Oncogene (2002) 21, 909–920

www.nature.com/onc

c-Myc initiates illegitimate replication of the *ribonucleotide reductase R2* gene

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The mechanisms through which the oncoprotein c-Myc initiates locus-specific gene amplification are not understood. When analysing the initiation mechanism of c-Myc-dependent amplification of the mouse *ribonucleotide reductase R2* (*R2*) gene, we observe c-Myc-dependent initiation of illegitimate DNA replication of the *R2* gene. We demonstrate multiple simultaneous c-Myc-induced *R2* replication forks, whereas *R2* normally replicates with a single fork. In contrast, *cyclin C* replicates with only a single replication fork irrespective of c-Myc deregulation. In addition to *de novo* replication forks, c-Myc also initiates bi-allelic replication of *R2*, abrogating its normal mono-allelic replication pattern. Moreover, several chromosomal regions also display c-Myc-induced illegitimate replication profiles. Thus, c-Myc can act as an illegitimate replication-licensing factor that promotes *de novo* replication initiation and illegitimate replication timing that adversely impacts upon genomic stability.
Oncogene (2002) **21,** 909–920. DOI: 10.1038/sj/onc/1205145

Keywords: c-Myc; genomic instability; amplification; DNA replication; *ribonucleotide reductase R2*

Introduction

Gene amplification has been studied in *in vitro* models, mostly using DNA synthesis-inhibiting drugs (Wahl *et al*., 1979; Brown *et al*., 1983; Johnston *et al*., 1983; Knudson, 1986; Meinkoth *et al*., 1987; Schimke, 1988; Tlsty *et al*., 1989; Smith *et al*., 1990, 1992; Hamlin *et al*., 1991). Gene amplification is thought to initiate through either replication- or segregation-driven mechanisms. The replication-driven model [also termed the 'onion-skin' model] postulates that rereplication of a gene occurs within a single cell cycle. This model allows for the formation of intrachromosomal amplification. It can also account for the formation of extrachromosomal DNA molecules that are released from their chromosomal locations by recombination. A well-known example is the amplification of chorion genes during *Drosophila* oogenesis (Spradling, 1981). The segregation-driven models include the 'deletion plus episome' and the 'chromatid exchange' models (for review see Stark, 1993). During the segregation-driven process, genes may be deleted from their chromosomal location (Morris and Thacker, 1993; Carroll *et al*., 1988) subsequently becoming extrachromosomal elements, episomes, or double minutes. Examples of this model are the ribosomal DNA (*rDNA*) amplicons in *Tetrahymena*. *rDNA* amplification in this organism involves specific breaks next to inverted repeats (Butler *et al*., 1996). In mammalian cells, the segregation-driven model includes breakage-bridge-fusion cycles that are instrumental in the generation of drug-induced amplicons (Ma *et al*., 1993; Poupon *et al*., 1996). In agreement with this model are data demonstrating that a double strand break is sufficient to initiate gene amplification (Pipiras *et al*., 1998). Thus, initiating events in drug-induced gene amplification involve chromosome breakage, deletion, inverted duplication, and terminal fusions (Wahl *et al*., 1979; Ruiz and Wahl, 1988; Hamlin and Ma, 1990; Smith *et al*., 1990, 1992; Ma *et al*., 1993; Poupon *et al*., 1996; Pipiras *et al*., 1998; Singer *et al*., 2000).

The initiating events in non-drug-dependent gene amplification are not known. Previous work has established that c-Myc deregulation initiates locus-specific gene amplification (Denis *et al*., 1991; Mai, 1994; Mai *et al*., 1996a, 1999; Kuschak *et al*., 1999), which is reversible *in vitro* (Mai *et al*., 1996a), and karyotypic instability (Mai *et al*., 1996a; Felsher and Bishop, 1999a), which is reversible *in vivo* (Felsher and Bishop, 1999b). c-Myc-dependent gene amplification is associated with the binding of c-Myc/Max heterodimers to E-boxes located on c-Myc target genes (Mai and Jalava, 1994; Wells *et al*., 1996). c-Myc/Max heterodimers in cell-free extracts from proliferating cells and *in vivo* were shown to bind two adjacent 5′-flanking E-box motifs of the *dihydrofolate reductase* (*DHFR*) gene (Mai and Jalava, 1994; Wells *et al*., 1996) and to four E-boxes located in the 5′-flanking region of *cyclin D2* (*CCND2*) (Mai *et al*., 1994). In these studies, c-Myc/Max heterodimer binding was correlated with

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Received 13 July 2001; revised 19 October 2001; accepted 31 October 2001

cellular proliferation and DNA synthesis. Regulatable c-Myc overexpression preceded enhanced binding of c-Myc/Max heterodimers to the 5′-flanking E-box motifs in both *DHFR* and *CCND2* (Mai, 1994; Mai *et al*., 1996a, 1999). Recently, the mouse *ribonucleotide reductase R2 (R2)* gene has been identified as a c-Myc amplification target gene: Transient c-Myc overexpression leads to chromosomal and extrachromosomal amplification, and rearrangements of the active *R2* gene (Kuschak *et al*., 1999).

Previous data have not addressed the initiation mechanism of c-Myc-dependent gene amplification. Here, we demonstrate that overexpression of c-Myc initiates illegitimate DNA replication and overrides

