The Activity of Transcription Factor IIIC1 Is Impaired during Differentiation of F9 Cells*

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Differentiation in vitro of mouse F9 embryonal carcinoma (EC) cells to the parietal endoderm (PE) mimics processes of development of the early mouse embryo. This differentiation is accompanied by a dramatic down-regulation of all genes transcribed by RNA polymerase III (pol III). Complementation of extracts from cells, differentiated for various time periods with purified pol III transcription factors show for the first time that TFIIIC1 can substantially restore this impaired transcription, particularly in the early stages of differentiation. At later stages (day 7) the TBP (TATA-binding protein)-TAF complex, TFIIIBβ, may also become limiting, which can contribute to but cannot account for the reduced transcription of type 2 promoters in PE cells. Because TFIIIBβ is not required for the expression of type 3 promoters, other components must necessarily be involved, and our results show that U6 transcription can significantly be reactivated by TFIIIC1. By employing a variant type 3 promoter construct, which essentially requires a mutant form of TBP (TBP-DR2), we show that TBP is not limiting in PE extracts. The partial purification of pol III transcription factors from PE and EC cells revealed that TFIIIC2 activity could be purified from both cell types, whereas TFIIIC1 activity was dramatically reduced in extracts from PE cells.

Gene products of RNA polymerase III (pol III) are essential components of central cellular processes such as translation (5 S rRNA, tRNA), protein transport (7SL RNA), and mRNA processing (U6 snRNA), and it is required that their expression be coordinately regulated. At present, however, little is known about the molecular mechanisms underlying this crucial cross-regulation. Previous experiments have shown that the activity of various pol III transcription factors is regulated in the course of differentiation, induction of cellular proliferation, environmental changes (e.g. heat shock), or viral infection (reviewed in Ref. 1).

The genes transcribed by pol III are subdivided into three groups, type 1, type 2, and type 3, based on promoter structure and the basal pol III transcription factors required for their expression. Human TFIIIC can be subdivided into two components: TFIIIC1 and TFIIIC2 (2, 3). TFIIIC2 is a multisubunit protein required for both type 1 and type 2 promoters. It represents the RNA binding activity and consists of five polypeptides (reviewed in Ref. 4). TFIIIC1 likewise represents a multimeric protein of which the subunits have, however, not yet been cloned. TFIIIC1 is required as an initiation factor for the transcription of all pol III genes (3) reviewed in Ref. 4), and only the C1 component of TFIIIC is required for the expression of type 3 promoters. The exact mechanism by which TFIIIC1 is incorporated into the transcription complex and exerts its effect is not clear at present, but it has been proposed that it plays a role in the recruitment of pol III (5).

TFIIIB is a pol III transcription factor involved essentialy in initiation of transcription. It has previously been shown (6–9) that different forms of human TFIIIB exist, designated as hTFIIIBa and -β by Teichmann and Seifart (6). Human TFIIIBβ is required for transcription initiation of type 1 and type 2 pol III promoters and represents a TBP-TAF complex. TFIIIBa is a multiprotein complex that does not, however, contain TBP as an integral part; expression of type 3 promoters in vitro hence requires the addition of TBP. Although the number and identity of TAFs in human TFIIIB have not been fully established, one of the TAFs that has been characterized in some detail is TFIIIB90, which is part of the human TFIIIBβ complex. Recently, additional polypeptides of human TFIIIB have been identified: hTFIIIB′, which is the human homologue of yeast TFIIIB component B′ (10), human BRFU (11, 12) and TFIIIB50 (13). These latter components are constituents of TFIIIBa and are required for the expression of the U6 and 7SK genes.

