Analysis of U1 Small Nuclear RNA Interaction with Cyclin H*

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TFIIH is a general transcription and repair factor implicated in RNA polymerase II transcription, nucleotide excision repair, and transcription-coupled repair. Genetic defects in TFIIH lead to three distinct inheritable diseases: xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy, with xeroderma pigmentosum patients being highly susceptible to skin cancer. Earlier data revealed that the cyclin H subunit of TFIIH associates with U1 small nuclear RNA, a core-splicing component. In addition to its role in RNA processing U1 small nuclear RNA also regulates diverse stages of transcription by RNA polymerase II both in vivo and in vitro, including abortive initiation and re-initiation. Here we identify structural components of U1 and cyclin H implicated in the direct interaction and show how they affect function. Because of unique features of cyclin H we have developed a new methodology for mapping RNA interaction with the full-length cyclin H polypeptide based on electrospray ionization tandem mass spectrometry. We also demonstrate the importance of U1 stem-loops 1 and 2 for the interaction with cyclin H. Functional assays implicate the identified interaction with U1 in regulation of the activity of the cyclin H associated kinase CDK7.

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3 The abbreviations used are: RNAPII, RNA polymerase II; CDK, cyclin-dependent kinase; CAK, CDK-activating kinase; CTD, carboxyl-terminal domain; snRNA, small nuclear RNA; RIP, RNA immunoprecipitation; aa, amino acids.

Earlier studies have described the modulation of P-TEFb kinase activity by an interaction with the non-coding RNA 7SK and the HEXIM (MAQ1) polypeptide (14, 15). Following these observations we described a non-coding RNA interaction with TFIIH and the interacting RNA was identified as U1 snRNA (U1) (16). The interaction with U1 has been shown to modulate biochemical activities of TFIIH at the initiation and re-initiation stages of transcription in vitro and to correlate with in vivo observations of coupling transcription with the proximity of the first intron to the promoter (16–19). U1 snRNA has been characterized as an important component of splicing machinery, implicated in early recognition of splice donor sites, and is also involved in the coupling of splicing with efficient transcription initiation and polyadenylation in vivo (12, 20).

The CDK7-associated cyclin (cyclin H) was identified by UV cross-linking as the subunit that mediates the interaction between TFIIH and U1 (16). Here we present biochemical data defining the structural and functional interaction between cyclin H and U1. Taking into consideration the unique structural properties of cyclin H we have developed a novel method of mapping protein-RNA interactions based on electrospray ionization tandem mass spectrometry. Our approach enabled us to conduct mapping analysis on the full-length soluble folded polypeptide and to analyze the importance of the mapped interaction in the functional enzymatic assay.

EXPERIMENTAL PROCEDURES

RNA Immunoprecipitation (RIP)—RIP was performed as described in Gilbert and Svejstrup (21) with the following modifications. 4 × 107 cells were cross-linked with 1% formaldehyde for 10 min and harvested in 4 ml of PBS. Glycine was added to 330 mM final concentration. Cells were re-suspended in 1 ml of RIP lysis buffer (50 mM HEPES, pH 7.5), 1 mM EDTA, 1% Triton X-100 and sonicated 10 times for 10 s.

