RNA Polymerase III Accurately Initiates Transcription from RNA Polymerase II Promoters * in Vitro*

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Background: RNA polymerase II (Pol II) and III (Pol III) transcription systems are reported to regulate non-overlapping subsets of genes. Pol III can initiate transcription from Pol II promoters with AT-rich sequence. The DNA template to nuclear extract ratio determines the predominant polymerase.

Results: Pol III can initiate transcription from Pol II promoters with AT-rich sequence. The DNA template to nuclear extract ratio determines the predominant polymerase.

Conclusion: Pol II and Pol III transcription initiation can overlap.

Significance: RNA polymerase specificity is variable and depends on the transcription conditions.

In eukaryotes, there are three major RNA polymerases (Pol) in the nucleus, which are commonly described as transcribing non-overlapping subsets of genes. Structural studies have highlighted a conserved core shared among all three transcription systems. Initiation of human Pol III from TATA box-containing Pol II promoters under conditions with impaired Pol II transcription activity have been described previously. RNA polymerase III and Pol II were found to co-localize at the promoters of the c-myc gene and the RPPH1 sRNA *in vivo*. Here, I report that Pol III can, like Pol II, initiate transcription from most tested Pol II core promoters when assayed with crude human nuclear extracts (HSK, SNF, or Dignam). Both polymerases initiate from the same transcription start site, and depend on a TATA box or AT-rich region but not the downstream promoter element (DPE) or the motif ten element (MTE). Moderate (~2-fold) changes in the ratio of DNA template to nuclear extract were sufficient to change Pol II-mediated transcription to a mixture of Pol II- and Pol III-, or to a solely Pol III-dependent initiation of transcription from Pol II promoters. Polymerase specificity is thus not fixed but a variable that depends on the properties of the promoter and the transcription conditions. These findings provide functional evidence for a close similarity between the Pol II and Pol III transcription complexes, and additionally explain previous controversies in the literature.

Gene expression is primarily regulated through transcription (1), a key step of which is the formation of transcriptionally competent preinitiation complexes. Eukaryotes contain three major nuclear RNA polymerases: RNA polymerase (Pol) II is devoted to the synthesis of ribosomal RNAs (with the exception of 5 S rRNA). Pol II synthesizes mRNA precursors as well as most snRNA and micro-RNAs, and Pol III synthesizes tRNAs, the 5 S rRNA, as well as other small RNAs involved in RNA processing and transport (2, 3). RNA polymerases are thus commonly regarded as transcribing non-overlapping subsets of genes (e.g. Refs. 4–6).

Here I report the ability of Pol III to accurately initiate transcription at most Pol II promoters *in vitro*, which suggests a functional conservation between the two transcription machineries. This notion is supported by structural studies, which highlighted the conserved core of all three eukaryotic transcription systems (3, 7, 8). Identical or homologous proteins for the 12 subunits of Pol II are also found in Pol I and III. All or most of the additional subunits present in Pol I and III are related to the independent Pol II basal transcription factors TFII F and TFII E, respectively (7–11). The TATA box-binding protein (TBP) was first identified as one key component of the Pol II initiation factor TFII D, which binds to AT-rich DNA sequences with high affinity (reviewed in Ref. 12). In addition, TBP is also present as a subunit of the Pol I transcription factor SL I (13), the Pol III transcription factors Br f1- TFI I IB (14), and other factors (15). TBP is conserved from archaea to humans and is an integral part of all three eukaryotic transcription machineries (15–18). The Pol II and Pol III transcription machineries may thus be more similar, both structurally and also functionally, than previously thought.

Specific DNA sequences interact with the basal transcription factors, which in turn recruit their respective RNA polymerases to the sites of transcription initiation. The minimal set of transcription factors required for Pol II assembly and specific initiation of transcription (at certain promoters) consists of TBP.
TFIIB, and the 30-kDa subunit of TFIIF (19). Likewise, the transcription initiation factors for Pol I and Pol III (SL1/Tif-1B and TFIIB, respectively) contain a TFIIB-related protein and TBP (or a TBP-related protein (Trf1) in *Drosophila*) counterparts (7–9, 20).

