RESEARCH ARTICLE

Reconstitution of human rRNA gene transcription in mouse cells by a complete SL1 complex

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ABSTRACT

An important characteristic of the transcription of a ribosomal RNA gene (rDNA) mediated by DNA-dependent RNA polymerase (Pol) I is its stringent species specificity. SL1/TIF-IB is a key complex for species specificity, but its functional complex has not been reconstituted. Here, we established a novel and highly sensitive monitoring system for Pol I transcription to reconstitute the SL1 activity in which a transcript harboring a reporter gene synthesized by the influenza virus RNA-dependent RNA polymerase. Using this monitoring system, we reconstituted Pol I transcription from the human rDNA promoter in mouse cells by expressing four human TAFIs in the SL1 complex. The reconstituted SL1 active transcription in mouse A9 cells carrying an inactive human chromosome 21 that contains the rDNA cluster. Chimeric SL1 complexes containing human and mouse TAFIs could be formed, but these complexes were inactive for human rDNA transcription. We conclude that four human TAFIs are necessary and sufficient to overcome the barrier of species specificity for human rDNA transcription in mouse cells.

KEY WORDS: Ribosomal RNA gene, Transcription, Species specificity, RNA polymerase I, SL1, TIF-IB

INTRODUCTION

One of the most important rDNA transcription issues to be addressed is the molecular mechanism of its stringent species specificity (Heix and Grummt, 1995). Three decades ago, Grummt et al. demonstrated that in vitro transcription of human and mouse rDNAs requires completely homologous extracts (Grummt, 1981; Grummt et al., 1982), i.e. human rDNA is not transcribed in mouse cell extracts and vice versa. This distinct promoter recognition specificity of Pol I is mediated by a multi-subunit factor called SL1 in humans and TIF-IB in mice. SL1/TIF-IB recruits Pol I on the rDNA promoter through RRN3/TIF-IA and stabilizes the binding of the upstream binding factor (UBF) at the rDNA promoter (Friedrich et al., 2005; Moss and Stefanoivsky, 2002; Russell and Zomerdijk, 2006). Pol I, UBF and TIF-IA are functionally interchangeable between humans and mice, whereas the SL1/TIF-IB complex must be derived from the same species to support rDNA transcription (Bell et al., 1990; Heix and Grummt, 1995; Learned et al., 1985; Mishima et al., 1982; Rudloff et al., 1994; Schnapp et al., 1993). The human SL1 complex contains the TATA-binding protein (TBP) and three TBP-associated factors known as TAFI-A (also known as TAFI-48 and TAFI-A), TAFI-B (also known as TAFI-63 and TAFI-B) and TAFI-C (also known as TAFI-110C and TAFI-C) (Comai et al., 1992). Among these SL1 components, TBP is interchangeable between humans and mice, indicating that TAFIs are responsible for the promoter selectivity of SL1/TIF-IB (Rudloff et al., 1994). UV cross-linking experiments have demonstrated that TAFI-A and TAFI-B bind to both the homologous and heterologous promoters, suggesting that a binding of SL1/TIF-IB to the heterologous promoter precludes a formation of productive initiation complexes (Rudloff et al., 1994). There are contradictory reports about reconstituting SL1/TIF-IB activity in vitro. It has been reported that the three recombinant human TAFIs and the TBP are necessary and sufficient to reconstitute a transcriptionally active human SL1 complex (Zomerdijk et al., 1994). In contrast, three mouse recombinant TAFI-A and the TBP complex did not reconstitute the TIF-IB activity in an in vitro transcription system (Heix et al., 1997). Later, a further TAFI-A, TAFI-D (also known as TAFI-41 and TAFI-D1), was identified from the TBP-antibody affinity-purified SL1 fraction (Gorski et al., 2007). TAFI-D co-migrates with TBP on SDS-PAGE, so it remained unidentified for more than 10 years following the initial identification of the SL1 complex (Comai et al., 1992). TAFI-D is involved in rDNA transcription in vivo (Gorski et al., 2007). Thus, we hypothesize that TAFI-D is a final component in reconstituting the human SL1 activity in mouse cells and overcoming the rDNA transcription species barrier.

To examine this hypothesis, we established a novel and highly sensitive monitoring system for Pol I transcription. The products synthesized by Pol I are not translated to proteins. We tried to overcome this problem using the influenza virus RNA-dependent RNA polymerases (RdRP). Influenza A virus belongs to the Orthomyxoviridae family, and its genome comprises eight single-stranded RNAs of negative polarity (Naito et al., 2007). The viral RNA (vRNA) is associated with the viral RdRP, comprising PB1, PB2 and PA subunits, and nucleoprotein (NP), forming viral ribonucleoprotein (vRNP) complexes (Nagata et al., 2008; Portela and Digard, 2002). The transcription promoter and the replication signal of the viral genome exist at the 3′ and 5′ untranslated terminal regions (UTRs) of each segment. Transcription of the influenza virus genome is initiated by the cap snatching mechanism (Li et al., 2001). The viral RdRP polyadenylates the nascent RNA chain, possibly by a slippage
mechanism at the adenylation signal which consists of five to seven uracil residues located near the 5’ terminal of vRNA (Poon et al., 1999). Thus, the synthesized RNA has the authentic eukaryotic mRNA structure. An influenza-virus-like particle generation system was established based on the transfection of plasmid DNAs containing the viral genes under the control of the Pol I promoter and terminator (Neumann et al., 1999; Zobel et al., 1993). In this system, an influenza virus genome RNA of an exact size and orientation is synthesized by cellular Pol I. When the viral coding region is replaced with a reporter gene (see Fig. 1A), the reporter gene is expressed through the viral system described above. We examined the mechanism of rDNA transcription. In particular, we evaluated its species-specific property using this novel and highly sensitive assay system, as we considered the possibility that the species-specificity contradiction might be caused by reconstituted Pol I with an activity less than the detectable level of conventional assay systems.