Samples were adjusted to 50 mM HEPES (pH 7.5), 1 mM EDTA, 0.5% Triton X-100, 25 mM MgCl2, 5 mM CaCl2, and 60 units of DNase I (Roche Applied Science) and 120 U RNase Inhibitor (Promega) were added. Samples were incubated at 37 °C for 15 min then adjusted to 20 mM EDTA. Samples were precleared three times with 20 μl of protein G-Sepharose 4 Fast Flow beads (Amersham Biosciences) for 2 h at 4 °C. 15 μl of antibodies (cyclin H (C-18) sc-609 (Santa Cruz Biotechnology) (no antibodies for negative control) were bound to 20 μl of protein G beads. Samples were incubated for 2 h at 4 °C with antibody bound beads. Beads were washed once in 1 ml of binding buffer (50 mM HEPES, 0.5% Triton X-100, 25 mM MgCl2, 5 mM CaCl2, 20 μM EDTA), once in FA500 (50 mM HEPES, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate), once in LiCl buffer (10 mM Tris, 250 mM LiCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA), and once in TES (10 mM Tris, 10 mM NaCl, 1 mM EDTA). 75 μl of RIP elution buffer (100 mM Tris (pH 7.8), 10 mM EDTA, 1% SDS) was added for 10 min incubations at 37 °C. This was repeated and eluate pooled. 150 μl of eluate was adjusted to 200 mM NaCl, 20 μg of proteinase K (Roche Applied Science) was added and samples incubated for 1 h at 42 °C and then 1 h at 65 °C. Phenol/chloroform 5:1 (pH 4.7) (Sigma) was used to
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extract RNA, which was precipitated in ethanol and analyzed by reverse transcription-PCR with the primers TACTTACCTGGCAGGGGAG and CACTACCCAAATTATGCAG.

RNA Bandshifts—A titration (0, 50, and 100 fmol) of recombinant cyclin H (expressed from pET28b in Escherichia coli BL21 strain) diluted in binding buffer (50 mM Tris (pH 8), 150 mM NaCl, 10% glycerol, 0.01% Triton X-100), 1 μl (5 fmol) of 32P-labeled U1, 1 μg of nonspecific competitor (poly(dl-dC)-poly(dl-dC) (Amersham Biosciences)), and 16 μl of binding buffer were incubated for 1 h on ice (20 μl final volume). The samples were loaded on a 6% polyacrylamide 0.5 × TBE (89 mM Tris borate (pH 8.3), 2 mM EDTA) native gel and run at 70 volts. The gel was dried and visualized using a phosphor imaging screen (Eastman Kodak Co.) and scanner (Amersham Biosciences).

For competition assays 1 μl of a titration of non-labeled RNA (0.5, 50 fmol) was added to 100 fmol of cyclin H, 5 fmol of 32P-labeled U1, 1 μg of nonspecific competitor in 20 μl of binding buffer on ice for 1 h. U1 and mutant U1, a stem-loop 2 deletion mutant (Δ51–91) (22), were used as competitors. Wild-type U1 was in vitro transcribed from a PCR product derived from U1 plasmid (16) (deletion mutants were made using ExSite mutagenesis kit (Stratagene)).

Reconstituted Pull-down Assay—10 pmol of recombinant His6-tagged cyclin H and 1 pmol of 32P-labeled RNA (U1, ASL1-(Δ18–48), ΔSL2-(Δ51–91), ΔSL3-(Δ93–118), or ΔSL4-(Δ140–165)) were incubated in 1 ml of pull-down buffer (50 mM Tris (pH 8), 500 mM NaCl, 0.05% Triton X-100). After 2-h incubation at 10 °C 15 μl of nickel-primed Sepharose beads (Amersham Biosciences), preblocked with 50 μg of Saccharomyces cerevisiae tRNA (Sigma), were added. After 1-h incubation at 10 °C the beads were washed four times for 5 min in 1 ml of pull-down buffer. 30 μl of wash buffer and 40 μl of phenol/chloroform 5:1 (pH 4.7) (Sigma) was added to the beads, vortexed, and centrifuged at 13,000 × g for 5 min. 20 μl of the aqueous phase was removed and added to 20 μl of formamide loading dye (95% formamide, 0.1% bromophenol) and analyzed on 6% polyacrylamide TBE UREA gel as above. For competition assays a titration (10, 100 pmol) of peptides (peptides 1 and 2 or [Glu1]fibrinopeptide B (Sigma) for negative control) was added to the beads in 20 μl of water after the final wash. After 1-h incubation at 4 °C the beads were washed in 1 ml of wash buffer, and the RNA was eluted and visualized as above.

