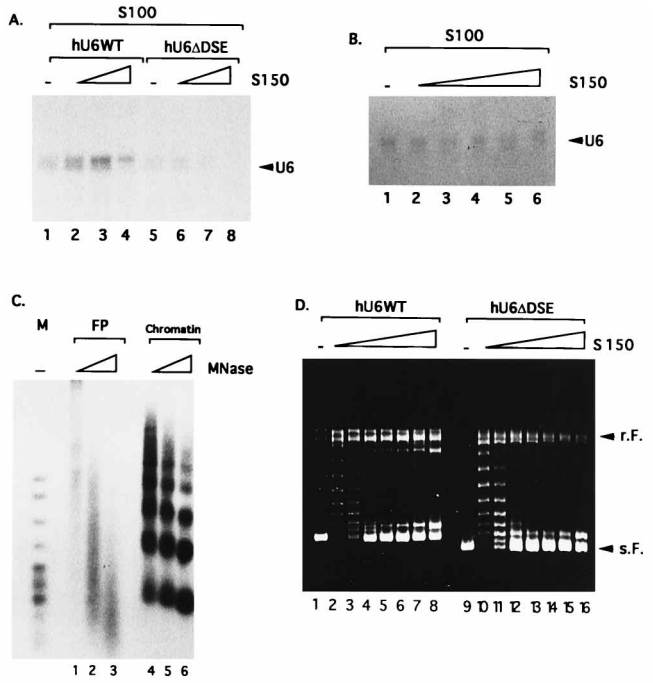

FIG. 2. The DSE is necessary for transcriptional activation of the nucleosomally organized U6 gene. (A) Plasmid DNA (500 ng) harboring the human U6 snRNA gene was assembled into chromatin with 5, 15, and 40 μl of S150 extract derived from *X. laevis*, and transcription was started by addition of 8 μl of HeLa S100 extract and nucleotides. After further incubation for 2 h, RNA was isolated and loaded onto a 6% sequencing gel. Lanes 1 to 4, wild-type (WT) U6 gene; lanes 5 to 8, DSE-deficient U6 mutant; lanes 1 and 5, transcription of naked DNA; lanes 2 to 4 and 6 to 8, transcription of U6 minichromosomes. (B) Plasmid DNA (500 ng) harboring the human wild-type U6 snRNA gene was incubated with 2, 5, 15, 40, and 50 μl (lanes 2 to 6, respectively) of S150 extract derived from *X. laevis* without prior reconstitution, and transcription was started by addition of 8 μl of HeLa S100 extract and nucleotides. Lane 1, transcription of 500 ng of U6 plasmid DNA without addition of *Xenopus* extract. (C) Micrococcal nuclease (MNase) cleavage pattern of the reconstituted wild-type U6 samples. Lanes 1 to 3, 500 ng of naked U6 DNA digested with 0.25, 0.5, and 1 U, respectively, of MNase for 1 min at room temperature; lanes 4 to 6, 500 ng of plasmid U6 wild-type DNA assembled into chromatin as depicted in panel A and subsequently digested with 2, 4, and 6 U, respectively, of MNase. Lane M depicts a radioactively labelled size standard (pBR322; *Msp*I-digested fragments of 622, 527, 404, 307, and 242 bp are clearly visible, whereas the smaller fragments are not well resolved due to the limited resolution of the agarose gel). DNA was isolated, blotted onto positively charged nylon membranes, and probed with the U6 wild-type fragment containing U6 sequences from −232 to +289. (D) Topological analyses of U6 minichromosomes reconstituted under the conditions described for panel A. After incubation of 500 ng of U6 plasmid DNA with 0, 2, 5, 10, 15, 40, 50, and 70 μl of S150 extract, the DNA was extracted and topoisomers were separated as described in Materials and Methods. Lanes 1 to 8, U6 WT; lanes 9 to 16, DSE-deficient U6 mutant. r.F. and s.F. denote the relaxed and supercoiled DNA form, respectively.

gate transcription of the human U6 snRNA gene when packaged into chromatin, we assembled U6 minichromosomes by incubating U6 plasmid DNA with S150 extract derived from *X. laevis* as described previously (37). It is evident that the S150 extract led to formation of nucleosomes on the U6 gene with an average spacing of 160 bp, as visualized by digestion with micrococcal nuclease and comparison with appropriate size markers (Fig. 2C, lanes 4 to 6). To further prove the nucleosomal organization of the template, we performed topology assays, which showed a stepwise change in superhelical density for both templates caused by increasing numbers of reconstituted nucleosomes (Fig. 2D). Previous experiments using this extract showed a strong repressive influence on 5S gene transcription after chromatin reconstitution (41). As shown in Fig. 2A, the reconstitution of nucleosomes on the U6 plasmid DNA led to different transcriptional effects depending on the promoter sequences contained in the template. Interestingly, when the wild-type U6 promoter including all promoter elements was used in the chromatin assembly reaction, the transcription rates were enhanced compared to the control (lanes 2 to 4 versus lane 1). The reverse effect, a strong transcriptional repression, was observed with a truncated version of the U6 gene lacking the DSE (lanes 6 to 8). To exclude experimental artifacts, conceivably related to stimulatory activities in the S150 extract, the same amounts of extract were added as a control at the beginning of transcription without prior reconstitution. Under these conditions, no stimulatory influence of the S150 extract could be observed (Fig. 2B, lanes 2 to 6).

