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RNA ligands generated against complex nuclear targets indicate a role for U1 snRNP in co-ordinating transcription and RNA splicing

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Abstract RNA ligands were generated against various gene products present in HeLa nuclear extract. Functional profiling was performed to identify RNA ligands that modulate RNA polymerase II (pol II)-mediated transcription. Unexpectedly, four of the eight inhibitor ligands identified by this screen contained an 11-nucleotide sequence identical to the 5′-splice site of eukaryotic pre-mRNAs. Such ligands were shown to impede pre-initiation complex assembly on a cytomegalovirus promoter. In addition, U1 small nuclear ribonucleoprotein particles (snRNP) and pol II had been co-immunoprecipitated in the absence of transcription. These results suggest a role for U1 snRNP in co-ordinating transcription and RNA splicing.

Key words: RNA ligand; U1 small nuclear ribonucleoprotein particle; RNA polymerase II; Transcription; Splicing

1. Introduction

In eukaryotes, nearly all pre-mRNAs are exclusively transcribed by RNA polymerase II (pol II) and undergo splicing and other processing events to become templates for protein synthesis. The initiation of transcription by pol II, which represents a key step for the regulation of gene expression, involves numerous basal transcription factors and diverse transacting regulators [1]. Increasing evidence has indicated that the transcription and processing of pre-mRNA are co-ordinated events in the nucleus. Pol II appears to interact with a variety of RNA processing factors and to mediate RNA processing co-transcriptionally [2–14]. In these studies, however, RNA processing factors have been perceived to act restrictively 'downstream' of transcription initiation even though they are found in close proximity with the transcription machinery [15,16].

Recently, it has been established that an iterated enrichment process (systematic evolution of ligands by exponential enrichment, SELEX) from libraries of random nucleic acid molecules can generate RNA or DNA ligands with high affinity and specificity for various targets [17–19]. This present study shows that small RNA molecules (RNA ligands) isolated from a combinatorial selection abolish transcription initiation from a pol II promoter through interaction with U1 small nuclear ribonucleoprotein particles (snRNP) and that pol II and U1 snRNP associate in the absence of transcription. These results yield new insights into the connection between transcription and RNA processing in that U1 snRNP is involved in the co-ordination of these events.

2. Materials and methods

2.1. Selection

The initial pool of DNA templates for RNA molecules was constructed by annealing two chemically synthesized overlapping DNA oligos: template oligo containing a section of 40 random nucleotides (nt) and constant flanking sequences on either side (template oligo, 5′-GAGGAAGAGGGATGGGN$_{40}$CATAACCCAGAGGTCGAT-3′), and complementary 5′-DNA oligo (5′-PR, GGGGGAATTCTAATACGACTCACTATAGGGAGAGAGGAAGAGGGATGGG), which were converted into double-stranded (ds) DNA by DNA polymerase I (Promega). In later rounds, the DNA templates were regenerated from RNA molecules by reverse transcriptase (Roche) and polymerase chain reaction (PCR) with primers 5′-PR and 3′-PR (GGGGGGATCCAGTACTATCGACCTCTGGGTTATG). The RNA molecules were synthesized by T7 RNA polymerase (Roche) in vitro and purified on a 10% polyacrylamide/7 M urea gel after the DNA templates were removed by DNase I digestion (Promega), and quantitated with a UV spectrometer (Beckman). In other experiments, RNA molecules (RNA 9A, 9B and 9C) with mutated sequences of a selected ligand (RNA 9) were generated by T7 RNA polymerase with the same methods from dsDNA templates obtained through PCR with primers 5'PR and 3'PR and synthetic DNA oligos (RNA 9A, 5′-GAGGAAGAGGGATGGGAGTCCACGAGCGTATCCAGTATCCACAGACATAACCCAGAGGTCGAT-3′; RNA 9B, 5′-GAGGAAGAGGGATGGGGTAGGTAAGTACAATCCAGTATCCACAGACATAACCCAGAGGTCGAT-3′; RNA 9C, 5′-GAGGAAGAGGGATGGGAGTCCACGAGCGTAGGTAAGTACAATAGACATAACCCAGAGGTCGAT-3′, respectively). In the first round, HeLa nuclear extract (200 μg) and RNA (10 nmol, transcribed from dsDNA templates of around 6×10^{14} different sequences) were combined in a volume of 1 ml of incubation buffer (20 mM HEPES, pH 7.9; 100 mM KCI; 0.2 mM EDTA; 0.5 mM DTT; 20% glycerol). The HeLa nuclear extract was prepared as previously described [20]. In later rounds, nuclear proteins (20–25 μg), and RNA (~1 nmol) were incubated in a volume of 100 μl. The unbound RNA molecules were found to be rapidly degraded in the HeLa nuclear extract while the bound RNA molecules remain intact. For each of the first eight rounds of selection, RNA/protein binding was assayed with a gel mobility-shift assay in a wide range of protein concentrations and variable time of incubation, and the integrity of RNA ligands was detected by denaturing gel electrophoresis. Particular protein concentrations and time of incubation were chosen to remove 90% of the input RNA molecules. From the ninth to fourteenth rounds, a gel mobility-shift assay was used to isolate the bound RNA molecules from each pool. The mixture of ^{32}P-labeled RNA,