The minimal DNA sequence required for accurate transcription of a gene is termed the core promoter. There is no universal core promoter motif, and promoters may contain several or none of the known motifs (21, 22). The TATA box, which is bound by TBP, was the first core promoter motif identified and is shared between Pol II and Pol III promoters. In Pol III promoters, the TATA box is accompanied by the proximal sequence element (PSE), which serves as a binding site for the transcription factor SNAPc (23). Both PSE and factor SNAPc are also utilized by Pol II for the initiation of transcription of a small set of genes. The primary determinant distinguishing Pol II from Pol III promoters is the presence of a TATA box at its canonical location within the Pol III promoters (23–25). Under special conditions, however, the TATA box by itself is sufficient to recruit the Pol III transcription machinery (26).

This article reports Pol III to accurately initiate transcription from most tested Pol II promoters *in vitro*. Initiation of Pol III from TATA-containing Pol II promoters has been previously demonstrated with fractionated human nuclear extract that lacks TFIIID (27), an essential factor for Pol II initiation. In the absence of TFIIID, human TBP-containing Brf1-TFIIIB stably binds the TATA box and recruits Pol III (28). Transcription of TATA-containing Pol II promoters by Pol III was also described in low salt crude nuclear extract with low Pol II activity (29).

To my surprise, I found Pol III to accurately initiate transcription from Pol II TATA box or AT-rich sequence containing core promoters in *in vitro* transcription assays with unfractionated high salt human nuclear extracts (HSK, SNF, or Dignam). The analyzed Pol II promoters did not contain any known Pol III core promoter motifs besides the TATA box, or an upstream AT-rich sequence, which were essential to recruit both Pol II and Pol III. Modest (~2-fold) changes in the ratio of nuclear extract to DNA template were sufficient to shift from Pol II initiation, to a mixture of Pol II and III initiation, to sole initiation of transcription by Pol III. This transition from Pol II to Pol III-mediated transcription suggests that polymerase specificity is not constant, but instead depends on the properties of the promoter and transcription conditions.

**EXPERIMENTAL PROCEDURES**

**DNA Sequences and Templates**—Double-stranded core promoter sequences were inserted into PstI and XbaI sites of pUC119. If denoted “+T,” a Pol III-specific double terminator sequence (5’-TTTTTTTTGACTGACTTTTTTTT-3’) was additionally inserted between PstI and HindIII sites of pUC119. The utilized core promoter sequences of human calmodulin 2 (Calm2), adenosylmethionine decarboxylase 1 (AMD1), T-cell lymphotropic virus type 1 (HTLV-I), and adenovirus major late (AdML) were from −50 to +50 in respect to the +1 transcription start site, the human RNU6–1 RNA (U6, Pol III) promoter was from −125 to +50, the *Drosophila melanogaster* hunchback Promoter 2 (hbp2) was from −50 to +50 or, if denoted in the figure, from −303 to +432. The DNA sequence from −2 to +48 of the G element G3A (G) promoter was described previously (30). When indicated, the TATA box region was substituted with CGTCCGGA (mTATA) and the downstream promoter element (DPE) with GTACATA (mDPE).

**Human Nuclear Extract**—HeLa nuclear extracts (HSK) were prepared as described (31) with modified Buffer HEG containing 25 mm HEPES-K+ (pH 7.6), 0.1 mm EDTA, 10% (v/v) glycerol, 0.01% NP40, 0.1 mm KCl, 1 mm DTT, 1 mm benzamidine-HCl, 0.2 mm PMSF, 1 mm Na3S2O5, and 10 mm β-mercaptoethanol. Dignam nuclear extracts were prepared as described previously (32). The nuclei for the human adaption of the soluble nuclear fraction extract (SNF) were obtained as described for HSK. The nuclei were then resuspended in 0.5 ml of HEMG20 (25 mm HEPES-K+ (pH 7.6), 12.5 mm MgCl2, 0.1 mm EDTA, 20% (v/v) glycerol, 0.1 mm DTT) with either 0.4 mKGluc (SNF-KGluc (2.4 mg/ml of protein)) or 0.1 mKCl (SNFKCl (2.6 mg/ml)) for each gram of nuclei and spun in a SW28 rotor at 24,000 rpm for 1 h at 4°C, as described in Ref. 33. The clear second layer was utilized for transcription reactions.

