Dynamic interaction of Y RNAs with chromatin and initiation proteins during human DNA replication

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Accepted 15 February 2011
Journal of Cell Science 124, 2058-2069
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doi:10.1242/jcs.086561

Summary
Non-coding Y RNAs are required for the initiation of chromosomal DNA replication in mammalian cells. It is unknown how they perform this function or if they associate with a nuclear structure during DNA replication. Here, we investigate the association of Y RNAs with chromatin and their interaction with replication proteins during DNA replication in a human cell-free system. Our results show that fluorescently labelled Y RNAs associate with unreplicated euchromatin in late G1 phase cell nuclei before the initiation of DNA replication. Following initiation, Y RNAs are displaced locally from nascent and replicated DNA present in replication foci. In intact human cells, a substantial fraction of endogenous Y RNAs are associated with G1 phase nuclei, but not with G2 phase nuclei. Y RNAs interact and colocalise with the origin recognition complex (ORC), the pre-replication complex (pre-RC) protein Cdt1, and other proteins implicated in the initiation of DNA replication. These data support a molecular ‘catch and release’ mechanism for Y RNA function during the initiation of chromosomal DNA replication, which is consistent with Y RNAs acting as replication licensing factors.

Key words: DNA replication, Y RNA, Licensing factor, Non-coding RNA

Introduction
Accurate replication of chromosomal DNA is fundamental for the propagation of the genome during the eukaryotic cell division cycle and for maintaining its integrity. A key regulation step is initiation, when two DNA replication forks are established at each replication origin (reviewed in Bell and Dutta, 2002; Kearsey and Cotterill, 2003; Takeda and Dutta, 2005; DePamphilis et al., 2006; Aladjem, 2007). In this evolutionarily conserved process, the replication proteins origin recognition complex (ORC), Cdc6, Cdt1 and MCM2–MCM7 associate in a stepwise manner with replication origins in the G1 phase of the cell cycle, forming the pre-replicative complex (pre-RC). Initiation at these sites then depends on the activities of CDK and DDK protein kinases, and involves the recruitment of further proteins, including CDC45, GINS, MCM10 and replication protein A (RPA), to form the pre-initiation complex (pre-IC), while Cdc6 and Cdt1 are displaced. Finally, DNA primase and polymerases are recruited to begin DNA synthesis, marking entry into S phase. Additional levels of control have evolved in metazoan organisms (Arias and Walter, 2007), and proteins such as DUE-B, high mobility group A (HMGA)1a and the Ku autoantigen have been implicated in the initiation of DNA replication in mammalian cells (Norseen et al., 2008; Rampakakis et al., 2008; Thomae et al., 2008; Chowdhury et al., 2010).

Non-coding RNAs have recently emerged as additional factors involved in the regulation of the initiation of eukaryotic DNA replication in several different species (reviewed in Krude, 2010). In extracts from human somatic cells, reconstitution of chromosomal DNA replication in isolated cell nuclei requires a class of non-coding RNAs termed Y RNAs (Christov et al., 2006; Gardiner et al., 2009; Krude et al., 2009). These are small, structured stem-loop RNAs of between 69–112 nucleotides in length, which are conserved in vertebrate evolution (Pruijn et al., 1993; Farris et al., 1995; Mosig et al., 2007; Perreault et al., 2007). Humans have four Y RNAs (hY1, hY3, hY4 and hY5 RNAs), whereas other vertebrates have between one and four. Non-human vertebrate Y RNAs can functionally substitute for human Y RNAs in a cell-free DNA replication system (Gardiner et al., 2009), and all human Y RNAs can substitute for each other (Christov et al., 2006). This functional redundancy results from an evolutionarily conserved double-stranded RNA domain present in all vertebrate Y RNAs, which is essential and sufficient for Y RNA function in DNA replication (Gardiner et al., 2009). Additional studies have shown that the execution point for Y RNA function during chromosomal DNA replication is the initiation step: degradation of hY3 RNA inhibits the de novo initiation of new DNA replication forks in the mammalian cell-free system, but it does not influence progression rates of already established DNA replication forks (Krude et al., 2009).

The molecular mechanism by which Y RNAs function in the initiation of mammalian chromosomal DNA replication is currently unclear. DNA replication is a structure-bound process in the cell nucleus (Berezney et al., 2000), yet Y RNAs are predominantly soluble and their intracellular localisation is controversial (Pruijn et al., 1997). In nucleation and cell fractionation experiments, Y RNAs were found to be almost exclusively soluble and cytoplasmic in Xenopus laevis oocytes and mammalian cells (O’Brien et al., 1993; Peek et al., 1993; Simons et al., 1994). By contrast, Y RNAs were detected by in situ hybridisation in both the cytoplasm and the nucleus of mammalian cells (Matera et al., 1995; Farris et al., 1997). It is thus unknown whether Y RNAs associate with nuclei during DNA replication, or whether they exert their function in solution. Here, we have addressed this question by studying the
association of fluorescently labelled Y RNAs with template nuclei and with DNA replication proteins during DNA replication in a human cell-free system. Our results suggest a ‘catch and release’ mechanism for Y RNA function, by which Y RNAs associate with unreplicated chromatin prior to initiation, are essential for initiation, and then dissociate from these sites once DNA replication has initiated there.