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Abbreviations: Pol II, RNA polymerase II; CTD, carboxyl-terminal domain; snRNP, small nuclear ribonucleoprotein particles; SELEX, systematic evolution of ligands by exponential enrichment; RT/PCR, reverse transcription/polymerase chain reaction; nt, nucleotide(s); ds, double-stranded; CMV, cytomegalovirus

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HeLa nuclear proteins (25 μg) and tRNA (20 μg) was incubated at 37°C for 30 min and loaded on a 5% non-denaturing polyacrylamide gel (acrylamide:bis-acrylamide, 80:1). Radiolabeled RNA species that migrated more slowly than free RNA on the gel were isolated, eluted from gel by shaking in sodium acetate (0.3 M), extracted by phenol, precipitated by ethanol and converted into dsDNA template through reverse transcription (RT)/PCR for the next round of selection. For RT/PCR, the first DNA strand was synthesized with RNA molecules and 3′-PR using a reverse transcription kit (Roche), followed by PCR with 3′-PR and 5′-PR using a PCR kit (Life Technologies). Through this selection process, RNA ligands appeared to be generated to a variety of targets present in the extract as evidenced by the appearance of numerous shifted RNA complexes that appeared in later rounds of selection (data not shown). For cloning, the dsDNA templates from the fourteenth round were ligated into pUC19 vector (New England Biolab) through *Eco*RI and *Bam*HI restriction sites, which was used to transform DH5α *Escherichia coli* competent cells (Life Technologies). For imaging, gels were either dried for autoradiography with a Storm 840 Phosphorimager (Molecular Dynamics), or directly exposed to Fuji X-ray films that were processed with a Kodak film processor.

2.2. Pol II transcription and pre-initiation complex analysis

Various competitor RNAs including tRNA, Pool 1 and 14 RNA, and RNA ligands (35, 70 and 105 pmol) were added to fresh HeLa nuclear extract (45μg; Promega) with RNasin (40 U; Roche) and pre-incubated at 30°C for 15 min. *Bam*HI linearized DNA template (200 ng) containing a cytomegalovirus (CMV) promoter and human β-globin exons 1, 2 and introns, rNTPs (0.4 mM), Mg^{2+} (3 mM) and [α-^{32}P]GTP (10 μCi, 800 Ci/mmol, Amersham) in 44% transcription buffer (20 mM HEPES, pH 7.9; 100 mM KCl; 0.2 mM EDTA; 0.5 mM DTT; 20% glycerol) were added to initiate transcription in a total volume of 25 μl at 30°C (60 min). An RNA loading control (~700 nt) generated by T7 in vitro transcription was added to the pol II transcription reactions just prior to RNA extraction by phenol. The transcripts were resolved on a 6% polyacrylamide/7 M urea gel. For post-initiation inhibition of pol II transcription by ligands, the experimental conditions were as described above except that samples were pre-incubated in parallel before and after the addition of RNA ligands. After the globin DNA template and HeLa nuclear extract were pre-incubated at 30°C for 30 min, increasing amounts of RNA 9 (35, 70 and 105 pmol) were added to the mixture which was incubated at 30°C for another 30 min. Transcriptions were started by adding the rest of the transcription mix, and total RNA was extracted 45 min after transcription initiation.

