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Abstract: It is known that transcription can induce DNA recombination, thus compromising genomic stability. RECFQ5 DNA helicase promotes genomic stability by regulating homologous recombination. Recent studies have shown that RECFQ5 forms a stable complex with RNA polymerase II (RNAPII) in human cells, but the cellular role of this association is not understood. Here, we provide evidence that RECFQ5 specifically binds to the Ser2,5-phosphorylated C-terminal repeat domain (CTD) of the largest subunit of RNAPII, RPB1, by means of a Set2-Rpb1-interacting (SRI) motif located at the C-terminus of RECFQ5. We also show that RECFQ5 associates with RNAPII-transcribed genes in a manner dependent on the SRI motif. Notably, RECFQ5 density on transcribed genes correlates with the density of Ser2-CTD phosphorylation, which is associated with the productive elongation phase of transcription. Furthermore, we show that RECFQ5 negatively affects cell viability upon inhibition of spliceosome assembly, which can lead to the formation of mutagenic R-loop structures. These data indicate that RECFQ5 binds to the elongating RNAPII complex and support the idea that RECFQ5 plays a role in the maintenance of genomic stability during transcription.

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RECQ5 helicase associates with the C-terminal repeat domain of RNA polymerase II during productive elongation phase of transcription

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ABSTRACT

It is known that transcription can induce DNA recombination, thus compromising genomic stability. RECQ5 DNA helicase promotes genomic stability by regulating homologous recombination. Recent studies have shown that RECQ5 forms a stable complex with RNA polymerase II (RNAPII) in human cells, but the cellular role of this association is not understood. Here, we provide evidence that RECQ5 specifically binds to the Ser2,5-phosphorylated C-terminal repeat domain (CTD) of the largest subunit of RNAPII, RPB1, by means of a Set2–Rpb1-interacting (SRI) motif located at the C-terminus of RECQ5. We also show that RECQ5 associates with RNAPII-transcribed genes in a manner dependent on the SRI motif. Notably, RECQ5 density on transcribed genes correlates with the density of Ser2-CTD phosphorylation, which is associated with the productive elongation phase of transcription. Furthermore, we show that RECQ5 negatively affects cell viability upon inhibition of spliceosome assembly, which can lead to the formation of mutagenic R-loop structures. These data indicate that TAR results as a consequence of replication fork collapse at R-loops (3). These data suggest that TAR results as a consequence of replication fork collapse at R-loops (3).

INTRODUCTION

The numerous processes that occur in the nucleus during cell proliferation have to be tightly coordinated to ensure genome integrity and faithful genome propagation. Transcription is known to stimulate DNA recombination, thus affecting genome stability (1). This phenomenon, called transcription-associated recombination (TAR), has been linked to replication fork pausing that results from the convergence of transcription and replication. TAR has also been linked to the formation RNA : DNA hybrids (R-loops) between the nascent transcript and the template DNA strand, which increases the susceptibility of the non-transcribed strand to damage or to the formation of secondary structures that impair replication fork progression (1). R-loops are formed when the co-transcriptional assembly of mRNA-particle complexes is impaired (1). For example, it has been shown that inactivation of the human SR protein ASF/SF2, which is required for spliceosome assembly, results in DNA fragmentation, cell-cycle arrest and genomic instability as a consequence of R-loop formation (2). ASF/SF2 depletion also leads to accumulation of stalled replication forks, and chromosome breaks caused by ASF/SF2 deficiency occur specifically in S-phase, preferentially at gene-rich regions (3). These data suggest that TAR results as a consequence of replication fork collapse at R-loops (3).

RECQ5 belongs to the RecQ family of DNA helicases that play critical roles in the maintenance of genomic stability and cancer suppression (4). Recent studies in mammalian cells have established RECQ5 as an important anti-recombination factor that acts by controlling the assembly of the RAD51 filament on single-stranded DNA (ssDNA), which catalyses the homology search and strand invasion during homologous recombination (HR) (5,6). RECQ5 binds directly to the RAD51 recombinase and disrupts the RAD51-ssDNA filament in a reaction driven by ATP hydrolysis, thus preventing homologous duplex invasion during HR (6). In accordance with this finding, RECQ5-deficient cells versus RECQ5-proficient cells show an increased efficiency of HR-mediated DNA double-strand break (DSB) repair, an elevated frequency of sister chromatid exchange, a prolonged persistence of RAD51 foci in response to DNA damage and an increased rate of chromosomal...
rarrangements (5,6). Moreover, RECQ5 has been shown to accumulate at sites of DSBs and sites of replication arrest in a manner dependent on the MRE11–RAD50–NBS1 complex, a key player in DNA damage signaling and repair (7).