**Results**

**hY RNAs associate rapidly with euchromatin in late G1 phase nuclei**

To enable a direct analysis of whether Y RNAs associate with nuclear structures during chromosomal DNA replication, we generated Alexa-Fluor-conjugated Y RNAs (Fig. 1). Each of the four human Y RNAs was synthesised in vitro from plasmid expression constructs using SP6 RNA polymerase (Christov et al., 2006; Gardiner et al., 2009), and these purified hY RNAs were covalently coupled at their 3′-ends to an Alexa-Fluor dye (Fig. 1A). We then tested whether these Alexa-Fluor-conjugated hY RNAs still reconstituted the initiation of chromosomal DNA replication in a human cell-free system (Fig. 1B). In this system, late G1 phase template nuclei initiate semi-conservative chromosomal DNA replication when incubated with purified Y RNAs in the presence of two essential fractions from human cell extracts, termed QA and ArFT (which contain all of the soluble proteins required for the initiation of DNA replication) (Christov et al., 2006; Gardiner et al., 2009). Each of the four Alexa-Fluor-conjugated hY RNAs increased the proportion of replicating late G1 phase nuclei to the same extent as observed with unconjugated hY1 RNA (Fig. 1B). This increase is statistically significant (t-tests, \( P<2.4 \times 10^{-5}, n=6–14 \)). These data indicate that conjugation of Y RNAs to Alexa-Fluor dyes at their 3′-ends does not inactivate their function as DNA replication initiation factors.

In the next experiments, we used confocal fluorescence microscopy to investigate whether these fluorescent hY RNAs associate with template nuclei in this cell-free system (Fig. 1C,D). Alexa-Fluor-488-conjugated hY3 and hY5 RNAs associated with the late G1 phase nuclei, within 1 minute of incubation, in a non-uniform distribution. RGB line profiles across representative nuclei show that the amounts of chromosomal DNA (red) inversely correlated with the amounts of associated hY3 and hY5 RNAs (green; Fig. 1C,D). Areas of predominant Y RNA association therefore correspond to areas of decondensed DNA, which are characteristic for euchromatin. The pattern of nuclear association changed over time as hY RNAs were gradually lost from euchromatic sites during the 120 minutes of incubation, and hY RNAs also became detectable in nucleoli after 60 minutes. Alexa-Fluor-conjugated hY1 and hY4 RNAs showed similar patterns (data not shown).

To determine whether the association of Y RNAs with the nuclei is specific under these conditions, we also synthesised and analysed Alexa-Fluor-594-conjugated human 5S ribosomal RNA (supplementary material Fig. S1A). As shown for unconjugated 5S rRNA (Christov et al., 2006), Alexa-Fluor-594-conjugated 5S rRNA did not reconstitute the initiation of chromosomal DNA replication (supplementary material Fig. S1B). In contrast to hY RNAs, 5S rRNA did not detectably associate with a nuclear structure in late G1 phase template nuclei under these conditions (supplementary material Fig. S1C), indicating that the observed association of Y RNAs with chromatin is specific.

In conclusion, these time-course experiments show that hY RNAs associate rapidly with euchromatin in late G1 phase template nuclei under experimental conditions that support the initiation of DNA replication in vitro.

**hY1 colocalises with hY3 and hY4 RNAs, but not with hY5 RNA**

Next, to determine whether the four different hY RNAs colocalise with each other during this reaction, we co-incubated Alexa-Fluor-594-conjugated hY1 RNA with Alexa-Fluor-488-conjugated hY3, hY4 or hY5 RNAs (Fig. 2). Both hY1 and hY3 RNAs localised to the same intranuclear sites on euchromatin at 1 minute and 15 minutes of incubation. These sites showed variations in the hue of the yellow signal in the merged images, suggesting that the relative amounts of hY1 and hY3 RNAs present at individual sites varied to some extent. hY4 RNA showed a similar colocalisation with hY1 RNA on euchromatic sites, and small additional amounts of hY4 RNA were also present in nucleoli.

By contrast, hY5 RNA did not colocalise with hY1 RNA under these conditions (Fig. 2). After 1 minute of incubation, most hY1 RNA associated with euchromatin whereas hY5 RNA was excluded from these sites and was enriched in the nucleoli. After 15 minutes of incubation, hY5 RNA was not detected in the nucleus any more, whereas hY1 RNA was still present at its euchromatic sites. Therefore, the additional presence of hY1 RNA in this reaction changes the localisation of hY5 RNA (cf. Fig. 1D). This suggests that different hY RNAs have different binding affinities for euchromatin sites, and that when present together, hY1 outcompetes hY5 RNA for binding to these sites.

**The loop domain is required for the targeting of hY RNAs to euchromatin**

The major difference between Y RNAs is the internal loop domain (Mosig et al., 2007; Perreault et al., 2007). Therefore, this domain might contribute to the relative binding affinities of different Y RNAs to chromatin. In the next set of experiments, we tested this hypothesis by analysing the association of mutant hY RNAs to chromatin (Fig. 3).

The structure of hY1 RNA with its evolutionarily conserved domains is shown in Fig. 3A as a reference. The lower stem domain is formed by the pairing of the 3′- and 5′-ends of the RNA up to a central helical bulge, providing a binding site for the Rho60 protein (Chen and Wolin, 2004). The adjacent double-stranded upper stem domain is essential and sufficient for the function of Y RNAs in chromosomal DNA replication (Gardiner et al., 2009). The central loop domain is predominantly single-stranded, rich in polypyrimidine tracks and contains small secondary stem loops. Finally, the single-stranded 3′-polyuridine tail provides a binding site for the La protein (Wolin and Cedervall, 2002).