Pol II binding to the pre-initiation complex was analyzed on an immobilized dsDNA containing the CMV promoter. The CMV promoter DNA was generated by PCR with an upstream vector-specific primer (5′-AGTCGTGACGTGAATTACG-3′) and a biotinylated DNA oligo specific for β-globin exon 2 (5′-biotin-GGATATCGC-GACTCACCACCAACTTCATCCACGTTC-3′), and immobilized on streptavidin-coated agarose beads (Pierce). The transcription pre-initiation complex was assembled on the immobilized CMV promoter DNA in the presence or absence of HeLa nuclear proteins (45 μg) and RNA competitors (105 pmol each). Promoter bound proteins were eluted with 1×sodium dodecyl sulfate (SDS) loading buffer (50 mM Tris–Cl, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), resolved on a 10% polyacrylamide/SDS gel and transferred onto a nitrocellulose membrane (Schleicher&Schuell) for Western blot analysis with anti-pol II (N-20, Santa Cruz Biotech).

2.3. Pol III transcription

The in vitro transcriptions by pol III were performed according to previously described methods [21] with modifications. Briefly, each reaction contained *Bam*HI linearized plasmid DNA (200 ng) with a human U6 RNA gene promoter and an 1170 bp ribozyme construct at downstream, HeLa nuclear extract (45 μg), rNTP (0.4 mM each), RNasin (40 U; Roche), Mg^{2+} (3 mM), [α-^{32}P]GTP (10 μCi, 800 Ci/mmol, Amersham) and 16 mM creatine phosphate in 44% transcription buffer (20 mM HEPES, pH 7.9; 100 mM KCl; 0.2 mM EDTA; 0.5 mM DTT; 20% glycerol) in a volume of 25 μl. The transcriptions were carried out at 30°C for 60 min. The effects of RNA ligands (105 pmol each) as well as α-amanitin (1, 50 and 100 μg/ml, Roche) on pol III activity, and transcripts were analyzed with the same methods for pol II as described above.

2.4. Oligonucleotide-directed cleavage by RNase H

Targeted depletion of U1 and U3 RNA sequences by RNase H cleavage was mediated by a deoxyoligonucleotide (anti-U1, 5′-TTCAGGTAAGTACTCA-3′) specific for the 5′-terminal U1 RNA [22] and one (anti-U3, 5′-TGGTTTTCGGTGCTCT-3′) specific for a putative single-stranded region of U3 RNA [23]. For RNase H cleavage reactions, HeLa nuclear extract (45 μg), RNasin (40 U; Roche), deoxyoligonucleotides (200 ng), RNase H (1 U, Roche), Mg^{2+} (3 mM) and transcription buffer (20 mM HEPES, pH 7.9; 100 mM KCl; 0.2 mM EDTA; 0.5 mM DTT; 20% glycerol) in a volume of 15 μl were incubated at 30°C for 45 min. The snRNA-depleted nuclear extract samples were tested for transcription activities as well as the effect by RNA ligands.