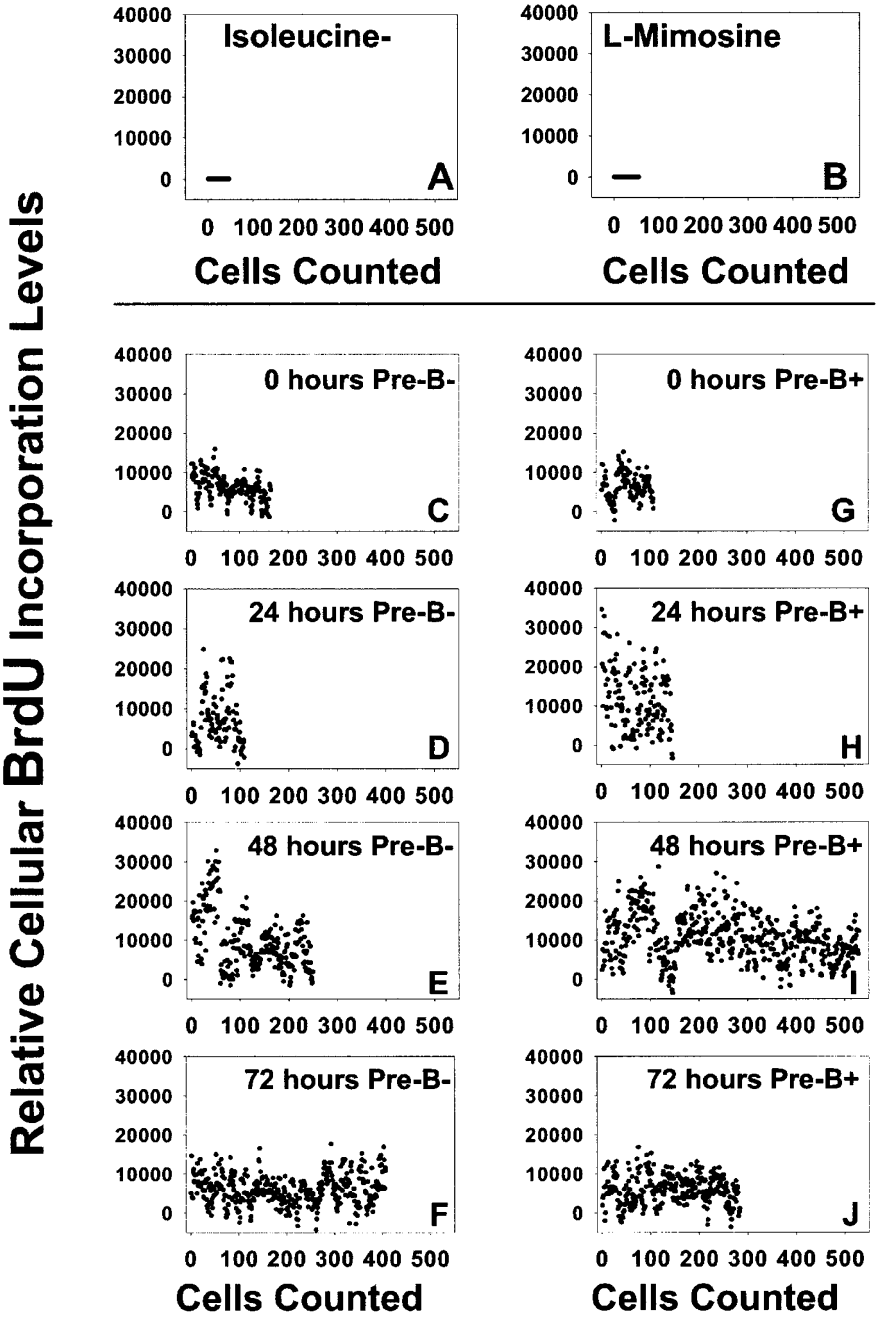


Figure 1 Bromodeoxyuridine incorporation into Pre-B− and Pre-B+ cells. BrdU was pulsed into Pre-B cells during synchronization and at 0, 24, 48, and 72 h after mock or 4-HT activation. Cells incorporated no BrdU following a 48 h incubation in isoleucine-depleted (ILE-) medium (**A**) or after 12 h incubation in medium containing 400 mM L-mimosine (**B**). After mock or 4-HT activation, low BrdU was taken up in either Pre-B− cells (**C**) or Pre-B+ cells (**G**). Significant BrdU incorporation differences were observed at 24 hours, where Pre-B+ (**H**) cells took up more BrdU than Pre-B− cells (**D**) ($P<0.0001$) and at 48 h where Pre-B+ (**I**) cells took up more BrdU than Pre-B− cells (**E**) ($P=0.0159$). No significant differences in BrdU uptake between Pre-B− and Pre-B+ cells were observed at 72 h (**F, J**)

replication timing of the *R2* gene, which precedes the previously described c-Myc-induced amplification and rearrangement of *R2* (Kuschak *et al*., 1999). In an experimental mouse Pre-B cell system (Mai *et al*., 1999; Kuschak *et al*., 1999) that harbors a 4-hydroxytamoxifen- (4-HT) regulatable Myc-ER^{TM} construct (Little-