A reconstituted mononucleosome occupies the distal promoter region of the U6 gene. For further analyses of the rather unexpected results described above, we attempted to characterize the ability of distal U6 sequences to assemble nucleosomes. For this purpose, linear DNA fragments harboring U6 wild-type sequences from −232 to +289 were reconstituted into chromatin as described in Materials and Methods. After generation and purification of mononucleosomes over a 5 to 30% glycerol gradient (Fig. 3A), we performed a DNase I footprinting analysis. As shown in Fig. 3B, the nucleosomal

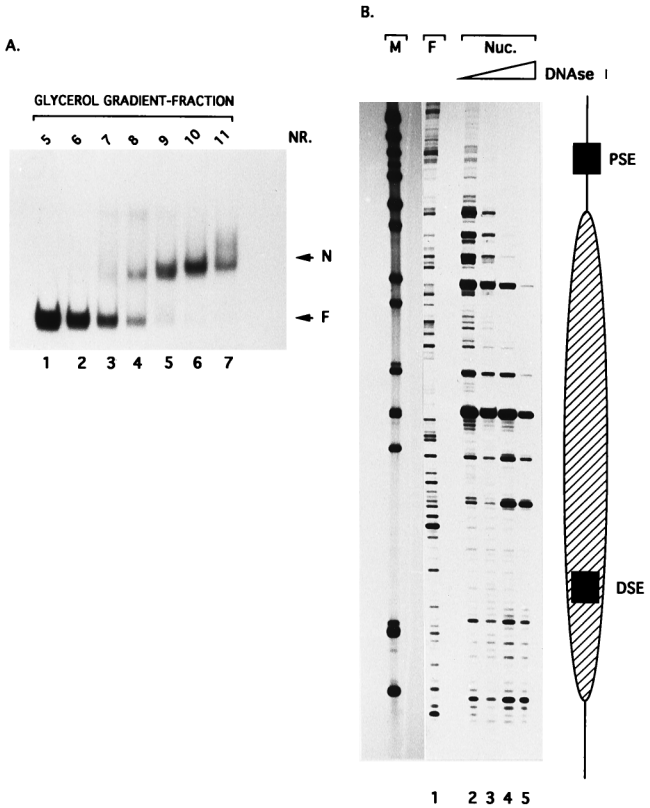

FIG. 3. DNase I footprint analysis of U6 mononucleosomes. (A) A fragment of the human U6 gene harboring sequences from −232 to +289 was reconstituted into chromatin, and the reconstituted constructs were purified by glycerol gradient centrifugation. (B) U6 mononucleosome fraction 9 (40,000 cpm) was treated with 20, 40, 60, and 70 ng of DNase I (lanes 2 to 5). Lane F denotes the fragments obtained from digestion of naked DNA. Lane M depicts a standard marker (pBR322; *Msp*I digest) for gel calibration showing fragments of 26, 34, 67, 76, 90, 110, 123, 147, 160, 180, 190, 201, 217, and 238 nt. The hatched oval on the right shows the position of the nucleosome.

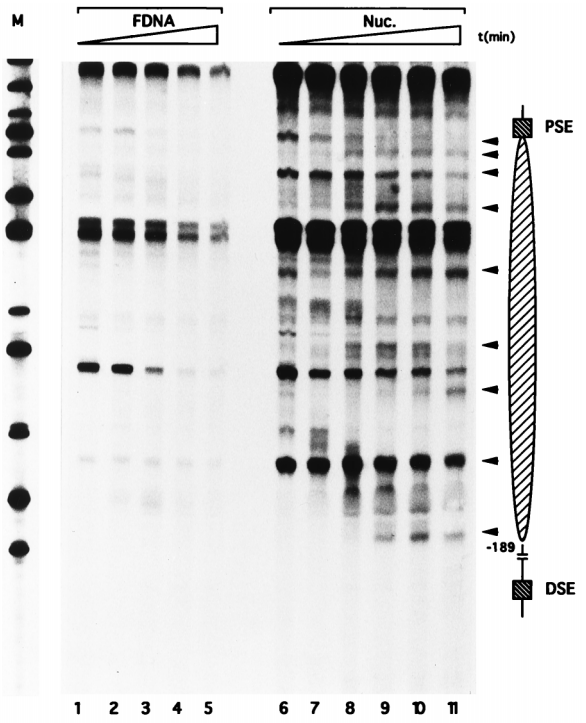

FIG. 4. ExoIII footprint of a U6 mononucleosome. A total amount of 10^5 cpm of the 5′-end-labelled U6 fragment from −243 to −44, either incorporated in a mononucleosome or as free DNA, was treated with ExoIII and processed as described in Materials and Methods. Lanes 1 to 5, free DNA (FDNA); lanes 6 to 11, U6 mononucleosome (Nuc). The oval as well as the arrows next to the figure depict the region of the nucleosomal U6 gene where the main ExoIII stops occurred. Lane M depicts a standard marker (pBR322; *Msp*I digest) for gel calibration showing fragments of 67, 76, 90, 110, 123, 147, 160, 180, 190, 201, 217, and 238 nt.