**In Vitro Transcription Analysis**—Human transcription reactions were performed at a total volume of 50 μl. Unless specified otherwise, 7 μl of HSK (90 μg; 13 mg/ml of protein) and 500 ng of supercoiled DNA template (10 μg/ml, 237 fmol) were incubated in a buffer containing 20 mm HEPES-K+ (pH 7.6), 50 mm KCl, 0.02 mm EDTA, 2% (v/v) glycerol, 6 mm MgCl2, 4% PEG (15–20 kDa), 0.5 mm DTT, 10 mm P-creatine, and 3 mm ATP for 75 min at 30°C to allow formation of the preinitiation complex. Transcription was started by addition of 4 μl of 5 mm NTPs (0.4 mm each final), and terminated, if not denoted otherwise, after 20 min with 100 μl of transcription stop buffer (20 mm EDTA, 200 mm NaCl, 1% (w/v) SDS, 0.3 mg/ml of glycogen). Five μl of 2.5 mg/ml proteinase K was added and incubated for 15–20 min at ~21°C (room temperature). For single round transcriptions, 300 μg/ml of heparin was added (34). All reactions with tagetitoxin RNA polymerase III inhibitor (TagetinTM, Epicenter) were carried out with limiting (0.02 mm) GTP. Tagetitoxin was added prior to preinitiation complex assembly (35), whereas α-amanitin was added shortly before addition of the NTPs. If not specified otherwise, α-amanitin was utilized at a concentration of 4 ng/μl to distinguish transcription by Pol II and III (36, 37). Nucleic acids were extracted using standard phenol-chloroform purification and subsequent EtOH precipitation. Primer extension analysis was performed with a 5’-32P-labeled M13 reverse sequencing primer (5’-AGCGGATA-AACAATTTCACACAGGA-3’), located 60 bp downstream of the PstI cloning site, thus generating a 116-nucleotide primer extension product for promoters extending to +50°) as described in Ref. 38. Primer extension of the transcripts from the 0.71-kb hbp2 promoter fragment was done with a hbp2-specific primer (5’-GCATCATACGCACGTGCGCCAGC-3’). The extended *Drosophila* promoter was utilized to avoid primer extension of endogenous human transcripts found in nuclear extracts.

Reverse transcription products were quantified with a Typhoon PhosphorImager and ImageQuant software (GE Healthcare). All shown experiments were carried out at least three times with different HSK extracts.
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Dignam nuclear extract (85 μg of protein; 9 mg/ml of protein) in vitro transcriptions were performed with the reaction conditions described for HSK, but with a 15-min transcription time. For SNF transcriptions, 250 ng (5 ng/μl, 118 fmol) of DNA template in 1 μl of TE and 21 μl of hSNF or transcription buffer (12.5 mM HEPES-K+ (pH 7.6), 100 mM KCl, 6.25 mM MgCl2, 0.05 mM EDTA, 10% (v/v) glycerol, 0.05 mM DTT) as specified, were incubated at 30 °C for 75 min to allow formation of the preinitiation complex and transcription initiated by addition of 3 μl of 5 mM NTPs. Transcription time, stop, RNA isolation, and primer extension were performed as described for HSK transcriptions.

In vitro transcription reactions with Drosophila SK (39) extracts were carried out as previously described (40). Briefly, 250 ng of supercoiled DNA templates (10 ng/μl, 118 fmol) were transcribed with the indicated amount of nuclear extract for 45 min at ~21 °C (room temperature) in a 25-μl final volume containing 32.5 mM HEPES-K+ (pH 7.6), 50 mM KCl, 0.05 mM EDTA, 5% (v/v) glycerol, 6.25 mM MgCl2, 4% PEG (15–20 kDa), 0.5 mM DTT, 10 mM P-creatine, 0.005% Nonidet P-40, 3 mM ATP, and 0.5 mM rNTPs.

Transcription Start Site Mapping—Mapping was performed with the Sequenase 2.0 DNA Sequencing Kit (Affymetrix).

RESULTS

Multiple Polymerases Can Utilize RNA Pol II Promoters in Vitro—The fungal toxin α-amanitin is an efficient inhibitor of transcriptional elongation. Human Pol II is highly sensitive to α-amanitin (50% inhibition at 0.018 ng/μl), whereas Pol III is only mildly sensitive to α-amanitin (50% inhibition at ~20 ng/μl), and RNA polymerase I is insensitive (37). This toxin is thus commonly used to distinguish the transcripts of the three eukaryotic polymerases.