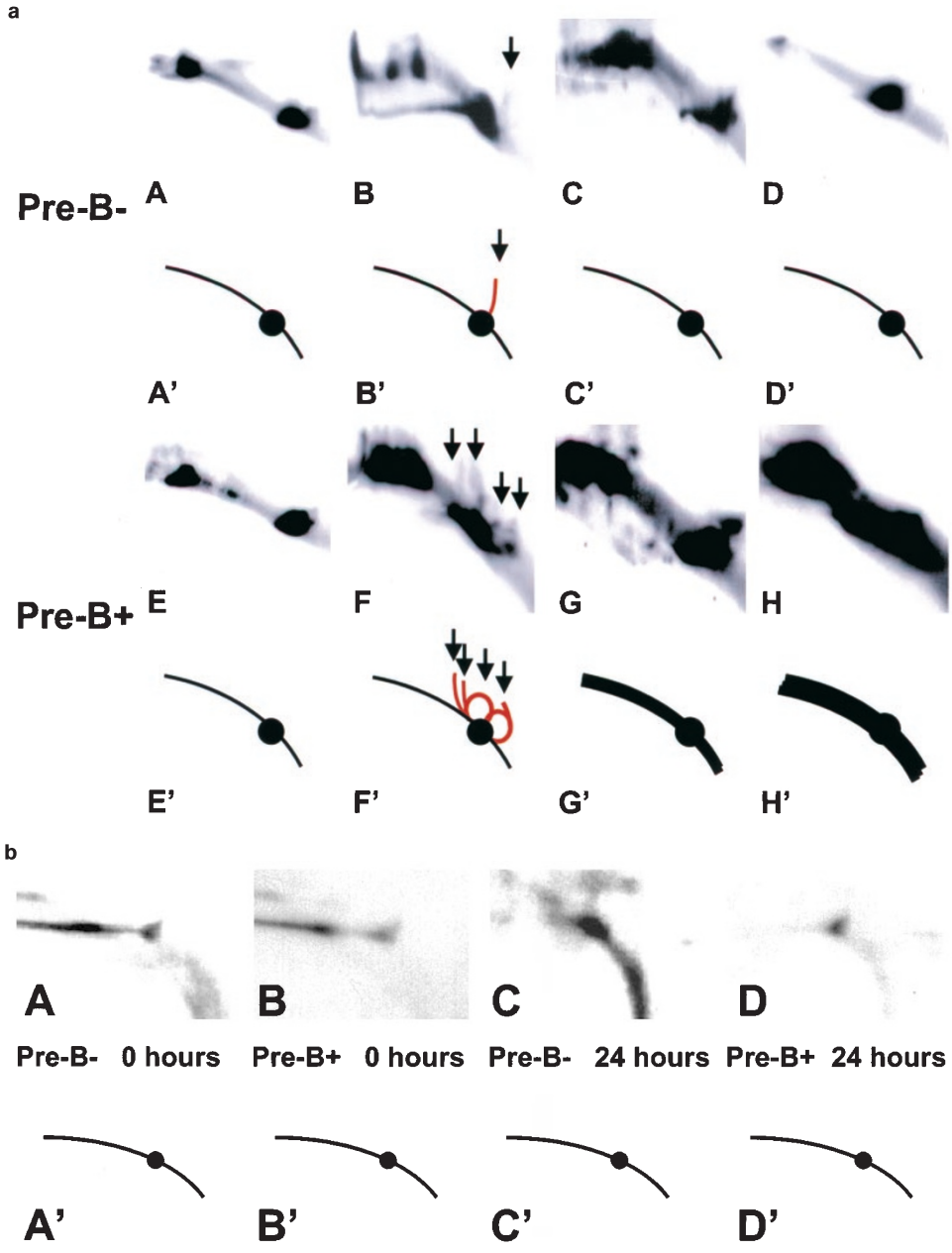
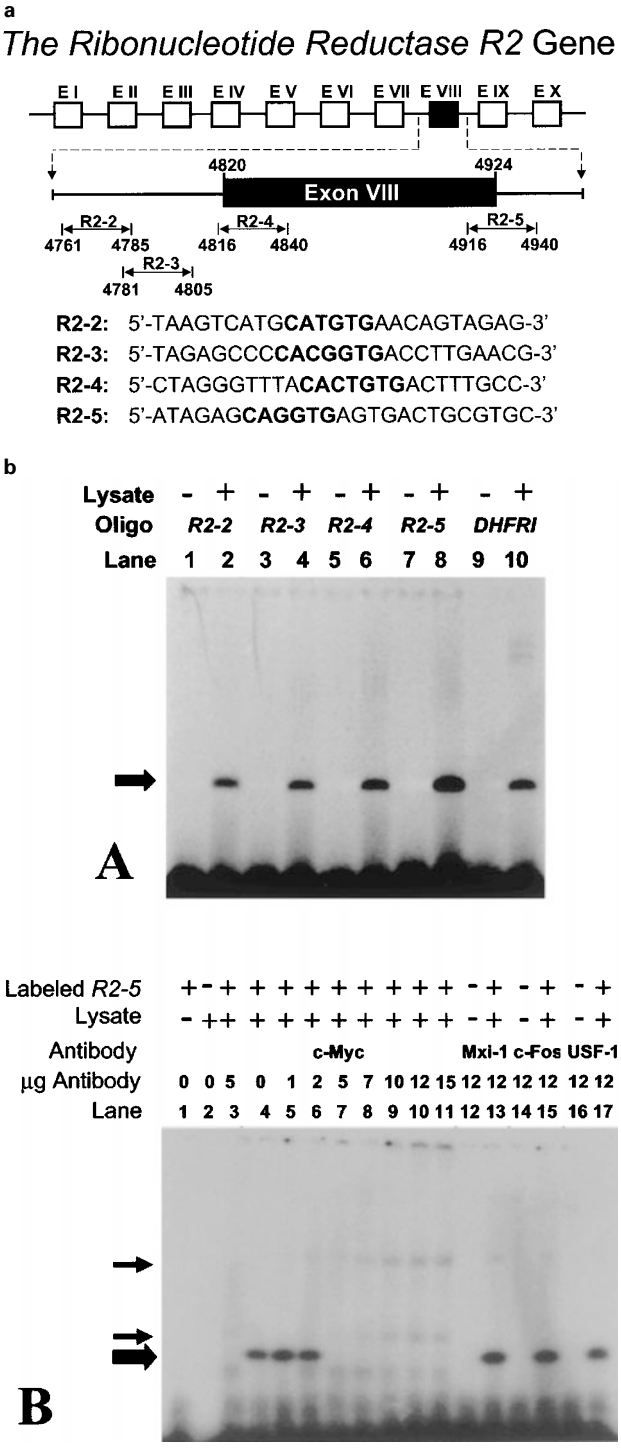