organization of the gene became visible after DNase I treatment (lanes 2 to 5). Prominent bands reflecting strong histone-DNA interactions, as evidenced by the modular appearance of 10-bp fragments, could be observed in the promoter region centered between the PSE and DSE. In individual reconstitution experiments, the nucleosomal position differed and occasionally the DSE was not covered by the nucleosome. However, in all cases nucleosomes were assembled with high affinity. Moreover, the nucleosome was always located upstream from the PSE and the rotational phasing was maintained, although the positions of the nucleosomal borders varied. It is therefore evident that the distal U6 promoter region between the DSE and PSE contains sequences with high affinity for the organization of nucleosomes but less pronounced signals for their exact positioning. It is conceivable that in the nuclear context, upstream sequences or nucleosomes help to fix the U6 nucleosome at a more defined position.

To further verify that a nucleosome occupies the distal promoter region between the DSE and PSE, we performed ExoIII footprinting experiments. For this purpose, *Sac*I/*Hin*dIII fragments carrying U6 sequences from −243 to −44 were labelled with [γ-^{32}P]ATP and recut with *Xba*I. As compared to naked DNA (Fig. 4, lanes 1 to 5) digestion of the nucleosomal fragments exerts ExoIII stops at positions different from those occurring in the naked DNA (Fig. 4, lanes 6 to 11). A prominent stop at position −189 reflects the 5′ boundary of the nucleosome. This finding extended the results obtained by DNase I footprinting, showing that the region between the DSE and PSE is preferentially assembled into a nucleosome, although the exact borders of the nucleosome may vary.

The nucleosome in the distal promoter region between the DSE and PSE facilitates transcription of the linear U6 DNA fragments. As a next step, we attempted to investigate the functional consequences of the nucleosome placed in the distal promoter region. First we tested whether the transcriptional results obtained upon transcription of U6 plasmid DNA reconstituted into chromatin with *X. laevis* S150 extract could be reproduced when linear templates with a defined nucleosome position were employed for transcription. Figure 5A shows the results of these experiments. To compare the wild-type U6 gene with the DSE-deficient mutant, both linear templates were transcribed in a reconstituted system (see Materials and Methods). Comparison of the U6 wild-type nucleosomal fragment (Fig. 5A, lanes 4 to 6) with the nonnucleosomal control fragment (Fig. 5A, lanes 1 to 3) clearly demonstrates enhanced transcription in the case of the U6 mononucleosome. Furthermore, this effect appeared to be dependent on the presence of the DSE, since in the case of the DSE-less mutant a repression upon nucleosome assembly rather than an activation of transcription was achieved (compare lanes 7 to 9 with lanes 10 to 12 [Fig. 5A]). To rule out a stimulatory activity within the histone fraction, required for nucleosome reconstitution, this fraction was concomitantly added at the beginning of the transcription reaction of the naked template DNA. As demon-

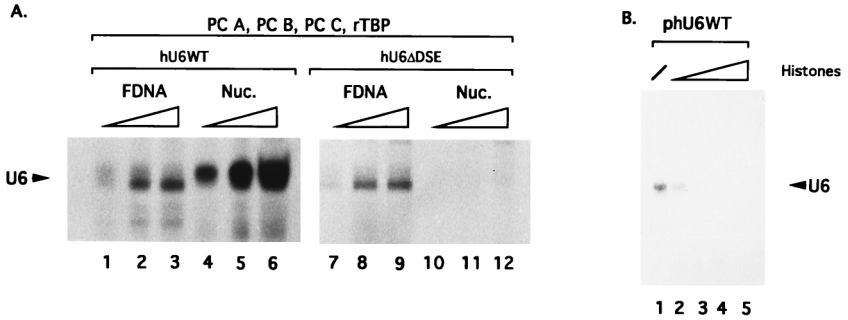

FIG. 5. Nucleosome reconstitution on U6 fragment DNA leads to a facilitated transcription which is not due to an activity within the histone fraction. (A) Lanes 1 to 3, transcription of 50, 125, and 250 ng, respectively, of nucleosome-free U6 wild-type (WT) DNA containing sequences from −232 to +289; lanes 4 to 6, transcription of 50, 125, and 250 ng, respectively, of nucleosomal U6 WT DNA. Lanes 7 to 9 and 10 to 12, amounts of DNA as above with the exception that the DSE-deficient mutant was used. After the reconstitution reaction was completed, the transcription reaction was started by adding the recombinant TBP and phosphocellulose (PC) fractions derived from a HeLa S100 extract as indicated at the top of the figure. (B) Lane 1, control transcription of 100 ng of U6 WT plasmid DNA without addition of core histones; lanes 2 to 5, addition of 0.8, 2, 4, and 6 μg, respectively, of core histones at the beginning of transcription.