In the present study, we established a viral RdR Pol-based reporter system to monitor Pol I transcription. Using this monitoring system, we succeeded in reconstituting Pol I transcription from human rDNA in mouse cells by exogenously expressing human TAF1A, B, C and D in the SL1 complex, but not with human TAF1A, TAF1B and TAF1C. Exogenous expression of four TAFIs not only induced transcription from the human rDNA promoter in a reporter plasmid but also reactivated human rDNA in mouse cells containing the human chromosome 21 carrying an inactive human rDNA cluster. These results indicate that four TAFIs in human SL1 are necessary and sufficient to reconstitute a transcriptionally active SL1 and overcome the barrier of species specificity during rDNA transcription in mouse cells.

RESULTS
A novel reporter assay system for transcription by Pol I
We developed a novel and sensitive reporter assay system to monitor transcription by Pol I in order to precisely analyze the species barrier for rDNA transcription (Fig. 1A) by modifying the original method (Zobel et al., 1993) for the influenza-virus-like particle generation system. In this system, engineered vRNA containing a firefly luciferase (Luc) gene or an EGFP gene sandwiched between 5’- and 3’-terminal cis-acting regulatory regions is synthesized by Pol I under the control of the promoter and terminator in transfected cells. PA, PB1, PB2 and NP are supplied from expression vectors under the control of the Pol II promoter (Neumann et al., 1999). mRNA encoding Luc is synthesized by the influenza viral RdR Pol using RNA transcribed by Pol I as a template and the cellular cap structure as a primer (Li et al., 2001; Poon et al., 1999). The synthesized

**Fig. 1.** An RdR Pol-based reporter assay system for the transcription by Pol I. (A) A schematic representation of the influenza virus RNA-dependent RNA polymerase (RdR Pol)-mediated Pol I reporter assay system. The influenza virus model RNA genomes are expressed from phPoll-vNS-Luc or phPoll-vNA-EGFP (denoted ‘Luc. or EGFP’) by Pol I. The influenza virus RdR Pol amplifies the synthesized RNA and transcribes mRNA from the model viral RNA of negative polarity. (B) Dependency of the Pol I reporter assay system on viral polymerases and NP. The Luc activity was normalized to the Renilla luciferase activity expressed under the control of the SV40 promoter, a typical Pol II promoter. AU, arbitrary units. Results are mean ± s.d. obtained from three independent experiments. (C) Evaluation of Pol I reporter systems. 3 Pol.+NP indicates transfection of plasmids containing RdR Pol components and NP. IRES and PolyA indicate an internal ribosome entry site and a poly(A) signal, respectively. (D) Expression of EGFP in the RdR Pol-based system. HeLa cells were co-transfected with plasmids expressing PA, PB1, PB2 and NP, and phPoll-vNA-EGFP (left panels). Transfection was also carried out with omission of the plasmid encoding NP (right panels).
RNA is a typical form of eukaryotic mRNA and is thereby subjected to translation after its transport from the nucleus to the cytoplasm. First, we examined whether this reporter system was dependent on viral components (Fig. 1B). phPolI-vNS-Luc contains an Luc gene of reverse orientation sandwiched between 5′- and 3′-terminal UTRs of the influenza virus (A/WSN/33) segment 8, which encodes a nonstructural (NS) protein. As expected, the Luc activity was detected in HeLa cells expressing all components of this system (Fig. 1B, lane 6). Although phPolI was originally called pHH21 or pPolI in a previous report (Neumann et al., 1999), we designated this plasmid as phPolI because it makes it easy to distinguish plasmids carrying the human or mouse rDNA promoter. Next, we evaluated this system by comparing it with an internal ribosome entry site (IRES)-mediated reporter system (Hannan carrying the human or mouse rDNA promoter. Next, we constructed a reporter system expressing EGFP. The EGFP signal was observed in the presence of four viral components, whereas it was not observed in the absence of NP (Fig. 1D).

**Evaluation of the RdR Pol-based reporter system for Pol I**

Next, we confirmed the reliability of the RdR Pol-based reporter system for monitoring transcription by Pol I. We constructed three reporter plasmids carrying the human rDNA promoter with mutations at residues that abrogate activity (Grummt, 1982; Grummt, 1998; Haltiner et al., 1986; Jones et al., 1988; Kishimoto et al., 1985; Miller et al., 1985; Moss and Stefanovsky, 1995). As shown in Fig. 2A,B, phPolI-ΔCore-vNS-Luc and phPolI-ΔUCE-vNS-Luc lacked the core and upstream control elements of the human rDNA promoter, respectively. G residues at −7 and −16 relative to the transcription initiation site are quite important for the transcription of the mammalian rRNA gene. These G residues are replaced with A residues in phPolI-G-7,-16A-vNS-Luc. The RdR Pol-mediated Luc activity driven by phPolI-ΔCore-vNS-Luc in HeLa cells was completely eliminated (Fig. 2C, lanes 4 and 5). In addition, the RdR Pol-mediated Luc activity driven by phPolI-ΔUCE-vNS-Luc and phPolI-G-7,-16A-vNS-Luc in HeLa cells were <10% of that driven by phPolI-vNS-Luc (Fig. 2C, lanes 6–9). These results were consistent with previous reports about the properties of the rDNA promoter.

