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A homologous recombination defect affects replication-fork progression in mammalian cells

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Summary

Faithful genome transmission requires a network of pathways coordinating DNA replication to DNA repair and recombination. Here, we used molecular combing to measure the impact of homologous recombination (HR) on the velocity of DNA replication forks. We used three hamster cell lines defective in HR either by overexpression of a *RAD51* dominant-negative form, or by a defect in the *RAD51* paralogue *XRCC2* or the breast tumor suppressor *BRCA2*. Irrespectively of the type or extent of HR alteration, all three cell lines exhibited a similar reduction in the rate of replication-fork progression, associated with an increase in the density of replication forks.

Importantly, this phenotype was completely reversed in complemented derivatives of *Xrcc2* and *Brca2* mutants. These data reveal a novel role for HR, different from the reactivation of stalled replication forks, which may play an important role in genome stability and thus in tumor protection.

Supplementary material available online at <http://jcs.biologists.org/cgi/content/full/121/2/162/DC1>

Key words: Homologous recombination, Replication, Mammalian cells, Breast cancer, Unchallenged cells

Introduction

Replication forks are routinely arrested by a broad variety of stresses (Hyrien, 2000; Shechter and Gautier, 2004). The relationships between homologous recombination (HR) and DNA replication have been well documented in cells challenged with strong genotoxic stresses. Indeed, HR reactivates replication forks arrested at DNA lesions (Kuzminov, 1995). In mammalian cells, prolonged inhibition of replication progression generates DNA double-strand breaks and stimulates HR (Saintigny et al., 2001). Consistently, the pivotal HR protein *RAD51* localizes at arrested replication forks (Sengupta et al., 2003; Sengupta et al., 2004). Finally, treatment with cisplatin reduces replication kinetics, an effect that is abrogated by mutation of the HR gene *Xrcc3* (Henry-Mowatt et al., 2003).

By contrast, the impact of HR on the replication dynamics in unchallenged mammalian cells is still unexplored. This prompted us to use the molecular combing approach to measure the rate of fork progression and the density of initiation events in cells affected in HR, in absence of other additional stress. We analyzed three hamster cell lines with different defects in HR: one expresses a dominant-negative form of *Rad51* (V79SMRAD51), the other two are mutated either in the *Rad51* paralogue *Xrcc2* (*xrcc2*) or in the breast tumor suppressor *Brca2* (*brca2*). The consequences of these genetic modifications for gene conversion and sensitivity to genotoxic stress have been extensively studied in mammalian cells (Daboussi et al., 2005; Johnson et al., 1999; Kraakman-van der Zwet et al., 2002; Lambert and Lopez, 2000; Lambert and Lopez, 2001; Lambert and Lopez, 2002; Liu et al., 1998; Thacker et al., 1995). We also studied complemented cell-lines derived from *Xrcc2* and *Brca2* mutant cell lines. Altogether, our results reveal a

novel role for HR in the control of replication dynamics, different from the reactivation of stalled replication forks, and further support the link between HR and replication for the maintenance of genetic stability.

Results and Discussion

Impact of HR deficiency on replication-fork progression

Molecular combing assays were carried out in the three different HR-deficient cell lines and their corresponding controls, described in Table 1. Newly synthesized DNA was labeled in vivo by two successive pulses with IdU then CldU (20 minutes each). The combed DNA molecules were uniformly stained blue using anti-DNA antibody and the thymidine analogs were revealed by green and red fluorescence (see Materials and Methods). Analysis of the replication labeling revealed two types of signal: symmetric labeling (equal length of red and green tracks) and asymmetric labeling. The latter pattern most often coincides with the end of the DNA molecules, as indicated by the blue staining and, thus, corresponds to broken molecules (Fig. 1A). We focused on symmetric labeling and, therefore, on full-size replication tracks. Moreover, this allows to specifically measure the kinetics of replication elongation rather than the reactivation of arrested replication forks.