Figure 2 Illegitimate *R2* replication occurs within 24 hours of c-Myc deregulation. The 2D gel separates restricted DNA in the first dimension by differential mass, whereas in the second dimension, the DNA is separated based on mass and hydrodynamic volume. Hence, in the second dimension the shape (single, double, symmetric or asymmetric shape of forks as well as replication bubbles) will affect migration as will the mass of a strand of DNA as it undergoes replication (i.e. as the mass of DNA increases from *n* to 2*n*). (**a**) 2D gel electrophoresis (Dijkwel *et al*., 1991) and Southern analyses of non-activated (Pre-B−) (A–D) and 4-HT-activated (Pre-B+) cells (E–H) at 0, 24, 48 and 72 h after synchronizing and seeding into complete medium (Wang *et al*., 1998). The membranes were probed with a 1487 bp *Pst*I *R2* cDNA fragment (Thelander and Berg, 1986). Schematic illustrations depict the replication status of the *R2* locus at each corresponding time point (A′–H′). Replication forks in schematic are drawn in red. *R2* pseudogenes (top left:A–H) are not affected by c-Myc activation (see also Kuschak *et al*., 1999) and were not highlighted in the schematic illustrations. A single arrow indicates a replication fork emerging from the active *R2* locus in Pre-B− cells (B, B′), while four arrows point to simultaneous replication forks emerging from the active *R2* gene locus in Pre-B+ cells (F, F′) at 24 h. No replication forks are present at the 0 (A, E), 48 (C, G) or 72 h (D, H) time points. (**b**) *Cyclin C* replication is unaltered by c-Myc. 2D membranes of genomic DNA from synchronized Pre-B− and Pre-B+ cells (Wang *et al*., 1998) were probed with 400 and 500 bp *Eco*RI *cyclin C* cDNA fragments (Li *et al*., 1996) at 0 and 24 h time points (A, B and C, D, respectively). Schematic illustrations depict the replication status of *cyclin C* at the 0 and 24 h time points (A′–D′)

wood *et al*., 1995), we demonstrate the initiation of locus-specific DNA over-replication of the *R2* gene, located on band A of mouse chromosome 12 (Khan *et al*., 1994). In contrast, a control gene, *cyclin C (CCNC)*, remains unaffected by deregulated c-Myc levels, indicating that c-Myc overexpression initiates illegitimate DNA overreplication in a subset of genes. In addition, we show that c-Myc deregulation initiates the bi-allelic replication of the *R2* gene thereby abrogating the normal mono-allelic replication profile of *R2*. Furthermore, our data indicate that c-Myc-induced illegitimate DNA replication expands beyond the *R2* gene on chromosome 12. *De novo* replication profiles and timing can set the stage for genomic instability and can predispose cells to further genomic alterations and to neoplasia.