A number of recent proteomic studies have revealed that RECQ5 forms a stable complex with RNA polymerase II (RNAPII) in human cells (7–9). The RECQ5–RNAPII interaction is direct and is mediated by the largest subunit of RNAPII, RPBl (8). Knockdown of the RECQ5 transcript in human cells has been found to increase the transcription of several genes (9). Likewise, RECQ5 has been shown to inhibit RNAPII transcription in an in vitro system reconstituted using purified proteins (10). Despite these findings, the function of RECQ5 during the RNAPII transcription cycle remains elusive.

Here, we provide evidence that RECQ5 associates with RNAPII during the productive elongation phase of transcription through direct binding to the C-terminal repeat domain (CTD) of RPBl. Moreover, we show that depletion of RECQ5 reduces the cellular sensitivity to dioxpyrin, a plant-derived bisnaphthoquinonoid, which interferes with spliceosome assembly, presumably by inhibiting DNA topoisomerase I (Top1)-mediated phosphorylation of ASF/SF2, and hence is likely to promote formation of R-loops during RNAPII transcription (3,11). These findings are discussed in light of a possible role for RECQ5 in promoting genomic stability at sites of RNAPII transcription.

MATERIALS AND METHODS

Plasmids, proteins and antibodies

The vector pTXB1 (New England Biolabs) was used for bacterial expression of wild-type and mutant forms of human RECQ5 as fusions with the self-cleaving chitin-binding domain (CBD) tag. Construction of these plasmids was previously described (12). The expression vector for RECQ5 Δ908–954 (ΔSRI) was constructed in the same way as the vector for RECQ5 Δ640–653 (Δ908–954). Point mutations in RECQ5 were made using QuickChange Site-Directed Mutagenesis Kit (Stratagene). The vector pLexA-Km (Dualsystems) was used for construction of the yeast two-hybrid bait plasmids carrying different parts of the human RECQ5 cDNA. RECQ5 cDNA was amplified by PCR and ligated in pLexA-Km via EcoRI and SalI sites in frame with a LexA DNA-binding domain. Internal deletions and point mutations of RECQ5 were subcloned from the plasmid encoding the LexA-RECQ5 into the BglII sites of pACT (Clontech). Among the 1.4 × 10⁷ transformants tested (Clontech). Among the 1.4 × 10⁷ transformants tested, 198 positive clones were found. Clones carrying the bait plasmid encoding the LexA-RECQ5 411-991 and a randomly primed human peripheral blood cDNA library cloned into the BglIII sites of pACT (Clontech). Among the 1.4 × 10⁷ transformants tested for histidine prototrophy and β-galactosidase staining, 198 positive clones were found. Clones carrying the bait and prey plasmids were tested for β-galactosidase activity using a pellet X-gal (PXG) assay as previously described (14).

Yeast two-hybrid assay

The yeast two-hybrid screen for proteins that interact with RECQ5 was carried out using Saccharomyces cerevisiae strain L40 (MATa trp1 leu2 his3 lys2::lexA-HIS3 ura3::lexA-lacZ), which was sequentially transformed with the bait plasmid encoding the LexA-RECQ5 and a randomly primed human peripheral blood cDNA library cloned into the BglIII sites of pACT (Clontech). Transformants tested for histidine prototrophy and β-galactosidase staining, 198 positive clones were found. Clones carrying the bait and prey plasmids were tested for β-galactosidase activity using a pellet X-gal (PXG) assay as previously described (14).