The aim of the work presented here was to investigate how pol III transcription is down-regulated during differentiation of F9 cells. After the induction of differentiation in these cells, it has previously been reported that the expression of pol III genes is generally impaired (14–17). The target protein, by which this repression of pol III transcription is mediated, is controversial in the literature. Based on the reduction of activity in the PCB fraction of differentiated cells (15, 17), TFIIIB or one of its TAF components (TFIIIB90) has been considered the target for this regulation. Because TFIIIB90 is a component of TFIIIBβ but not of TFIIIBa, however, it cannot be considered as the only target by which the expression of all pol III genes, including those coding for U6 RNA, is repressed. Using extracts from F9 cells differentiated in vitro for 5 days, we previously showed (16) that the activity of phosphocellulose fraction C was reduced. Based on a more efficient purification protocol and characterization of individual components of the pol III transcription system, we show here for the first time that the
activity of TFIIIC1, which is a transcription factor required for the expression of all pol III genes, is impaired during the differentiation of F9 cells.

**EXPERIMENTAL PROCEDURES**

**Plasmids**

The plasmids pUVAI, pUmU60.34, and pUPDR2 contained single copies of the genes coding for VAI RNA, mouse U6 snRNA, and a TBP-DR2-dependent transcript, respectively (18–20).

**Buffers**

Buffer A contained 20 mM HEPES (pH 7.9), 10% (v/v) glycerol, 3 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride. Buffer B contained 20 mM Tris-HCl (pH 7.9), 10% (v/v) glycerol, 5 mM MgCl₂, 3 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride. All fractions employed were dialyzed against buffer B including 60 mM KCl before use, except for the polymerase fraction (see below). After dialysis, the total protein concentration of each fraction was determined by a colorimetric protein assay (Bio-Rad).

**Preparation of Cytoplasmic Extract (HEK S100) and Purification of Transcription Factors**

**Cytoplasmic Extract (S100)**

Cytoplasmic extract from human embryonic kidney cells (HEK S100) was prepared as described by Weil et al. (21). The S100 extract was dialyzed against buffer A including 100 mM KCl and was subsequently chromatographed through phosphocellulose as described previously (22).

**PCC Fraction**

The PCC fraction (350 mM-600 mM KCl) was used to prepare human TFIIIC1, TFIIIC1-like, TFIIIC2, and PBP as described previously (3) with the following modifications.

**PBP/hTFIIIC2**—The PCC fraction was dialyzed against buffer B containing 60 mM KCl and loaded onto a MonoQ column (MQ, American Biosciences, Inc.) at 5 mg of protein/ml of bed volume. After washing the column with the same buffer, bound proteins were eluted with a linear gradient from 60 to 450 mM KCl. Fractions eluting at 300–330 mM KCl contained PBP and were devoid of TFIIIC1 and TFIIIC2 as well as TFIIIBα and RNA pol III. TFIIIC2 activity eluted from 350 to 380 mM KCl and was devoid of TFIIIC1, TFIIIBβ, and RNA pol III.

**hTFIIIC1-like/hTFIIIC**—Fractions eluting from 250 to 290 mM KCl in buffer B from MonoQ (see above) were diluted to 200 mM KCl in buffer B and applied to an EMD-SO₃⁻-Fractogel column (ESF; Merck, Darmstadt) at 5 mg of protein/ml of bed volume. After washing the column with the same buffer, bound proteins were eluted with a linear gradient from 200 to 600 mM KCl and a 1 M KCl step. hTFIIIC1 activity eluted from this column at 450 to 500 mM KCl.

**TFIIIC1-like/hTFIIIC**—Fractions eluting from 150 to 190 mM KCl in buffer B from the MQ gradient (TFIIIC0) (3) were applied to an EMD-SO₃⁻-Fractogel column at 5 mg protein/ml bed volume. After washing the column with 200 mM KCl in buffer B, bound proteins were eluted with a linear gradient from 200 to 600 mM KCl. TFIIIC1-like activity eluted from 500 to 550 mM KCl. The activity, which stimulates U6 transcription (TFIIH), eluted from 360 to 420 mM KCl.