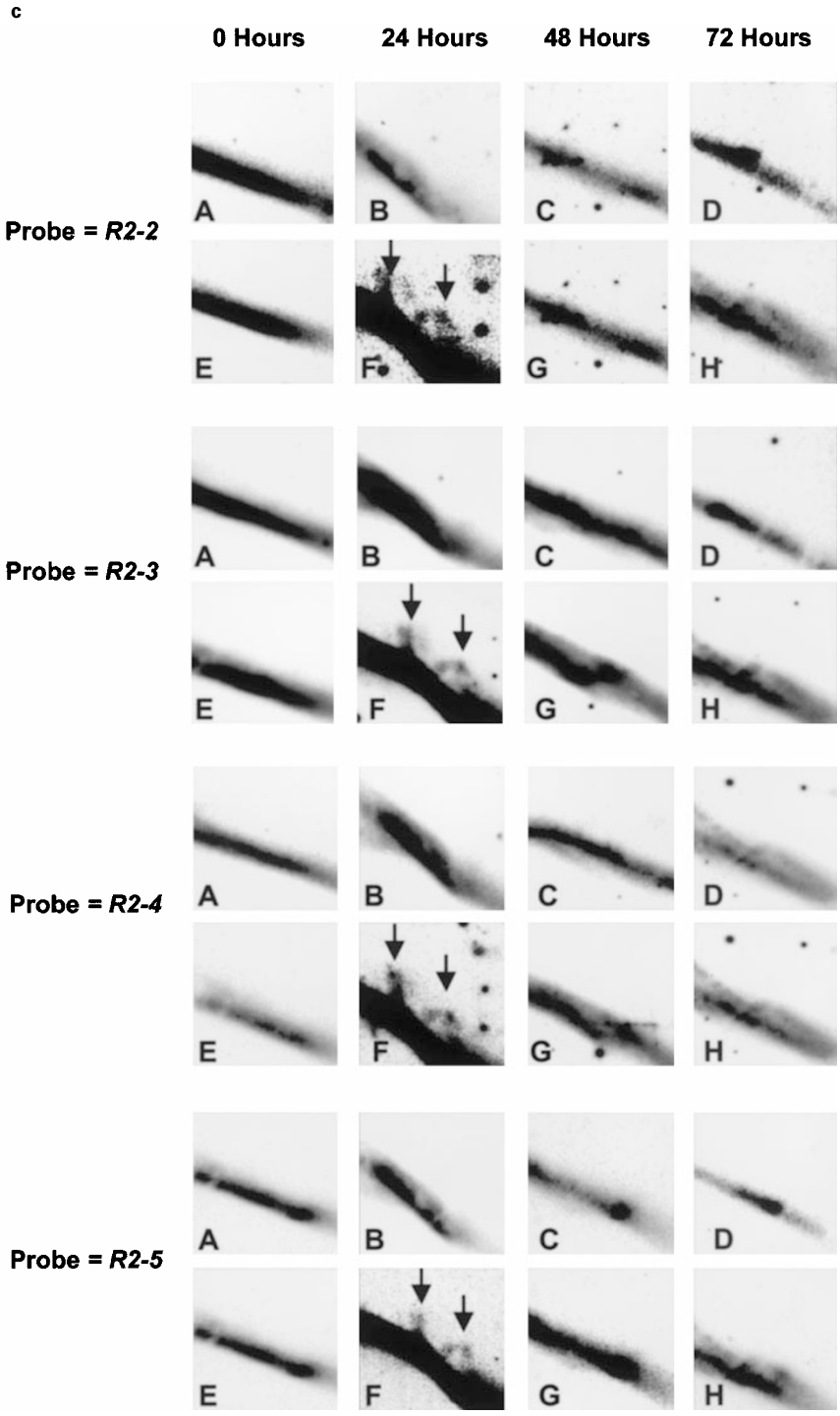


Figure 3 c-Myc interaction with *R2* E-boxes precedes initiation of *de novo* rereplication (**a**) The mouse *ribonucleotide reductase R2 (R2)* gene. This figure is a schematic representation of the mouse *R2* gene (Thelander and Berg, 1986). It indicates the E-box-containing oligonucleotides and shows their relative location within the flanking region of exon VIII of the *R2* gene. *R2–2* spans nucleotides 4761–4785, *R2–3* spans nucleotides 4781–4805, *R2–4* spans nucleotides 4816–4840, and *R2–5* spans nucleotides 4916–4940. The non-canonical E-box motifs are highlighted in bold. (**b**) c-Myc and Max interact with E-box motifs in the *R2* gene. (A) Proteins complex formation with *R2* oligonucleotides. Whole cell extracts (Mai and Jalava, 1994) from NIH3T3 cells bind each of the four $^{[32]}$P-5′-end-labeled *R2* oligonucleotides (**a**) and the $^{[32]}$P-5′-end-labeled *DHFRI* oligonucleotide (Mai and Jalava, 1994). The *R2* oligonucleotides were loaded in the absence (lanes 1, 3, 5 and 7) or presence of 5 μg of cell extract (lanes 2, 4, 6 and 8). *DHFRI* was loaded into lanes 9 and 10 in the absence (lane 9) and presence (lane 10) of extract. The arrow indicates the position of a DNA/protein complex common to the *R2* and *DHFRI* oligonucleotides (B) Anti-c-Myc antibody disrupts *R2* oligonucleotide/protein complex formation. Protein binding to *R2* E-box containing oligonucleotides (**a**) is disrupted in the presence of increasing concentrations of anti-c-Myc antibody, but unaffected by control antibodies and shown in a representative EMSA experiment using the *R2–5* oligonucleotide. Similar data were obtained with each of the other *R2* oligonucleotides (not shown). Lane 1: *R2–5* alone;

(*continued overleaf*)

Results

Our previous work described the locus-specific chromosomal and extrachromosomal amplification, and rearrangement of the mouse *ribonucleotide reductase R2* gene 72 h after transient c-Myc deregulation (Kuschak *et al*., 1999). Our current work focuses on the initiation mechanism of c-Myc-dependent *R2* gene amplification. In this study we used cultured mouse Pre-B cells that harbor a 4-hydroxytamoxifen- (4HT) responsive Myc-ER^{TM} construct (Materials and methods).

Cell cycle arrest and synchronization and Myc-ER^{TM} activation

To study the effects of c-Myc on the initiation of locus-specific DNA amplification, we analysed the initiation of *R2* gene amplification in cell cycle synchronized Myc-ER^{TM}-activated (Pre-B+) cells, while using cell cycle synchronized mock-activated (Pre-B−) cells as controls (Materials and methods). To demonstrate Myc-ER^{TM} activation and cell cycle synchronization, we carried out quantitative fluorescent immunostaining, bromodeoxyuridine (BrdU) incorporation, and determined the mitotic indices (Materials and methods). Nuclear c-Myc levels were measured during each experiment to ensure successful Myc-ER^{TM} activation. Nuclear c-Myc protein levels were found significantly higher in 4-HT-treated, c-Myc-activated Pre-B+ than in mock treated Pre-B− cells 24 h after Myc-ER^{TM} activation ($P<0.005$). We also measured BrdU incorporation to determine whether Myc-ER^{TM}-activated cells showed increased rates of DNA replication. Cell cycle synchronization was evident by the absence of BrdU incorporation during the synchronization process when cells were incubated in isoleucine-depleted (ILE-) medium (Figure 1a) or in medium containing 400 mM L-mimosine (Figure 1b). BrdU incorporation was then measured at 0, 24, 48 and 72 h after mock or 4-HT treatment. Pre-B+ cells incorporated significantly more BrdU than Pre-B− cells at 24 h ($P<0.0001$) (Figure 1D,H) and 48 h ($P=0.0159$) (Figure 1E,I) as indicated by Kruskal-Wallis tests, marking enhanced DNA replication rates in c-Myc-deregulated cells. Significant changes were not evident at 72 h (Figure 1F,I). Moreover, we examined whether c-Myc deregulated cells exhibited enhanced cellular proliferation rates. At 24 h, we observed a twofold increase in the mitotic index of Pre-B+ cells (3.4% in Pre-B+ vs 1.7% in Pre-B− cells). Together, these data indicate Myc-ER^{TM} activation, increased BrdU incorporation and cellular proliferation within 24 h of Myc-ER^{TM} activation.

c-Myc induces de novo *replication of* R2 *in Pre-B cells*

Next, we examined whether c-Myc-dependent amplification of the *R2* gene occurred through a replication-mediated mechanism using a strictly controlled experimental system. Genomic DNA from cell cycle synchronized Pre-B− and Pre-B+ cells (Wang *et al*., 1998) was analysed by neutral-neutral two-dimensional (2D) gel electrophoresis (Dijkwel *et al*., 1991) at 0, 24, 48 and 72 h. The DNA samples were digested with *Hind*III, leaving the active *R2* gene uncut (Thelander and Berg, 1986). We found a single replication fork in Pre-B− cells at the active *R2* gene 24 h after synchronization and seeding into complete medium (Figure 2a (B,B′), arrow). In contrast, in c-Myc-activated Pre-B+ cells we observed several replication forks originating simultaneously at 24 h from the active *R2* gene (Figure 2a (F,F′), arrows). We found an increase in *R2* gene copy number in Pre-B+ cells 48 and 72 h after Myc-ER^{TM} activation (Figure 2a (G,G′,H,H′) (see also Kuschak *et al*., 1999). c-Myc upregulation did not alter the replication profile of all genes. This is evident from the unaltered replication pattern observed for a control gene, *cyclin C (CCNC)* (Li *et al*., 1996) (Figure 1b), indicating that c-Myc-mediated illegitimate replication functions in a gene- and chromosomal region-specific context.