Far western assay

Far western assays were performed as described previously (15). Briefly, duplicate samples of ~1.5 μg of the purified proteins were separated on a 4–20% Criterion gel (Bio-Rad). One half of the gel was stained with Coomassie brilliant blue and the second half was transferred to nitrocellulose membrane. The membrane was incubated overnight at 4°C in blocking/renaturation buffer containing 1× PBS, 3% non-fat dry milk, 0.2% Tween 20, 0.1% PMSF, 5 mM NaF and 2 mM dithiothreitol. The nitrocellulose was then probed with ≥300 000 cpm [32P]labeled GSTyCTD fusion protein that had been hyperphosphorylated with CTDK-I for 4 h at 4°C. After extensive washing, the nitrocellulose was air-dried and subjected to autoradiography.

Immobilized CTD peptide binding assay

Synthetic biotinylated peptides were dissolved in PBS and incubated with 300 μl of TetraLink Tetrameric Avidin Resin (Invitrogen) for 45 min at room temperature (RT). The peptide concentrations in the column output, flow-through and wash fractions were monitored by
absorbance at 280 nm and these values were used to approximate the amount of peptide on the column. Generally, 30–50 µg of biotinylated peptide was conjugated to the 300 µl column. The peptide columns were stored in PBS at 4°C and were stable for ~2 months. Approximately 10–15 µg of purified protein and 50 mg insulin were dissolved in PBS for a final volume of 500 µl as the output; 450 µl of the output was applied to the peptide resin and incubated for 20 min at RT with mixing every 5 min. The flow-through and two washes with half-column volumes of PBS were collected. The resin was then extensively washed with 5 ml of buffer containing 25 mM HEPES (pH 7.6), 8% glycerol, 150 mM NaCl and 0.1 mM EDTA (HGNE150). The bound protein was then eluted with four half-column volumes of HGNE300 and four half-column volumes of HGNE1000. The resin was regenerated with 5 ml HGNE1000 and 5 ml PBS. Samples were analyzed by SDS–PAGE followed by Coomassie blue staining.

CBD pull-down assay

CBD-tagged RECQ5 and its variants were produced in *Escherichia coli* BL21-CodonPlus(DE3)-RII cells (Stratagene) and immobilized on chitin beads (20 µl; New England Biolabs) as previously described (7). Beads were incubated for 2 h at 4°C with a total extract from HEK293 T cells (600 µg of protein) in a volume of 500 µl of buffer TN2 [50 mM Tris–HCl (pH 8), 120 mM NaCl, 20 mM NaF, 15 mM sodium pyrophosphate and 0.5% (v/v) NP-40] supplemented with 1 mM benzamidine, 0.2 mM PMSF, 0.5 mM sodium orthovanadate and protease inhibitor cocktail (Roche) and then were washed three times with buffer TN2. Bound proteins were released from the beads by addition of 25 µl of 3× SDS-loading buffer followed by incubation at 95°C for 7 min. Eluted proteins were separated by 10% SDS–PAGE and analyzed by western blotting using the indicated antibodies. RECQ5 was detected by Ponceau S staining.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP-IT Express kit from Active Motif (Carlsbad, CA, USA) according to manufacturer’s instructions. Briefly, HeLa or 293 (mock or GFP-RECQ5 transfected) cells grown to 70-80% confluency were harvested and seeded in a 96-well plate described above. One day after transfection, siRNA-treated cells were harvested and seeded in a 96-well plate at a density of 10 000 cells/well in a volume of 100 µl of DMEM containing fetal calf serum and penicillin/streptomycin. Next day, cells were treated with different concentrations of diospyrin (compound D1; obtained from Dr Banasri Hazra of Jadavpur University, Kolkata) ranging from 0 to 100 µM. Experiments were performed in triplicate and the values were calculated and reported as mean ± SEM.

Quantitative real-time PCR

The amount of immunoprecipitated DNA in each ChIP reaction was measured by quantitative real-time PCR (qPCR) using a Roche LightCycler 480 Real-Time PCR System and a Roche LightCycler 480 DNA SYBR Green I Master. Primer sequences used for the qPCR reactions are listed in Supplementary Table S2. Specificity of target amplification was confirmed by agarose gel electrophoresis and melting curve analysis. For each primer pair, a series of 5-fold dilutions of input DNA were used to generate a linear standard curve in which the crossing point was plotted versus log10 of template concentration. Primer pair efficiency was calculated from these data as $E = 10^{[(Ct_{input} - Ct_{im})/slope]}$, and primer pairs with $E > 1.8$ were used for qPCR. For data analysis, Pfaffl’s method was used (16). Fold enrichment of immunoprecipitated target regions was expressed as ratio of the amount of DNA estimated for a specific antibody versus the amount of DNA estimated for the control IgG antibody. All qPCR reactions were performed at least six times using DNA template obtained from two independent ChIP experiments. The data were plotted using GraphPad Prism software as mean ± SEM.