Mutant hY1 RNAs were prepared that contained a terminal deletion of the lower stem domain (ΔLS RNA), an internal deletion of the loop domain (ΔLP RNA), or deletions of both lower stem and internal loop domains (ΔLS/ΔLP RNA). We synthesised these deletion mutants in vitro and, following purification, coupled them to Alexa Fluor 488 (Fig. 3B). The Alexa-Fluor-conjugated mutant ΔLP, ΔLS or ΔLS/ΔLP RNAs all supported the initiation of DNA replication in vitro (Fig. 3C), similar to their unconjugated forms (Gardiner et al., 2009).

Each of these three fluorescent mutant hY1 RNAs associated with chromatin in vitro within 1 minute of incubation (Fig. 3D). The relative preference for intranuclear sites in these mutants,
Fig. 1. Human Y RNAs associate with euchromatin in vitro. (A) Synthesis of Alexa-Fluor-conjugated hY RNAs. The four hY RNAs were synthesised by in vitro transcription, purified by anion-exchange chromatography and covalently coupled to Alexa Fluor hydrazides at their 3′-termini as indicated. RNAs were separated on a denaturing 8% polyacrylamide gel and stained with SYBR Gold. M indicates 100-nucleotide RNA ladder. (B) Alexa-Fluor-conjugated hY RNAs support DNA replication in vitro. Template nuclei from late G1 phase human cells were incubated with fractions QA and ArFT in the presence of the indicated hY RNAs (Christov et al., 2006). Replication buffer and unfractionated cytosolic extract (S100) served as negative and positive controls, respectively. Proportions of replicating nuclei were determined by immunofluorescence microscopy. Mean values ± s.d. are shown for each experiment. (C,D) Dynamic association of hY RNAs with late G1 phase nuclei in vitro. Alexa-Fluor-488-conjugated hY3 RNA (C) and hY5 RNA (D) were incubated with late G1 phase nuclei in the presence of fractions QA and ArFT for the indicated times. Nuclei were fixed and fluorescent hY RNA was directly visualised by confocal microscopy. DNA was counterstained with propidium iodide. Single channels and merged images of Alexa-Fluor-488-conjugated hY3 and hY5 RNAs (green) and chromosomal DNA (red) are shown of representative nuclei. RGB line profiles across nuclei are shown on the right (green, hY RNAs; red, DNA). The analysed profiles are boxed on the corresponding merged RGB images. Scale bars: 10 μm.
Chromatin association of non-coding Y RNAs

Fig. 2. Colocalisation of human Y RNAs. Alexa-Fluor-conjugated hY RNAs were co-incubated with late G1 phase nuclei in the presence of fractions QA and ArFT for 1 minute and 15 minutes, as indicated. Nuclei were fixed and fluorescent hY RNAs were directly visualised by confocal microscopy. Single channels and merged images of Alexa-Fluor-594-conjugated hY1 RNA (red) and Alexa-Fluor-488-conjugated hY3, hY4 and hY5 RNAs (green) are shown for representative nuclei. Scale bar: 10 μm.

However, differs from that of full-length hY1 RNA. Deletion of either lower stem or loop resulted in a more homogeneous initial association across the nuclei, including the nucleoli. Furthermore, the double mutant had an additive phenotype, showing preferential initial binding to central heterochromatic sites including the nucleoli, in addition to their association with euchromatin. These data suggest that both lower stem and loop domains contribute to the selective association of Y RNAs with euchromatin.

To corroborate these findings independently, we co-incubated hY1 and ΔLS/ΔLP RNAs and compared their association in the same nuclei (Fig. 3E). Under these competitive conditions, hY1 RNA preferentially associated with euchromatic sites, whereas the double mutant bound chromatin without site specificity.

We conclude that the upper stem domain of Y RNAs (i.e. ΔLS/ΔLP RNA), which is essential and sufficient for Y RNA function in DNA replication (Gardiner et al., 2009), is capable of interacting with chromatin across the nucleus, albeit without specificity for particular sites. The loop and lower stem domains, which are not essential for DNA replication (Gardiner et al., 2009), contribute to the intranuclear localisation of the upper stem domain by providing selectivity for binding to euchromatin. By that molecular mechanism, Y RNAs can localise preferentially to chromosomal DNA at euchromatic sites in late G1 phase nuclei. Because chromosomal DNA replication initiates at discrete euchromatic sites at the G1 to S phase transition, we investigated the intranuclear localisation of hY RNAs in the context of DNA replication.

hY RNAs dissociate from replicated DNA

First, we tested whether hY RNAs associate with sites of DNA replication in the cell-free system (Fig. 4). DNA replication foci were labelled by incorporation of digoxigenin-dUTP into nascent DNA and visualised by confocal immunofluorescence microscopy. Alexa-Fluor-488-conjugated hY3 RNA associated with chromatin in the late G1 phase template nuclei within 1 minute of incubation, before DNA replication became detectable in vitro (Fig. 4A, top row). By 15 minutes, DNA replication had initiated in 28% of nuclei and by 120 minutes this number had increased further to 35%. In both non-replicating and replicating nuclei, hY3 RNA remained associated but did not seem to colocalise with discrete DNA replication foci. When analysed at higher resolution (Fig. 4B), hY3 RNA was not detected at discrete DNA replication foci, but was still concentrated at sites of unreplicated DNA. RGB line profiles confirmed that peak positions of hY3 RNA and DNA replication foci do not overlap with each other (Fig. 4A, bottom panels). We observed identical patterns for hY1, hY4 and hY5 RNAs (supplementary material Fig. S2). These data suggest that Y RNAs bind to unreplicated chromatin, and become displaced from these sites as a result of DNA replication.