**PCB Fraction**

The PCB fraction (350 mM KCl) was used to prepare TFIIIBα, TFIIIBβ, and RNA pol III by EMD-DEAE-Fractogel chromatography (EDF; Merck, Darmstadt) as described (3, 10). TFIIIBβ was further purified by EMD-SO₃⁻-Fractogel chromatography as described (3). The polymerase fraction was also applied to EMD-SO₃⁻-Fractogel and was step-eluted. The polymerase activity eluted in the 400–650 mM KCl fraction and was free of TFIIIBβ, TFIIIC1, and TFIIIC2 activities. 0.1 mg/ml bovine serum albumin was added, and the polymerase fraction was diluted with glycerol to a final concentration of 50% (v/v) glycerol. All fractions were stored at −80 °C.

**Recombinant Human Wild-type TBP and TBP-DR2**

Recombinant human wild-type TBP and TBP-DR2 were expressed in *Escherichia coli* and purified as described (20, 23).

**F9 Cell Line and Culture Conditions**

F9 EC cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% (v/v) penicillin-streptomycin-cinch solution (100 units/ml), and 3.7 mg of sodium hydrogen carbonate/ml. Every 2 to 3 days, cells were split by a factor of 1.5 to 1.10, seeded into new tissue culture flasks, pretreated with a sterile 1% gelatin solution. Differentiation was induced by the addition of 10⁻³ M retinoic acid, 10⁻³ M cAMP, and 10⁻⁴ M 3-isobutyl-1-methylxanthine (all from Sigma) in the same medium. Every 2 days, the medium containing the above mentioned additives was changed, and cells (PE) were harvested after 3, 4, 5, 6, and 7 days, respectively.

**In Vitro Transcription**

The *in vitro* transcription mixtures contained the appropriate protein fractions, 1 µg of plasmid DNA, 600 µM ATP, CTP, and UTP, 30 µM GTP, 3 µCi of [α-³²P]GTP (Amersham Biosciences, Inc.), 20 units of ribonuclease inhibitor (RNase Block, Stratagene), 60 mM KCl, 20 mM Tris-HCl (pH 7.9), 10% glycerol, and 5 mM MgCl₂ in a final volume of 50 µl. After a 90-min incubation at 30 °C, the RNA was purified and loaded onto a denaturing 7 M urea, 6% polyacrylamide gel. The gel was analyzed by autoradiography with intensifying screens and for quantitative analysis by a Fuji FLA-3000 Bio Imaging Analyzer.

**RESULTS**

**Polymerase III Transcription Is Reduced in the Course of Differentiation of F9 Cells**—To investigate the regulation of pol III-mediated gene expression in the course of differentiation of F9 embryonal carcinoma cells (EC) to parietal endoderm (PE), cellular extracts were prepared from such cells at various time points (3, 4, 5, and 6 days) after induction of differentiation by cAMP/retinoic acid, referred to in this article as PE(3), PE(4), PE(5), and PE(6) (see “Experimental Procedures”). The transcriptional activity of these extracts, analyzed by *in vitro* transcription of the VAI gene as template, was compared with that of an extract from undifferentiated EC cells. The results in Fig. 1 show that the decrease of pol III transcription upon differen-
tiation of F9 cells is a slow process reaching its maximum after 5 to 6 days. Three days after induction of differentiation, microscopic inspection revealed changes of the cellular morphology, which was accompanied by the reduction of transcriptional activity to about 75% of the levels detected with EC cell extracts (Fig. 1, compare lanes 1 and 2). Four days after addition of cAMP/retinoic acid, the transcriptional activity reached 30% of that in EC cells (compare lanes 1 and 5). After day 6 of differentiation a further change was observed in morphology (compact cells with long extremities). At this time point virtually no transcriptional activity (2%, lane 11) was observed in the extract of these cells. It was possible, however, to reactivate these extracts by the addition of partially purified phosphocellulose fraction PCB (lanes 3, 6, 9, and 12) and PCC (lanes 4, 7, 10, and 13) obtained from human cells (“Experimental Procedures”). In the course of these experiments it was observed that although fraction PCB partly restored transcription, supplementation with fraction PCC always led to a significantly higher degree of stimulation (see Table 1).