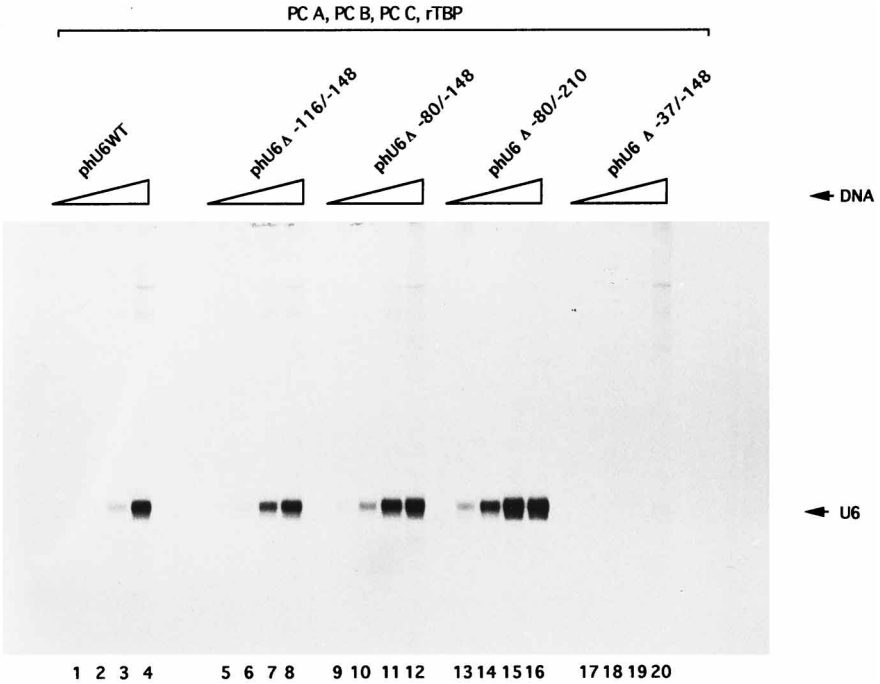

FIG. 6. Increased transcriptional efficiency by stepwise deletion of DNA sequences located between the DSE and PSE. The transcription reactions were conducted with 50, 100, 200, and 400 ng of DNA, from left to right in each set of four lanes. Transcriptions were performed essentially as described in Materials and Methods. PC, phosphocellulose; rTBP, recombinant TBP; WT, wild type.

strated in Fig. 5B (lanes 2 to 5), addition of the histones to the transcriptional apparatus without a prior reconstitution reaction led to an efficient inhibition of transcription, possibly as a consequence of histone-DNA precipitates formed under conditions of low ionic strength. Hence, it became clear that transcriptional activation of the U6 gene was not due to activities possibly contaminating the histone fraction.

Transcription of the human U6 gene is increased upon progressive deletion of the promoter region between the DSE and PSE. The experimental results shown above demonstrate that the 5′ distal region of the U6 gene is capable of assembling a nucleosome. Furthermore, increased transcription as a direct consequence of nucleosome reconstitution could be observed. Based on these findings, it appears feasible that a DNA loop structure could be formed upon chromatin reconstitution, leading to juxtapositioning of the DSE and PSE. This assumption was further confirmed by results shown in Fig. 6. In these experiments, mutants of the U6 gene, in which DNA sequences between the DSE and PSE had been deleted, were transcribed without prior nucleosome reconstitution. It is known that deletion of similar sequences in the case of the 7SK gene results in an enhanced interaction of Oct-1 and the PSE-binding protein (PBP) (also designated PTF) (26). This facilitated communication of the factors results in increased transcriptional rates. In the case of the U6 deletion constructs, we observed a similar stimulatory effect on transcription compared to the wild type (Fig. 6, lanes 1 to 4 versus lanes 5 to 16). Moreover, deletion of the PSE sequence resulted in a strong decrease of transcription efficiency (Fig. 6, lanes 17 to 20) due to the removal of the binding site for PBP. This finding reflects the necessity for Oct-1 to interact with a bound PBP molecule to activate U6 transcription. In summary, the deletion of intervening DNA sequences between the DSE and PSE provides the same stimulation of transcription as the packaging of the wild-type U6 DNA promoter into a nucleosome. As a next step, we tested how reconstitution of nucleosomes onto the U6 mutants carrying deletions between the DSE and PSE would influence transcription.