Next, we tested this hypothesis by measuring the amount of Alexa-Fluor-488-conjugated hY3 RNA associated with replicating and non-replicating nuclei (Fig. 4C). About half of the associated hY3 RNA dissociated from non-replicating nuclei during the first 60 minutes of incubation. An additional, statistically significant amount of hY3 RNA dissociated from replicating nuclei, indicating that DNA replication stimulates Y RNA displacement from chromatin. We corroborated this finding by measuring the amounts of chromatin-associated hY3 RNA in the presence of the DNA replication inhibitor aphidicolin (Fig. 4D). In the presence of aphidicolin, no DNA replication foci were detectable in the nuclei (Fig. 4A, bottom row), and a significantly increased amount of hY3 RNA remained associated with the nuclei when compared with the untreated control group (Fig. 4D).

From these data, we conclude that Y RNAs associate with unreplicated euchromatin in G1 phase nuclei. Furthermore, once DNA replication is initiated in a Y RNA-dependent manner, DNA synthesis significantly stimulates the dissociation of Y RNA from replicating chromatin, including the discrete sites of DNA replication foci. We tested next whether the lower stem and loop domains are required for the displacement of Y RNAs from replicated DNA. The three mutant RNAs, ΔLS, ΔLP and ΔLS/ΔLP, which all support the initiation of DNA replication in this system, bound to unreplicated chromatin quickly, then dissociated from sites of replicated DNA, and remained detectable only on unreplicated DNA (Fig. 5). Therefore, the loop and lower stem domains are not required for the dynamic displacement of Y RNAs from replicated DNA.

Taken together, these observations suggest that Y RNAs are not a constituent of active DNA replication fork progression complexes at replication foci, nor do they remain on chromatin once it has
been replicated. They bind to chromatin, however, before replication initiates, including those sites where initiation takes place. To identify a molecular link between Y RNAs and the established DNA replication machinery, we went on to investigate the interaction of human DNA replication initiation and elongation proteins with immobilised hY RNAs.

**hY RNAs interact with DNA replication initiation factors**

To obtain immobilised hY RNAs, we chemically coupled the oxidised 3’-termini of purified hY RNAs covalently to agarose beads. These hY RNA beads were used for pull-down experiments of hY RNA-interacting proteins from human cell extracts (Fig. 6). Extracts were first pre-cleared with uncoupled agarose beads and then adsorbed to affinity beads coupled to one of the four hY RNAs. Uncoupled agarose beads were used as negative controls. Following incubation, the beads were extensively washed with physiological buffer and associated proteins were detected by western blotting (Fig. 6).

The well-established Y RNA binding proteins Ro60 and La (Wolin and Cedervall, 2002; Chen and Wolin, 2004) interacted with all four hY RNAs under these conditions, but not to uncoupled agarose beads (Fig. 6A).

We discovered several novel interactions between hY RNAs and DNA replication initiation proteins with this approach (Fig. 6B). A subset of proteins constituting the pre-RC associated with hY RNAs. The ORC2 and ORC3 subunits of the ORC interacted with all four hY RNAs. The ORC4 and ORC6 subunits strongly interacted with hY1, hY3 and hY5 RNAs, but showed reduced interaction with hY4 RNA when compared with the other three hY RNAs. This suggests that individual ORC subunits might contribute differently to the association of the ORC complex with Y RNAs. Furthermore, Cdt1 and Cdc6 proteins associated with all four hY RNAs. We also detected interactions of all four hY RNAs with the DNA unwinding element binding protein DUE-B, the two subunits of the Ku autoantigen, Ku70 and Ku80, the single-stranded DNA binding protein RPA and, albeit very weakly, the proliferating cell
Finally, we detected interactions of HMGA1a with hY1 and hY3, but not with hY4 and hY5 RNAs. In contrast to these initiation proteins, we did not detect strong interactions between hY RNAs and proteins of the CMG and replisome complexes (Fig. 6C). Of the CMG complex, all six subunits of the MCM2–MCM7 heterohexameric complex, CDC45 and all four human GINS subunits did not interact detectably with hY RNAs (Fig. 6C). DNA polymerases α, δ, and e, MCM8, MCM10 and And-I also did not interact with hY RNAs. Several regulators of DNA replication, including cyclin A, CDK2 and geminin, also did not interact (Fig. 6C). It is important to note, however, that we cannot exclude very weak interactions between hY RNAs and these latter proteins, which might have fallen short of the detection threshold for western blotting.

Taken together, these pull-down experiments establish a novel molecular link between Y RNAs and proteins of the DNA replication initiation machinery, including the pre-RC, DUE-B and Ku, but not the elongation machinery.

hY RNAs colocalise with DNA replication initiation factors on unreplicated chromatin

In the next set of experiments, we assessed the physiological significance of these molecular interactions by investigating the intranuclear localisation of DNA replication initiation proteins in
comparison with hY1 RNAs and DNA replication foci in the cell-free system (Fig. 7). Alexa-Fluor-594-conjugated hY1 RNA was incubated with late G1 phase template nuclei and fractions QA and ArFT for 1 minute and 60 minutes, respectively. After fixation, hY1 RNA was visualised directly, whereas candidate proteins and DNA replication foci were detected by indirect immunofluorescence microscopy. In pilot experiments, we discovered that the majority of available primary antibodies contained a ribonuclease activity that degraded Y RNA (supplementary material Fig. S3), thus preventing any colocalisation studies. We therefore inactivated this RNase activity with ribonuclease inhibitor for each antibody prior to immunofluorescence microscopy (supplementary material Fig. S3).