TFIIIC1 Activity Is Limiting for pol III Transcription in Differentiating F9 Cells—To characterize the limiting activity in phosphocellulose fraction C, cytoplasmic extracts from PE(6) cells were supplemented with individual fractions of a PCC MonoQ gradient from HEK cells (Fig. 2). This chromatographic technique clearly separates pol III transcription factors TFIIIC1-like (IIIC0), TFIIIC1, PBP, and TFIIIC2 (3, 11). The cytoplasmic extract (S100) from cells differentiated for 6 days (lane 1) was devoid of pol III transcription activity but could be reactivated by the addition of a PCC fraction from HEK cells (lane 26). Supplementation of the PE(6)-S100 extract with individual fractions of the gradient demonstrated the presence of two peaks of activity. The elution points of these peaks are characteristic for those of TFIIIC1-like (lane 7-9) and TFIIIC1 (lanes 13-16), whereas fractions of the gradient containing TFIIIC2 (lanes 20-23) revealed no stimulation.

The effects of purified TFIIIC1-like (IIIC0), TFIIIC1, TFIIIC2, and hTFIIIBβ were analyzed more systematically in S100 extracts from EC cells (lanes 1-11) as well as from PE(4) (lanes 12-22) and PE(6) cells (lanes 23-33). The results shown in Fig. 3A were quantitatively evaluated in Fig. 3B. It is evident that the reactivation of transcription activity in PE(4)- and PE(6)-S100 was observed mainly by fractions containing TFIIIC1 (lanes 16 and 17 and 27 and 28) or TFIIIC1-like (lanes 13-15 and 24-26) but not by TFIIIC2 (lanes 22 and 33). Supplementation with increasing amounts of the IIIBβ fraction alone did not significantly stimulate transcription in extracts from PE(4) cells (lanes 18-20) and only moderately stimulated transcription in extracts from PE(6) cells (lanes 29-31).

Quantitative evaluation of the results (Fig. 3B) showed that TFIIIC1-containing fractions mediated only a small effect on transcription when added to S100 extracts from EC cells. In contrast, the addition of 2 μg of TFIIIC1 to S100 extracts from PE cells differentiated for 4 days stimulated transcription by about 10-fold (Fig. 3B). Under these conditions, the addition of 5 μg of TFIIIBβ alone had no effect, and the pairwise addition of 5 μg of TFIIIBβ and 2 μg of TFIIIC1 only reached the level of TFIIIC1 alone (about 11-fold; Fig. 3B). The situation was somewhat different when extracts from cells differentiated for 6 days were examined. The addition of 2 μg of TFIIIC1 led to a 40-fold increase of transcription. If added simultaneously with 5 μg of TFIIIBβ, the degree of stimulation even rose to 60-fold.

These results indicate that up to 4 days of differentiation, cells are not deficient in TFIIIBβ activity, whereas TFIIIC1 (or TFIIIC1-like) is clearly the limiting factor. At day 6 of differentiation, TFIIIBβ also becomes limiting because the combined supplementation with purified TFIIIBβ and TFIIIC1 leads to a stimulation exceeding that achieved with TFIIIC1 alone (Fig. 3B).

The relationship between the addition of TFIIIBβ and TFIIIC1 was investigated in more detail by comparing the activities of extracts from cells differentiated for 6 (Fig. 4A, lanes 2–7) and 7 days (lanes 8–15). The results confirmed that transcription in S100 extracts was significantly reduced after 6 days (lane 2) and was virtually abolished after 7 days. Even higher amounts of extract did not yield a detectable signal (lanes 8–10). Transcription in these extracts could be reactivated efficiently by the addition of purified TFIIIC1 (lanes 5 and 13) but not by purified TFIIIBβ alone (~2-fold, lanes 6 and
Quantitative evaluation of these experiments (Fig. 4) showed that the stimulating effect of simultaneously adding TFIIIC1 and TFIIIB was even more pronounced in extracts prepared at day 7 than it was in extracts from day 6 PE cells. This indicates that TFIIIB activity declines more slowly and only becomes limiting after day 6, whereas that of TFIIIC1 is already limiting at day 4. At day 7 of differentiation microscopic examination revealed a partial necrosis of the cells, and to avoid possible artifacts, all subsequent experiments were conducted after 6 days of differentiation.