Chromatin reconstitution on U6 mutant constructs leads to an inhibition of transcription. In order to examine properties of nucleosomally organized U6 mutants, plasmid DNA harboring the deleted U6 genes was assembled into chromatin with the *X. laevis* S150 extract. As already shown in Fig. 2, the wild-type U6 minichromosomes were transcribed in a facilitated manner (Fig. 7A, lanes 2 to 6). However, transcription of the mutant U6 constructs was progressively inhibited by formation of chromatin (Fig. 7A, lanes 7 to 24). The results shown in Fig. 7, lanes 1, 7, 13, and 19, reenforce the findings shown in Fig. 6, and they show that progressive deletion of sequences between the DSE and PSE leads to an enhancement of transcription. Additionally, to ensure complete packaging of two plasmid U6 mutants into nucleosomes, the different topoisomers were separated by agarose gel electrophoresis after chromatin reconstitution. As shown in Fig. 7B, the increase in superhelical density upon generation of nucleosomes is clearly visible on both templates (compare lanes 1 to 7 with lanes 8 to 14).

Translationally phased nucleosomes on human U6 wild-type DNA. To define whether the facilitated transcription of U6 wild-type minichromosomes is really due to a specific nucleosomal organization within the distal region of the U6 promoter, we carried out an indirect end-labelling analysis. After reconstitution of chromatin with *X. laevis* S150 extract, the samples were digested with micrococcal nuclease and cut with *Eco*RI (see Materials and Methods for details). The hybridization of blotted DNA fragments with an *Apa*LI/*Eco*RI probe revealed a regular signal pattern (Fig. 8, lanes 5 and 6). Although it should be noted that the comparatively low level of resolution of this assay does not allow the exact allocation of cuts at the single-base-pair level, it is apparent that strong

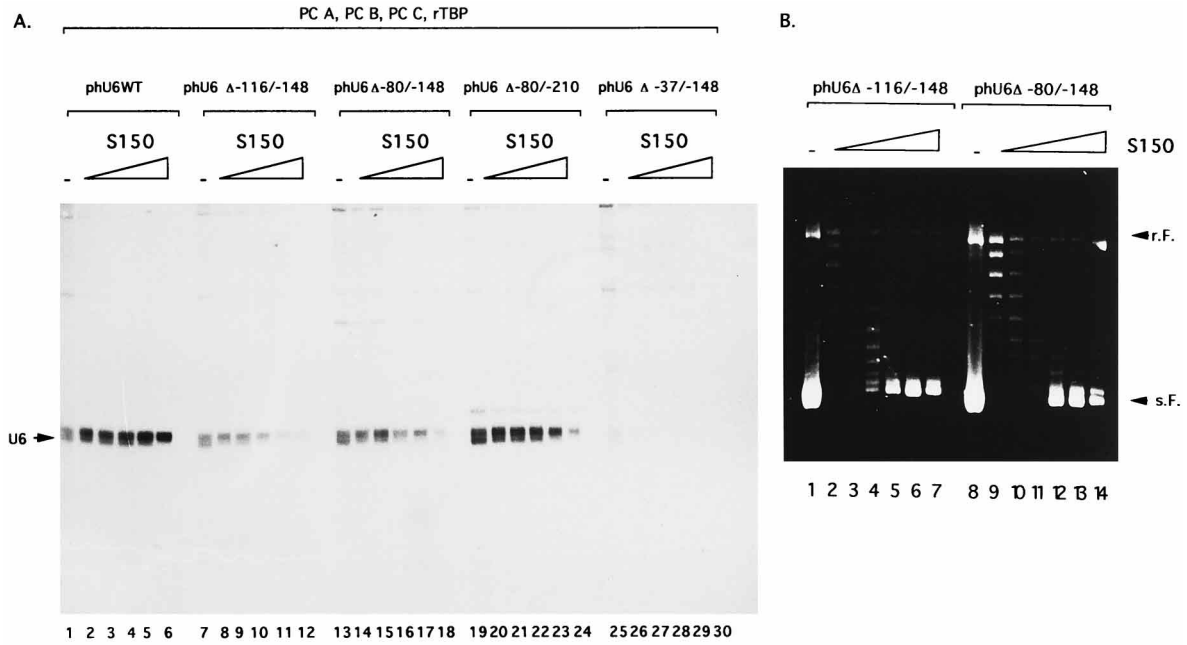

FIG. 7. Chromatin assembled on plasmid DNA harboring the U6 deletion mutants inhibits transcription. (A) Plasmid U6 DNA (500 ng) was assembled into chromatin with 4, 6, 8, 16 and 40 μl of S150 extract. After the reconstitution reaction was completed, the transcription reaction was started by adding the recombinant TBP (rTBP) and phosphocellulose (PC) fractions derived from a HeLa S100 extract as indicated at the top of the figure. Lanes 1, 7, 13, 19, and 25 depict the nucleosome-free control transcription reactions. WT, wild type. (B) Topological analysis of two U6 mutants as shown in panel A. After chromatin reconstitution the topoisomers were processed as described in Materials and Methods.

micrococcal cuts appeared at approximate positions of −220, −190, −80, −40, −20, and +160. The cuts at −220 or −190 and −80 or −40 reflect the possibility that nucleosome positioning may not occur only in a single fashion but could be a result of two alternative translational phases within the entire plasmid population. Moreover, the small differences in the sizes of fragments generated by micrococcal nuclease cuts in the vicinity of the regulatory distal promoter elements may be due to mechanical sliding of nucleosomes during the experimental procedure. However, it should be noted that in the region between −190 and −80, no micrococcal cuts appeared. This finding indicates the presence of a nucleosome in this region. The chromatin structure of the U6 deletion mutants remains to be established and will be dealt with in future experiments.