The pre-RC proteins ORC2 and Cdt1 localised to many small discrete foci across the nuclei after 1 minute of incubation, before DNA replication initiated (Fig. 7A). At this time point, hY1 RNA generally overlapped with these proteins in the euchromatin, but there were also discrete sites of these pre-RC proteins that did not contain hY1 RNA, predominantly located in peripheral and nucleolar heterochromatin. The Y RNA-interacting proteins DUE-B, Ku70 and Ku80 were also concentrated at euchromatin sites that overlapped with hY1 RNA (Fig. 7A).

In addition, we analysed the localisation of hY1 RNA in comparison with the pre-RC protein MCM2 and the pre-IC protein CDC45 (Fig. 7A), even though they did not associate with hY RNAs in our pull-down assays (cf. Fig. 6). MCM2 and CDC45 were also found at discrete sites across the nuclei with a preferential enrichment in euchromatin that also overlapped with sites of hY1 RNA (Fig. 7A). Therefore, pre-RC and pre-IC proteins colocalise with hY1 RNA on euchromatin sites before DNA replication initiates.

Next, we investigated the localisation of hY1 RNA and pre-RC proteins in comparison with sites of DNA replication following initiation in vitro (Fig. 7B). After an incubation of 60 minutes, both Cdt1 and MCM2 proteins were detected with hY1 RNA at overlapping central sites in the nuclei. By contrast, DNA replication foci were detected at distinct sites in early replicating euchromatin, which did not overlap with hY1 RNA, MCM2 or Cdt1. Therefore, hY1 RNA, MCM2 and Cdt1 proteins are all absent from sites of replicated DNA, yet they are still present at sites that have not been replicated.

Taken together, our in vitro results show that several DNA replication initiation proteins that biochemically associate with hY RNAs also colocalise with hY1 RNA on unreplicated chromatin, and dissociate from these sites when they are replicated. In the final set of experiments we aimed to corroborate these findings in intact human cells.

**Dynamic association of endogenous hY RNAs with G1 phase nuclei**

To analyse the association of endogenous hY RNAs with cell nuclei before and after DNA replication in vivo, we synchronised EJ30 cells in the G1 and G2 phases of the cell cycle (Fig. 8A). We fractionated the cells and measured the amounts of hY RNAs present in the soluble and nucleus-associated fractions (Fig. 8B). As shown before (Christov et al., 2006; Christov et al., 2008), hY1
and hY3 were the most abundant Y RNAs in the cells, and overall amounts were only 1.4-, 1.7-, 1.3- and 0.7-fold higher in G1 phase cells compared with G2 phase cells for hY1, hY3, hY4 and hY5 RNA, respectively (data not shown). After fractionation, about 10% of total RNA was associated with cell nuclei in both G1 and G2 phase cells (Fig. 8B). Strikingly, between 20% and 70% of the hY RNAs were associated with the nuclei in G1 phase cells. By contrast, only 4–10% of hY RNAs were associated with G2 phase nuclei, demonstrating that hY RNAs associate with unreplicated G1 phase nuclei and become dissociated from replicated G2 phase nuclei in intact human cells.

Discussion

Y RNAs are essential for the initiation step of chromosomal DNA replication in a mammalian cell-free system (Christov et al., 2006; Gardiner et al., 2009; Krude et al., 2009). Here we have addressed the issue of whether Y RNAs associate with a nuclear structure during this process. We have found that hY RNAs associate with chromatin before the initiation of DNA replication, interact with proteins of the pre-replication complex, and dissociate from sites of replicated DNA. These data suggest a molecular mechanism for Y RNA function during DNA replication, which is consistent with the original licensing factor model for once-per-cell-cycle control of eukaryotic DNA replication (Laskey et al., 1981; Blow et al., 1987).

Y RNAs bind to euchromatin dynamically

Previous reports on the intracellular localisation of Y RNAs are controversial, suggesting cytoplasmic, nuclear and nucleolar locations (O’Brien et al., 1993; Peek et al., 1993; Simons et al., 1994; Matera et al., 1995; Farris et al., 1997; Pruijn et al., 1997). Our data show that hY RNAs associate rapidly and preferentially with euchromatin from a soluble pool before DNA replication initiates, and dissociate again from sites of replicated DNA after initiation has occurred. Consistent with earlier ultrastructural work (Matera et al., 1995; Farris et al., 1997), we have detected hY RNAs associated with nucleolar structures upon longer incubation in vitro. It is therefore likely that Y RNAs associate with euchromatin in late G1 phase nuclei, which is consistent with the observation that the four hY RNAs are functionally redundant for the initiation of chromosomal DNA replication in vitro (Christov et al., 2006; Gardiner et al., 2009). In co-incubation experiments, however, only hY1, hY3 and hY4 RNAs show a high degree of overlap at euchromatin sites, whereas hY5 RNA preferentially binds to nucleoli. This might point towards an additional role for hY5 RNA, which is consistent with the literature.
Firstly, Gendron and colleagues showed that Ro RNPs containing hY5 RNA preferentially localise to nuclei in cultured human cells (Gendron et al., 2001). More recently, Hogg and Collins demonstrated that hY5 RNA co-purifies with ribosomal protein L5 and its binding partner 5S rRNA (Hogg and Collins, 2007). They suggest that hY5 RNA might play a specialised role in 5S rRNA surveillance and quality control of ribosome biogenesis. As ribosome biogenesis takes place in the nucleoli, our observation would therefore be consistent with a dual function of hY5 RNA in both DNA replication and RNA surveillance. In support of dual Y RNA function, we have recently shown that Y RNAs can support different functions, depending on associated RNP proteins (Langley et al., 2010).