During routine testing, I noted that transcription from diverse Pol II core promoters was only modestly sensitive to 4 ng/μl of α-amanitin, when 10 ng/μl of DNA template was transcribed with HSK extract at 0.5 mg/ml of protein in vitro (Fig. 1A). Both, α-amanitin-sensitive and -insensitive transcripts were initiated from the same +1 transcription start site. The α-amanitin-sensitive transcript displayed an additional weaker start site at −1, relative to the main transcription start site (Fig. 1B). This indicated either the existence of an α-amanitin-resistant Pol II in the nuclear extract, or that multiple polymerases are capable of transcribing the Pol II promoters.

To distinguish between the two scenarios, I titrated the nuclear extract in the presence or absence of α-amanitin and quantified the difference in transcriptional activity. Transcription by a single α-amanitin-resistant Pol II should exhibit a constant ratio of α-amanitin-sensitive to -insensitive transcripts, whereas competition between multiple RNA polymerases should vary with extract concentrations if one polymerase transcription system is more limiting or affine than the other.

I found that the ratio of α-amanitin-sensitive to -insensitive transcripts increased with higher extract concentrations in vitro (Fig. 1C). Notably, the level of α-amanitin-resistant transcription was greater at lower extract concentrations (0.25–0.5 mg/ml). With higher concentrations of nuclear extract (>1 mg/ml) the transcriptions became highly sensitive to α-amanitin. Similar results were obtained with crude human nuclear extract prepared according to Dignam et al. (32) (Fig. 2A), or with an adapted human version of the SNF extract (33) (Fig. 2B) where α-amanitin-resistant transcription was stronger in low salt nuclear extracts. This suggests that multiple polymerases initiate transcription from Pol II promoters.

This observation was made with human but not with Drosophila SK extract. Neither low extract concentrations nor high amounts of DNA template resulted in Pol II-independent initiation of transcription from Pol II promoters in Drosophila extracts (Fig. 2C).

Human Pol III and Pol II Accurately Initiate Transcription from Pol II Promoters in Vitro—To characterize the Pol II-independent transcriptional activity at Pol II promoters, I titrated α-amanitin to in vitro transcription reactions under low-extract non-Pol II conditions (0.5 mg/ml of HSK). As a control,
the Pol III promoter from the human U6–1 snRNA gene was included. Transcription initiating from both Pol II and Pol III promoter templates displayed identical sensitivity to higher concentrations of α-amanitin, indicating that Pol III initiates transcription from Pol II promoters (Fig. 3A, quantified in B). RNA polymerase III transcription was stronger in low salt (0.1 M KCl) SNF. C, transcription with a Drosophila nuclear extract is sensitive to 4 μg/ml of α-amanitin at different concentrations of extract. AdML, adenovirus major late promoter; AMD1, human adenosylmethionine decarboxylase 1 gene promoter; Act87E, Drosophila Actin 87E gene promoter.

FIGURE 2. α-Amanitin-insensitive transcription of Pol II promoters in diverse human, but not Drosophila nuclear extracts. A, in vitro transcription reactions with Dignam nuclear extract. 5 ng/μl of DNA template were transcribed with increasing amounts of nuclear extract in the presence or absence of 4 ng/μl of α-amanitin. B, transcription reactions with an adapted version of human SNF extracts containing KCl or potassium glutamate (see “Experimental Procedures”). 10 ng/μl of DNA template (118 fmol) were transcribed with varying amounts of SNF in the presence and absence of 4 ng/μl of α-amanitin. RNA polymerase III transcription was stronger in low salt (0.1 M KCl) SNF. C, transcription with a Drosophila nuclear extract is sensitive to 4 μg/ml of α-amanitin at different concentrations of extract. AdML, adenovirus major late promoter; AMD1, human adenosylmethionine decarboxylase 1 gene promoter; Act87E, Drosophila Actin 87E gene promoter.