As shown in Fig. 1B, the replication signals appear to be shorter and more frequent in HR-defective cells. In order to quantify this effect, 150 full-size tracks were measured for each cell line. The mean lengths of these tracks were significantly lower in the three HR-deficient cells than in control V79 and V79 Puro cells ($P < 0.001$), indicating that the replication speed slows down in HR mutants. The mean replication speed was between 0.97 and 1.07

Table 1. Cell lines and derivatives

Cell line	Origin	Plasmid transfected or chromosome transferred
V79-puro	V79	Empty expression vector (pCDNA3-puro) (Daboussi et al., 2005)
V79 SMRad51*	V79	Vector coding for SMRad51 (pCDNA3-puro) (Daboussi et al., 2005)
<i>Xrcc2</i>	IRS1: V79 deficient in <i>Xrcc2</i> (Thacker et al., 1995)	Empty expression vector (pCDNA3-puro) (Daboussi et al., 2005)
<i>Xrcc2</i> complemented	IRS1 (<i>XRCC2</i> complemented) (Tambini et al., 1997)	Expression of <i>XRCC2</i> cDNA
V79	V79 (from which V-C8 was derived)	
<i>Brca2</i> (V-C8)	V79-deficient in <i>Brca2</i> (Kraakman-van der Zwet et al., 2002; Wiegant et al., 2006)	
<i>Brca2</i> complemented	V-C8#13 (Kraakman-van der Zwet et al., 2002; Wiegant et al., 2006)	The human <i>BRCA2</i> is expressed from human chromosome 13

*The dominant-negative SMRad51 is a chimera gene made by the fusion of the N-terminal part of yeast RAD51 to the entire mouse *Rad51* cDNA (Lambert and Lopez, 2000). V79SMRad51 is a hamster V79 cell line expressing the dominant-negative SMRad51.

Kb/minute in the controls, and between 0.77 and 0.83 Kb/ minute in HR-deficient lines (Fig. 1C). For 20-minute pulse labeling, this corresponds to a difference of ~4 Kb.

To verify that this reduced replication speed actually results from HR alteration, we performed a second set of experiments to compare the velocity of replication forks in *Xrcc2*- and *Brca2*-deficient mutants, and in their complemented counterpart. Importantly, the mean replication speeds found in *Xrcc2* and *Brca2* mutants, as well as in control V79 cells, were not statistically different in the two independent series of experiments (supplementary material Fig. S1). As shown in Fig. 1C, the replication speed was similar in complemented cells, and in control V79 and V79 Puro cell lines ($P=0.5$ for *Xrcc2* complementation; $P=0.42$ for *Brca2* complementation) but significantly different from that of their respective parental HR-defective cell line ($P<0.001$). Hence, the replication phenotype of HR mutants appears fully reversed in complemented cells.

The slow fork progression observed in HR-deficient cells could result either from the accumulation of endogenous damage or from the presence of slow-replicating zones and/or DNA structures that are difficult to replicate. However, the former hypothesis appears unlikely here. First, because we took only symmetrical tracks into account, putative replication stalling should occur on each fork during both pulses (i.e. every 20 minutes) to produce a genome-wide effect, which would correspond to an unlikely high frequency of replication arrests. Second, the extent of replication delay was not correlated with the magnitude of sensitivity to genotoxic stress: *xrcc2*- and *brca2*-defective cells are much more sensitive to genotoxic stress than SMRad51-expressing cells (Daboussi et al., 2005; Kraakman-van der Zwet et al., 2002). In spite of this, the speed of replication was reduced to similar levels in all cell lines. Third, it has been shown that adducts on DNA decrease replication elongation kinetics and defects in HR restore replication kinetics (Henry-Mowatt et al., 2003). Here, we demonstrate that, in unchallenged cells, defects in HR do not accelerate but rather slow down replication kinetics, suggesting that the defect in replication is not exclusively connected with damaged DNA. Thus, our results strongly suggest that HR exerts a global effect on replication speed rather than a localized rescue of stalled forks. This conclusion is supported in molecular terms by the description of physical interactions between HR proteins (RAD51 and RAD52) and the MCM complex (Shukla et al., 2005). This conclusion does not exclude the possibility that slow replication in HR-deficient cells

could be restricted to a particular subset of genomic loci or in particular population of the cells. If this comes true, this would result in moderate effect on the global measurement of genome replication but would imply that the impact of HR on the subset of genomic loci or in the sub-population is much more important.