c-Myc/Max heterodimer interaction with R2 *E-boxes*

To understand the role of c-Myc in the initiation of illegitimate *R2* replication, we investigated the direct interaction of c-Myc/Max heterodimers with E-boxes in the *R2* gene. A cluster of four non-canonical (see Blackwell *et al*., 1993) E-boxes was identified in the regions flanking exon VIII of the mouse *R2* gene (Figure 3a). To examine c-Myc/Max binding at these four sites, we designed oligonucleotides and performed electromobility shift assays (EMSA), competition, supershift, and glutathione-S-transferase (GST)-fusion protein experiments. Established positive and negative binding sites were used as internal controls. The positive control oligonucleotide, *DHFRI*, was shown to bind c-Myc/Max heterodimers in EMSA and footprinting experiments (Mai and Jalava, 1994; Wells

lane 2: anti-c-Myc antibody alone; lane: 3 *R2–5* and NIH3T3 cell extract; lanes 4–11 contain in addition 0, 1, 2, 5, 7, 10, 12 or 15 μg of anti-c-Myc antibody, respectively, lanes 12–17 show control antibodies in the presence and absence of protein extracts. Lanes 12 and 13: 12 μg of anti-Mxi-1 (anti-Mad-2) antibody, lanes 14 and 15: 12 μg of anti-c-Fos antibody, lanes 16 and 17: 12 μg of anti-USF antibody. The large arrow indicates the complex seen in (**a**). The small arrows indicate supershifted complexes. (**c**) 2D-analyses with E-box-containing *R2* oligonucleotides. The *R2* E-box motifs are part of c-Myc-induced *de novo* replication profiles. The 2D membranes described in Figure 2 were re-probed with each of the four radiolabeled *R2* oligonucleotides. No replication forks were detected in the Pre-B− cells at any of the 0–72 h time points when probed with any of the oligonucleotides. In contrast, two replication forks are detected with each of the four *R2* oligonucleotides in Pre-B+ cells at the 24 h time point (arrows)

et al., 1996). The negative control oligonucleotide, *DHFRm*, is a point-mutated version of *DHFRI* that is unable to bind c-Myc/Max heterodimers (Mai and Jalava, 1994). Cell extracts from proliferating cells showed complex formation with each of the *R2* and *DHFRI* oligonucleotides (Figure 3b (A), arrow). Competition experiments confirmed these findings and showed that each *R2* E-box-containing oligonucleotide as well as *DHFRI*, but not with *DHFRm*, is able to bind the same protein complexes (data not shown). To conclusively prove c-Myc/Max binding to each of the *R2* oligonucleotides, we carried out supershift experiments using an anti-c-Myc antibody (Mai and Jalava, 1994). We observed that *R2-5* oligonucleotide/protein complexes were supershifted in the presence of increasing amounts of anti-c-Myc antibody (Figure 3b (B), lanes 4–11, small arrows). No complex disruption or supershifting was detected in the presence of control antibodies such as anti-Mxi-1 (Figure 3b, lane 13), anti-c-Fos (Figure 3b, lane 15) and anti-USF-1 (Figure 3b, lane 17). Complex disruption was observed in experiments with *R2–2*, *R2–3*, and *R2–4*, as was the inability to disrupt complexes with control antibodies (not shown). In addition, GST-Myc and GST-Max fusion proteins (Mai and Mårtensson, 1995) formed complexes with each of the *R2* oligonucleotides (not shown). Together, these data demonstrate the specific interaction of the c-Myc/Max proteins with the *R2* E-box sequences.

R2 *E-boxes are part of c-Myc-induced illegitimate* R2 *replication profiles in Pre-B cells*

To determine whether the four *R2* E-box-containing oligonucleotides were part of c-Myc-induced illegitimate *R2* replication profiles, we carried out neutral–neutral 2D gel electrophoresis. Using genomic DNA from synchronized Pre-B− and Pre-B+ cells, we examined the hybridization signals of each of the four *R2* E-box-containing oligonucleotides (Figure 3c). In Pre-B+ cells, at the 24 h time point each *R2* oligonucleotides hybridized to two replication forks simultaneously (Figure 3c, arrows). In contrast, in Pre-B− cells, probing with the *R2* E-box-containing oligonucleotides detected no replication forks at any of the 0, 24, 48 or 72 h time points (Figure 3c). Together these data indicate that each of the *R2* E-boxes is part of the c-Myc-dependent illegitimate *de novo R2* replication profiles in Pre-B+ cells.

c-Myc-induced de novo *replication profiles on mouse chromosome 12*

We surmized that *de novo* replication of *R2* would be detectable at the chromosomal level, specifically on band A of mouse chromosome 12, where the mouse *R2* gene is located (Khan *et al*., 1994). Thus, we assessed directly whether the presence of c-Myc-dependent *de novo* replication forks was paralleled by an increase in BrdU incorporation into the *R2* locus on mouse chromosome 12 (band A) during the initiation of *R2* DNA replication. Using a pulse of BrdU incorporation into Pre-B− and Pre-B+ cells *in vivo*, we examined a potential increase either in relative signal intensity of BrdU at chromosome 12 band A, and/or an increase in the length of the BrdU signal in Pre-B+ chromosomes 12 (Materials and methods).

Pre-B− cells showed a single peak of BrdU incorporation into one *R2* allele at the 24 h time point (Figure 4a (B,E)). In contrast, Pre-B+ chromosomes 12 showed increased BrdU signal intensity ratios in one or both bands A of chromosome 12 ($P=0.0480$) (Figure 4a (D,F) and b). Furthermore, the Pre-B+ metaphases displayed increased length of BrdU incorporation signals in one or both bands A of one or both chromosomes 12 ($\log_{10}$ ratio $P=0.0133$) (Figure 4a (D,F) and b). This suggests that under normal, non-c-Myc activating conditions, the *R2* gene replicates its two alleles at different times, i.e., one allele replicates before the other. Asynchronous replication timing patterns for *Igf2, Igf2r, H19* and *Snrpn* in the mouse was described by Kitsberg *et al*. (1993). Later, Simon *et al*. (1999) showed that asynchronous replication of imprinted genes is established in the gametes and maintained during the course of development. In contrast, c-Myc-upregulated Pre-B+ cells can replicate their *R2* loci simultaneously in a bi-allelic fashion, indicating that c-Myc overexpression can result in disruption of allelic replication timing (Figure 4c (C,E,F)). In addition, it is worth noting that many other chromosomes in Pre-B+ metaphases displayed c-Myc-dependent alterations in their BrdU incorporation profiles when compared to their Pre-B− controls (Figure 4a (A–D)), suggesting that c-Myc can initiate additional illegitimate replication profiles. Thus, c-Myc upregulation results in the overreplication of *R2* and in alterations in replication timing of the *R2* alleles. Moreover, the inappropriate expression of c-Myc protein enhances replication beyond the chromosomal region that carries *R2* and can also affect additional chromosomal sites.