The association of hY RNAs with nucleoli at later stages of the in vitro incubation could also be linked to their function in the initiation of DNA replication. Nuclear DNA is replicated late in S phase and the cell-free system does not support initiation of DNA replication at these sites during a standard 3-hour incubation in vitro (Keller et al., 2002). The association of hY RNAs with nucleolar sites might thus be due to a specific association of hY RNAs with unreplicated nucleolar chromatin under these conditions, in preparation for later initiation at these sites. Systematic mutation of the domains of hY1 RNA established that both the loop and terminal stem domains confer specificity for hY1 RNA binding to euchromatin. The upper stem domain, which is essential and sufficient for the DNA replication function of vertebrate Y RNAs (Gardiner et al., 2009), binds to chromatin indiscriminately. Therefore, our experiments provide a novel and auxiliary function for the non-essential loop and lower stem domains in the context of regulating the DNA replication function of Y RNAs. Consistent with a model of modular RNP function (Hogg and Collins, 2008), it appears likely that these two domains provide a means for Y RNA diversification and their specialisation into associating with different nuclear domains. In particular, the loops of hY1, hY3 and hY4 RNAs could confer the preferential interaction with euchromatin in late G1 phase nuclei. This, in turn, could facilitate the targeting of the functional determinant present in the upper stem to early replicating euchromatin sites at the G1 to S phase transition.
Y RNAs interact with initiation proteins

Despite an essential role of Y RNAs for chromosomal DNA replication in mammalian cells (Christov et al., 2006; Christov et al., 2008; Gardiner et al., 2009; Krude et al., 2009; Langley et al., 2010), molecular links with the established DNA replication machinery have not been reported to date. We have shown here that all four human Y RNAs interact with DNA replication initiation proteins, including ORC, Cdt1, Cdc6, DUE-B and Ku and colocalise with these proteins on unreplicated chromatin before the initiation of chromosomal DNA replication in vitro. By contrast, replication fork proteins such the CMG complex (CDC45, MCM2–MCM7 and GINS), DNA polymerases and associated factors do not interact with Y RNAs at the molecular level. DNA replication forks in S phase cell nuclei are clustered into discrete replication foci (Berezney et al., 2000), and Y RNAs are not present at these foci. These molecular interactions therefore build upon our previous data showing that Y RNAs are essential for the initiation step of DNA replication but not for chain elongation synthesis at replication forks (Krude et al., 2009).

Our biochemical data show that Y RNAs interact with the pre-RC proteins ORC, Cdt1 and Cdc6, but not with MCM2–MCM7. We showed by confocal fluorescence microscopy that ORC2, Cdt1 and MCM2, as well as the pre-IC protein CDC45, are present with hY RNAs at overlapping intranuclear sites on unreplicated chromatin before initiation of DNA replication in vitro. These data suggest that Y RNA function might be associated with origin specification and/or activation, pre-RC or pre-IC assembly, but not with DNA helicase activity of the CMG complex after initiation.

ORC has already been shown to interact with other non-coding RNAs, which regulate initiation of DNA replication in distinct eukaryotic systems (Mohammad et al., 2007; Norseen et al., 2008; Norseen et al., 2009). Thus our data add to the concept of ORC as a target for regulation by non-coding RNAs during initiation.

The architectural chromatin protein HMGA1a has been shown to recruit ORC to viral, and possibly cellular, replication origins in an RNA-dependent manner (Norseen et al., 2008; Thomasa et al., 2008). HMGA1a interacts with only hY1 and hY3, and not with hY4 or hY5 RNAs, yet all four hY RNAs are active during the initiation of DNA replication. Therefore, the interaction between HMGA1a and Y RNAs is unlikely to be essential for the initiation of chromosomal DNA replication in our system. Nevertheless, it remains a possibility that HMGA1a plays an auxiliary role in this process via an interaction with hY1 and hY3 RNAs, perhaps at a subset of chromosomal sites.

The DUE-B protein binds to DNA unwinding elements (DUEs) at human replication origins and is implicated in the unwinding step of origin activation (Casper et al., 2005; Chowdhury et al., 2010). DUE-B binding to origins is dependent on ORC, and is in turn required for the loading of CDC45 (Chowdhury et al., 2010). Like Y RNAs, DUE-B is released from the DNA template during initiation (Chowdhury et al., 2010). Interestingly, DUE-B has structural similarity to aminoaicyl-tRNA editing enzymes (Casper et al., 2005; Kemp et al., 2007), which might account for its molecular interaction with the highly structured hY RNAs. As with depletion of Y RNAs (Christov et al., 2006; Christov et al., 2008; Gardiner et al., 2009), depletion of DUE-B leads to an inhibition of DNA replication in cell-based and cell-free assays (Casper et al., 2005). A possible theory, therefore, is that the interaction between Y RNAs and DUE-B is functionally relevant for origin activation.