Helicase assay

PAGE-purified DNA and RNA oligonucleotides used for helicase assays were purchased from Microsynth. The sequences are: deoxy-18 (d18): 5'-tcc cag tca cga cgt tgt-3'; ribo-18 (r18): 5'-ucc cag uca cgu ugu-3'. The oligonucleotides were 5'-end labeled with T4 polynucleotide kinase and γ[32P]ATP (GE HealthCare) and annealed to M13mp2 ssDNA at a 1 : 1 (mol/mol) ratio in the presence of 20 mM Tris–HCl (pH 7.4) and 150 mM NaCl. For helicase assays, RECQ5 at concentrations ranging from 0 to 360 nM was incubated either with 1 nM M13mp2/d18 or with 1 nM M13mp2/r18 substrate for 30 min at 37°C in 10 µl of 33 mM Tris–acetate buffer (pH 7.9) containing 10 mM Mg-acetate, 66 mM K-acetate, 0.1 mg/ml BSA and 2 mM ATP. Helicase reactions were stopped by addition of 2.5 µl of 5× stop dye (100 mM EDTA, 60% glycerol, 1.5% SDS, 0.1% bromophenol blue and 0.1% xylene cyanol). Products were separated by electrophoresis on 15% native polyacrylamide gels in TBE buffer and visualized by autoradiography.

Cell viability assay

Cell viability was measured using Resazurin Fluorometric Cell Viability Assay Kit (Biotium, Inc.) according to the manufacturer’s instructions. Briefly, HeLa cells were seeded at the confluence of 20% in 10-cm plates. After 24 h, cells were transfected with appropriate siRNA as described above. One day after transfection, siRNA-treated cells were harvested and seeded in a 96-well plate at a density of 10 000 cells/well in a volume of 100 µl of DMEM containing fetal calf serum and penicillin/streptomycin. Next day, cells were treated with different concentrations of diospyrin (compound D1; obtained from Dr Banasri Hazra of Jadavpur University, Kolkata) ranging from 0 to 100 µM. Experiments were performed in triplicate and the values were calculated and reported as mean ± SEM.
carried out in hexaplicates for each drug concentration. After 24 h, cells were washed gently once with pre-warmed PBS, and then a mixture of resazurin and DMEM (100 μl) in a ratio 1:10 was added in to each well. After 4 h of incubation at 37°C, cell viability was monitored by measuring fluorescence with excitation wavelength at 540 nm and emission wavelength at 590 nm in a SpectraMax reader M5 (Molecular Devices). The fluorescent signal generated from the assay is directly proportional to the number of living cells in the sample. The percentage of survival of diospyrin-treated cells was calculated relative to mock (DMSO)-treated cells and plotted using GraphPad Prism as mean ± SD

RESULTS
RECO5 binds to the CTD of RNAPII through a conserved motif

In a yeast two-hybrid screen for proteins that interact with the non-conserved C-terminal region of human RECO5 (amino acids 411–991 fused to the LexA DNA-binding domain), we isolated a clone from a human peripheral blood cell cDNA library that encoded the CTD of the largest subunit of RNAPII, RPB1 (amino acids 1266–1970). This domain consists of evolutionary conserved repeats of the heptapeptide sequence YSPTSPS that serve as a binding platform for proteins involved in transcription and mRNA processing (17–19). Thus, our finding suggests that RECO5 is a novel RNAPII CTD-binding protein.

During transcription, the CTD undergoes dynamic phosphorylation on serine residues, producing different phosphorylation patterns that predominate in individual stages of the transcription cycle and determine the recruitment of a specific set of proteins (17–19). Using the MotiScan software, we found that the extreme C-terminal region of RECO5 (amino acids 910–950) contains a putative phospho-CTD (P-CTD)-binding motif, called a Set2–Rpb1-interacting (SRI) domain (Figure 1A). This motif was initially identified as a Ser2,5 P-CTD-binding module in the yeast methyltransferase Set2, which catalyses histone H3 lysine 36 methylation during transcription (20). NMR studies on the SRI domains of the yeast Set2 and its human homologue SETD2 have revealed a novel CTD-binding fold consisting of a left-turned three-helix bundle (21,22). RECO5 appears to contain only helices 1 and 2 that form the CTD-binding surface.