2.5. Immunoprecipitation, RNA affinity chromatography and Western blot

For immunoprecipitation, 1 μg of a murine anti-U1 70k antibody (Santa Cruz Biotech) or IgG (Sigma) was incubated with or without 45 μg HeLa nuclear extract, and precipitated by 4 μl of flurry protein G-Sepharose beads (Pharmacia) in 50 μl IP buffer [24] on a slow rotating shaker at 4°C for 4 h. The beads were washed twice with 50 μl IP buffer. For RNA-based affinity chromatography, HeLa nuclear proteins were loaded onto columns containing streptavidin–agarose beads without RNA ligands (beads) or tagged with RNA ligands 5 or 9. The ligands were appended to the beads by hybridizing them to a biotinylated 2′-*O*-methyl-RNA oligonucleotide (5′-biotin-AGUACUAUCGACCUCUGGGUUAUG-3′) that is complementary to part of the ligand's 3′-constant sequence. After washing, the bound proteins were eluted with a DNA oligonucleotide (3′-PR) complementary to the entire 3′-constant sequence of the RNA ligands. Protein samples from the affinity chromatography or immunoprecipitation were boiled in SDS loading buffer, resolved on a 10% polyacrylamide/SDS gel and electrically transferred onto a nitrocellulose membrane (Schleicher&Schuell). Western blot analysis was carried out with an ECL kit (Amersham). The membrane was pre-blocked with 5% blocking reagent (Amersham) before being probed with a rabbit anti-pol II monoclonal antibody (N-20, Santa Cruz Biotech) specific to an N-terminal epitope of the largest subunit of pol II, or with an anti-U1 70k antibody (Santa Cruz Biotech).

3. Results and discussion

Combinatorial selection of RNA ligands against crude cellular extracts through SELEX provides the possibility of obtaining arrays of ligands for numerous cellular targets from the same selection. These ligands may be used to characterize the target molecules involved in various activities (e.g. [24]). To determine if RNA ligands had been generated that could impede pol II-mediated transcription, the DNA templates for round 14 (see Section 2) were cloned and RNAs from 60 random clones were screened for their activity in an in vitro transcription assay. Among the 60 RNA molecules, eight were found to strongly inhibit pol II transcription from a CMV promoter. To determine if this inhibition was dose-dependent, the activity of the RNA ligands was analyzed over a range of concentrations. As shown in Fig. 1A, RNA 9, 13, 15 and 43 strongly repressed transcription in a dose-dependent manner, while RNA 5 as well as pool 1 RNA and several others (e.g. RNA 22, 35 and 47, data not shown) showed only a slight effect on transcription at these concentrations. Several other ligands (RNA 30, 32, 48 and 58) also showed similar inhibition (data not shown). Unexpectedly, four of the inhibitors contain an 11 nt consensus sequence identical to the 5′-splice site sequence found in eukaryotic pre-mRNAs (Fig. 1B), while neither the non-inhibitors nor the other inhibitors contain this consensus. The transcription inhibition appeared to be pol II-specific, because these RNA ligands (RNA 9, 13, 15 and 43, lanes 2–5, Fig. 1C) showed no effect on the transcription by pol III from a human U6 RNA gene