From the results presented in Fig. 1 it was deduced that phosphocellulose fraction B was capable of partially complementing transcription activity in extracts from differentiating F9 cells. In contrast, the results shown in Figs. 3 and 4 demonstrate that purified TFIIIB was not primarily the limiting component. Because this issue is a subject of controversy in the literature (15–17), we further purified the PCB fraction by EDF anion exchange chromatography and subsequently by cation exchange chromatography on ESF gradients. Individual fractions were analyzed in two separate experimental conditions assaying for the activity of either TFIIIC1 (Fig. 5A) or TFIIIB (Fig. 5B), respectively. The same protein fractions were also tested for their ability to reconstitute transcription in extracts from differentiated F9 cells. The results clearly showed that the reconstituting activity co-elutes with the activity of TFIIIC1 (corresponding to fraction 12 of the gradient, 450–480 mM KCl) but not with the activity of TFIIIB (fractions 2, 4, and 6, 290–360 mM KCl). Characterization of fractions of this
gradient by immunoblots (data not shown) revealed that TBP eluted in fraction 4, which corresponds to the elution point of TFIIIB/H9252, whereas fraction 12, corresponding to TFIIIC1, was devoid of TBP. These results indicate that phosphocellulose fraction B and TFIIIB/H9252 eluted from EDF are contaminated with TFIIIC1 activity. In contrast, the TFIIIC1 fraction, eluted from MQ, is devoid of TFIIIB/H9252 (data not shown).

We have previously shown that differentiation of F9 cells also led to a drastic impairment of U6 gene transcription, which could be reactivated by supplementation with phosphocellulose fraction C (16). These results were confirmed and extended by the data presented in Fig. 6, documenting that only the TFIIIC1 fraction (lanes 14–16) was able to substantially restore U6 transcription in extracts obtained from PE(6) cells (lanes 9–16) or PE(4) cells (data not shown). TFIIU, PBP, or TFIIIBa alone (lanes 10–12) were not able to reactivate U6 transcription in extracts from cells differentiated for 6 days. However, the possibility cannot be excluded that one or more of these factors may also be limiting, because in the absence of sufficient quantities of TFIIIC1 such a conceivable deficit would remain undetected. In agreement with our previous results (6), the addition of TFIIIBβ had no effect on U6 transcription (lanes 5 and 13), and hence TAF TFIIIB90 cannot be the limiting component for transcription of the U6 gene in PE cells.

Because it has been reported (17) that TBP also declined during F9 differentiation, we investigated this question by experiments using a variant type 3 promoter, which is not recognized by wild-type TBP (Fig. 6, lanes 17–28). This promoter instead essentially requires a mutant form of TBP (TBP-DR2), whereas all of the remaining transcription factors (TFIIIB/H9252, PBP, TFIIIC1, and TFIIIC2) are identical to those involved in the expression of the U6 gene (20). Using the TBP-DR2-dependent promoter, we were able to analyze the transcription of 5'-regulated pol III genes regardless of the endogenous TBP activity in the F9 extracts. The results showed that in the absence of TBP-DR2 no transcription was observed in extracts from EC (lanes 17 and 18) or PE(6) cells (lanes 23 and 24). The addition of TBP-DR2 resulted in transcription of this construct by EC extracts (lane 19) but not by PE(6) extracts (lane 25). These results demonstrate that TBP is not the limiting component in PE extracts, which required additional complementation with a TFIIIC1 fraction for active transcription (lanes 26–28).