We also constructed the pmPolI-vNS-Luc reporter plasmid, which carries the mouse rRNA gene promoter instead of the human rRNA gene promoter in phPolI-vNS-Luc. Although pmPolI was originally called as pHMP1 in a previous report (Turan et al., 2004), we redesignated this plasmid pmPolI because it makes it easy to distinguish plasmids carrying the human or mouse rDNA promoter. As shown in the human reporter system, we constructed three reporter plasmids carrying mutations in the mouse rDNA promoter that abrogate activity (Fig. 2D,E). There was no RdR Pol-mediated Luc activity driven by pmPolI-ΔCore-vNS-Luc in mouse NIH3T3 cells (Fig. 2F, lanes 4 and 5). The RdR Pol-mediated Luc activity driven by pmPolI-ΔUCE-vNS-Luc and pmPolI-G-7,-16A-vNS-Luc in mouse NIH3T3 cells was dramatically lower compared with that by pmPolI-vNS-Luc (Fig. 2F, lanes 6–9). These results clearly demonstrate that our RdR-mediated reporter system detects Pol I activity.

The transcription rate of rDNA by Pol I is closely correlated with the cell growth rate and depends on the cell culture conditions (Grummt, 1999). We examined RdR Pol-mediated Luc activity in HeLa cells maintained with 0.5%, 2% and 10% serum (supplementary material Fig. S1A). The Pol I transcription level, as monitored by the Luc activity, increased in a serum-concentration-dependent manner, as previously reported by conventional assay systems monitoring the incorporation of [3H]uridine into the 45S rRNA precursor (Stefanovsky et al., 2006). Furthermore, we confirmed that the RdR Pol-based reporter system responded to exogenous expression of UBF, an essential transcription factor for transcription by Pol I (supplementary material Fig. S1B,C; Grummt, 1999). CX-5461 inhibits Pol I via the disruption of SL1 recruitment on the rDNA promoter (Drygin et al., 2011). Treatment with CX-5461 reduced the Luc activity in the RdR Pol-based reporter system when normalized to c-Myc- or β-actin-encoding mRNAs as previously described (Drygin et al., 2011) (supplementary material Fig. S1D). Taken together, we conclude that this RdR Pol-based reporter system is highly sensitive and useful for analyzing the transcription mechanism by Pol I.

**In vivo reconstitution of human rDNA transcription in mouse cells**

We used the RdR Pol-based reporter system to uncover the molecular mechanism underlying the species-specific transcription by Pol I. phPolI and pmPolI, used in Fig. 3, contain the human and mouse Pol I promoters, respectively (Neumann et al., 1999; Turan et al., 2004). In human HeLa cells, the Luc activity was observed in the presence of viral RdR Pol components and NP (Fig. 3, 3Pol+NP) when the human Pol I vector (phPolI-vNS-Luc) was transfected. In contrast, the Luc activity was not detected when the mouse Pol I vector (pmPolI-vNS-Luc) was transfected (Fig. 3A, upper panel). Furthermore, the mouse reporter, but not the human reporter, showed Luc activity in mouse NIH3T3 cells (Fig. 3A, lower panel). These results indicate that species-specific transcription by Pol I is reproduced in the RdR Pol-based reporter system.

To reconstitute the human SL1 activity in mouse cells, we cloned four human TAFIs (hTAFIs) and performed immunoblotting using extracts from 293T cells transfected with vectors encoding hTAFIs (Fig. 3B). The effect of hTAFs on transcription from the human rDNA promoter was examined in NIH3T3 cells using the RdR Pol-based reporter system for Pol I. The human rDNA promoter was not transcribed in NIH3T3 cells without the human SL1 complex or even in the presence of the three factors: hTAFIA, hTAFIB, and hTAFIC (Fig. 3C, lanes 3 and 5). In sharp contrast, with hTAFIA, hTAFIB, hTAFIC and hTAFID (Fig. 3C, lanes 4 and 6) Luc activities were detected from the human reporter (phPolI-vNS-Luc). Next, NIH3T3 cells were co-transfected with the human reporter plasmid in the presence of all combinations of hTAFIs (Fig. 3D). No combination of the three hTAFIs reconstituted the efficient transcription activity driven by the human rDNA promoter (Fig. 3D, lanes 3–6), although a low transcription level was observed when either hTAFIA or hTAFIB was omitted. Importantly, human rDNA transcription was not at all stimulated in NIH3T3 cells when a single factor or any combination of two factors were expressed (Fig. 3D, lanes 7–16). These results indicate that the four hTAFIs are required for maximum transcription from the human Pol I promoter in mouse cells.
addition, hTAFIC and hTAFID played an essential role in the SL1 activity and species specificity (Fig. 3D, lanes 3 and 4), and mouse TAF IA (mTAF IA) and mTAF IB could partially replace human cognate proteins for human rDNA transcription in mouse cells (Fig. 3D, lanes 5 and 6). This finding is consistent with that from UV crosslinking experiments stating that TAF IA and TAFIB bind to both homologous and heterologous promoters (Rudloff et al., 1994). As a control, we examined the effect of all combinations of hTAFIs on the mouse reporter system in NIH3T3 cells (supplementary material Fig. S2). Exogenous expression of the four hTAFIs did not increase the level of the mouse Pol I activity but rather interfered with mouse Pol I activity. Taken together, the four hTAFIs in human SL1 are necessary and sufficient to reconstitute a transcriptionally active SL1 and overcome the barrier of species specificity in human rDNA transcription in mouse cells.

