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A novel activity enhances promoter escape of RNA polymerase I

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ABSTRACT

We have characterized a novel transcriptional activity from HeLa cells that is required for ribosomal gene transcription by RNA polymerase I. This activity has a native molecular mass of 16 kDa and does not bind to conventional chromatographic resins. Single-round and immobilized-template experiments revealed that initiation complex formation is independent of the novel activity. Functional studies showed that it stimulates the transition from initiation to elongation, promoter escape. Thus the novel activity does not resemble the mouse initiation/elongation factor TIF-IC but is a true novel entity.

Introduction

The three DNA dependent RNA polymerases in eukaryotes are dependent on specific subsets of transcription factors to recognize the promoter, to initiate transcription, for promoter escape, to elongate and to terminate transcription. Yeast and mammal RNA polymerase II transcription factors are well defined [1,2] and in yeast the minimal set of transcription factors needed to initiate *in vitro* transcription of RNA polymerase I has been described [3]. The components of the mammalian RNA polymerase I transcription apparatus still await silver-gel definition. Mouse RNA polymerase I transcription depends on the four transcription factors TIF-IA, TIF-IB, TIF-IC, and UBF [4] and TFIIH has just been shown to be essential for Pol I transcription [5]. All these transcription factors have functional homologs in the yeast *Saccheromyces cerevisiae* despite the initiation/elongation factor TIF-IC that has not been cloned yet [6]. Human rDNA transcription needs a minimal set of two transcription factors UBF and SL1, the TIF-IB homolog in addition to the active form of RNA polymerase I that is associated with the TIF-IA homolog hRRN3 [7]. Quite recently two additional components of the basal transcription factor SL1 have been described [8,9] indicating that the components of the basal transcription apparatus of mammalian RNA polymerase I have not yet been characterized exhaustively. Here we show the purification and characterization of a novel, hitherto undescribed activity of RNA polymerase I that is not identical with TIF-IC and that enhances promoter escape.

Abbreviations: Pol I, RNA polymerase I; TIF, transcription initiation factor; UBF, upstream binding factor; SL1, selectivity factor 1; TFIIH, transcription factor IIH; hRRN3, human rRNA synthesis defective 3; RPA116, RNA polymerase I subunit 116; TAF110, TBP-associated factor 110; CSB, Cockayne syndrome B; XPB, xeroderma pigmentosum B; cdk 7, cyclin dependent kinase 7; TCA, trichloracetic acid; NTP, nucleotide triphosphates.

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Materials and methods

Cell growth. HeLa cells were grown in DMEM supplemented with 10% FCS and penicillin/streptomycin. Transcriptionally active nuclear extracts were prepared from logarithmically growing mammalian cell culture as described [10].

Antibodies. Western blotting was performed with antibodies directed against RPA116, TAF110, TIFIA, and UBF (all kind gift of I.Grummt), CSB [11] XPB and cdk7 (Santa Cruz).

In vitro transcription. All *in vitro* transcription assays were performed as described [11].

Protein purification. Following equilibration of the DEAE-Sepharose CL-6B column with buffer AM100 [12], nuclear extracts from HeLa cells were loaded (10 mg protein/1 ml matrix). The column was washed with the buffer AM100 and bound proteins were eluted with the buffer AM280. The fraction DEAE FT was loaded on the Heparin-Sepharose column (10 mg protein/1 ml matrix) and the flow-through fraction HS FT was collected. Following equilibration of Q-Sepharose, SP-Sepharose or CM-Sepharose with the buffer AM100 the resin was loaded with proteins (10 mg/1 ml matrix). The protein/matrix solution was overhead rotated for 15 min at 4 °C and centrifuged for 10 min at 1000 rpm at 4 °C. The fraction SP FT was incubated with Phosphocellulose P11 equilibrated with the buffer AM100 (0.24 mg/1 ml matrix). Supernatant fraction P11 FT.

Glycerol-gradient sedimentation. Linear 12.5–30% glycerol (4.2 ml) gradients were prepared with a Gradient Maker in Beckman SW50 centrifuge tubes. The fraction CM FT (0.3 ml per

gradient) was applied on the top of glycerol gradient. Following ultracentrifugation of the samples for 16 h (45 000 rpm, 4 °C), the fractions (12 × 375 μl) were collected from top to the bottom and tested in the *in vitro* transcription assay, precipitated with TCA and resolved on a 8–20% gradient gel.

Results

Identification of a novel activity

Initiation of human ribosomal RNA gene transcription requires beside the polymerase multible initiation factors. By chromatography of nuclear extracts over DEAE-Sepharose a significant enrichment of RNA polymerase I and transcription factors can be achieved [12]. To concentrate the RNA polymerase I transcription apparatus we performed a DEAE chromatography and eluted the bound proteins with AM280. The DEAE280 fraction was not sufficient to direct transcription (Fig. 1A) unless it was supplemented with the flowthrough (FT) of the DEAE column. Western blot analysis revealed the presence of all known transcription factors of RNA polymerase I in the DEAE280 fraction (Fig. 1B), indicating that an unknown component of RNA polymerase I is separated by DEAE chromatography.