RNA-Protein Cross-link and RNA Purification—50 pmol of recombinant cyclin H and 50 pmol of U1 were mixed in 84 μl of 25 mM HEPES, 10% glycerol, 0.05% H and L alanine and 0.06 ng) or RNase T1 (Roche Applied Science) (0.25, 0.5, 0.75, and 1.0 unit) were added. After 15-min incubation at 4 °C, 2 μl of 6 × RNase stop buffer (1% Sarkosyl, 100 mM NaCl, 10 mM EDTA, 25 μg/ml tRNA) was added. 12 μl of formamide loading dye was added, and the samples were analyzed on an 8% polyacrylamide TBE urea gel, which was visualized as above.

Recombinant CDK7/Cyclin H Kinase—Recombinant CDK7 and cyclin H (expressed as above) from pET28b in E. coli BL21 strain. Both polyepitides were mixed together and bound to a 1-ml nickel-Sepharose HiTrap column (Amersham Biosciences) in the presence of 8 M urea, renatured under a decreasing 10-mL gradient of urea, and eluted with a gradient of imidazole.

Purification of Native Kinase—20 μl of protein G-conjugated Sepharose beads (Sigma) and 15 μl of antibodies (cyclin H (C-18) sc-609 (Santa Cruz Biotechnology) or preimmune rabbit serum for the negative control) were bound in 1 ml of binding buffer (50 mM Tris (pH 8), 150 mM NaCl, 0.05% Triton X-100). After 1-h incubation 20 μl of HeLa cell extract was added. After 2-h incubation at 10 °C, the beads were washed three times for 5 min in 1 ml of binding buffer and once in 1 ml of kinase buffer (20 mM Tris (pH 7.8), 100 mM NaCl, 10 mM MgCl2). Kinase Assay—15 μl of kinase buffer, 1 μl of 1 mM ATP, 1 μl of [γ-32P]ATP (Amersham Biosciences), and 1 μl of CTD peptide (10 mg/ml) were added to the recombinant or native kinase preparations. After 1-h incubation at 30 °C 20 μl of 2 × SDS loading dye was added, and the samples were analyzed on a 16% SDS-polyacrylamide Tris-glycine gel, which was visualized as above. For competition assays with the native kinase, after the final wash in binding buffer, a titration (10, 50, and 100 pmol) of peptides (peptides 1 and 2 or [Glu1]fibrinopeptide B (as above)) was added to the beads in 20 μl of water. After 1-h incubation at 4 °C the beads were washed in 1 ml of kinase buffer, the wash was removed and the kinase assay carried out as above.

RESULTS

Formation of a Specific Complex between Cyclin H and U1—We have confirmed the specificity of the reported interaction between cyclin H and U1 (16). First, we have used a newly developed RIP assay to test this interaction in vivo (21). As shown in Fig. 1A, immunoprecipitation with cyclin H antibodies from cultured HeLa cells following the short range formaldehyde cross-linking results in specific isolation of U1 RNA, detected by RT-PCR (Fig. 1A).

The specificity of the in vivo interaction between cyclin H and U1 has been tested by two independent approaches: RNA bandshift and affinity co-precipitation. First, we analyzed complex formation between the affinity-purified soluble recombinant cyclin H and synthetic 32P-labeled U1 (Fig. 1B) by RNA bandshift (electrophoretic mobility shift assay). As shown in Fig. 1C, a stable reversible complex is observed in the presence of the nonspecific competitor poly(dl-dC)-poly(dl-dC) (Amersham Biosciences). It is important to emphasize that an excess of free probe was present in the binding reaction and that we have observed only one single complex under those conditions. The 32P-labeled U1 probe was also specifically competed out of the complex by an excess of non-
and 32P-labeled U1, we were able to affinity purify the U1 on nickel-primed Sepharose beads via the His6-tag of the interacting cyclin H. Therefore we were interested in developing a method to map the protein-RNA interaction in solution without introducing any terminal deletions.