Transcriptional reactivation of inactive human rDNA in A9 cells
As shown above, the RdR Pol-based reporter system was sensitive for detecting Pol I activity. However, we could not completely exclude the possibility that human rDNA was transcribed in mouse cells by an unexpected effect between RdR Pol and the four hTAFIs. To exclude this possibility and directly show the function of the human SL1 in mouse cells, we used mouse A9 cells carrying a single intact human chromosome 21 (A9ch21 cells), which contains human rDNAs, but not any human TAF I genes (Cuthbert et al., 1995). Human rDNA transcription could not be detected in A9ch21 cells (Fig. 4A, left panel). Because the inactive human rDNA promoter region was reported to be highly methylated (Guetg et al., 2012; Santoro and Grummt, 2001), we examined the DNA methylation pattern on human rDNAs in A9ch21 cells. Fig. 4B shows the DNA methylation pattern on human rDNAs in A9ch21 cells. The underlined G residues at −7 and −16 are replaced with A residues in pmPolI-G-7,-16A-vNS-Luc. (F) The activity of mouse rDNA promoters containing mutations. NIH3T3 cells were co-transfected with plasmids encoding viral components (3 Pol+NP) and reporter plasmids as indicated below each lane. Results in C and F are mean ±s.d. obtained from three independent experiments. + and ++ indicate 30 ng and 90 ng of plasmid DNA, respectively, AU, arbitrary units.
(HpaII) and -insensitive (MspI) restriction enzymes. Although human rDNAs were transcriptionally inactive in A9ch21 cells, 40% of human rDNA promoters were unmethylated, and the methylation status of the human rDNA promoter was not significantly different from that of the mouse rDNA promoters in A9ch21 cells (Fig. 4B). Moreover, the epigenetic profile on the human rDNA promoter in A9ch21 cells was examined by chromatin immunoprecipitation (ChIP) assays. Both acetylation and trimethylation at histone H3 lysine 9 (H3K9) on human rDNAs were slightly higher than those on mouse rDNAs in A9ch21 cells (Fig. 4C). In addition, the amount of UBF bound to the human rDNA promoter in A9ch21 cells was quite low. However, micrococcal nuclease (MNase) digestion assays demonstrated that the human rDNA promoter region was more resistant to MNase than the mouse rDNA promoter in A9ch21 cells comprising both active and inactive rDNAs (Fig. 4D). These results suggest that inactive human rDNAs are not assembled into a typical heterochromatin structure in mouse cells, but they rather just form compact chromatin structure resistant to nuclease.

Next, we examined the effect of exogenous hTAFIs expression on transcription from the human rDNA promoter in A9ch21 cells. First, we confirmed that the SL1 activity was reconstituted by four exogenously expressed hTAFIs in A9ch21 cells (Fig. 4E). We then examined transcription from human rDNA in A9ch21 cells in the presence of the four hTAFIs. Total RNA was prepared from A9ch21 cells transiently expressing hTAFIs, followed by quantitative RT-PCR using primer sets for human 45S pre-rRNA. The inactive human rDNA in mouse cells was reactivated by four exogenously expressed hTAFIs (Fig. 4F). Human pre-rRNA could not be observed in A9ch21 cells in the absence of TAF ID (Fig. 4G, lane 2). These results indicate that human rDNA transcription was reconstituted by four hTAFIs in mouse A9ch21 cells in addition to that monitored by the RdR Pol-based reporter system.

**Formation of a chimeric SL1 complex**

Human rDNA transcription in mouse cells required four hTAFIs, whereas three hTAFIA, hTAFIB and hTAFIC did not confer transcription, as shown in Figs 3 and 4. It is possible that hTAFIA, hTAFIB and hTAFIC cannot interact with mouse TAFID (mTAF ID). To examine this hypothesis, Flag-tagged mTAFID was co-expressed with HA-tagged hTAF IA, hTAF IB and hTAFIC, followed by immunoprecipitation with anti-Flag antibody. HA-tagged hTAF IA, hTAF IB and hTAFIC co-immunoprecipitated with Flag-tagged mTAFID, as they were with Flag-tagged hTAFID (Fig. 5A). Endogenous TBP was also co-immunoprecipitated with Flag-tagged hTAFID and mTAFID (Fig. 5B). These results indicate that mTAFID is able to form a chimeric SL1 complex, including TBP and hTAF IA, hTAF IB and hTAFIC, in agreement with a previous report (Heix et al., 1997). Furthermore, Flag-tagged mTAFID formed a chimeric SL1 complex with HA-tagged hTAF IA, hTAF IB and hTAFIC, and endogenous TBP did not confer transcription of human rDNA (Fig. 5C, lane 4). In addition, no combination of three hTAFIs and a single mTAFI conferred maximum transcription of human.
rDNA, which was driven by four hTAFIs (supplementary material Fig. S3A). These data suggest that any chimeric SL1 complex comprising three hTAFIs and a single mTAFI cannot form functional SL1 complexes.