The abundant heterodimeric Ku protein (made up of Ku70 and Ku80 subunits) is involved in many fundamental cellular processes including DNA repair, transcription and DNA replication (Tuteja and Tuteja, 2000; Downs and Jackson, 2004). In addition to its affinity for DNA ends and hairpins, Ku also binds to RNA (Yoo and Dynan, 1998; Tuteja and Tuteja, 2000), which could account for its strong molecular interaction with the hairpin-rich hY RNAs observed here. Ku has been shown to bind DNA replication origins in human cells and reduced levels of Ku result in cell growth defects and reduced rates of initiation of DNA replication (Sibani et al., 2005; Rampakakis et al., 2008). Therefore, Y RNAs and Ku might act in the same pathway regulating initiation of DNA replication in vertebrate somatic cells. There is a caveat to an essential role for Ku, however, because Ku80 knockout mice are viable, despite showing growth defects (Nussenzweig et al., 1996; Zhu et al., 1996).

The single-stranded DNA binding protein RPA and the sliding clamp PCNA are both required for the initiation of human chromosomal DNA replication in vitro (Szüts et al., 2003; Szüts et al., 2005). RPA is involved in the DNA unwinding step of initiation and both proteins are required for the recruitment of DNA polymerases to activated origins in eukaryotes. Therefore, both proteins might interact with Y RNAs during initiation. However, both RPA and PCNA are also required for DNA chain elongation synthesis and consequently localise to DNA replication foci in this system (Szüts et al., 2003; Szüts et al., 2005). These dual roles for RPA and PCNA therefore necessitate further analysis to assess how far their interaction with Y RNAs is functionally relevant for the initiation step.
Molecular mechanism for Y RNA function in DNA replication

Data presented here allow us to propose a molecular mechanism for Y RNA function during DNA replication. As shown here, soluble Y RNAs first associate with initiation-competent, unreplicated euchromatin in G1 phase. Most likely, this involves molecular interactions between Y RNAs and proteins of the initiation complex. Because several initiation proteins interact with Y RNAs, it is likely that Y RNAs interact with a protein complex rather than a single protein. Secondly, Y RNAs are essential for the initiation step of chromosomal DNA replication, as we have shown previously (Christov et al., 2006; Gardiner et al., 2009; Krude et al., 2009). This function is mediated by the upper stem domain of Y RNAs, which is evolutionarily conserved in vertebrates. Finally, once DNA synthesis is initiated in a Y RNA-dependent manner, Y RNAs dissociate from replicated chromatin sites. The fact that Y RNAs were not detected at replication foci and that their displacement is stimulated by DNA replication argues that they are either displaced from these sites or degraded.

Thus, Y RNAs employ a ‘catch and release’ mechanism for their essential function during the initiation of chromosomal DNA replication. The dynamic association of Y RNAs with unreplicated chromatin and their functional requirement for the initiation step of DNA replication mirrors that of the DNA replication licensing factors Cdt1 (RLF-B) and MCM2–MCM7 (RLF-M) (Krude et al., 1996; Thommes et al., 1997; Tada et al., 2001). Their binding to unreplicated chromatin during G1 and S phase discriminates replicated from unreplicated sections of the genome and ensures that chromosomal DNA is replicated once and only once during each cell cycle (Blow and Dutta, 2005; DePamphilis et al., 2006; Aladjem, 2007). Y RNAs are thus consistent with the ‘activator’ factor as proposed in the original model of replication licensing for once-per-cell-cycle control of eukaryotic DNA replication (Laskey et al., 1981; Blow et al., 1987).

Materials and Methods

Cell culture and fractionation

Human EJ30 bladder carcinoma cells were cultured and synchronised as described (Krude et al., 1997; Krude, 2000). Cytosolic and nuclear extracts of asynchronously proliferating human HeLa cells were obtained from Cibitbiotech (Mons, Belgium). Fractionation of the cytosolic extract into protein fractions QA and ArFT was carried out by anion exchange chromatography on Q Sepharose and arginine Sepharose as described (Szüts et al., 2003; Szüts et al., 2005).

For a quantification of endogenous hY RNAs, synchronised G1 and G2 phase EJ30 cells were fractionated into nuclei and cytosolic extract by hypotonic treatment and dounce homogenisation, followed by centrifugation at 3000 g (Krude et al., 1997). Nuclei were washed in hypotonic buffer and subsequently extracted in hypotonic buffer containing 0.5 M NaCl (Krude et al., 1997). Total RNA was isolated from the nuclear and cytosolic extracts by phenol extraction and ethanol precipitation, and quantified by nanodrop spectrophotometry. Y RNAs in these extracts were quantified by RT-PCR as detailed previously (Christov et al., 2006), using serial dilutions of pure hY cDNA plasmids as calibrators.

Synthesis and purification of wild-type and mutant Y RNAs

Wild-type and mutant hY RNAs were synthesised in vitro as described (Christov et al., 2006; Gardiner et al., 2009). Sequences of the mutant Δ5S and Δ5P RNAs were as published (Gardiner et al., 2009). Mutant Δ5S/Δ5P was identical to USL RNA (Gardiner et al., 2009) with two additional uridine residues at its 3′-terminus. RNAs were purified by anion exchange chromatography on a MonoQ column (Amersham Biosciences). RNA was loaded in 50 mM sodium acetate, pH 5.2; washed in 50 mM sodium acetate (pH 5.2) containing 0.4 M NaCl; and eluted with a linear 0.4–1 M NaCl gradient in 50 mM sodium acetate, pH 5.2. The size and purity of all in vitro-synthesised RNA was confirmed using 8 M urea denaturing polyacrylamide gel electrophoresis and staining with SYBR Gold (Invitrogen) as described (Christov et al., 2006). Multimeric 100-nucleotide RNA molecules were used as molecular weight markers (Roche).