Activation of U6 transcription upon chromatin assembly depends on wild-type sequences between −148 and −116. To investigate whether special features of the U6 wild-type sequence between the DSE and PSE are crucial for alleviated transcription, we constructed a reinsertion mutant. As described in Materials and Methods, the wild-type sequence between −148 and −116 was replaced by irrelevant pUC18 sequences of similar length. As already shown in Fig. 7, transcription of the U6 deletion mutant lacking sequences from −148 to −116 was significantly repressed by chromatin reconstitution (Fig. 9A, lanes 7 to 10). Although transcription of the reinsertion mutant was less repressed (Fig. 9A, lanes 12 to 15), activation could not be restored to the level observed for the wild-type U6 gene (compare lanes 12 to 15 with lanes 2 to 5 [Fig. 9A]). To clarify these unexpected results, we conducted computer analyses with the CURVATURE software developed by Shpigelman and coworkers (38) which revealed that the wild-type sequences in the distal promoter region lead to a significant degree of curvature (Fig. 9B, top). When sequences between −148 and −116 were deleted and replaced by an irrelevant pUC18 sequence, this feature of curved DNA was lost, resulting in a rigid stretch of DNA (Fig. 9B, bottom). This observation could possibly provide a molecular explanation for the functional data shown in Fig. 9A, as it might be difficult for this artificial sequence to be correctly packaged into a nucleosome.

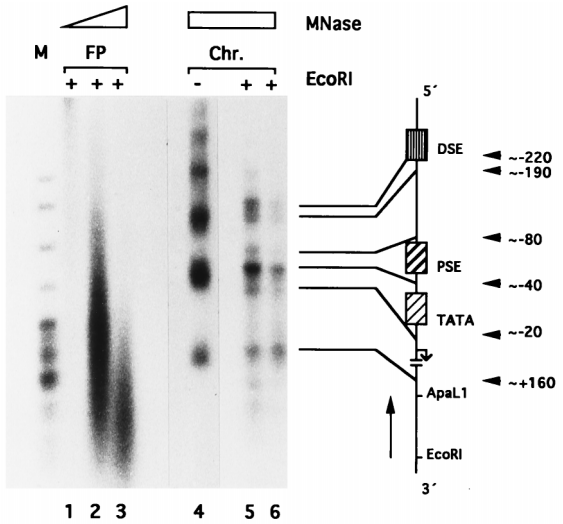

FIG. 8. Determination of translationally phased nucleosomes on wild-type U6 DNA. Samples containing 500 ng of plasmid U6 DNA were reconstituted into chromatin and subsequently treated with micrococcal nuclease. Lanes 1 to 3, 0.25, 0.5, and 1 U, respectively, of free DNA; lanes 4 to 6: 4 U each of the reconstituted chromatin. After purification of the DNA fragments, a restriction with 5 U of *Eco*RI was conducted (lanes 1 to 3, 5, and 6). Electrophoresed and blotted fragments were hybridized against an *Eco*RI/*Apa*I probe derived from the wild-type U6 gene.

