Regulation of RNA Polymerase I Transcription in Response to F9 Embryonal Carcinoma Stem Cell Differentiation*

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Hadi M. Alzuherri and Robert J. White‡
From the Institute of Biomedical and Life Sciences, Division of Biochemistry and Molecular Biology, Davidson Building, University of Glasgow, Glasgow G12 8QQ, United Kingdom

Dramatic changes in the patterns of transcription are a common feature of early development. We have used F9 embryonal carcinoma cells as a model system to study gene regulation during an early stage of murine embryogenesis. We find that transcription by RNA polymerase I decreases when F9 cells differentiate into parietal endoderm. The reduced rate of transcription is associated with a down-regulation of several components of the class I transcription apparatus. The most substantial change involves the essential factor SL1, which is a multisubunit complex that contains the TATA-binding protein and three TATA-binding protein–associated factors (TAFs). The abundance of two of these TAFs, TAFI95 and TAFI48, decreases during F9 cell differentiation. Developmental regulation of a specific class of genes may therefore be achieved through changes in the availability of TAFs.

The early stages of mouse development involve rapidly changing patterns of transcription, which play a key role in embryogenesis. These events are inaccessible to study at the biochemical level because of the difficulty in obtaining homogeneous cell populations in sufficient numbers. One way to circumvent this problem involves the use of embryonal carcinoma (EC)† cell lines, which mimic events within the early embryo. A well characterized example is the F9 EC cell line, which can be induced to differentiate into parietal endoderm (PE) by treatment with retinoic acid and cAMP (1). Differentiation of F9 cells is accompanied by a dramatic decrease in the rate of transcription by RNA polymerase (pol) III (2). This response appears to provide an accurate reflection of regulatory phenomena that occur in early development because in situ hybridization has demonstrated that the abundance of pol III transcripts decreases substantially when PE forms in mouse embryos (3). By using F9 cells as a model system, it has been possible to demonstrate that the decrease in pol III transcription that accompanies differentiation into PE reflects a specific decrease in the abundance of a transcription factor called TFFIIB (2, 4).

Ribosomogenesis requires equimolar amounts of each of the rRNAs. Because 5 S rRNA is made by pol III, whereas the remaining rRNAs are made by pol I, these polymerases are frequently regulated coordinately (5, 6). We have therefore investigated whether the decrease in pol III transcription that occurs during F9 cell differentiation is accompanied by a similar change in pol I activity. Our results indicate that PE cells contain less rRNA than F9 EC cells, and this reflects a reduced rate of transcript initiation. Changes are observed in several components of the pol I transcription machinery. PE cells display a slight decrease in the catalytic activity of the polymerase itself. They also show a slight reduction in the level of the basal factor UBF, which binds to rRNA promoters and stimulates expression by folding the DNA and excluding repressor proteins (7, 8). An additional role for UBF is to interact with an essential initiation factor called SL1 or TIF-IB and stabilize its association with the promoter (5, 8, 9). SL1/TIF-IB is a complex containing the TATA-binding protein (TBP) and three TBP–associated factors (TAFs) (5, 10). The abundance of two of these TAFs, TAFI95 and TAFI48, is significantly depressed following F9 cell differentiation. As a consequence, the activity of SL1/TIF-IB is severely limiting for pol I transcription in PE cell extracts. We conclude that the differentiation of F9 EC cells is accompanied by changes in several components of the pol I transcription machinery, which result in decreased production and steady-state levels of the large rRNA.

EXPERIMENTAL PROCEDURES

Cell Culture—F9 cells were cultured and differentiated as previously described (2).

RNA Extraction and Analysis—Total cellular RNA was extracted using TRI reagent (Sigma), according to the manufacturer’s instructions. Agarose gel electrophoresis, Northern transfer, and hybridization were conducted as previously described (2). Primer extension analysis was carried out by the method of Carey et al. (11). The primer was 5′-CAGGCCAACCGCGACAGACCCAAG-3′, which is complementary to bases +100 to +122 of the mouse rRNA gene.

Preparation of Extracts and Protein Fractions—Whole cell extracts were prepared according to the method of Manley et al. (12) and also by a more direct freeze-thaw procedure (13). Both approaches yielded similar results.