FIGURE 3. RNA polymerase III accurately initiates transcription from RNA polymerase II promoters in crude nuclear extract in vitro transcription assays. All transcription reactions were performed with unfractionated high salt human nuclear extract (HSK). A, effect of different concentrations of α-amanitin upon transcription under Pol III-favoring conditions (0.50 mg/ml of HeLa extract, 10 ng/μl of DNA template). U6, promoter of the human U6–1 snRNA gene, which is transcribed by RNA polymerase III. B, plots of data from A. For each promoter, the amount of transcriptional activity in the absence of α-amanitin was defined to be 100%. C, α-amanitin-resistant transcription can be blocked by the Pol III-specific inhibitor tagetitoxin. Transcription reactions were performed under Pol III-favoring conditions (0.50 mg/ml of HeLa extract) or Pol II-favoring conditions (2 mg/ml of HeLa extract). Where indicated, α-amanitin (4 μg/ml) and/or tagetitoxin (18 μM/30 units) were included in the reactions. AdML, adenovirus major late promoter; AMD1, human adenosylmethionine decarboxylase 1 gene promoter.
which reflect prior observations that SL1 binding is solely mediated by its TAFs (9).

Transcription by human Pol III, unlike Pol II, can be terminated by a stretch of four or more thymine nucleotides (42, 43). This Pol III-specific property can be exploited to distinguish between Pol II- and Pol III-dependent transcription. I therefore cloned a Pol III-specific double terminator (\(5'\)-TTTTTTTGT-CAGTACTTCTTTTTT-3\(') downstream of the core promoter templates utilized in the preceding in vitro transcription assays. This further extended the primer extension product by 24 nucleotides, enabling a two-template assay consisting of both a Pol III terminator-containing and terminator-lacking template. Addition of the Pol III terminator (\(5'\)-T) to Pol II promoters made the synthesis of transcripts reading through the inserted terminator sensitive to low concentrations of \(\alpha\)-amanitin (4 ng/\(\mu\)l), indicating that these transcripts were exclusively mediated by Pol II. At both low and high concentrations of extract, the Pol III terminator efficiently terminated Pol II-independent transcripts (Fig. 4A). This provided an additional line of evidence that Pol III initiates transcription from Pol II promoters and permitted the relative quantification of Pol II- and Pol III-dependent transcription. Titration of nuclear extracts confirmed that transcription by Pol III was predominant at lower extract concentrations, whereas Pol II transcription was foremost at higher concentrations (Fig. 4B, quantified in C). RNA polymerase II-dependent transcription at low extract concentrations could be achieved by limiting the template DNA concentration (data not shown). On the other hand, Pol III-mediated transcription from Pol II promoters could be achieved with high extract concentrations (1.8 mg/ml) by increasing the DNA template concentration (Fig. 4D). This effect was not observed when utilizing an inactive, TATA-mutated version of the same core promoter (mTATA, Fig. 4E). The absence of Pol III-mediated transcription at higher extract concentrations is thus unlikely due to nonspecific DNA-binding proteins or putative inhibitors present in the nuclear extract. Taken together, although only a small percentage of template DNA is commonly utilized for productive transcription (44), the available DNA template seems to be limiting for Pol III initiation from Pol II promoters.

**Polymerase Dynamics**—The remarkably sharp transition from Pol III- to Pol II-dependent transcription indicated the involvement of other factors besides the DNA to nuclear extract ratio. I therefore investigated the polymerase reinitiation rate by varying the transcription times for Pol II- and Pol III-mediated transcription. Prolonged transcription times,

FIGURE 4. The ratio of DNA template to nuclear extracts determines the initiation of Pol II or III from Pol II promoters. A, a Pol III-specific poly(dT) terminator distinguishes between Pol II and Pol III transcription and increases the primer extension length, which permits a two-template assay with the same primer. Two-template transcription assays were performed under Pol III (0.50 mg/ml of HeLa extract) or Pol II favoring (1.8 mg/ml of HeLa extract) conditions, with constructs that either lack or contain a Pol III-specific terminator downstream of the core promoter. B, determination of the fraction of transcription mediated by Pol III. The two-template transcription was performed with different amounts of HeLa extract with 10 ng/\(\mu\)l of DNA template. C, plots of the data in B. The % Pol III transcription was defined to be: 100% × (amount of transcription in the absence of terminator) / (amount of transcription in the presence of terminator). D, increased DNA template concentrations promote Pol III transcription with 1.8 mg/ml of HeLa extract. E, titration, as in D, with the TATA-mutated DNA template. AdML, adenovirus major late promoter; AMD1, human adenosylmethionine decarboxylase 1 gene promoter.
The TATA Box or an AT-rich Sequence Is Sufficient to Recruit Human Polymerase II and III to Polymerase II Promoters—DNA sequence analysis of the Pol II promoters used in this study revealed the absence of typical Pol III promoter elements Box A/B/C, and PSE (4, 45). It was notable, however, that the Pol II promoters from which Pol III initiated contained either a TATA box or an AT-rich sequence around bp −30 relative to the transcription start site. To investigate whether these sequences are required to recruit Pol II and/or Pol III, the TATA box was inactivated by substitution with TCGTCCGA (mTATA). With both low and high concentrations of nuclear extract, elimination of the TATA box abolished all transcriptional activity (Fig. 6A). As shown in Fig. 6B, the 0.71-kb promoter fragment of hbp2 was efficiently transcribed by Pol III with low but not high HSK extract concentrations. As the extended promoter fragment does not contain any known Pol III motifs in conjunction to the TATA box, the findings argue that the TATA box by itself is sufficient to recruit both human Pol II and Pol III to Pol II promoters, even using crude nuclear extracts.