Chimeric SL1 complexes did not bind to the human rDNA promoter

UV crosslinking experiments have demonstrated that TAFI A and TAFI B bind to both homologous and heterologous promoters (Rudloff et al., 1994). This report encouraged us to examine whether the mouse SL1/TIF-IB complex and chimeric complex are associated with the human rDNA promoter in mouse A9 cells carrying the human chromosome 15 (A9ch15 cells). The four exogenously expressed human TAFIs activated transcription of human rDNA in A9ch15 and A9ch21 cells (supplementary material Fig. S3B). The reactivation level of human rDNA transcription in A9ch15 cells by the four hTAFIs was 9.1-fold higher than that of A9ch21 cells (supplementary material Fig. S3C). Therefore, we used A9ch15 cells instead of A9ch21 cells for additional ChIP assays. We performed ChIP assays using anti-TBP antibody. TBP bound to the mouse rDNA promoter, but not to the human rDNA promoter, in mouse A9ch15 cells (Fig. 6A, left panel). However, TBP bound to the human rDNA promoter in HeLa cells (Fig. 6A, right panel). These results clearly indicate that the mouse SL1/TIF-IB complexes cannot associate with the human rDNA promoter. As shown in Fig. 6B, the four exogenously expressed human TAFIs recruited TBP on the human rDNA promoter in A9ch15 cells. In contrast, chimeric SL1 complexes and four mTAFIs could not recruit TBP on the human rDNA promoter in A9ch15 cells compared with the four hTAFIs. Furthermore, the occupancy of UBF at the human rDNA promoter increased slightly in A9ch15 cells in the presence of the four exogenous human TAFIs (Fig. 6C), which is in good agreement with a report stating that human SL1 stabilizes the binding of UBF at the human rDNA promoter (Friedrich et al., 2005). A large amount of RPA194, the largest subunit of Pol I, was also recruited on human rDNA in mouse A9ch15 cells by the four exogenously expressed human TAFIs. We conclude that SL1 complexes comprising four human TAFIs are required for the recognition of the human rDNA promoter.

DISCUSSION

In this report, we describe an RdR Pol-based reporter system for transcription by Pol I. The RdR Pol-based reporter system showed high sensitivity, because RNA synthesized by Pol I is amplified and converted into translatable mRNA by RdR Pol. Transcription of human rDNA was reconstituted in mouse cells by exogenously expressing four TAFIs from the human SL1 complex. In addition, we demonstrated that silent human rDNA transcription in mouse A9ch21 cells was reactivated by the four exogenously expressed human TAFIs. These results suggest that the four human TAFIs are necessary and sufficient to overcome the species-specific barrier of human rDNA transcription in mouse cells.
It has been reported that the human SL1 activity can be reconstituted in vitro by the three recombinant hTAF5, hTAF3A, hTAF3B and hTAF3C, in addition to recombinant TBP, with a relatively higher background (Zomerdijk et al., 1994). Those authors used DNA-affinity-purified UBF and recombinant hTAF3A, hTAF3B, hTAF3C and TBP for reconstituting in vitro transcription by Pol I. Furthermore, it has been shown that UBF interacts with hTAF3D in vivo and in vitro (Gorski et al., 2007). Based on those results, it is quite likely that a low level of hTAF3D, which was associated with either DNA-affinity-purified UBF or the purified Pol I fraction, was present in their in vitro reaction. This assumption is supported by a report that mouse TIF-1B activity is not reconstituted by recombinant protein of three mTAF5, mTAF3A, mTAF3B and mTAF3C, in addition to recombinant TBP and UBF (Heix et al., 1997). Taken together, we conclude that hTAF3D is the putative final component in completing the SL1 activity.

The four mouse TAF3A, TAF3B, TAF3C and TAF3D proteins show 90%, 87%, 75% and 68% similarity, respectively, to their human counterparts, according to BLAST2 (NCBI) analyses. Their similarity is in good agreement with the observation that TAF3A and TAF3B are partially replaceable for human cognate proteins, whereas TAF3C and TAF3D have crucial roles in species specificity, as shown in Fig. 3D. We found that mouse Flag-tagged TAF3D forms a complex with human HA-tagged TAF3A, TAF3B, TAF3C and TBP (Fig. 5). This result is partially supported by a report that mouse and human TAF3s (hTAF3A,
hTAF1B and hTAF1C can form chimeric TBP–TAF1 complexes (Heix et al., 1997). It appears that subtle differences between individual TAFIs might affect the overall conformation of the chimeric complex between TBP and the TAF1S, which could be transcriptionally inactive, as mentioned by Heix and Grummt (Heix and Grummt, 1995). Our results clearly indicate that the human SL1 activity requires four hTAFs, at least in mouse cells. For further analysis of the molecular mechanism of the promoter selectivity by Pol I, SL1/TIF-IB activity should be reconstituted in vitro by four TAFIs and TBP recombinant proteins.

Although the human SL1 activity was reconstituted in mouse cells, the four mouse TAF1S did not confer transcription of mouse rDNA in HeLa cells (supplementary material Fig. S4). These results are consistent with a report that the human SL1 can reprogram mouse nuclear extracts during in vitro transcription from the human rDNA promoter (Learned et al., 1985), but TIF-IB cannot reprogram human nuclear extracts during in vitro transcription from the mouse rDNA promoter (Schnapp et al., 1991). TIF-IB requires mouse Pol I to reprogram human nuclear extracts, whereas mouse SL1/TIF-IB, human UBF and human Pol I can reconstitute transcription from the mouse rDNA promoter in vitro (Bell et al., 1990; Schnapp et al., 1991). It is assumed that there is an unknown factor(s) preventing TIF-IB activity in HeLa cells, suggesting that the species barrier(s) to rDNA transcription might be different between humans and mice.