Chromatography of Extracts from EC and PE(6) Cells Identifies TFIIIC1 as the Limiting Activity—Supplementation experiments with purified human transcription factors from HEK cells identified TFIIIC1 as the limiting component in cellular extracts derived from differentiated F9 cells (Fig. 2–4 and 6). To further compare the activities of TFIIIBβ, TFIIIC1, and TFIIIC2 in EC versus PE cells, the transcription factors were purified from these cells on an analytical scale. For this purpose 150 mg of cytoplasmic extracts from EC and PE(6) cells...
Fig. 5. Separation of TFIIIBβ from TFIIIC1 by EMD-SO₄²⁻ cation exchange chromatography. TFIIIBβ fractions (EMD-DEAE, 230–380 mM KCl) containing the PE-stimulating activity were further purified by EMD-SO₄²⁻ chromatography with a gradient from 200 to 600 mM KCl. The resulting fractions (BEDF/SO₄²⁻) were analyzed either in a TFIIIC1-free in vitro transcription system (A, lanes 1–13) or in a TFIIIBβ-free transcription system (B, lanes 1–13) reconstituted with PCB (30 µg) and MQ-TFIIIC2 (2 µg) or in a TFIIIBβ-free transcription system (B, lanes 1–13) reconstituted with PCC (20 µg). As positive controls, purified MQ-TFIIIC1 (A, lanes 13) or purified TFIIIBβ (B, lane 13) was tested in the corresponding transcription systems. C, to identify the activity that stimulates the S100 from PE(6) cells, 100 µg of this extract were supplemented with individual fractions from the EMD-SO₄²⁻ gradient and analyzed by in vitro transcription of the VAI gene.

were purified according to the established protocol. After phosphocellulose chromatography, step-eluted fractions PCA (100 mM KCl), PCB (350 mM KCl), PCC (600 mM KCl), and PCD (1 M KCl) were obtained. The resulting PCB fractions, which contained TFIIIBβ as well as RNA polymerase III, were dialyzed against buffer B and chromatographed in parallel on EDF anion exchange columns eluted with a gradient from 60 to 450 mM KCl (BEDF). Fig. 7, A and B, reveals the activity of different fractions of the gradient in appropriately reconstituted in vitro transcription systems. The results clearly show that activity is found in a region of the gradient associated with TFIIIBβ (270 mM KCl) for both cases. The experiment is based on two parallel column runs, and comparative quantitation is possible therefore only as a first approximation. Based on the positive controls (lanes 13 in Fig. 7, A and B) it can be estimated that TFIIIBβ is reduced about 2-fold, demonstrating that TFIIIBβ (or one of its components) is certainly not inactivated in these cells.

The phosphocellulose fractions containing TFIIIC1 and TFIIIC2 from EC and PE(6) cells were further purified by MonoQ anion exchange chromatography according to the established purification scheme and tested for activity in complementation assays. The multiprotein complex TFIIIC2 could be isolated from both cell types with comparable activity (compare lanes 4–6 in Fig. 8, A and B). In contrast, transcriptionally active TFIIIC1 could only be isolated from undifferentiated EC cells and not from corresponding fractions obtained from cells differentiated for 6 days (compare lanes 6–8 in Fig. 8, C and D). These results clearly show the lack of TFIIIC1 activity in PCC MonoQ fractions of differentiated cells and confirm the results obtained from the above mentioned supplementation experiments.

DISCUSSION

In the course of in vitro differentiation of F9 embryonal carcinoma cells to parietal endoderm, pol III expression progressively declines. This down-regulation can be monitored by in vitro transcription in extracts from cells differentiated for various time periods, and 6 days after induction of differentiation it reaches a residual level of only ~2% in comparison to that of the undifferentiated cells (Fig. 1). It could previously be shown that all class III genes are affected by this process (15, 16).

TFIIIB (or one of its TAFs) was discussed previously as the main target through which this transcription regulation is mediated (15, 17). Alzuherri and White (17) investigated cells that were differentiated for periods of 5–9 days and found that extracts from these cells could be reactivated by ~5-fold by a TAF-containing fraction obtained by chromatography of a PCB fraction over immobilized TBP. According to these authors (17) this fraction still revealed multiple polypeptides upon silver staining so that the identity of the stimulating activity remained uncertain in these studies. Because our complementation experiments showed that reactivation of transcription in these extracts could be obtained with both phosphocellulose fractions B and C, the question remained whether TFIIIB is the only component involved. By supplementation with highly purified pol III transcription factors, we attempted to clarify this question.