The DPE and Motif Ten Element (MTE) Are Exclusively Utilized by Polymerase II—Pol III transcription was also observed to lesser extents from some TATA-less promoters. As the extended promoter fragment does not contain any known Pol III motifs in conjunction to the TATA box, the findings argue that the TATA box by itself is sufficient to recruit both human Pol II and Pol III to Pol II promoters, even using crude nuclear extracts.

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The DPE and Motif Ten Element (MTE) Are Exclusively Utilized by Polymerase II—Pol III transcription was also observed to lesser extents from some TATA-less promoters. To investigate a role of the DPE and MTE in Pol III recruitment, the human Calm2, and the DPE in the transposable element G3A were mutated. Unlike G3A, Calm2 contains an AT-rich sequence upstream of the transcription start site (−35 TGGTT-TATGA −26). As with TATA box promoters, Pol III-dependent transcripts from Calm2 were detectable when low amounts of extracts were used. No initiation of transcription by Pol III was observed for G3A. Mutation of the DPE eliminated Pol II but not Pol III transcripts (Fig. 6C). RNA polymerase III recruitment is thus MTE/DPE-independent.
RNA Pol IIIInitiation from RNA Pol II Promoters

RNA Polymerase III Initiating from Polymerase II Promoters Explains Previous Controversies in the Literature—Transcription of Pol II promoters by α-amanitin-insensitive Pol II, or extracts with unusually high resistance to inhibition, has been reported previously. Most notably, Piras et al. (46) demonstrated Pol II–independent transcription of the HTLV-1 promoter, which depended on TBP. In contrast, Lenzmeier and Nyborg (47) provided several lines of evidence that HTLV-1 was exclusively served by Pol II, even when utilizing the same extract as Piras et al. (46). Given that Lenzmeier and Nyborg (47) used a 3.5-fold higher extract to DNA ratio than Piras et al. (46), I hypothesized that this conflicting result was caused by the different ratios of DNA template to nuclear extract concentration utilized. To test this hypothesis, the HTLV-1 promoter was transcribed with low (Pol III-favoring) and high (Pol II-favoring) concentrations of nuclear extract in the presence or absence of 4 ng/μl of α-amanitin and tagetitoxin or Pol III inhibitor. Consistent with both Piras et al. (46) and Lenzmeier and Nyborg (47), HTLV1 transcription was resistant to α-amanitin at low extract concentrations, whereas it was exclusively provided by Pol II with higher extract concentrations (Fig. 7A). The conflicting results could therefore be explained by differences in the ratio of DNA to nuclear extract, and thus a hidden Pol III transcription activity. However, given the transcription conditions and that Pol II outcompetes Pol III, it is more likely that HTLV-1 is transcribed by Pol II in vitro.

DISCUSSION

This study has demonstrated that both human Pol II and Pol III initiate transcription in vitro from most examined Pol II promoters. The Pol III transcriptional activity did not require fractionation of the nuclear extract and was characterized in the commonly used crude nuclear extracts (HSK, SNF, and Dignam). The investigated Pol II promoters did not contain recognizable Pol III promoter motifs. RNA polymerase III–mediated transcriptions was accurately initiated and depended upon a TATA box or an upstream AT-rich sequence. RNA polymerase III recruitment to AT–rich Pol II promoters is thus specific, in contrast to previous reports (48). The +1 transcription start site was conserved between Pol II and Pol III.