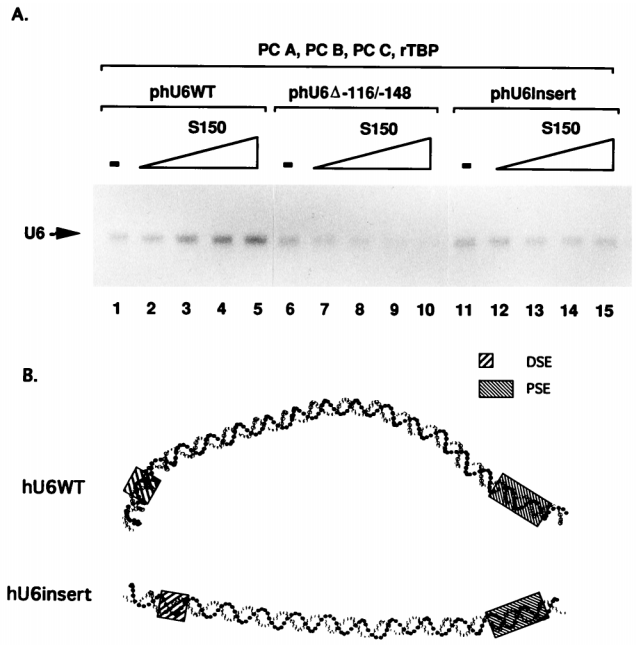

FIG. 9. U6 wild-type sequences between −148 and −116 are necessary for transcriptional activation upon chromatin assembly. (A) Plasmid U6 DNA (500 ng) was assembled into chromatin with 4, 8, 16, and 40 μl of S150 extract. Lanes 1 to 5, wild-type (WT) U6 DNA; lanes 6 to 10, hU6Δ−116/−148; lanes 11 to 15, insertion mutant of the U6 gene (hU6Insert). After the reconstitution reaction was completed, the transcription reaction was started by adding the recombinant TBP (rTBP) and phosphocellulose (PC) fractions derived from a HeLa S100 extract as indicated at the top of the figure. Lanes 1, 6, and 11 depict the nucleosome-free control transcription reactions. (B) Computer analyses of curvature for U6 sequences between −232 and −43 for the wild type (top) and the reinsertion mutant (bottom) where sequences between −116 and −148 were replaced by pUC18 DNA. Regulatory elements and their positions on the DNA are indicated in the figure.

DISCUSSION

The main goal of this study was to gain insight into the transcriptional regulation of a nucleosomally organized human U6 gene. During the past decade, a great deal of information has been collected concerning genes which precisely position nucleosomes. The 5S genes of different species have been the most intensively investigated with regard to their chromatin structure (10, 12, 13, 33, 41). Additionally, other well known examples like the yeast PHO5 gene (1), the mouse mammary tumor virus long terminal repeat (29, 34), the *Drosophila* adh gene (16), the *X. laevis* vitellogenin B1 gene (36), and the *Drosophila* hsp26 and hsp27 genes (24, 32) have been investigated in this context. Multiple reasons seem to exist for the observed increased ability of certain DNA sequences to precisely position nucleosomes (8, 39). Recently, it was shown that CTG repeats occuring in some genetic disorders favor the assembly of nucleosomes, thereby providing the strongest naturally occurring nucleosome positioning sequences known so far (9, 48).

To identify DNA sequences within the human U6 gene which preferentially position nucleosomes, we assembled linear DNA fragments of the U6 gene into mononucleosomes by a standard protocol, as described in Materials and Methods. To our knowledge, the human U6 snRNA gene has hitherto not been investigated in this respect. Determination of translational and rotational phasing by DNase I and ExoIII footprinting revealed a nucleosomal occupancy of the distal U6 promoter region between the DSE and PSE. In both cases, prominent histone-DNA contacts showed that the 3′ border of the nucleosome was located upstream of the PSE, although its exact position could vary slightly. The 5′ border of the reconstituted mononucleosome in the vicinity of the DSE accordingly differed between individual experiments, but in most cases the DSE was only partially covered by the histone octamer. Such variations could be explained by sliding of the nucleosome in the course of the experimental process. It is also conceivable that upstream nucleosomes or DNA sequences contribute to a more defined position of this nucleosome in a native chromatin context.

To investigate whether nucleosomes are equally positioned on U6 wild-type minichromosomes, we performed indirect end-labelling experiments. Analyses of micrococcal cuts in the promoter region of the gene showed preferential cuts in the proximity of positions −220, −190, −80, −40, −20, and +160, determined within the limits of resolution of an agarose gel. In the experiment shown in Fig. 8, two cuts appeared near the DSE (−220 and −190) and PSE (−80 and −40). From these findings, we concluded that the nucleosomal positioning on plasmid DNA varies slightly for presently unknown reasons.

Consequences of chromatin reconstitution for transcription of the human U6 gene. The control of eukaryotic gene expression is governed not only by promoter elements close to the transcription start site but also by enhancers, which can be located many thousands of base pairs away from the promoter (25, 31). One can imagine that enhancer action in vivo is mediated by the formation of a DNA loop structure, which in the eukaryotic nucleus could be generated by DNA winding into chromatin. Up to now, there are only few examples of facilitated gene transcription upon in vitro chromatin reconstitution. In the case of the *X. laevis* vitellogenin B1 gene, a positioned nucleosome shortens the distance between the binding sequences for the estrogen receptor and nuclear factor I, leading to a juxtaposition of both elements (36). Consequently, the transcriptional rate is enhanced through formation of this static loop. A similar mechanistic model was recently described for the *D. melanogaster* hsp26 gene (24). In this case, a nucleosome was shown to be positioned between two heat shock elements in vivo. This also leads to stimulated gene expression to an extent which is similar to that observed after deletion of the DNA sequences between the heat shock elements.

Apart from studies with the yeast U6 gene, which demonstrated a functional role for TFIIIC by rescuing transcription after chromatin assembly (4), nothing is known about the human U6 gene in this respect. It is important to emphasize that the vertebrate U6 gene possesses no B-box element as is present in the yeast U6 gene. To study the effects of chromatin on human U6 gene transcription, plasmid DNA was assembled into minichromosomes. In contrast to results obtained with the ribosomal 5S gene and several examples of genes transcribed by Pol II, nucleosome assembly in the case of the U6 gene leads to transcriptional stimulation. It should be stressed that this stimulation depends on the presence of the DSE. A U6 mutant, in which the DSE had been deleted, did not show efficient transcription after chromatin reconstitution. The possibility that DSE binding activities in the S150 extract are responsible for the stimulatory effect on U6 transcription could be excluded in control experiments (Fig. 2B). Collectively, the results show that U6 chromatin structure generated by in vitro reconstitution is responsible for transcriptional stimulation.