Coupling of hY RNAs to Alexa-Fluor fluorophores and agarose beads

Purified RNA was oxidised at the 3′-end with NaIO4. For coupling to fluorophores, oxidised RNA was incubated with a fivefold molar excess of Alexa Fluor 488 or Alexa Fluor 594 hydrazide (Invitrogen). The coupling reaction was quenched by reduction with NaBH4, and Alexa-Fluor-conjugated hY RNAs were isolated by ethanol precipitation. For coupling to agarose beads, oxidised RNA was incubated with adipic acid dihydrazide agarose (Sigma) at a final concentration of 1 mg/ml. This reaction was also quenched with NaBH4.

RNA binding assays and western blotting

Human cell extracts were pre-depleted with 6B Sepharose beads (Sigma) for 1 hour at 4°C. Pre-depleted extracts were then incubated with RNA-coupled agarose beads or 6B Sepharose beads equilibrated in RNA binding buffer (20 mM HEPES pH 7.8, 100 mM potassium acetate, 2 mM MgCl2, 1 mM DTT). After binding, beads were extensively washed with binding buffer and associated proteins were eluted by denaturation. Proteins were analysed by SDS-PAGE and gel electrophoresis followed by western blot analysis. For western blotting, the following primary antibodies against human proteins were used: anti-Ro60, La (both from Euroimmun, Huntingdon, UK); ORC2, ORC3, ORC6, HMGAla (all from Aloy Schepers, Department of Gene Vectors, Munich, Germany); ORC4 (sc-19726, Santa Cruz Biotechnology); Cdt1 (ab22716 and ab31574, Abcam); Cdc6 (K0069-3S, MBL); DU-E-B (Michael Leffak, Wright State University, Dayton, OH); Ku70, Ku80 (both from Stephen Jackson, University of Cambridge, UK); RPA (pAb-RPA1) (Szüts et al., 2003); PCNA (PC10, sc-56, Santa Cruz Biotechnology); MCM2 (ab6153, Abcam); MCM7 (sc-9966, Santa Cruz Biotechnology); Pan-MCM (BD Pharmingen); Psf1, Psf2, Psf3, Slid (all from Stephen Bell, University of Oxford, UK); Primase p58, Pol alpha p68, Pol alpha p166, Pol delta p124, CDC45 (Heinz-Peter Nasheuer, National University of Ireland, Galway), Pol epsilon p261 (Frank Grosse, University of Jena, Germany); MCM8 (sc-47117, Santa Cruz Biotechnology); MCM10, And-1 (both from Anindya Dutta, University of Virginia, Charlottesville, VA); CDK2 (sc-163, Santa Cruz Biotechnology); Cyclin A (sc-53227, Santa Cruz Biotechnology); Gemini (ab12147, Abcam).

DNA replication in vitro and localisation assays

DNA replication reactions were performed as described (Christov et al., 2006). To study the nuclear localisation of Alexa-Fluor-conjugated hY RNAs in vitro, DNA replication reactions contained Alexa-Fluor-488- and Alexa-Fluor-594-conjugated hY RNAs at 300 ng and 900 ng per reaction, respectively. This difference was to compensate for a difference in signal strength between these fluorophores. To analyse DNA replication reactions, nuclei were fixed and spun onto polylysine-coated glass coverslips. Alexa-Fluor-conjugated hY RNAs were detected directly. Digoxigenin-labelled and Alexa Fluor-488- or Alexa Fluor-633-conjugated goat anti-mouse and anti-rabbit IgG-F, and F A fragments (Invitrogen) were used as secondary antibodies. Confocal fluorescence microscopy was performed on a Leica SP1 microscope using 63× lens magnification with additional optical zoom for low- and high-resolution analysis, respectively. Individual channels were recorded sequentially, except for localisations of hY RNAs on DNA or replication foci, which were recorded simultaneously.

Computing and image analysis

DNA secondary structures were calculated using the Mfold v3.2 RNA folding algorithm (web server at: http://www.bioinfo.rpi.edu/applications/mfold/cgi- bin/rna-form1.cgi) under default conditions (Mathews et al., 1999; Zuker, 2003). The t-tests (two-tailed, two-sample of unequal variance) and analysis of variation (ANOVA, single factor, between groups, α=0.05) were performed using Microsoft Excel. ImageJ (NIH, software) was used to calculate the integrated pixel density of individual nuclei on original greyscale TIFF files of single-channel confocal images with ImageJ v1.43r software (http://rsweb.nih.gov/ij), using plugins obtained from MacBiophotonics (http://www.mabiophotonics.ca). Background subtraction was applied with two standard deviations from the mean prior to analysis. RGB line profiles were obtained from merged images using ImageJ v1.43r software using plugins from MacBiophotonics.

We thank Cath Green and Sebastian Klingle for critical reading of this manuscript, Rachael Walker for help with flow cytometry and Stephen P. Jackson, Aloys Schepers, Michael Leffak, Stephen D. Bell, Heinz-Peter Nasheuer, Frank Grosse and Anindya Dutta for reagents. This work was supported by Cancer Research UK (project grant C1471/A10308) and by...
Supplementary material available online at http://jcs.biologists.org/cgi/content/full/124/12/2058/DC1