Phosphocellulose chromatography was carried out by the method of Segal et al. (14), except that the final high salt elution was made using 1.2 M KCl instead of 1.0 M. SL1/TIF-IB was present in this 0.6–1.2 M KCl step fraction (PC-D), which contained ~1% of recovered protein. UBF and TFFIIB were present in the 0.35–0.6 M KCl step fraction (PC-C). Heparin gradient chromatography of SL1/TIF-IB was carried out as previously described (15). Human UBF was fractionated by gradient chromatography of a HeLa nuclear extract on Q-Sepharose, performed as previously described (16), and it was found to elute at 500 mM KCl, away from the bulk of protein. HeLa UBF was also fractionated by heparin chromatography of a PC-C fraction to give the Chepl1.0 fraction (13).

Transcription Assays—Transcription reactions were carried out as previously described (2), except that pBR322 was not included, the incubations were for 60 min, and α-amanitin was present at a final concentration of 200 μg/ml. The pol I template was pMrWT, which...
Down-regulation of Pol I Transcription during F9 Cell Differentiation

The Level of Pol I Transcripts Decreases After F9 Cell Differentiation—Total RNA was extracted from undifferentiated F9 EC cells and from cells that had been induced to differentiate into PE by culture in the presence of retinoic acid and dibutyryl cAMP. The abundance of the 28 S rRNA product of pol I was then compared in F9 EC and PE cells by Northern blot analysis (Fig. 1A). After differentiating for 5 days (lane 2), the 28 S rRNA was found to be 2.7-fold less abundant than in undifferentiated EC cells (lane 1), and after 7 days of differentiation its level had decreased by 4.0-fold (lane 3). This effect is specific, because the steady-state level of tRNA remains unchanged, as shown previously (2).

Large rRNA is synthesized as a 47 S precursor molecule, the 5′ end of which is degraded rapidly (22). Because the sequences at the start of the primary transcript are highly unstable, their level in the cell reflects the rate of ongoing initiation by pol I. Primer extension analysis with various amounts of total RNA was used to quantitate the 5′ end of the primary rRNA transcript and test whether its abundance is altered by differentiation (Fig. 1B). PhosphorImager quantitation revealed that the level of the pre-rRNA 5′ sequences is 3–5-fold higher in EC cells relative to PE cells. We conclude that a decrease in the rate of transcript initiation can account for the reduced steady-state levels of large rRNA in differentiated PE cells.

Pol I Transcription Apparatus Becomes Less Active following F9 Cell Differentiation—To compare the activity of the pol I transcription apparatus before and after differentiation, whole cell extracts were prepared and assayed for their ability to initiate transcription on a rRNA gene template. EC cell extracts were found to support much higher levels of pre-rRNA synthesis than extracts prepared in parallel from PE cells (Fig. 2A). This was the case with several sets of extracts that had been prepared using either of two different extraction protocols. EC cell extracts were more active than PE cell extracts when these assays were carried out over a range of protein/DNA ratios. Quantitation of these experiments indicated that the activity of the pol I transcription initiation apparatus is 6–9-fold higher in EC extracts than in PE extracts. It is unclear why the EC/PE differential is greater in vitro than in vivo. As a control for the specificity of these effects, we assayed the ability of F9 cell extracts to support pol II initiation from an HPRT promoter. Previous work has demonstrated that HPRT transcription is not decreased after F9 cell differentiation (2). Consistent with this finding, we found that EC and PE extracts initiate transcription at the HPRT promoter with comparable efficiencies (Fig. 2B). We conclude that the observed decrease in initiation by pol I is a specific regulatory event.

The Low Rate of Pol I Transcription Initiation in PE Cells Is Not Caused by an Excess of Dominant Repressor—The above-described results suggest that the ability of pol I to initiate transcription is greater in EC than in PE cells. To determine whether this reflects the production of an excess of dominant repressor following differentiation, we performed mixing experiments (Fig. 3). Titrating increasing amounts of PE extract into a constant amount of EC cell extract did not diminish the level of transcription that was obtained using EC extract alone. This mixing approach therefore provided no evidence for a dominant repressor of pol I transcription that is present in PE extracts in stoichiometric excess. We therefore addressed the possibility that the reduced rate of rRNA synthesis following differentiation is the result of a decrease in the activity of one or more components of the pol I basal transcription apparatus.