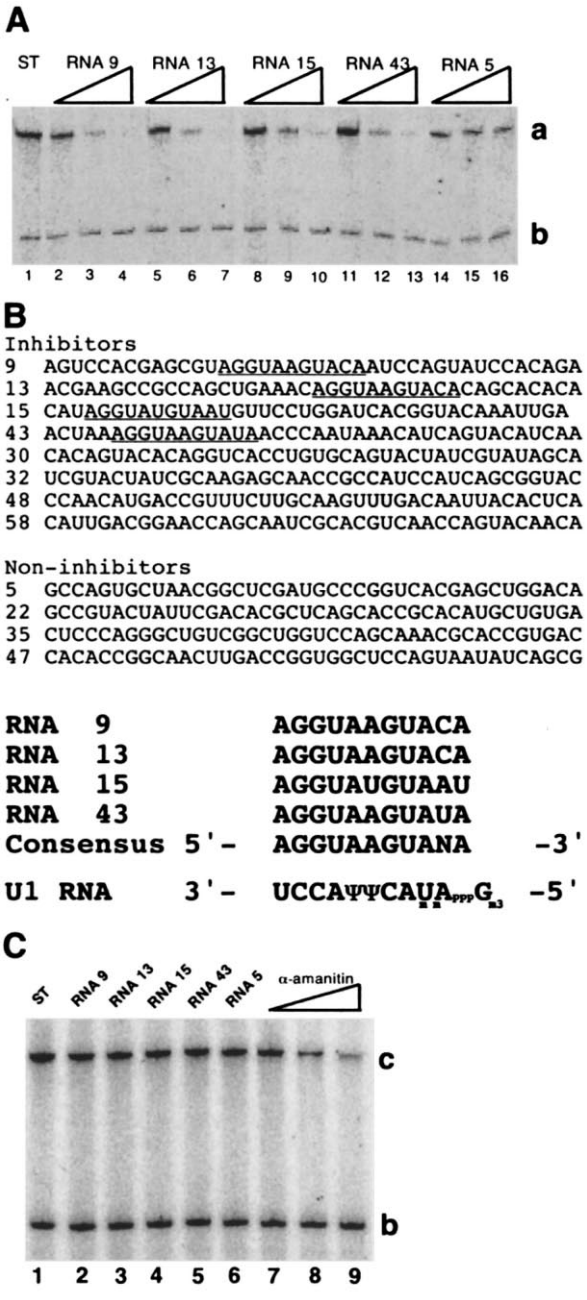

Fig. 1. Inhibition of in vitro transcription of β-globin sequences from the CMV promoter by RNA ligands. A: RNA ligands (35, 70 and 105 pmol) were added to HeLa nuclear extract (45 μg) with RNasin (40 U) and pre-incubated at 30°C for 15 min. DNA template, rNTPs, Mg^{2+} and ^{32}P-GTP were added to start transcription in a total volume of 25 μl at 30°C for 60 min. Band a, β-globin transcript (1100 nt) derived from the DNA template with the CMV promoter; band b, RNA loading control (700 nt) which was generated by T7 in vitro transcription and added to the CMV transcription reactions just prior to RNA extraction for electrophoresis on a 6% polyacrylamide/7 M urea gel. ST, control with no RNA ligands. B: Sequences of RNA ligands (random region only) isolated from pool 14. The four inhibitors (RNA 9, 13, 15 and 43) contain an 11 nt consensus sequence at variable positions (underlined), which is identical to the eukaryotic 5′-splice site. The other four inhibitors (RNA 30, 32, 48 and 58) do not contain this consensus. Non-inhibitors (RNA 5, 22, 35 and 47) showed very limited effect on pol II activity. Bottom, comparison of the 5′-end of U1 snRNA and the consensus of the inhibitor RNAs. Ψ, pseudouridine; m, methyl group; G_{m3}, trimethyl guanosine cap. C: Effects of RNA ligands on pol III activity. RNA ligands (105 pmol each) were added to HeLa nuclear extract (45 μg) with RNasin (40 U) and pre-incubated at 30°C for 15 min. The rest of the transcription mix were added to start transcription in a total volume of 25 μl at 30°C for 60 min. Band c, a ribozyme transcript (1170 nt) derived from the DNA template with a human U6 RNA gene promoter; band b, RNA loading control (700 nt), from the same batch but twice the amount as used in Fig. 2B, added before RNA extraction for electrophoresis on a 6% polyacrylamide/7 M urea gel. Lane 1, ST, without RNA ligands; lanes 7–9, in the presence of α-amanitin (1, 50 and 100 μl/ml, respectively).

promoter at the concentration that pol II had been strongly inhibited. The activity of pol III was confirmed by its insensitivity to low concentrations of α-amanitin (lanes 7–9, Fig. 1C), which is characteristic to the pol III in human and other organisms [21,25,26].

This study has focused on determining how the RNA ligands with the 5′-splice site abrogate pol II transcription. To determine whether U1 snRNP associates with pol II, U1 snRNP was immunoprecipitated from the HeLa nuclear extract with a monoclonal antibody raised against the U1 70k subunit, and the immunoprecipitates were subject to Western blotting with an antibody specific for pol II. As shown in Fig. 2A, the largest subunit of pol II was detected with the antibody (N-20) specific for an epitope at the amino-terminus of the subunit. In separate experiments, purified RNA 5 (non-inhibitor) and RNA 9 (inhibitor) were affixed to the streptavidin-crosslinked beads via a biotin linkage and used to purify proteins recognized by these ligands from HeLa nuclear extract. U1 snRNP and pol II were co-purified through the affinity chromatography using columns tagged with RNA 9, but not with RNA 5 or beads alone (data not shown). These results indicate that U1 snRNP associates with pol II in vitro in the absence of transcription and that the RNA ligands do not appear to impede this association.