Our strategy was to form a U1-cyclin H complex in solution, cross-link the protein to RNA, and follow it by tryptic digestion of the protein and purification of RNA with the cross-linked peptide (Fig. 2). In the final stage the peptide was identified by electrospray ionization tandem mass spectrometry. From several choices of cross-linking techniques we employed chemical cross-linking with formaldehyde, which had the advantage of reversibility (25), allowing us to identify unmodified peptides.

By using formaldehyde cross-linking and affinity purification of RNA-containing products with PHOS-Select iron affinity gel (Sigma) we isolated and identified two peptides (peptide 1 (HWTFSSEEQLAR9–21 aa)) and peptide 2 (IALTDAYLYTPSQQATAILSSASR198–223 aa)); these peptides were specific for the U1-cyclin H cross-linked complex (Fig. 3A, marked in red). Importantly, despite the fact that the two specific peptides are distant from each other when superimposed on the three-dimensional structure of cyclin H they form a single interface (Fig. 3B, marked in red) (PDB ID: 1KXU (www.pdb.org)) (26–28).

To test whether the identified peptides are functionally implicated in the complex formation with U1, we tested their effect on the interaction. The 32P-labeled U1-cyclin H complex was formed and affinity-purified on nickel-primed Sepharose via the His6-tag on the cyclin H polypeptide as described earlier (Fig. 1D). The U1-cyclin H complex was disrupted by the presence of the identified peptides in the reconstituted pull-down assay (Fig. 3C). This strongly suggests that these peptides are indeed involved in the interaction of cyclin H with U1.

Mapping U1 Interaction Sites—U1 is 165 nucleotides in length and comprises three stem-loop structures in a cloverleaf conformation (SL1-(18–48), SL2-(51–91), and SL3-(93–118)) followed by a 3′ single-stranded region and a fourth 3′ stem-loop (SL4-(140–165)) (29) (Fig. 4A). Previous studies on multiple protein interactions of U1 clearly indicated the importance of the various stem-loop structures for the specificity of the interaction (20). We designed a set of deletion mutants, eliminating each of the stem-loop structures (Fig. 4, A and B), and tested them in the reconstituted cyclin H pull-down assay (Fig. 4B). The result revealed low yields of 32P-labeled U1 stem-loop 2 deletion mutant (ΔSL2-(Δ51–91)), affinity-purified via cyclin H interaction (Fig. 4B, lane 10), It was significantly reduced when compared with wild-type U1 and the other stem-loop deletion mutants (ΔSL1-(Δ18–48), ΔSL3-(Δ93–118), and ΔSL4-(Δ140–165) (Fig. 4B, lanes 7, 9, 13, and 14). We concluded that stem-loop 2 was implicated in the interaction with cyclin H.
To further support the evidence for stem-loop 2 involvement in the interaction, we repeated RNA bandshifts (see Figs. 1C and 4C) in the presence of various specific competitors. With the titration range of 0.5 to 50 fmol, wild-type U1, but not the stem-loop 2 deletion mutant mutU1 (22), was able to compete out the complex (Fig. 4C, compare lanes 4 and 6). This further implicated stem-loop 2 in the specific interaction with cyclin H.

To further delineate the properties of the U1 interaction with cyclin H, we also employed RNase footprinting. 5′-End 32P-labeled U1 was digested with titrated amounts of RNase A and RNase T1 after formation of the complex with cyclin H. The resulting cleavage pattern revealed specific protected and hypersensitive sites. As shown in Fig. 5, footprinting analysis identifies protected areas within stem-loops 1 and 2 (Fig. 5, compare lane 2 with lanes 3–5) as well as a hypersensitive site within stem-loop 3 (Fig. 5, compare lanes 2 and 3 with lanes 4 and 5).

On its own the result of the footprinting experiment could not distinguish between the direct effect of cyclin H interaction and the indirect effect, i.e. a particular structural change within U1, induced by interaction with cyclin H. Nevertheless, protection of stem-loop 2, as observed in the footprinting experiment (Fig. 5, lanes 3 and 5), implicates the loop in the direct/indirect interaction with cyclin H and supports our earlier conclusion for the affinity purification of U1-cyclin H complexes (Fig. 4B) and for mutant competition of the U1-cyclin H complex in the bandshift assay (Fig. 4C). Overall, stem-loop 2 of U1 snRNA is clearly implicated in the interaction with cyclin H.