Discussion

Earlier work had implicated c-Myc in the enhanced replication of *Simian Virus 40 (SV 40)* in human lymphoma cells: Elevated levels of c-Myc protein allowed for over-replication of the virus (Classon *et al*., 1987; Henriksson *et al*., 1988). More recently, it was shown that c-Myc overexpression can lead to DNA endoreduplication and polyploidy (Li and Dang, 1999; Gandarillas *et al*., 2000). Li and Dang (1999) examined the role of c-Myc in the replication process in human and rodent cells. They observed that c-Myc was able to promote endoreduplication in the presence of a mitotic block. Myc and endoreduplication were reported by Gandarillas *et al*. (2000), who showed that the upregulation of c-Myc protein levels led to endoreduplication in keratinocytes prior to their terminal differentiation.

Our current work also points to a function of c-Myc in replication. In contrast to previous data that described c-Myc-induced overall endoreduplication, we find non-random illegitimate replication events that are initiated by c-Myc deregulation. Using a regulatable Myc-ER^{TM} system (Littlewood *et al*., 1995) that was stably introduced into in a Pre-B lymphocyte line (Mai *et al*., 1999; Kuschak *et al*., 1999), we demonstrate that c-Myc initiates locus- and chromosomal region-specific illegitimate replication events that affect the timing and extent of DNA replication (Figure 1–4). The locus-specific example studied here is *R2*, where we observe *de novo* re-replication profiles and altered timing following c-Myc deregulation. In contrast, *cyclin C* was unaltered under identical experimental conditions. We studied mouse chromosome 12 (band A), with enhanced DNA replication following c-Myc deregulation, as an example of c-Myc-dependent chromosomal region-specific changes in replication. In addition, we noted that many other chromosomes showed region-specific alterations in their replication profiles.

Based on our present data describing c-Myc-dependent illegitimate replication initiation, we propose that c-Myc protein overexpression illegitimately licenses the use of multiple replication initiation sites of *R2* that are silent under normal, non-c-Myc-deregulated conditions. Simultaneously, c-Myc enhances replication of a chromosomal region that expands beyond the *R2* locus on mouse chromosome 12 and induces enhanced replication of additional regions on other chromosomes. Such illegitimate replication affects the stability of *R2* and presumably other genes in this and additional chromosomal regions, setting the stage for both intrachromosomal and extrachromosomal amplification that involves *R2* (Kuschak *et al*., 1999) and other genes (Mai, 1994; Mai *et al*., 1996a, 1999). Global changes in replication

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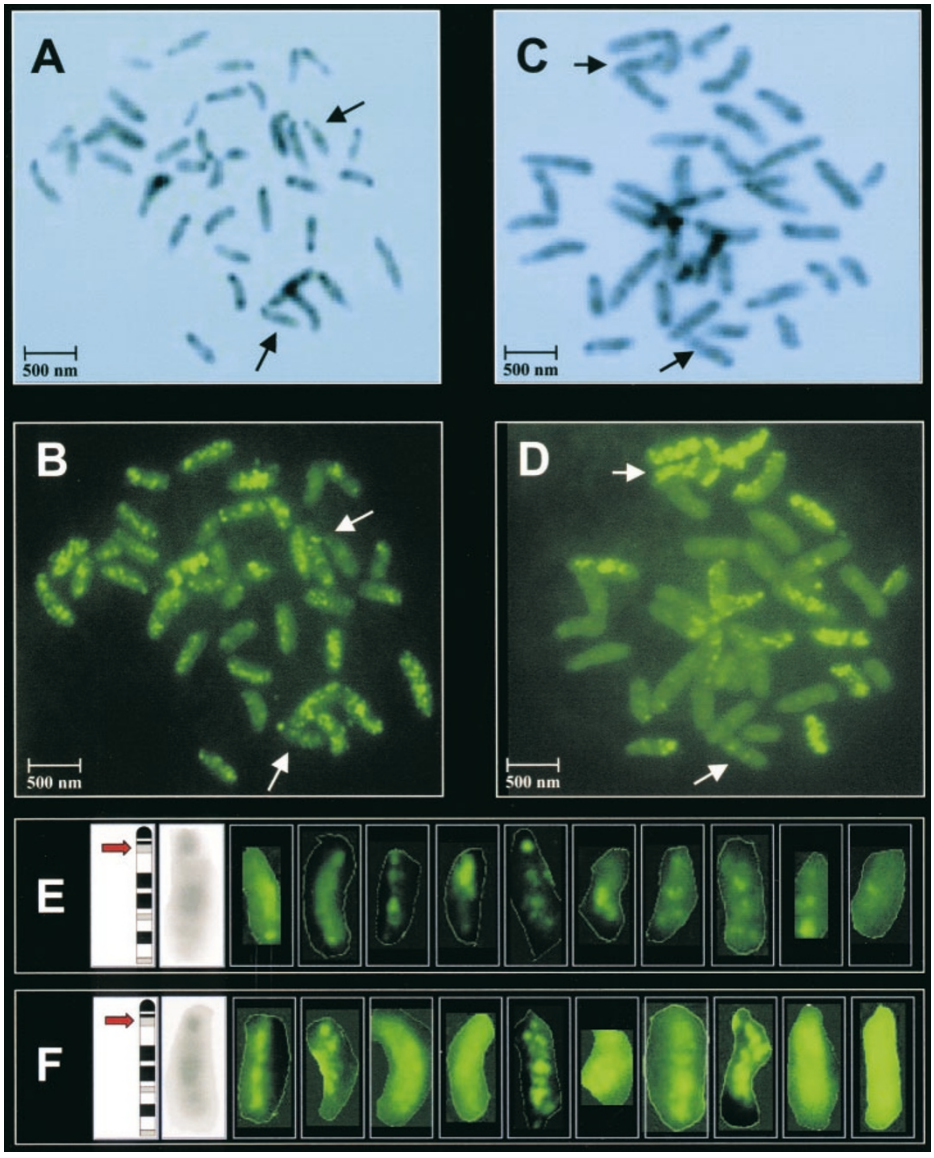