It is important to keep in mind that the incorporation of nucleotide analogs might affect replication, which results in a replication stress that could be toxic. In the experiments described here, control and mutant cell lines were submitted – in parallel – to the same conditions, and such toxicity was not observed during the short time course (two 20-minute pulses each) of each experiment. However, it is still possible that the HR defect amplifies and reveals a replication stress generated by the use of nucleotide analogs, which was not detectable in wild-type cells. Nevertheless, the data presented here reveal a novel connection between HR and replication, i.e. a role of HR in the progression of replication forks during elongation. Indeed, this is a new role for HR on replication elongation, different from the well-established role on the reactivation of replication forks that have been blocked.

Defects in HR lead to increased firing of replication origins

The slowing down of fork progression has been repeatedly correlated with an increase in the density of initiation events (for reviews, see Gilbert, 2007; Taylor, 1977). Particularly, this association has been observed in different situations, such as perturbation of the nucleotide pools (Anglana et al., 2003), and ATR or Chk1 inactivation (Marheineke and Hyrien, 2004).

Thus, as a confirmation of the above results, we investigated the impact of HR deficiency on the density of active replication forks. Fork density was determined on 7-10 Mb of DNA fibers,

Table 2. Density of replication forks

Cell line	Number of forks per Mb	Inter-origin distance (Kb)
V79	9.3	43.5
V79 SMRAD51	11.7	31.7
<i>Xrcc2</i>	11.4	31.1
<i>Xrcc2</i> complemented	8.8	43.9

Fork density was determined on 7-10 Mb of DNA fibers labeled with anti-DNA antibody, bearing at least one elongation signal. Inter-origin distance was measured on fibers bearing at least two signals.