To show that the putative SRI motif of RECO5 is important for RECO5 binding to the CTD of RNAPII, we deleted it (amino acids 908–954) from the bait plasmids carrying the full-length RECO5 and RECO5 411–991,
respectively. The resulting constructs were tested for yeast two-hybrid interaction with the CTD prey plasmid isolated in our yeast two-hybrid screen. We found that neither of these mutants interacted with RNAPII CTD (Figure 1B and C). In contrast, the full-length RECQ5 as well as RECQ5 411-991 showed positive yeast two-hybrid interaction with CTD (Figure 1B and C). A previous study demonstrated that human CTD was phosphorylated when expressed in yeast (23). Thus, our data suggest that RECQ5 binds to the P-CTD of RNAPII by means of the SRI domain.

It has been shown that RECQ5 binds to RNAPII through a region including amino acids 396–617 (9). Using a series of internal deletion variants of RECQ5, we mapped more precisely the boundaries of this domain to amino acids 515 and 640, respectively (Supplementary Figure S1). Hereafter, this domain is referred to as the Internal RNAPII-interacting (IRI) domain. A deletion of the proximal part of the IRI domain of RECQ5 that spans amino acids 515–568 (ΔIRI) completely abolished binding of RECQ5 to the hypophosphorylated form of RNAPII (RNAPIIA) (Supplementary Figure S1C, Lane 4). In contrast, yeast two-hybrid analysis indicated that the RECQ5 ΔIRI mutant bound to the RNAPII CTD to a similar extent as wild-type RECQ5 (Figure 1B and C). Likewise, an N-terminally truncated variant of RECQ5 of amino acids 541–991 was found to interact with the RNAPII CTD in the yeast two-hybrid system, although it was defective in interacting with RNAPIIA (Figure 1B and C, and data not shown).

**RECQ5 specifically binds to Ser2,5-phosphorylated CTD heptapeptide repeats**

As an initial test of P-CTD binding, purified recombinant versions of RECQ5 were subjected to SDS–PAGE and far western analysis using a 32P-labeled GSTyCTD fusion protein phosphorylated on Ser2 and Ser5 by yeast CTDK-I (binding probe is expected to carry ~25 doubly phosphorylated repeats). The full-length RECQ5 bound very well to the P-CTD fusion protein (Figure 2A, Lane 1), whereas RECQ5 deleted for the SRI domain did not bind appreciably (Figure 2A, Lane 3). Likewise, a C-terminal fragment containing the SRI domain (residues 675–991) bound to P-CTD-like full-length RECQ5, but a similar fragment lacking the SRI domain did not show any binding (Figure 2A, Lanes 4 and 5). Notably, RECQ5 missing only the IRI domain bound P-CTD almost as well as the wild-type protein (Figure 2A, Lane 2 versus 1). These data indicate that RECQ5 binding to the P-CTD requires the SRI domain.

To check whether the SRI domain of RECQ5 has the same binding specificity as the SRI domain of Set2 (22), synthetic biotinylated peptides with three consensus CTD heptapeptide repeats were immobilized on avidin-coated beads to create affinity resins. Because the full-length RECQ5 showed significant background interaction with the TetraLink resin, we used the C-terminal fragment of RECQ5 with and without the SRI domain for the peptide-binding experiments. Binding was evaluated with four different phosphorylated peptides: (i) a non-phosphorylated peptide (NP); (ii) a peptide with phospho-serines at position 2 of each repeat (2P3); (iii) a peptide with phospho-serines at position 5 of each repeat (5P3); and (iv) a peptide with phospho-serines at positions 2 and 5 of each repeat (2,5P3) (Figure 2B). The RECQ5 675-991 protein bound well to the resin with the 2,5P3 peptide: the protein was depleted from the column flow-through fraction and was only eluted from the resin by 1 M NaCl (Figure 2C, top panel). In contrast, RECQ5 675–991 did not bind detectably to resins with the NP, 2P3 or 5P3 peptides (Figure 2C, top panel). Moreover,
RECOQ5 675-991ΔSRI was unable to bind to the 2,5P3 column, confirming that the SRI domain of RECOQ5 is required for interaction with the phosphorylated CTD (Figure 2C, bottom panel). These results substantiate that the SRI domain of RECOQ5, like the SRI domain of Set2, binds directly and specifically to CTD repeats with phospho-serines at positions 2 and 5.