To determine if the 5′-splice site is required for the transcription inhibition by the RNA ligands, several mutated sequences of RNA 9 with or without the 5′-splice site were tested for an effect on pol II transcription. As shown in Fig. 2B, the transcription inhibition was abolished when the 11 nt 5′-splice site sequence was deleted from RNA 9 (RNA 9A, lanes 2–4), but was not affected when an 11 nt adjacent sequence was removed from either side of the 5′-splice site (RNA 9B, lanes 5–7; RNA 9C, lanes 8–10). Experiments were also conducted to determine if the 5′-terminal sequence of U1 RNA, which recognizes the 5′-splice site of pre-mRNAs, is required for transcription inhibition by RNA 9. The 5′-terminal sequence of U1 RNA as well as a single-stranded region of U3 RNA [23] was depleted through oligonucleotide-directed cleavage by RNase H [22]. As shown in Fig. 2C, depletion of the 5′-terminal sequence of U1 RNA abolished the transcription inhibition by RNA 9 (lanes 2–4), while removal of a U3 RNA sequence showed no effect (lanes 5–7) compared to when both U1 and U3 RNAs were intact (lanes 8–10). These results indicate that the inhibition of pol II transcription by the RNA ligands containing a 5′-splice site is mediated through interaction with the pol II-associated U1 snRNP.

To determine the mechanism of transcription inhibition, RNA 9 was added to transcription reactions prior to and following pre-initiation complex assembly on the CMV pro-