Several Components of the Pol I Transcription Apparatus Are Down-regulated during F9 Cell Differentiation—To determine whether differentiation is accompanied by a change in the catalytic capacity of pol I, we carried out random polymerization assays using poly(dA-dT) as template. EC extracts were found consistently to have slightly higher pol I catalytic activi-

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2 H. Alzuberry and R. J. White, unpublished data.
ity than PE extracts that were prepared in parallel. However, the difference between matched pairs of extracts was only 1.6–2.2-fold. Although this change in pol I activity may contribute to the observed regulation of rRNA synthesis, it seems insufficient to account fully for the reduced levels of pol I transcription that are found in PE cell extracts.

To examine whether differentiation affects the abundance of UBF, we carried out Western blots with EC and PE cell extracts (Fig. 4A). The amount of UBF was found to decrease by 1.7–3.1-fold after differentiation, with an average difference of ~2.5-fold. Similar changes in UBF levels were also detected when EC- and PE-derived fractions were compared after chromatography. To examine whether there is a generalized reduction in the level of transcription factors following F9 cell differentiation, the same extracts were probed for the presence of TFIIB, and the amount of this basal pol II factor was found to remain virtually constant when the EC and PE cell extracts were compared (Fig. 4B). We also found that the abundance of the pol III factor TFIIIC does not decrease when F9 cells differentiate (2, 4). We conclude from these data that the observed reduction in the level of UBF is a specific phenomenon.

The Activity of SL1/TIF-IB Decreases When F9 Cells Differentiate and Is Limiting for Pol I Transcription in PE Cell Extracts—If the reduced transcriptional activity of PE cell extracts is caused by a lack of UBF, it should be possible to stimulate expression by adding more UBF. However, we found that titrating in partially purified UBF made little difference to the rate of pol I transcription in PE-derived extracts (Fig. 5A). In contrast, an SL1-containing fraction tested in parallel produced a substantial increase in the rate of rRNA synthesis. Indeed, this fraction was sufficient to raise the rate of transcription in a PE cell extract to levels obtained using EC cell extracts. These data suggest that there is a relative excess of UBF activity following F9 cell differentiation, despite its decrease in abundance. Instead, the transcription of rRNA genes appears to be limited in PE extracts by a lack of SL1/TIF-IB activity. A rather different response was obtained when EC cell
The steady-state level of 28S rRNA is significantly lower in F9 PE cells than in the undifferentiated EC progenitors. This decrease reflects a reduction in the activity of the pol I transcription apparatus and a diminished rate of transcript initiation from F9 EC cells, efficient transcription of the mouse gene was obtained, whereas the corresponding fraction from PE cells was significantly less active when tested in parallel (Fig. 6B). Although 0.36 μg of the EC-derived fraction was sufficient to give a signal in this assay (lane 2), approximately four times more of the PE-derived fraction was required to cross the detection threshold (lane 7). We conclude that F9 cell differentiation is accompanied by a significant decrease in the activity of SL1/TIF-IB.

DISCUSSION

Down-regulation of Pol I Transcription during F9 Cell Differentiation

FIG. 5. The activity of SL1/TIF-IB is limiting for pol I transcription in PE cell extracts. A, a pMrWT template (20 ng) was transcribed using 9 μg of EC (lane 1) or PE (lanes 2–7) cell extract. Reactions were supplemented with 1.5 or 2.6 μg of Q-Sepharose-fractionated human UBF (lanes 3 and 4, respectively) or with 0.6, 1.1, or 2.0 μg (lanes 5, 6, and 7, respectively) of the EC-derived SL1 fraction (PC-D). B, a pMrWT template (20 ng) was transcribed using 8 μg of EC (lanes 1–7) or PE (lanes 8–14) cell extract. Reactions were supplemented with 0.7 (lanes 2 and 9), 1.5 (lanes 3 and 10), or 2.6 μg (lanes 4 and 11) of Q-Sepharose-fractionated human UBF or with 0.5 (lanes 5 and 12), 0.9 (lanes 6 and 13), or 1.6 μg (lanes 7 and 14) of the EC-derived SL1 fraction (PC-D).

extracts were tested in the same way (Fig. 5B). Although adding the SL1/TIF-IB fraction produced a slight increase in transcription by the EC extract, the UBF fraction gave a much more dramatic stimulation (lanes 1–7). In contrast, when tested in parallel, PE extract again responded to SL1/TIF-IB but not to the UBF fraction (lanes 8–14). These results provide evidence for a shift in the balance of the pol I factors as F9 cells differentiate; the UBF fraction stimulates transcription in EC extracts but not in PE extracts, whereas the activity of SL1/TIF-IB becomes severely limiting after differentiation.