The SRI, but not IRI, domain of RECOQ5 mediates its binding to the hyperphosphorylated form of RNAPII

Next we compared the binding capabilities of wild-type and mutant forms of RECOQ5 to RNAPII holoenzyme from human cells. To do so, RECOQ5 proteins were produced in bacteria as fusions with a CBD, bound to chitin beads and incubated with a total extract of HEK293T cells. RNAPII binding was analyzed by western blotting using two different mouse monoclonal antibodies against the RNAPII CTD: (i) H5 that recognizes CTD repeats containing phospho-Ser2 and hence selectively detects the hyperphosphorylated form of RNAPII (IIO); and (ii) 7C2 that recognizes CTD repeats irrespective of their phosphorylation status and, therefore, can detect both RNAPIIO and RNAPIIa. As expected, wild-type RECOQ5 was found to bind both forms of RNAPII (Figure 3, Lane 3). RECOQ5 ΔIRI was impaired in binding to RNAPIIa but exhibited binding to RNAPIIO with an extent similar to that of wild-type RECOQ5 (Figure 3, Lane 4). On the contrary, RECOQ5 ΔSRI showed binding to RNAPIIa but was impaired in interacting with RNAPIIO (Figure 3, Lane 5). These data indicate that the binding of RECOQ5 to the hyperphosphorylated form of RNAPII is mediated by the SRI domain of RECOQ5.

RECOQ5 associates with RNAPII-transcribed genes within the region of productive elongation

RNAPII binds to the promoter with a CTD in the non-phosphorylated state. CTD phosphorylation on Ser5 is one of the first steps in transcription initiation and leads to the movement of the transcription complex to a promoter-proximal pausing site. The escape of RNAPII from the pausing site and subsequent productive elongation of the transcript is associated with Ser2 phosphorylation of the CTD repeats (19). To investigate the interaction between RECOQ5 and RNAPII during the transcription cycle, we used ChIP to characterize the distribution of RECOQ5 along constitutively expressed genes. We chose two genes, the ACTG1 gene encoding γ-actin (3.1 kb) and the DHFR gene encoding dihydrofolate reductase (30 kb) (Figure 4A and B, top panels), which were used previously to dissect the distribution of RNAPII and its phosphorylated isoforms along the transcriptional unit (24). Exponentially growing HeLa cells were crosslinked with formaldehyde. Isolated chromatin fraction was sonicated to shear the genomic DNA into small fragments ranging from 200 to 500 bp and subjected to immunoprecipitation using the following antibodies: (i) 8WG16 that specifically recognizes non-phosphorylated CTD repeats; (ii) ab5095 that specifically binds to phospho-Ser2 in CTD; and (iii) anti-RECOQ5 antibody raised against the C-terminal fragment of RECOQ5 spanning amino acids 675–991. After reversing the crosslinks, ChIP-enriched DNA fragments were subjected to qPCR analysis using primers amplifying the core promoter, different regions within the transcriptional unit and an intergenic region located downstream of each gene (Figure 4A and B, top panels). Amplicon sizes ranged between 109 and 174 bp. In agreement with the published data (24), we found that the non-phosphorylated RNAPII was bound exclusively to the core promoter of the tested genes (Figure 4). Ser2-phosphorylation of RNAPII was nearly absent at the core promoter and accumulated in the body of each gene (Figure 4). On both genes, the phospho-Ser2 signal dramatically dropped in the 3′-untranslated region (3′-UTR) and reached intergenic background levels (Figure 4). Although RECOQ5 could bind to the hypophosphorylated form of RNAPII, it showed a relatively low occupancy at the promoter region (Figure 4). Notably, the RECOQ5 distribution pattern was almost identical to that of phospho-Ser2-CTD (Figure 4). Thus, our data suggest that, during the RNAPII transcription cycle, RECOQ5 associates with the productive elongation complex most likely through binding to Ser2,5-P-CTD.