The level of SL1/TIF-IB in cells is too low to study the molecular mechanism of promoter recognition and species-specific transcription by Pol I (Heix et al., 1997). Thus, to confirm the exogenous expression of hTAF1S, we performed immunoblotting using extracts from 293T cells transfected with vectors encoding hTAF1S (Fig. 3B). Despite considerable efforts, exogenously expressed TAF1S were not detected by immunoblotting using NIH3T3, A9ch15, A9ch21 or HeLa cell extracts. In these cells, the abundance of SL1 might be under the control of an unknown mechanism, whereas 293T cells lack the possible mechanism, which keeps the SL1 level low. Similarly, the p53 tumor suppressor protein level and activity are controlled by a ubiquitin-proteasome pathway involving MDM2 (Hock and Vousden, 2014). The possible mechanism to maintain a low level of SL1 is currently an open question.

We used two mouse A9 cell lines (A9ch21 and A9ch15) carrying a human chromosome to show the SL1 activity in the presence of four human TAF1S. During the course of this study, we found that the level of human rDNA transcription in A9ch15 cells was 9.1-fold higher than that in A9ch21 cells in the presence of the four human TAF1S (supplementary material Fig. S3C). The human rDNA copy number in an A9ch15 cell was smaller than that in an A9ch21 cell (supplementary material Fig. S3D). Notably, the level of DNA methylation on the human rDNA promoter in A9ch15 cells was significantly lower than that in A9ch21 cells (supplementary material Fig. S3E). The level of DNA methylation on the promoter may determine the level of response to exogenous expression of the four human TAF1S. In addition, the maintenance of the DNA methylation level on the human rDNA promoter was independent of human rDNA transcription in mouse A9 cells. Noncoding promoter RNA (pRNA) synthesized by RNA polymerase I recruits PARP1 and NoRC complexes to rDNA, so pRNA is involved in the formation and maintenance of silent rDNA chromatin (Guet al et al., 2012; Mayer et al., 2006; Santoro et al., 2010). Nucleolar localization of NoRC in NIH3T3 cells is mediated by pRNAs synthesized from mouse and human rDNA proximal Pol I promoters (Mayer et al., 2008). The results shown in supplementary material Fig. S3 raise fundamental questions whether mouse pRNA is involved in DNA methylation on human rDNA promoter, and whether human pRNA is transcribed by mouse Pol I machinery. In conclusion, the A9 cell line carrying human chromosomes 15 and 21 may provide a good model system for studying rDNA epigenetics.

Studies on the mechanism of rDNA transcription mediated by Pol I have become a focus again. It is well known that the regulation of rDNA transcription is related to cancer cell proliferation (White, 2005). c-Myc, a representative transcription factor involved in tumorigenesis, binds to rDNA and increases rDNA transcription (Arabi et al., 2005; Grandori et al., 2005). Several protein kinases, including casein kinase II and ERK, have the ability to upregulate rDNA transcription through the phosphorylation of UBF and TIF-IA in cancer cells (Bierhoff et al., 2008; Lin et al., 2006; Panova et al., 2006; Stefanovsky et al., 2001). Thus, the Pol I transcription machinery is being re-evaluated as an emerging target for treating cancer (Drygin et al., 2010; Hein et al., 2013). It is possible that the RdR Pol-based reporter system for Pol I activity would be a convenient and highly sensitive tool for monitoring Pol I activity and screening inhibitors for Pol I transcription.

MATERIALS AND METHODS

Cell cultures and luciferase assay

HeLa cells, NIH3T3 cells, A9ch15 cells and A9ch21 cells were used for transfection experiments. They were maintained at 37°C in Dulbecco’s modified Eagle’s medium (Nissui) supplemented with 10% fetal bovine serum. In addition, A9ch15 cells and A9ch21 cells were cultured in the presence of 50 μg/ml hygromycin B (Invivogen) to keep the human chromosome in the mouse cells. The Luc and the Renilla Luc activities were determined using the luciferase assay reagent (Promega) and the Renilla Luciferase Assay System (Promega) according to the manufacturer’s protocol. The relative luminescence intensity was measured for 10 seconds with a MiniLumat (Berthold).

DNA transfection

Cells were transfected with DNA using Lipofectamine 2000 (Invitrogen) or GeneJuice (Novagen) transfection reagent kit according to the manufacturers’ instructions. For Fig. 1B, HeLa cells were co-transfected with 25 ng each of pCAGGS-PA, pCAGGS-PB1, pCAGGS-PB2, pCAGGS-NP (Kawaguchi et al., 2005; Naito et al., 2007), which leads to synthesis of PB1, PB2 and NP mRNAs, respectively, by Pol II, 90 ng of phPolI-vNS-Luc and 10 ng of pRL-SV40 (lane 6). Assays were also carried out under omission of either one of the plasmids encoding viral components, pCAGGS-PA (lane 2), pCAGGS-PB1 (lane 3), pCAGGS-PB2 (lane 4), or pCAGGS-NP (lane 5) or in the absence of all viral components (lane 1). For Fig. 1C, HeLa cells were co-transfected with phPol (40 ng, lane 1) or phPol-vNS-Luc (40 ng, lane 2) together with plasmids (38 ng for each) encoding the four viral components and pRL-SV40 (10 ng), phPol-IREs-Luc (190 ng, lane 3), phPol-IREs-Luc-PolyA (190 ng, lane 3) and pSV40-Luc (190 ng, lane 5), which were introduced by HeLa cells together with pRL-SV40 (10 ng). For Fig. 1D, HeLa cells were co-transfected with 80 ng of each plasmid expressing PA, PB1, PB2 and NP and 80 ng of phPol-vNS-EGFP (Fig. 1D, left panels). Transfection was also carried out with the omission of the plasmid encoding NP (Fig. 1D, right panels). For Fig. 2C, HeLa cells were transfected with one of phPol-based plasmids (90 ng), pRL-SV40 (10 ng) for expression of Renilla Luc, and plasmids (each 25 ng) expressing PA, PB1 and PB2 subunits, and NP. For Fig. 2F, NIH3T3 cells were transfected with pCHA-TAF1A, pCHA-TAF1B, pCHA-TAF1C and pCHA-TAF1D (25 ng each) as indicated below each lane in the presence of the plasmid set (3Pol+NP) for the RdR Pol-based reporter.
were found to be methylated, and the methylation sites were located