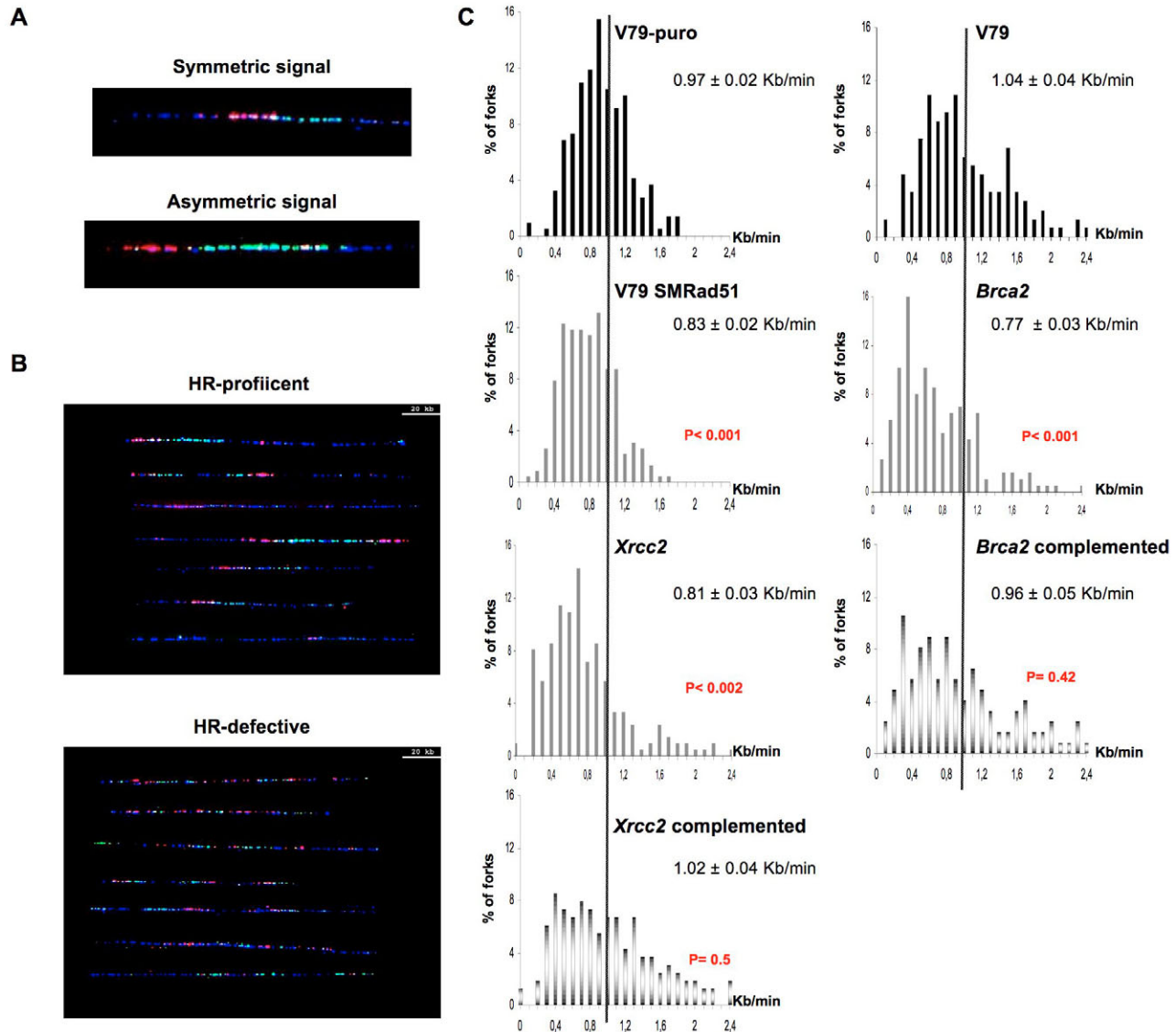


Fig. 1. Analysis of replication elongation by molecular combing. (A) Example of labeled molecules with symmetric and asymmetric replication signals. Blue, anti-DNA; green, anti-IdU; red, anti-CldU. (B) Typical examples of labeled molecules obtained from HR proficient (upper panel) or HR-defective cells (lower panel). (C) Percentage of forks traveling at the indicated speed (kb/minute) in the three HR-deficient cell lines, their respective parental cell line and complemented cell line derivatives. The analysis was performed on 150 DNA fibers with symmetric labeling. The dotted line corresponds to the mean rate in parental V79 or V79-puro cells. We compared the distributions of V79SM, Xrcc2-defective and Xrcc2-complemented cells with the distribution of V79. Consistently, there were no statistical differences between the complemented and the wild-type parental cell lines ($P=0.5$ for Xrcc2 and $P=0.42$ for Brca2). By contrast, the distributions between mutant and parental V79 or V79-puro or complemented derivatives were significantly different ($P<0.001$). There is no statistical difference between the complemented and parental cell lines ($P=0.5$ for Xrcc2 and $P=0.42$ for Brca2).