Effect of U1 Interaction on the CDK7/Cyclin H Kinase Activity—Having identified the sites of interaction between cyclin H and U1, we proceeded to analyze the effect of the interaction on the enzymatic activity of the kinase, of which cyclin H is a regulatory component. Within the framework of in vitro biochemical study the relevance of the identified interaction could only be justified by its effects on the catalytic activity involved.

Previous studies have demonstrated that the CDK7/cyclin H kinase forms a tripartite complex (CAK) with the assembly factor MAT-1, which in turn stabilizes the in vitro CDK7/cyclin H complex and renders it more active, even independently of T-loop phosphorylation (30–32). We therefore tested the effects of U1 on recombinant bipartite, tripartite, and native CDK7 kinases. Titration of U1 with recombinant (E. coli expressed) and renatured bipartite complex of CDK7 and cyclin H showed a consistent increase in the kinase activity when assayed against the CTD substrate (Fig. 6, A and B, lanes 2 and 3). Titration of U1 stem-loop 2 mutant mutU1 (22) did not affect the kinase activity (Fig. 6B, lanes 5–7). Bearing in mind the issue of low stability of the bipartite complex, we suspected that the presence of U1 may stabilize the interaction between the two subunits of the kinase. If that was the case, we
might expect to see very little effect of U1 in the presence of recombinant CAK (CDK7/cyclin H/MAT-1) complex. Indeed, a baculovirus-expressed tripartite complex showed little sign of U1 stimulating or repressing the kinase activity (data not shown).

However, the eukaryotic background of baculovirus expression system also alerted us to the potential presence of contaminants, either RNA or protein in nature, that could already be modulating the kinase activity. We therefore tested native CDK7 kinase that was immunoprecipitated from HeLa cell extracts that, as shown before, contained U1 (16) as well as a number of other potential regulatory factors. Immunopurification of CDK7 kinase, but not the control purifications, displayed very high specific kinase activity when assayed against the CTD substrate. We then disrupted the interaction between cyclin H and U1 with titrated amounts of competing peptides. As shown in Fig. 6, titration of the specific peptides, which have previously been shown to disrupt the interaction with U1 (Fig. 3C), dramatically impaired the activity of the native kinase (Fig. 6C, lanes 6–8). The control peptide showed no such effect. We concluded that in the context of the native immunopurified CDK7 complex, interaction with U1 played an important role in supporting the kinase activity. This could be attributed to the impact of the additional regulatory factors recruited to the native complex via the cyclin H or the U1 side of the interaction.

**DISCUSSION**

Cross-linking Methodology—Transcriptional cyclin-dependent kinases CDK7 and CDK9 appear to be subject to regulation mediated by the non-coding RNAs U1 and 7SK (14–16, 19, 33–35). Here we have conducted a biochemical analysis of the interaction between cyclin H and U1. A critical step in this analysis is based on a novel mass spectrometry based approach for mapping RNA-protein interactions. This method allows us to analyze the full-length polypeptide and to avoid deletion mutant analysis, which in the case of the cyclin H structure leads to the loss of structure and function of the peptide and the associated kinase activity (24). This new method allowed us to identify two peptides, which form a single interface on a free surface of cyclin H, away from the predicted CDK7 binding site (24, 26–28). Unambiguously, when added as competitors, these peptides specifically disrupt the interaction of U1 with cyclin H in the reconstituted complex.