TTGACCAGAGGGACCCCGG-3

GGTCCAATAGGAAC-3

TAAGACCGACAGGT-3

ATG-3

9

CCAGAGGGACCCCGG-3

AGGT-3

9

Construction of plasmid vectors
To construct phPoll-vNS-Luc, a DNA fragment of the Luc gene was amplified by PCR with primers, 5'-CTGTCGCCAGGGACAGG-3' and 5'-GGGGTGAATTCATTAGTACCCATGG-3'. The amplified DNA fragment was digested with EcoRI and XhoI, and then ligated into pEGFP-N1 to generate phPoll-EGFP. The amplified DNA fragment was then digested with HindIII and XhoI, and then ligated into pEGFP-N1 to generate phPoll-EGFP-NS-Luc.

Restriction enzyme and Mnase sensitivity assays
CpG methylation was assayed by digestion with HpaII (a methylation-sensitive restriction enzyme) and MspI (a methylation-insensitive restriction enzyme) as described previously (Gu et al., 2003; Santoro et al., 2002). Genomic DNA (500 ng) prepared from A9ch21 cells was digested with 20 units of HpaI or MspI for 3 hours at 37°C. Resistance to restriction enzymes was determined by quantitative PCR (qPCR) using the 3'-end DNA fragment amplified by PCR as a template.

Quantitative RT-PCR
Quantitative determination of pre-rRNA levels by RT-qPCR was carried out essentially as described previously (Murano et al., 2008). Total RNA was prepared with MagExtractor-RNA (TOYOBO) and treated with RNase-free DNase I (Invitrogen). For analysis of human 45S pre-rRNA, total RNA was subjected to reverse transcription with a primer, 5'-ACACACCCGTGCGCTCC-3', which corresponds to the 5' external transcribed spacer (ETS) of the human rRNA. The synthesized cDNA was quantitated by real-time PCR using FastStart SYB Green Master (Roch) in the presence of a primer set, 5'-CTGGCGTCTAGGGGCTTCTCGC-3' and 5'-CGCCTGCACGTCGACGAGCC-3'. The amplified DNA fragment was digested with EcoRI and XhoI, and then ligated into pEGFP-N1 to generate phPoll-EGFP.

Protein A-Sepharose
Protein A-Sepharose was prepared according to the manufacturer’s instructions.

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fragment was treated with NotI and cloned into the NotI site in phPollires-Luc, resulting in phPollires-Luc-PolyA. For expression with primers, 5'AAAAGGATCCATGAGGAAGCCGAGCTTG3'- and 5'AAAGAATTCATCTGGAGTCAGTCTG3', using cDNA derived from HeLa cells as a template. The amplified DNA fragments of UBfI and UBfII were digested with EcoRI and BamHI and cloned into the EcoRI and BamHI sites of plasmid pcDNA3.1(+)-Flag. To construct expression vectors for human TAFIA, TAFIB, TAFIC and TAFID, DNA fragments were amplified by PCR using cDNA prepared from HeLa cells. The primers used here were as follows: 5'-CTAGCTAGCATGATGTGGAGGAGGTGAAAGCGTTCA-3', 5'-CTAGCTAGCATGGACTCAGGACCCTCCACCTTCCCTCG3', and 5'-GGAAGATTCAGAAGGACATCTGTCCTGTA3'.

To construct expression vector for mouse TAFs, cDNA fragments were amplified by PCR using mouse cDNA prepared from A9ch21 cells. The primers used here were as follows: 5'-CTAGCTAGCATGAGTGATTTCAGTGAAGAATTAAAAGGGC-3', 5'-CTAGCTAGCATGGCTCAATCAGAAGG-3', and 5'-CTAGCTAGCATGGCTCAATCAGAAGG-3'. These cDNA fragments were cloned into the restriction site of pcDNA3.1(+)-Flag. To amplify fragments of UBF1 and UBF2, cDNAs of UBF1 and UBF2 were amplified by PCR using cDNA derived from HeLa cells as a template. The primers used here were as follows: 5'-CTAGCTAGCATGGATGTGGAGGAGGTGAAAGCGTTCA-3', 5'-CTAGCTAGCATGGACTCAGGACCCTCCACCTTCCCTCG3', and 5'-GGAAGATTCAGAAGGACATCTGTCCTGTA3'.

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Competing interests
The authors declare no competing interests.