Our results (Figs. 2 and 3) show for the first time that the addition of highly purified TFIIIC1 is able to substantially reactivate the impaired transcription activity in extracts of differentiated F9 cells. These results thus prove that TFIIIC1 is the main limiting activity for pol III-mediated transcription in these extracts, particularly in the early stages of differentiation.

These results are only apparently in contrast to those of Alzuherri and White (17), who reported a lack of reactivation of PE extracts after the addition of affinity-purified TFIIIC. The discrepancy between their study and our results can readily be reconciled by the fact that the B-box binding component only reflects TFIIIC2, and therefore no conclusions can be drawn with regard to the activity of TFIIIC1 in the different extracts. Our results also document that TFIIIC2 was not able to reactivate transcription in extracts of differentiated cells (Figs. 2 and 3) and hence was not limiting in PE cells.

Previous investigations showed a reduction of the binding activity of TFIIIC and PBP in phosphocellulose fraction C of differentiated F9 cells (16). This crude fraction contains TFIIIC1 in addition to the DNA-binding components TFIIIC2 and PBP, and because TFIIIC1 enhances the specific formation of protein-DNA complexes (3, 24), the reduction of the TFIIIC1...
activity in the course of differentiation can therefore also lead to a reduced formation of specific protein DNA complexes.

In agreement with previously published data (17), we found that extracts from differentiated cells can be stimulated ~2–5-fold by a crude PCB fraction from HEK cells. Further purification revealed that the stimulating activity in the PCB fraction co-elutes with TFIIIB upon anion exchange chromatography but could be separated from TFIIIB by subsequent cation exchange chromatography (ESF, Fig. 5, A–C). The fractions that reactivate pol III-mediated transcription in extracts from PE cells show TFIIIC1 activity in a reconstituted transcription system and, more importantly, contain no TFIIIB as evidenced by Western blot against TBP (data not shown). The presence of TFIIIC1 activity in the PCB fraction was previously reported and was defined as TFIIIC1 or TFIIIB, respectively (24). The TFIIIB fraction used by Alzuheri and White (17) to reconstitute transcription in differentiated F9 cells was purified by anion exchange chromatography (MonoQ), but the preparation is not described in detail in this paper. Instead, reference is made to an earlier paper from their laboratory (25) according to which the TFIIIB fraction was obtained by elution with an 0.48–0.56 M KCl from a MonoQ gradient. It is generally agreed, however, that this protein fraction does not represent the TBP-TAF components as evidenced by Western blot against TBP (data not shown). The presence of TFIIIC1 in their system, and therefore a direct comparison with our results is not possible.

The results from the complementation assays utilizing purified TFIIIB require particular attention. We used a highly purified TFIIIB fraction, which was free of contaminating TFIIIC1 activity, and showed that it had at most a 2-fold stimulating effect on pol III-mediated transcription in PE extracts (Figs. 3 and 4). Particularly in earlier stages of differentiation (until day 4), down-regulation of pol III transcription can almost be fully compensated by the re-addition of purified TFIIIB, which was free of contaminating TFIIIC1 activity (Fig. 4B). This effect is even more pronounced at day 7 of differentiation (Fig. 4B). These results demonstrate that TFIIIC1 is the limiting component for pol III-mediated transcription during early stages of F9 cell differentiation and suggest that at later stages the activity of TFIIIB is also reduced.

TFIIIB (or one of its TAFs) has been described to be the target by which regulation of pol III expression is achieved in other experimental systems. For example, by using murine fibroblasts either containing or lacking the retinoblastoma protein (Rb), it was demonstrated that Rb represses transcription