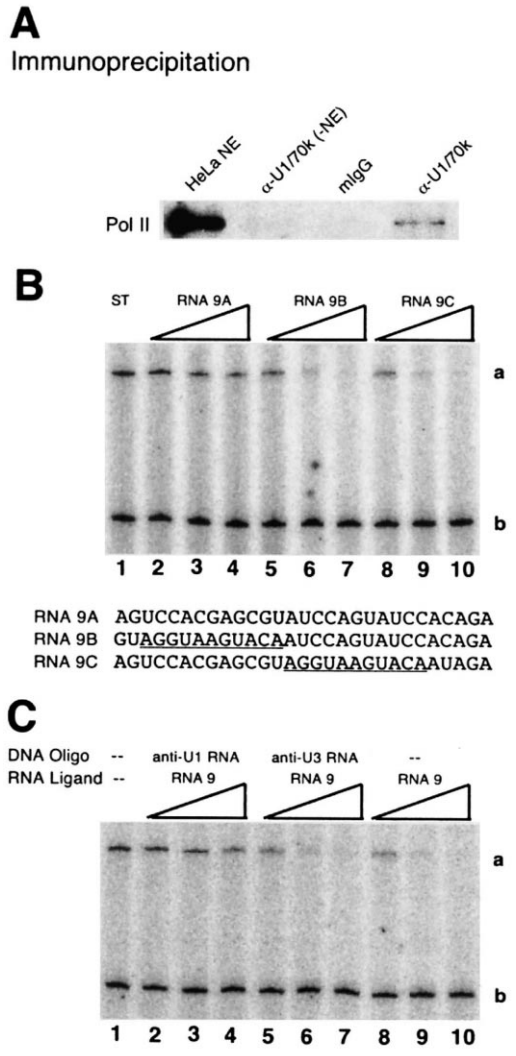

Fig. 2. Interaction of U1 snRNP and pol II. A: Co-immunoprecipitation of pol II and U1 70k from HeLa nuclear extract. A murine anti-U1 70k antibody (α-U1/70k, 1 μg) as well as mouse IgG (1 μg) was incubated with or without HeLa nuclear extract (45 μg), and bound proteins were immunoprecipitated with protein G–Sepharose beads. Western blot analysis was conducted using an antibody (N-20) specific to an N-terminal epitope of the largest subunit of pol II. HeLa NE, straight HeLa nuclear extract; mIgG, mouse immunoglobulin G. B: Effects of RNA molecules with mutated inhibitor RNA 9 sequences (RNA 9A, 9B and 9C) on pol II transcription. These RNA molecules either lack (RNA 9A, lanes 2–4) or contain (RNA 9B and RNA 9C; lanes 5–7 and 8–10, respectively) the 5′-splice site (underlined). Only sequences in the random region are shown. The methods are as described in Fig. 1A. ST, control without RNA ligands (lane 1). Band a, β-globin transcript; band b, loading control RNA. C: Effects of targeted depletion of snRNAs on the ligand-mediated inhibition of pol II transcription. U1- and U3-specific RNA sequences were depleted by RNase H cleavage directed by a deoxyoligonucleotide specific for the 5′-terminal U1 RNA (anti-U1, 5′-TTCAGGTAAGTACTCA-3′, lanes 2–4) or a putative single-stranded region of U3 RNA (anti-U3, 5′-TGGTTTTCGGTGCTCT-3′, lanes 5–7). HeLa nuclear extract (45 μg), RNasin (40 U; Roche), deoxyoligonucleotides (200 ng), RNase H (1 U, Roche), Mg^{2+} (3 mM) and transcription buffer (20 mM HEPES, pH 7.9; 100 mM KCl; 0.2 mM EDTA; 0.5 mM DTT; 20% glycerol) in a volume of 15 μl were pre-incubated at 30°C for 45 min before the addition of RNA 9 (35, 70 and 105 pmol) to the transcription reactions as described in Fig. 1A. Lane 1, control without RNA depletion and inhibitor; lanes 8–10, without RNA depletion.