After demonstrating an increased affinity of the distal promoter region for assembly of a histone octamer on either plasmid DNA or linear DNA fragments, we investigated which consequences follow for transcription of these fragments. The

results of these experiments agreed with those which were obtained with U6 plasmid DNA reconstituted with the S150 extract. They revealed an increased transcription after nucleosome assembly in the case of the wild-type U6 gene (Fig. 5A). Likewise, stimulation of U6 transcription upon nucleosome reconstitution was shown to be dependent on the DSE (Fig. 2A and 5A). However, it must be pointed out that determination of the rotational and translational phasing of nucleosomal fragments lacking the DSE has not been successful so far. The latter finding could have many reasons. It may be that nucleosome positioning on these fragments occurs in a random fashion based on the lack of specific positioning sequences as a direct consequence of the deletion. In fact, this would lead to transcriptional inhibition, but it complicates the investigation of rotationally phased nucleosomes.

Furthermore, to analyze the mechanism of transcriptional stimulation of U6 wild-type plasmid DNA after in vitro chromatin assembly, we constructed deletion mutants of the human U6 gene. These mutants contain stepwise deletions of the sequences between the DSE and PSE (Fig. 1). These deletions have dramatic consequences for the basic level of nonnucleosomal transcription of the U6 templates (Fig. 6). If the DSE is moved to the direct vicinity of the PSE, the transcriptional rates increase. This fact appears to be due to a facilitated interaction of both Oct-1 and PBP (PTF), as was demonstrated previously by Murphy et al. (26) for the 7SK gene. In contrast, reconstitution of these deleted constructs of the U6 gene into chromatin exerts strong inhibitory effects on transcription (Fig. 7). We presently do not know whether this loss of activation indeed follows from the inability of bound factors to interact due to incorrect spacing between the DSE and PSE on a nucleosome. It may be that inhibition could also result from the positioning of the TATA box near or within a nucleosome. If this occurs, the transcription might also be repressed. Although sequences located between the DSE and PSE contain no known factor binding sites, they may be important for correct nucleosome positioning and thus essential for transmission of the positive effects of chromatin on U6 transcription.

To clarify this question, we reinserted artificial sequences into the hU6 −116/−148 deletion mutant so that the original distance between the DSE and PSE was restored. Interestingly, this manipulation did not result in restoration of the positive influence of chromatin reconstitution on U6 transcription, which we observed for the wild-type U6 gene but not for the deletion mutants (Fig. 9A). This finding indicates that those sequences, which were originally located between the DSE and the PSE and were replaced by artificial vector sequences for construction of the reinsertion mutant, are of significance for U6 transcription in a chromatin context. We assume that these sequences contain strong nucleosome positioning signals, which may be essential to direct the stimulatory nucleosome to the correct position at the U6 promoter. This assumption is strengthened by computer analysis of the intrinsic topology of these sequences (CURVATURE software, originally described by Shpigelman and coworkers [38]), which revealed that the wild-type U6 promoter has a strong endogenous curvature that is not observed in the reinsertion mutant (Fig. 9B). The curvature of the wild-type U6 promoter resembles that of those parts of the mouse mammary tumor virus promoter which are known to contain strong nucleosome positioning signals (30). The lack of intrinsically curved sequences in the reinsertion mutant may interfere with correct nucleosome positioning, so that the activation of transcription is not observed in comparison to the wild type (Fig. 9A).

In conclusion, we propagated a model for transcriptional activation based on the formation of a specialized chromatin structure as described earlier for the *X. laevis* vitellogenin B1 gene (36) and the *D. melanogaster* hsp genes (24, 32, 42). Such a positive influence of chromatin on Pol III transcription extends former results obtained with the Pol II system and seems to reflect a property of chromatin to bring gene regulatory DNA elements into juxtaposition by winding longer DNA stretches around the histone octamer. We conclude that distal sequences of the U6 gene have high affinity for nucleosomes, since histone octamers are easily assembled over this region in vitro.

Although the experimental data for transcription of U6 wild-type chromatin templates clearly demonstrate that transcription is alleviated upon chromatin assembly, computer analysis revealed that the factor binding sites PSE and DSE are not necessarily in direct alignment with each other when the DNA is wrapped around a nucleosome which is positioned between these elements. However, the PSE binding activity is known to consist of at least four polypeptides of 43, 45, 50, and 190 kDa (14, 51) forming a large complex which may provide enough flexibility to interact with the octamer factor bound to the DSE in such a nucleosomal context. The question of whether a positioned nucleosome occupies the region between the DSE and PSE in vivo remains to be answered and will be investigated in future experiments.

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