FIG. 6. Complementation of 5′-regulated pol III gene transcription activity in cytoplasmic extracts from F9 PE(6) cells with TFIIIC1. 100 μg of F9 EC-S100 (lanes 1–8, 17–22) or PE(6)-S100 (lanes 9–16, 23–28) were supplemented either with TFIIIC1 (1 μg; ESF), PBP (2 μg; MQ), TFIIIB (5 μg; ESF), or increasing amounts of TFIIIC1 fraction (0.5–2 μg; MQ) as indicated and analyzed by in vitro transcription of the U6 gene (lanes 1–16). Samples shown in lane 17–28 contained pUPDR2 (see “Experimental Procedures”) as the DNA template and were supplemented either with wild-type TBP (wtTBP) (20 ng; lanes 18 and 24) or with TBP-DR2 (20 ng; lanes 19–22 and 25–28). Lanes 20–22 and 26–28 show the supplementation of S100 extracts from F9 EC and PE(6) cells in the presence of TBP-DR2 with a TFIIIC1 fraction (0.5–2 μg; MQ).

FIG. 7. Purification of active TFIIIBβ from S100 extracts of EC and PE(6) cells. TFIIIBβ activity was purified from 150 mg of S100 extracts of EC as well as PE(6) cells by PCB and EMD-DEAE chromatography as described under “Experimental Procedures.” The resulting fractions from both EDF gradients were analyzed in a TFIIIBβ-free in vitro transcription system based on purified TFIIIC1, TFIIIC2, and RNA polymerase III from HEK cells. Panel A shows the resulting activity from EC cells, and panel B reveals the activity prepared from PE(6) cells. As controls, the TFIIIBβ-free transcription system was tested either without (lane 12) or with the addition of purified hTFIIIBβ (lane 13).
expression after cell cycle arrest in G1 or G2/M also identified a comparable mechanism has also been reported for the repression of TFIIIB activity was also identified. In these experiments, modulation of TFIIIB activity was with (A and B, lane 10; C and D, lane 11) or with (A and B, lane 11; C and D, lane 12) the addition of purified hTFIIIC2 or hTFSIIC1, respectively.

Although TFIIIB remains incompletely characterized in higher organisms, there is general consensus that different TFIIIB complexes are required for the transcription of type 2 and type 3 pol III promoters. In previous studies addressing the regulation of pol III-transcribed genes, the different protein complexes TFIIIBa and TFIIIBβ have thus far been incompletely considered. Expression of the U6 snRNA gene is clearly repressed in the course of differentiation of F9 cells (16), and it has recently been reported that TFIIIB90 levels decrease substantially in the course of this process (17). According to these authors, the decrease in the abundance of this essential TAF seems sufficient to account for the down-regulation of class III gene expression that accompanies PE formation. However, because TFIIIB90 is not required for the transcription of type 3 pol III promoters (6, 10, 13, 29), the down-regulation of these genes cannot be mediated by a mere inactivation of TFIIIB90. Therefore, repression of all pol III genes during PE formation must necessarily involve additional components, and we show here (Fig. 6) that TFIIIC1 restores U6 transcription in PE cell extracts.

We have also studied the role of endogenous TBP in the course of F9 cell differentiation. By using a pol III promoter construct, which does not depend on wild-type TBP for its expression but instead utilizes a mutant form of TBP, we could show that TFIIIC1 but not TBP accounts for the down-regulation of the type 3 gene expression that accompanies PE formation.

In addition to RNA polymerase III and TBP, TFIIIC1 is essential for the transcription of all pol III genes including those regulated by 5'-located promoter elements (3, 30). The partial purification of the individual pol III transcription factors from EC and PE cells clearly shows a lack of TFIIIC1 activity in the extracts of differentiated cells. The reactivation of pol III transcription in PE extracts by complementation with TFIIIC1 is therefore not an indirect effect but shows that TFIIIC1 activity is lacking in these cells, whereas TFIIIBβ and TFIIIC2 remain active during the differentiation process. The molecular architecture of TFIIIC1 has hitherto not been deciphered, and its potential interactions with components of TFIIIB (e.g., TFIIIB150 as a subunit of TFIIIBβ and TFIIIBa) and/or pol III hence remain speculative. Future cloning of the subunits of TFIIIC1 will allow a direct quantitation of TFIIIC1 and its subunits by reverse transcription-PCR or Western blot analysis and will therefore be of particular importance.

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