bearing at least one elongation signal. Our results show a significant increase in the frequency of elongating forks in HR-defective cell lines, compared with control cell lines (Fig. 1B). The control cell line V79 presented 9.3 forks per Mb, whereas the Xrcc2 and V79SMRAD51 cell lines presented 11.4 and 11.7 forks per Mb, respectively ($P<0.01$) (Table 2). In Xrcc2 complemented cells, the density of replication forks was 8.8 forks per Mb, a value similar to that found in wild-type V79 cells ($P=0.3$). Reciprocally, we confirmed these data by measuring the intra-origin distances between origins present on a common DNA molecule. Consistently, the inter-origin distance dropped from 43.5 and 43.9 in wild-type and complemented cells, respectively, to 31.7 and 31.1 in SMRad51-expressing and Xrcc2-defective cells,

respectively (Table 2). Taken together, these data show an increase in the frequency of active replication forks in HR-defective cells. Alternatively, it is also possible that the data reflect an increase in the degree of replicon clustering in the HR-defective cells, rather than an actual compensation of the slow replication kinetics. Finally, these results do not support previous work reporting that the frequency of Y-shaped molecules (branched structures at replication forks), as measured by 2D-gel electrophoresis, is not altered in unchallenged Brca2-defective cells (Lomonosov et al., 2003). The authors concluded that defects in BRCA2 per se do not enhance origin firing or replication-fork progression. The differences in our study could result from the fact that molecular combing uses the incorporation of CldU and IdU, which might

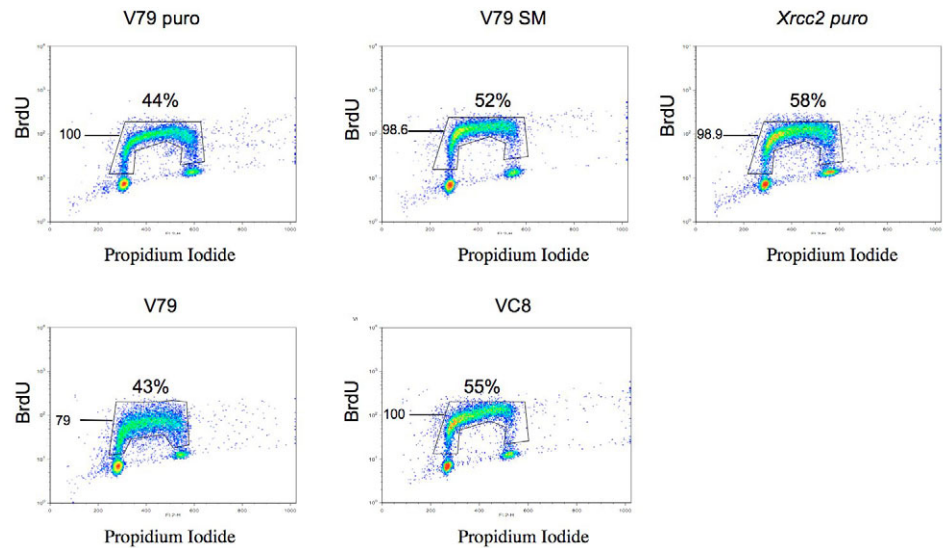


Fig. 2. FACS analysis of S-phase progression. DNA synthesis measured by BrdU incorporation. Graphs are representative of eight independent experiments. The mean BrdU fluorescence intensity and the percentage of cells in S phase are indicated for each cell line. The apparent increase in S-phase cells in HR-defective cells was not statistically significant.

constitute a replication stress that may be amplified by the HR defect, and/or that such particular metabolic conditions would be required to reveal an involvement of HR. In addition, the inconsistencies between the present data and the Y-shaped-molecule analysis could be explained by the fact that their analysis was restricted to the rDNA locus. Here, we studied three different cell lines with different HR defects by using molecular combing, a technique that allowed us to perform a genome-wide study of the replication dynamics. Importantly, the combing technique also permits a more direct measurement of the density of initiation events and of the rate of fork progression.

An increase in the density of active replication forks should compensate the lower replication elongation kinetics for the progression through S phase. We thus verified the above data by FACS analysis of control and HR-deficient cell populations that had been pulse-labeled with BrdU (Fig. 2). In eight independent experiments, both the distribution of cells within S phase and the efficiency of BrdU incorporation were not statistically affected by the HR status. Taken together, our results show that the slowing down of replication-fork progression is precisely compensated by the increase in the density of initiation events that take place in HR-defective cells.