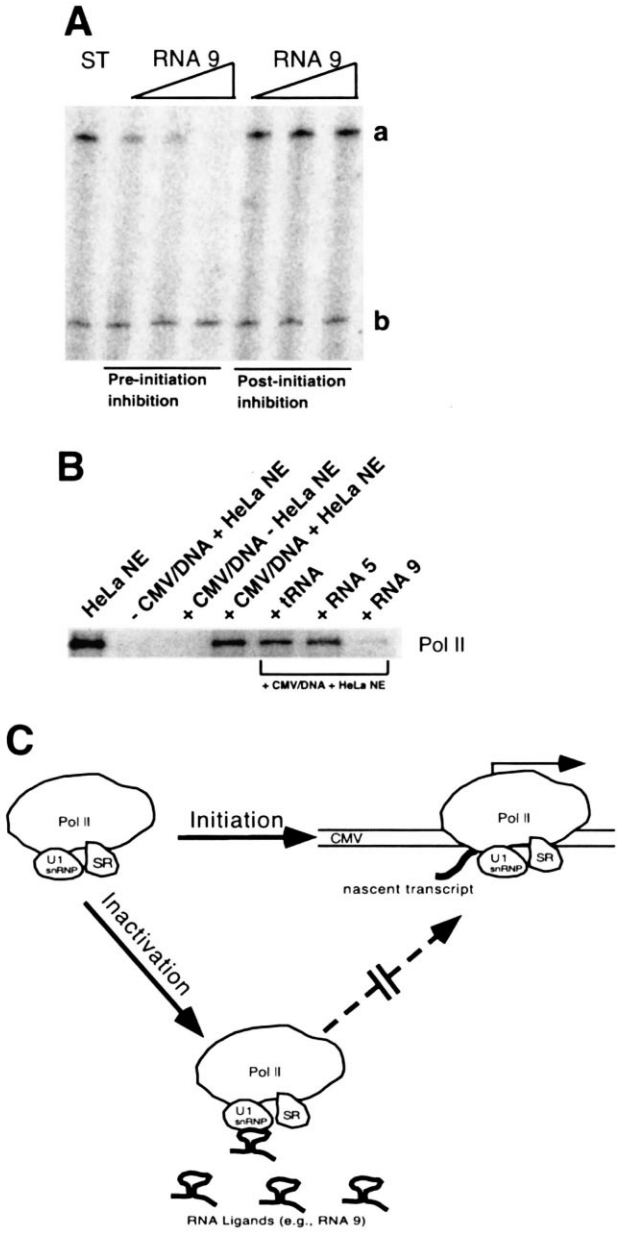

Fig. 3. Inhibition of transcription initiation by RNA ligands. A: Pre- and post-initiation inhibition of in vitro transcription from the CMV promoter by RNA 9. For analysis of the post-initiation inhibition, the globin DNA template and HeLa nuclear extract were pre-incubated at 30°C for 30 min. Then increasing amounts of RNA 9 (35, 70 and 105 pmol) were added to the mixture which was incubated at 30°C for another 30 min. Transcription was started by adding rNTPs and ^{32}P-GTP, and total RNA was collected 45 min after transcription initiation and analyzed as in Fig. 1A. The experimental procedure for pre-initiation inhibition was as described in Fig. 1A except that HeLa nuclear extract was pre-incubated before and after the addition of RNA 9, in parallel with samples for post-initiation inhibition. B: Inhibition of pol II binding to the pre-initiation complex by RNA ligands. The transcription pre-initiation complex was assembled on the immobilized CMV promoter DNA (CMV/DNA) in the presence (+HeLa NE) or absence (−HeLa NE) of HeLa nuclear proteins (45 μg) and RNA competitors (tRNA, RNA 5 and 9, 105 pmol each). Promoter bound proteins were analyzed by Western blotting with anti-pol II antibody (N-20). C: Model of RNA ligands-mediated inhibition of pol II transcription.

moter. As shown in Fig. 3A, RNA 9 effectively impeded pol II transcription when the ligand was added to the reactions prior to transcription initiation. However, once the pre-initiation complex was assembled on the promoter, RNA 9 was no longer effective. To investigate whether RNA 9 had affected pre-initiation complex assembly, a ligand–promoter competition experiment was performed. As shown in Fig. 3B, the binding of pol II to the CMV promoter was inhibited by RNA 9, while RNA 5 did not significantly affect pol II binding to the CMV promoter as compared to non-specific RNA competitors such as tRNA. These results indicate that RNA 9 did not adversely affect pol II activity, but specifically inhibited transcription initiation by blocking the recruitment of pol II to the promoter.

This study shows that U1 snRNP and pol II appear to be complexed before transcription initiation and through the early stage of RNA splicing, and that U1 snRNP may play an important role in co-ordinating transcription and RNA splicing. This notion is also supported by the observations that over-expression of a U1-specific protein subunit represses transactivation of pol II [27] and that pol II with a phosphorylated carboxyl-terminal domain (CTD) stimulates RNA splicing [14]. A recent study has also shown that a yeast U1-specific protein directly interacts with the phosphorylated CTD of pol II in the early spliceosome [28]. These observations strengthen the model that the CTD of pol II provides a 'platform' on which snRNPs and other splicing factors assemble to form the early spliceosome during transcription. Pol II appears to undergo an activation process (e.g. through dephosphorylation and phosphorylation) to initiate transcription [29]. In the present study, the activation of pol II for transcription initiation may be abolished by the binding of excessive nascent transcripts containing 5′-splice site (e.g. RNA 9 and other ligands) to the pol II-associated U1 snRNP. Such association may render the transcription machinery as a pseudo 'elongation complex' even when it is not engaged with DNA, and therefore prevent the activation of pol II for transcription initiation (Fig. 3C). Unlike putative transcription factors, the interactions of U1 snRNP and other RNA processing factors with the transcription machinery most likely serve to link the events of transcription and RNA processing, rather than to specifically activate or repress pol II activity in a promoter-dependent fashion. Therefore, these processing factors should be regarded as 'transcription co-ordinators'. The current study demonstrates that RNA ligands selected for various gene products present in crude cellular extracts may be utilized to identify novel functions of specific factors involved in complex molecular events. The author anticipates that this RNA ligand-based strategy will prove to be a valuable approach to the study of human proteomes.

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