Questions remain concerning technical issues with the choices of cross-linking techniques. The widely used technique of UV-mediated cross-linking (36) can be carried out with high efficiency in the presence of nucleotide derivatives, such as 5-bromo-UTP and 5-iodo-UTP (36). We took the precaution to monitor whether the introduction of these derivatives had an effect on the specificity of the U1-cyclin H complex formation. Following earlier detection of the complex using RNA bandshift assays (Fig. 1C), we tested the formation of the same complex with 32P-labeled U1 probes, synthesized with the incorporation of 5-bromo-UTP and 5-iodo-UTP. The bandshift assays revealed that the presence of the UTP derivatives in the RNA interfered with complex formation and overall reduced the yield of cross-linked product (supplemental Fig. 1). This result ruled out efficient UV-mediated cross-linking. The cross-link of choice, a reversible short range aldehyde cross-link, on the other hand proved successful. However, it is important to mention that within the mapping procedure the efficiency of tryptic digestion was badly affected by the cross-linking modification. Also, one should take into consideration potential masking of the tryptic digest site by bound RNA. Thus, we believe that our methodology benefited from having a highly pure two component system with high concentrations of both cyclin H and U1. We are now analyzing alternative reversible cross-linking techniques, which will allow us to obtain similar results in more complicated, low concentration ribonucleoprotein complexes.

Specific Interaction of CDK7 Kinase with U1—The described interaction of cyclin H with U1 has an important precedent. A second transcriptional kinase of the same class, CDK9, has been implicated in transcription elongation by phosphorylating the CTD of RNAII (37, 38). Importantly P-TEFb (CDK9/cyclin T) is regulated by interaction with non-coding RNAs. The RNA stem-loop structure of the human immuno-deficiency virus transcript, TAR, and the Tat protein are implicated in the activation of the P-TEFb kinase activity (39, 40). The 7SK RNA and HEXIM1 are implicated in the inactivation of this activity (14, 15, 33–35). More importantly, in both cases it appears that the cyclin sub-
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unit is involved. The TAR RNA interacts with cyclin T1 (23, 41–43), and 7SK stabilizes the interaction of HEXIM with cyclin T (33).

U1 snRNP—The observed coupling between the promoter proximal position of the first intron and promoter strength in vivo (17–19), and high levels of abortive initiation and re-initiation in vitro (16), raises interesting questions about the role of U1 snRNA. Assembly of the U1 snRNP involves recognition of U1 snRNA by the SMN complex (44, 45). This is followed by recruitment of the Sm heptameric protein ring, hypermethylation of the snRNA 5′ cap, 3′ trimming, and nuclear import (20). Once in the nucleus the U1 snRNP recruits three more proteins: the U1–70K protein interacts with stem-loop 1, the U1-A protein interacts with the loop of stem-loop 2, and the U1-C protein is probably attached via protein-protein interactions (20). The three-dimensional structure of the U1 snRNP has been determined by single-particle electron cryomicroscopy (20).

Despite the functional biochemical data concerning the interaction of TFIIH and U1 snRNA our current understanding of their association in vivo is limited. Determining how they interact in vivo will require functional analysis.

Here we have shown by footprinting assay that stem-loop 1 of U1 snRNA is implicated in interaction with cyclin H (Fig. 5). Interestingly this stem-loop has been shown to be involved in the recognition of U1 snRNA by the SMN complex (44). Importantly, analysis of the reconstituted re-initiation scaffold, after a U1-dependent second round of efficient transcription (16), reveals recruitment of the U1–70K subunit of U1 snRNP.4 The U1–70K subunit has also been identified in yeast two-hybrid analysis as a partner of Ioc4, which is responsible for release of RNA Polymerase II into transcription elongation (46).

Preliminary analysis of TFIIH 3′-5′ helicase activity, essential in promoter opening during initiation (4, 47–49), indicates that recruitment of U1 snRNP components, via U1 snRNA, can stimulate the helicase activity.4 We are interested in analyzing the step-by-step assembly of the TFIIH-U1 based complex in vivo. In our initial study we have isolated by conventional purification the most stable and abundant form of TFIIH-U1 based complex, after a U1-dependent second round of transcription and nuclear import during initiation (4, 47–49), indicates that recruitment of U1 snRNP could be recruited and retained in the transcriptional pre-initiation and re-initiation complexes via the TFIIH interaction with U1 snRNA.

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