We carried out complementation assays to test whether the activity of SL1/TIF-IB is diminished in PE cell extracts. The assay system exploited the fact that SL1 from humans is unable to function on a mouse rRNA promoter, whereas the other components of the human pol I transcription apparatus are fully active on a murine template (15, 17). A HeLa cell extract is therefore unable to transcribe a mouse rRNA gene unless a source of murine SL1/TIF-IB is provided. The addition of 2 μg of EC cell extract was sufficient to allow expression from the murine promoter in this system. In contrast, 10 μg of PE cell extract was required to give a comparable signal when tested in parallel (Fig. 6A). Because the HeLa extract used in this assay provides an excess of the other pol I factors, the relative inactivity of the PE cell extract must be because of a deficiency in the species-specific factor SL1/TIF-IB. As a further test of this conclusion, we used phosphocellulose chromatography to purify this factor approximately 100-fold from EC and PE cell extracts. These fractions were then compared for their ability to reconstitute transcription from the murine promoter in the complementation assay. When we added the SL1/TIF-IB fraction from F9 EC cells, efficient transcription of the mouse gene was obtained, whereas the corresponding fraction from PE cells was significantly less active when tested in parallel (Fig. 6B).

The Abundance of TAF95 and TAF48 Decreases Specifically When F9 Cells Differentiate—Western blots were carried out to compare the abundance of each of the four components of SL1/TIF-IB in EC and PE extracts. The overall level of TBP was found to be lower following differentiation (Fig. 7A). However, previous analyses have shown that this decrease in TBP is associated primarily with the down-regulation of TFIIB and that the TBP content of fractions containing SL1/TIF-IB and TFIID remains relatively constant (4). Nevertheless, the abundance of TAF48 (Fig. 7B) and TAF95 (Fig. 7C) was also found to be lower in the PE cell extracts than in the EC cell extracts. In contrast, the abundance of TAF68 remains unchanged following differentiation (Fig. 7D).

Western blot analysis was also conducted on phosphocellulose fractions containing SL1/TIF-IB that had been partially purified from EC and PE cells. As in the crude extracts, the fractions showed little change in the levels of TAF68 (Fig. 8A, upper). Furthermore the TBP content of these fractions changed little during differentiation (Fig. 8A, lower), as reported previously (4). However, both TAF95 (Fig. 8B) and TAF48 (Fig. 8C) were much less abundant in the fraction derived from PE cells compared with the equivalent EC-derived fraction, as seen in the crude extracts. For each subunit of SL1/TIF-IB, these results were confirmed using a second antisera raised against an alternative epitope (for TBP and TAF95, three separate antisera were used). The results suggest strongly that F9 cell differentiation is accompanied by a specific decrease in the abundance of two TAF components of SL1/TIF-IB.
pol II initiation at the HPRT promoter show little or no difference between EC and PE cells.

A previous study using rat L6 myoblasts has shown that the abundance of UBF decreases during myogenic differentiation (23). It may therefore be that a reduction in UBF levels is a common feature of differentiation in rodent cells. UBF is also a target for regulation during differentiation of the human monocytic cell line U937 (24). In U937 cells, however, UBF is regulated by interaction with the retinoblastoma protein, rather than a change in abundance (24). Genetic analysis has shown that ratinoblastoma protein is important for hematopoiesis but is not required for the early differentiation events that are mimicked by F9 cells (25).

Despite the changes in UBF, the reduced rate of pol I transcription that is found in PE cells appears to be caused primarily by a decrease in the availability of SL1/TIF-IB. UBF activity is in relative excess after differentiation, as shown by the fact that adding more UBF to PE extracts does not increase the level of expression. Instead, add-back experiments suggest that SL1/TIF-IB is the limiting component of the pol I transcription apparatus. We cannot be certain that SL1/TIF-IB is sufficient to restore active transcription to PE cell extracts, as our fractions contain traces of UBF. It is therefore possible that both SL1/TIF-IB and UBF are necessary to raise transcription in the PE cell extracts to levels obtained using EC cell extracts. Nevertheless, SL1/TIF-IB appears to be critical for this effect, because fractions that lack this factor but contain active UBF