Both transcription systems are mutually exclusive and compete for a common core promoter motif. The degree of Pol III transcription correlated with the quality of the TATA box or AT-rich region at bp −30 (relative to the +1 transcription start site), which were sufficient to recruit Pol III. Although TATA box–containing promoters strongly recruited Pol III, hCalm2 with “TGTTTATGA” (from −35 to −26, relative to the +1 transcription start site) only weakly supported Pol III transcription (Fig. 6C). Like the TATA boxes, AT-rich sequences are likely directly recognized by the TBP, which serves as the initial anchor for the Pol II and Pol III transcription machinery (49). Although TBP is part of the Pol II TFIIID complex, it is also part of the Pol III basal transcription factor Brf1-TFIIIB (14).

The Pol II preinitiation complex assembles faster on Pol II promoters than Pol III (Fig. 5B). However, once bound, Brf1-TFIIIB is stably committed to the TATA box of the Pol II promoter and cannot be replaced by the Pol II preinitiation complex (28). It is likely that Brf2-TFIIIB can function in a similar manner. By contrast, the DPE, which interacts with TAF6 and TAF9 of TFIIID (50), did not recruit Pol III.

TBP is conserved from archaea to humans. The structural conservation between the three eukaryotic RNA polymerase preinitiation complexes and their common use of TBP (7, 8, 15) could therefore lead to a TBP-mediated crossover function and facilitate TFIIIB-mediated Pol III transcription from TBP-recognized Pol II promoters. Such Pol III transcripts can serve as a template for protein synthesis in vivo (51). Thus, unless a gene contains poly(T) sequences, Pol III transcription could functionally complement mRNA or microRNA synthesis.

There is an emerging picture indicating that, even though the three eukaryotic RNA polymerases are commonly described as transcribing different subsets of genes, their core transcriptional machineries are structurally more alike than previously appreciated (7, 8). This may be reflected in my finding that both human Pol II and Pol III can accurately initiate transcription from a diverse subset of Pol II promoters. On the other hand, Pol III–dependent transcription initiation at Pol II promoters was found in human but not in Drosophila SK. Common use of TBP in the preinitiation complexes of Pol II and Pol III might cause crossover function of the polymerases. The substitution of TBP with Trf1 in some protostome Pol III transcription complexes (52) may therefore have been evolutionarily selected for increased specificity.

Genome-wide analysis of Pol III promoter occupancy in HeLa cells suggested promoter accessibility to be a major regulator of Pol III transcription (53). This was also observed in vitro. Mapping of Pol II and Pol III localization in human cell lines by ChIP-seq found Pol II to closely associate with Pol III genes throughout the genome (54). Co-occupancy of both polymerases was demonstrated for the promoters of the RPPH1 gene and U6–2 (24, 55), as well as for promoters of other ncRNAs (53, 56). The human c-myc promoter, for example, is transcribed by both Pol II and Pol III in vivo and in vitro (57), with the Pol III transcript being terminated early. Both Pol III promoter occupancy and the resulting small RNAs have the potential to function in gene regulation.

Consistent with the findings of this study, both polymerases initiate from the same start site of the c-myc promoter and
require a TATA box (57, 58). The functional separation of Pol II and Pol III may thus not be as strict as commonly described, but rather a function of the promoter and the cellular properties.

This encourages speculation that another function of chromatin is to protect Pol II promoters and their enhancers from cryptic Pol III transcription. Chromatin can actively prevent DNA accessibility, but can also maintain the global DNA to transcription factor ratio.

Chromatin is quickly reassembled after passing through the transcription machinery (59, 60), and hyperacetylation or lack of histone remodellers induces cryptic bidirectional transcription by Pol II (61, 62). It would be interesting to explore whether Pol III also contributes to these cryptic bidirectional transcripts.

In conclusion, this study highlights the property of human Pol III to accurately initiate transcription from Pol II promoter with a TATA box or an AT-rich sequence in a crude nuclear extract transcription system. The ratio of DNA template to nuclear extract determines whether Pol II, Pol III, or both polymerases initiated transcription from the analyzed Pol II promoters in vitro (Figs. 4 and 7). Polymerase specificity is thus not invariant but could be described as a function of the promoters’ properties and the transcription conditions. The functional separation of Pol II and Pol III transcription machineries may thus not be as distinct as commonly described.

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