The SRI, but not IRI, domain of RECOQ5 mediates its association with RNAPII-transcribed genes

To investigate which of the two RNAPII-binding domains of RECOQ5 is responsible for the observed accumulation of RECOQ5 at the coding regions of the ACTG1 and DHFR genes, the full-length RECOQ5, RECOQ5 ΔIRI, RECOQ5 ΔSRI and RECOQ5 ΔIRIΔSRI, respectively, were ectopically expressed in HEK293 cells as fusions with GFP (Supplementary Figure S2) and subjected to
ChIP analysis using an anti-GFP antibody. In control ChIP experiments, cells were transfected with empty vector expressing GFP only. The results obtained from the qPCR analysis clearly indicated that the SRI domain of RECQ5 was essential for the binding of RECQ5 to the coding regions of the ACTG1 and DHFR genes (Figure 5). In contrast, the IRI domain of RECQ5 was found to be dispensable for its association with these genes (Figure 5). To confirm our finding, we also examined the binding of RECQ5 and its mutants to the coding regions of two other genes, namely CDKN1A (encoding p21) and HSP70-2. As expected, GFP-tagged wild-type RECQ5 was largely enriched at the coding region of these two genes (Figure 5). The mutants lacking the SRI domain, RECQ5 ΔSRI and RECQ5 ΔIRIΔSRI, failed to accumulate at these regions, while the RECQ5 ΔIRI mutant showed similar fold of enrichment as wild-type RECQ5 (Figure 5).

Point mutagenesis studies on the SRI domain of human SETD2 identified several residues as critical for P-CTD binding (22). We generated mutations in equivalent residues of RECQ5 (F938L, K939A and R943A). Out of these, the R943A mutation completely abolished the interaction of RECQ5 411–991 with the CTD in the yeast two-hybrid system (Supplementary Figure S3A). Likewise, the R943A mutation abolished the association of RECQ5 with the coding region of the ACTG1 gene (Supplementary Figure S3B).

Taken together, our results indicate that out of the two RNAPII-interacting domains of RECQ5, the SRI domain is necessary and sufficient to mediate the association of RECQ5 with the coding regions of RNAPII-transcribed...
GENES. Moreover, these data imply that the recruitment of RECQ5 to the sites of transcription is dependent on Ser2,5 phosphorylation of the RNAPII CTD.

RECQ5 negatively affects cell viability upon inhibition of spliceosome assembly

To understand the functional role of RECQ5 during RNAPII transcription, we explored the possibility that RECQ5 is involved in the cellular response to co-transcriptionally formed R-loops. To this end, we first examined whether RECQ5 could unwind an RNA : DNA hybrid duplex prepared by annealing of a synthetic 18-mer RNA oligonucleotide to M13mp2 ssDNA. We found that RECQ5 failed to unwind this structure although it could unwind the corresponding DNA : DNA duplex (Supplementary Figure S4). This finding suggests that RECQ5 does not disrupt R-loops in vivo.

Next, we evaluated the effect of RECQ5 depletion on cell survival upon inhibition of spliceosome assembly, which leads to the formation of R-loops. To do so, RECQ5 was down-regulated in HeLa cells by RNA interference using two different siRNAs (Figure 6A). RECQ5-proficient and RECQ5-deficient cells were treated with diospyrin that blocks spliceosome assembly. It was shown that this Top1 kinase inhibitor caused the same phenotypic defects as ASF/SF2 depletion (3). Surprisingly, we found that RECQ5-proficient cells displayed a much higher sensitivity to diospyrin than RECQ5-deficient cells (Figure 6B). These data suggest that RECQ5 negatively affects cell viability in conditions leading to the formation of R-loops.