**Fig. 6.** The activity of SL1/TIF-IB is diminished following F9 cell differentiation. A, a pMrWT template (20 ng) was transcribed using 10 µg of HeLa nuclear extract supplemented with 2, 4, 6, or 8 µg of EC cell extract (lanes 2–5, respectively) or with 2, 4, 6, 8, 10, or 12 µg (lanes 7–12, respectively) of PE cell extract. B, a pMrWT template (20 ng) was transcribed using 10 µg of HeLa nuclear extract supplemented with 2, 4, or 6 µl (lanes 2–4, respectively) of an EC cell-derived SL1 fraction (PC-D) or with 6, 8, 10, 12, 14, or 16 µl (lanes 6–11, respectively) of the SL1 fraction (PC-D) derived from PE cells. Both PC-D fractions had a protein concentration of 0.18 mg/ml.

**Fig. 7.** The abundance of specific SL1 subunits decreases during differentiation. A, whole cell extract (56 µg) of EC (lane 1) or PE (lane 2) cells was resolved on an SDS-7.8% polyacrylamide gel and then analyzed by Western immunoblotting with antibody SL30 against TBP. B, whole cell extract (56 µg) of PE (lane 1) or EC (lane 2) cells and the HeLa cell PC-D fraction (5.6 µg, lane 3) were resolved on an SDS-7.8% polyacrylamide gel and then analyzed by Western immunoblotting with antibody against TAF48. C, SL1 prepared from an EC extract by gradient chromatography on heparin (24 µg, lane 1) and whole cell extract (56 µg) of EC (lane 2) or PE (lane 3) cells were resolved on an SDS-7.8% polyacrylamide gel and then analyzed by Western immunoblotting with antibody against TAF68.
produce no stimulation when added to the PE cell extract. The requirement for SL1/TIF-IB in these add-back experiments is consistent with the substantial down-regulation of this factor, which is detected both in complementation assays and also by immunoblotting.

Complementation assays with crude extracts or phosphocellulose fractions reproducibly show that the activity of SL1/TIF-IB decreases by 4-5 fold following differentiation. Despite this finding, the abundance of TAF$_{68}$ remains virtually constant, the maximal change observed being 1.2-fold. In contrast, substantial decreases are observed with TAF$_{95}$ and TAF$_{48}$. Accurate quantitation of the changes in these TAFs is difficult because of their low abundance following differentiation. When various extracts and fractions are compared, we find that the levels of TAF$_{95}$ and TAF$_{48}$ can decrease by anything from 3- to 13-fold after differentiation. It is difficult to be confident in these numbers, however, because of the very low signal obtained when Western blotting some PE cell extracts and fractions. Nevertheless, we are able to conclude that there is a specific and substantial reduction in the abundance of these subunits. SL1/TIF-IB is an essential component of the pol I machinery that is involved in promoter recognition and polymerase recruitment (5). Although murine SL1/TIF-IB activity has yet to be reconstituted from isolated subunits (21), both TAF$_{48}$ and TAF$_{110}$ (the human equivalent of TAF$_{95}$) are essential for transcription in the human system (26, 27). It therefore seems highly probable that the decreased abundance of TAF$_{48}$ and TAF$_{95}$ in PE cells can account for the reduction in SL1/TIF-IB activity that is detected by complementation assays.

To our knowledge, this constitutes the first report that SL1/TIF-IB is subject to developmental control. However, tissue-specific changes in TAFs have been described previously. Differentially human B cells contain a TAF component of TFIIID that is not found in other cell types and may contribute to B cell-specific patterns of pol II transcription (28). Furthermore, a set of TAFs becomes restricted to the developing nervous system during Drosophila development (29). Previous studies have also provided precedent for TAF regulation during differentiation of F9 cells. PE extracts were shown to contain substantially reduced levels of the pol III-specific TBP-containing complex TFIIIB (2, 4). This reflects a decrease in the abundance of a TFIIIB TAF called BRF (TFIIB-related factor) (4). As a result of this change, the rate of pol III transcription is considerably less in PE than in EC cells or extracts (2, 4). Because the pol III product 5 S rRNA is required in equimolar amounts for the synthesis of the 18 S rRNA, a reduction in the rate of pol III transcription must occur in PE cells.

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Down-regulation of Pol I Transcription during F9 Cell Differentiation

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