Author contributions
K.M. and K.N. designed research; K.M. and M.O. performed experiments; F.M., H.K., S.H., and Z.A. contributed reagents/materials/analysis tools; K.M. and K.N. wrote the paper.

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Supplementary material
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References

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Competing interests
The authors declare no competing interests.

Author contributions
K.M. and K.N. designed research; K.M. and M.O. performed experiments; F.M., N.H., S.H., and S.S. provided the plasmid vectors; R.F.N. provided a9 cell lines; and K.M. and K.N. wrote the paper.

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Supplementary material
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**Fig. S1.** Evaluation of the RdR Pol-based reporter system for Pol I. (A) Effect of the serum concentration on Pol I activity. The experimental scheme is shown in the upper panel. HeLa cells were transfected with plasmids for RdR Pol-based reporter system. HeLa cells were transferred to MEM supplemented with 0.5%, 2%, or 10% serum after 8 h post transfection. Black and grey boxes indicate human Pol I promoter activity (Luc) and SV40 promoter activity (renilla Luc), respectively. Each luminescence intensity was normalized by the amount of protein. (B) The schematic representation of UBF1 and UBF2. Black, grey, and striped boxes indicate homeo-domain like domain, HMG box, and acidic domain, respectively. UBF1 contains six HMG boxes in the middle of molecule. Mammalian cells express two UBF isoforms, UBF1 and UBF2, with molecular masses of 97 and 94 kDa, respectively. The biochemical activity of UBF2 is shown to be significantly lower than that of UBF1. (C) Response of the RdR Pol-based reporter system to exogenous expression of UBF. HeLa cells were transfected with phPolI-vNS-Luc (10 ng) and plasmids (each 10 ng) expressing PA, PB1, and PB2 subunits, and NP in the presence of 50 ng (lanes 2 and 8), 100 ng (lanes 3 and 9), and 200 ng (lanes 4 and 10) of pcDNA3-Flag-UBF1 or in the presence of 50 ng (lanes 5 and 8), 100 ng (lanes 6 and 9), and 200 ng (lanes 7 and 10) of pcDNA3-Flag-UBF2. The Luc activity was examined at 24 h post transfection and normalized by the amount of protein. The expression level of UBF1 and UBF2 were detected by immunoblotting using anti-Flag antibody (lower panel). (D) Effect of CX-5461 on the RdR Pol-based reporter system. Previously, it was shown that CX-5461 (Xcess Biosciences Inc.) can inhibit the Pol I activity using c-myc and actin-β mRNAs as a control (Drygin et al., 2011). According to the previous report, the Luc activity was normalized by the level of c-myc mRNA (left graph) or actin-β mRNA (right graph).
**Fig. S2.** Effect of hTAFIs on the mouse reporter system in NIH3T3 cells. Effect of all combinations of human TAFIs (hTAFIs) were examined by the RdR Pol-based reporter system for the mouse rDNA transcription in NIH3T3 cells. Exogenous expression of four hTAFIs gave no effect on or decreased the level of the mouse rDNA transcription in NIH3T3 cells, in contrast to the effect of hTAFIs on the human rDNA transcription in NIH3T3 cells (Fig. 3C and D).
Fig. S3. The human rDNA in A9ch21 cell and A9ch15 cell. (A) Human rDNA promoter activity detected by the RdR Pol-based reporter system. A9ch21 cells were transfected with combinations of pCHA-based plasmids encoding human TAFIs (HA-hA to HA-hD) and/or pCFlag-based plasmids encoding mouse TAFIs (Flag-mA to Flag-mD). (B) Human rDNA promoter activity detected by the RdR Pol-based reporter system in A9ch15 cell in the presence of four human TAFIs. (C) Re-activation of human rDNA transcription in A9ch21 cell and A9ch15 cell. Total RNA was prepared from A9ch21 cell transfected with pCHA as a control or pCHA-based plasmid set expressing four human TAFIs at indicated time post transcription. The level of human pre-rRNA was measured by quantitative RT-PCR and normalized by the level of mouse actin b. (D) Relative copy number of the human rDNA in A9ch21 and A9ch15 cell. (E) Methylation pattern on the mouse and human rDNA promoters in A9ch15 and A9ch21 cells.
Fig. S4. Fail to reconstitute mouse rDNA transcription in HeLa cells. (A) Exogenous expression of mouse TAF₈s (mTAF₈s). 293T cells were transfected with pCFlag-based plasmids encoding mouse TAF₈48/A (lane 2, mA), TAF₈68/B (lane 3, mB), TAF₈95/C (lane 4, mC), TAF₈41/D (lane 5, mD). The expression level of each exogenous mTAF₈s and human actin β were detected by immunoblotting using anti-Flag and anti-Actin β antibodies, respectively. (B) Four mouse TAF₈s failed to reconstitute transcription from the mouse rDNA promoter in human HeLa cells. HeLa cells were transfected with plasmids as indicated below each lane in the presence of plasmids encoding viral RdR Pol and NP (3Pol+NP), and pRL-SV40. phPolI-vNS-Luc is driven by the human rDNA promoter and used for positive control as human Pol I activity (lane 2). The Luc activity was normalized by the renilla Luc activity transcribed by Pol II. AU indicates arbitrary unit. (C) Any combinations of three human TAF₈s and single mouse TAF₁ failed to reconstitute transcription from the mouse rDNA promoter in HeLa cells. HeLa cells were transfected with plasmids as indicated below each lane in the presence of plasmids encoding viral RdR Pol and NP (3Pol+NP), and pRL-SV40.