The novel activity does not bind to chromatographic resins

To characterize the novel activity in more detail, we attempted to purify this activity from DEAE FT using conventional chromatographic procedures. The novel activity was monitored by its ability to stimulate transcription of inactive DEAE280 fractions. As a first resin we fractionated a DEAE FT fraction on Heparin-Sepharose that binds most of the RNA polymerase I factors. The transcriptional analysis revealed that the novel activity eluted in the flowthrough fraction of the Heparin column (Fig. 2A). Subsequently we tested anion and cation exchangers for their capability to bind the activity under different pH and salt conditions. We were not able to detect any specific interaction of the novel activity with any resin tested (data not shown). Thus we decided to develop a purification scheme basing on the binding of contaminating proteins to different resins whilst the activity remains in the flowthrough. This procedure enabled us to work in a "batch" method that allowed a fast and effective purification without dialysis steps. Purification of the flowthrough over Q-Sepharose and SP-Sepharose resins resulted in a 60% reduction of total protein content by retained activity in the specific transcription (Fig. 2B). In Western blot analysis of the SP flowthrough fraction was used to exclude the copurification of known RNA polymerase I transcription factors. None of the components of RNA polymerase I transcription coeluted in the tested fraction proving that the SP FT fraction habors a hitherto undescribed activity (Fig. 2C).

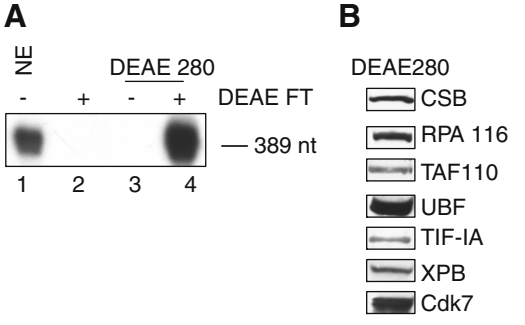

Fig. 1. Identification of a novel activity. (A) *In vitro* transcription with HeLa nuclear extract (NE) and after fractionation by DEAE chromatography. (B) The DEAE280 fraction containes all known RNA polymerase I transcription factors. Western blot analysis with antibodies against RPA116 (a subunit of Pol I), TAF110 (a subunit of SL1), TIFIA, UBF, CSB, XPB, and cdk7 (both subunits of TFIIH).

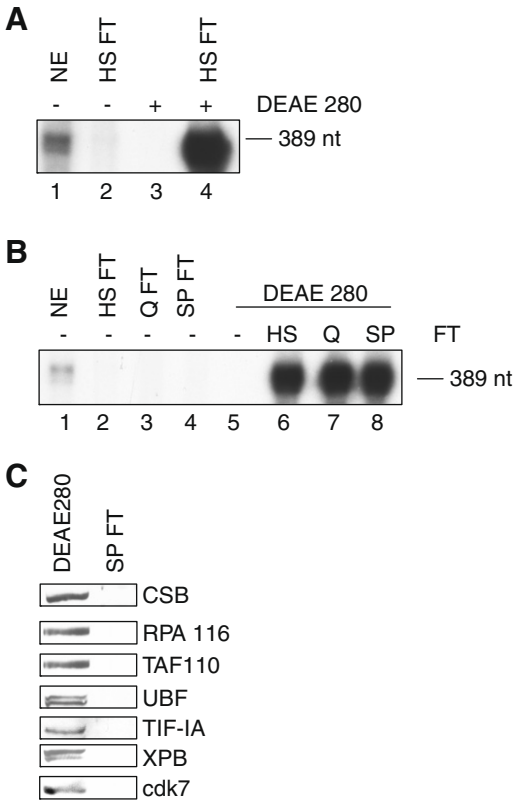

Fig. 2. Inert chromatographic characteristics of the novel activity. (A) The novel activity does not bind to Heparin–Sepharose. The flowthrough of Heparin–Sepharose chromatography was used to complement transcription with a DEAE 280 fraction. (B) The novel activity elutes in the flowthrough of strong ion exchangers. *In vitro* transcription analysis of the reconstitution of DEAE280 with HS/Q and SP flowthrough. (C) The flowthrough activity does not contain any known RNA polymerase I factor. Immunoblots of the fractions DEAE280 and SP FT were probed with antibodies directed against RPA116, TAF110, TIFIA, UBF, CSB, XPB, and cdk7.

Functional characterization of the novel activity

To investigate the role of the novel activity in transcription initiation, a Sarcosyl challenge experiment was performed. The sensitivity of transcription to this inhibitor reflects the formation of distinct multiprotein complexes at the promoter which allow a single round of transcription. The fully assembled Pol I initiation complex is resistant to 0.025% of Sarcosyl [11]. If the novel activity stimulates initiation complex formation more Sarcosyl-resistant complexes are formed during preincubation and a stimulation of single-round transcription is detectable. In the experiment displayed in Fig. 3A, preinitiation complexes were formed by preincubation of the template with the DEAE280 fraction in the presence (lanes 5 and 7) or absence of the purified activity (SP FT, lanes 4 and 6). After the preincubation period, Sarcosyl was added (lanes 6 and 7) and transcription was started. There is no stimulation of single round transcription detectable (compare lanes 6 and 7) indicating that the novel activity does not influence the efficiency of initiation complex formation.

As initiation complex formation is not stimulated by the novel activity, we asked if subsequent steps of the transcription cycle, i.e. promoter clearance and elongation are stimulated by this activity. To study this question in more detail, we performed experiments with immobilized template. Biotinylated rDNA template was immobilized by binding to streptavidin magnetic beads and