In conclusion, it has long been known that HR is required to reactivate previously arrested replication forks (Kuzminov, 1995) and in replication of damaged DNA matrix (Henry-Mowatt et al., 2003). Our data reveal a novel role for HR in the control of replication-fork speed and in the distribution of initiation events.

Materials and Methods

Cells and DNA manipulations

Cell lines were maintained at 37°C with 5% CO₂ in MEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 200 U/ml penicillin, 200 µg/ml streptomycin. All DNA manipulations were performed as described (Ausubel et al., 1999). Cell lines derived from V79 hamster cells (wild-type cells) or from ionising-radiation-sensitive (IRS1) cells, which are *xrcc2*-defective cells (Thacker et al., 1995). IRS1 cells complemented with *XRCC2* cDNA were also used (Tambini et al., 1997). Stable transfectants expressing *SMRAD51* were derived from these cells. As controls of selection, V79 and IRS1 cells were stably transfected with the empty expression vector pCDNA-puro (Lambert and Lopez, 2000), namely V79 puro and *xrcc2*. Finally, we also used the V-C8 cell line, defective in the *BRCA2* gene and its counterpart V-C8#13 complemented with chromosome 13 (Kraakman-van der Zwet et al., 2002; Wiegant et al., 2006) and the corresponding V79 parental cell line, corresponding therefore to a second wild-type V79 source.

BrdU incorporation

Cells were incubated for 20 minutes at 37°C with 10 µM BrdU. After fixation with 70% ethanol at -20°C, cells were re-suspended in 1 ml of 0.5 mg/ml pepsin in 0.1 M HCl, and 1 ml of 2 M HCl was also added. Cells were incubated for 40 minutes at 37°C, washed and the pellets were then re-suspended in 0.1 µg/µl anti-BrdU-fluorescein antibody (Becton Dickinson). After 30 minutes, 1 ml of the propidium iodide (PI)/RNase solution was added for a further 30 minutes. Cells (2×10^6) were analyzed in a FACScalibur (Becton Dickinson). The cell cycle distribution was analyzed by Mod Fit software.

Molecular combing

Molecular combing was performed as described (Michalet et al., 1997). The IdU and CldU labeling (20-minute pulse labeling each) protocol has been described by Anglana et al. (Anglana et al., 2003). The analogs were revealed by incubating coverslips for 1 hour with mouse anti-BrdU FITC antibody (1:5; Becton Dickinson) and rat anti-BrdU antibody (1:25; Seralab). Following washing with 0.5 M NaCl, 20 mM Tris pH 7.8 and 0.05% Tween, coverslips were incubated with secondary antibodies: Alexa-Fluor-488-conjugated goat anti-mouse (1:50; Molecular Probes) and Alexa-Fluor-594-conjugated goat anti-rat (Molecular Probes). After washing, coverslips were sequentially incubated with three antibodies: mouse anti-DNA (1:100; Argene Biosoft), rabbit anti-mouse Alexa-Fluor-350 (1:50; Molecular Probes), and goat anti-rabbit Alexa-Fluor-350 (1:25; Molecular Probes). 150 fibers with symmetrical green-red labeling were analyzed for each cell line. To measure the density of replication origins, DNA molecules were uniformly stained with anti-DNA antibody as described previously (Anglana et al., 2003). We focused on molecules bearing at least one symmetrical green-red signal, then added the length of enough fibers to reach a total of 7-10 Mb. We finally counted the number of forks traveling along this DNA length. Images were obtained and processed as described previously (Anglana et al., 2003).

Statistical analysis

Distributions of replication fork velocity and origin firing in HR-defective and wild-type cells were compared by means of Mann and Withney test. The non-parametric Kruskal-Wallis test was used to compare the fluorescence intensity of BrdU between defective and wild-type cells.

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