DISCUSSION

Genetic ablation of RECQ5 helicase in mice results in genomic instability and cancer susceptibility (6). Studies in mouse and human cells have suggested that RECQ5 regulates HR by promoting disassembly of the RAD51 presynaptic filament (6). However, RECQ5 was also found to interact with RNAPII in human cells, suggesting a role in transcription (7–9). In this work, we provide evidence that RECQ5 associates with RNAPII during the productive elongation phase of transcription through direct binding to the Ser2,5 P-CTD of RPB1 by means of a SRI motif located at the C-terminus of RECQ5. Although RECQ5 could also bind to hypophosphorylated form of RNAPII through an additional domain, herein referred as IRI domain, it showed relatively low occupancy at the promoter regions of RNAPII-transcribed genes, excluding a role for RECQ5 during transcription initiation. In addition, we show that RECQ5 negatively affects cell viability upon inhibition of spliceosome assembly, which can induce the formation of mutagenic R-loop structures. Moreover, we demonstrate that RECQ5 cannot unwind RNA : DNA duplexes in vitro, which excludes the possibility the RECQ5 directly disrupts R-loops. Together, these findings suggest that RECQ5 might play a role in the maintenance of genomic stability during RNAPII transcription.

Recently, Islam et al. (25) have also reported that the SRI domain of RECQ5 mediates its binding to the hyperphosphorylated form of RNAPII in human cells. In addition, these authors have found that the IRI domain of RECQ5 shares extensive homology with the so-called KIX domain identified as a protein interaction module in several RNAPII transcription regulators. In agreement with our results, they demonstrated that
mutations at conserved residues in the IRI/KIX domain of RECQ5 abolished the interaction of RECQ5 with RNAPII, but not with RNAPIIIO (25). However, in contrast to our observations, Islam et al. (25) found that the IRI/KIX domain of RECQ5 could bind to both RNAPII and RNAPIIIO. This discrepancy may arise from the use of different experimental approaches. Whereas we analyzed binding of RNAPII from a cell extract to beads coated with recombinant RECQ5 protein produced in bacteria, Islam et al. (25) monitored RECQ5–RNAPII complex formation in vivo by immunoprecipitation. Thus, it is possible that efficient binding of the IRI/KIX domain of RECQ5 to RNAPIII is dependent on a post-translational modification of RECQ5, which is absent if the protein is produced in bacteria. Nevertheless, our ChIP experiments clearly show that the IRI/KIX domain of RECQ5 does not play a role in the association of RECQ5 with the coding regions of RNAPII-transcribed genes, where the RNAPII CTD exists in the hypophosphorylated state. Further studies are needed to elucidate the complex mode of the RECQ5–RNAPII interaction. In particular, the site of interaction for the IRI/KIX domain of RECQ5 on RNAPII needs to be determined.

What is the exact role of RECQ5 during the elongation phase of RNAPII transcription? Our experiments with diospyrin suggest that RECQ5 might be involved in the suppression of the DNA-damaging effects of RNAPII transcription rather than in the repair of transcription-induced DNA damage. Interestingly, a recent study by Aygun et al. (10) showed that RECQ5 could directly inhibit RNAPII transcription in vitro in a manner dependent on its IRI/KIX domain, which led the authors to propose a model in which RECQ5 promotes genome stability by regulating RNAPII transcription itself. In line with this hypothesis, Islam et al. (25) demonstrated that the IRI/KIX, but not the SRI, domain of RECQ5 was required for suppression of sister chromatid exchange and resistance to camptothecin-induced DNA damage. However, it should be noted that the in vitro transcription system used by Aygun et al. (10) lacked CTD kinase activity responsible for CTD phosphorylation on Ser2 during transcription. Our experiments with the RECQ5 ΔSRI mutant have indicated that hyperphosphorylation of RNAPII prevents binding of RECQ5 to RNAPII via the IRI/KIX domain, suggesting that Ser2 CTD phosphorylation may alleviate the inhibitory effect of RECQ5 on RNAPII transcription so that RECQ5 could not affect RNAPII transcription during productive elongation in vivo. However, it is possible that a formation of an R-loop during transcription could trigger a domain interaction switch in the RECQ5–RNAPII complex to adopt the inhibitory arrangement, which would prevent further extension of the R-loop structure and hence diminish its DNA-damaging effect. Our finding of negative effect of RECQ5 on cell viability under conditions that favor R-loop formation is consistent with this hypothesis. Future work will clarify the molecular mechanism of this phenomenon to shed light on the biological processes that enforce genomic stability during transcription.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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