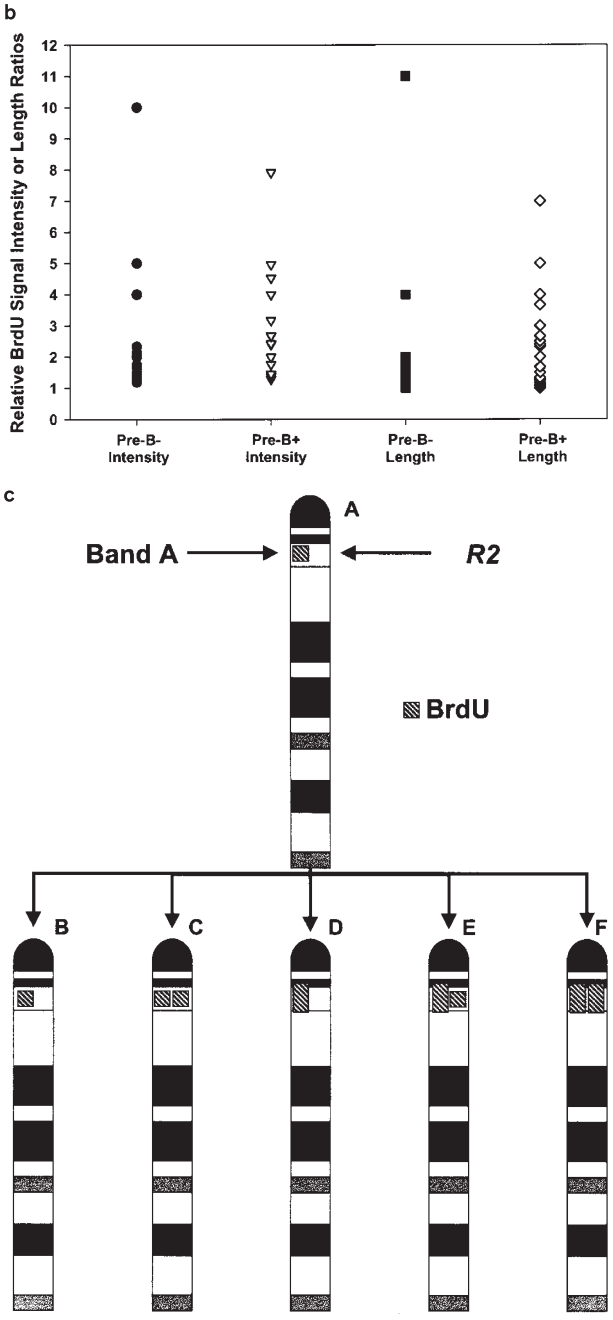

Figure 4 c-Myc induces enhanced BrdU incorporation into mouse chromosome 12 band A. (**a**) Pre-B+ cells show enhanced BrdU uptake in band A of chromosome 12 where *R2* is located. Arrows point to mouse chromosomes 12 in the respective metaphases (A–D). (A) and (B) show representative inverted DAPI and BrdU-stained metaphases from Pre-B− cells. (C) and (D) show representative inverted DAPI and BrdU-stained metaphases from Pre-B+ cells. (B) and (D) show Pre-B− and Pre-B+ metaphase chromosomes after BrdU pulse-labeling, illustrating an enhanced uptake of BrdU into band A of chromosome 12 in Pre-B+ cells (D). (E) and (F) show 10 representative Pre-B− and Pre-B+ chromosomes 12, respectively. Pre-B+ chromosomes 12 display elevated BrdU incorporation into both chromatids of chromosomes 12 (D, F), while Pre-B− cells show incorporation of BrdU into band A of one chromosome 12 chromatid (B, E). In (E) and (F), the red arrows show band A, where mouse *R2* is localized. A schematic overview of BrdU-profiles of chromosome 12 band A is given in (**c**). (**b**) c-Myc deregulation overrides allelic replication control. Summary of BrdU-incorporation data in Pre-B− and Pre-B+ chromosomes 12 band A. The data represent the relative BrdU incorporation intensities as well as the relative BrdU incorporation signal lengths. Relative BrdU intensity ratios, as measured for chromosome 12 (band A) of 34 individual chromatids, are shown for Pre-B− (black circles) and Pre-B+ cells (open triangles). Each symbol represents the ratio of signal intensity of the replicationally active allele intensity over the intensity of the inactive allele of chromosome 12. The signal intensities differ significantly between the chromosomes 12 of Pre-B− and Pre-B+ cells ($P=0.048$). Relative length ratios of BrdU-incorporation, as measured for 74 individual chromatids 12 (band A), are shown for Pre-B− (black squares) and Pre-B+ cells (open diamonds). Each symbol represents the ratio of signal length of the replicationally active allele signal length over the signal length of the inactive allele of chromosome 12. The BrdU signal length differences between Pre-B− and

(*continued overleaf*)

initiation and timing will contribute to the dynamics of karyotypic instability (Mai *et al*., 1996b; Felsher and Bishop, 1999a,b) via DNA breakage, deletions, chromosomal rearrangements, and fusions. It is evident that replication-driven mechanisms act early during c-Myc-dependent initiation of *R2* gene amplification. However, it is also likely that resulting DNA breakage at the *R2* locus and at other chromosomal sites, in addition to fusions, deletions and recombination events, will further add to the dynamics of genomic instability, setting the stage for early neoplasia. In conclusion, we propose that c-Myc can act as an illegitimate replication-promoting or replication licensing factor and initiate genomic instability of *R2* through replication-driven mechanism.

Materials and methods

Cell culture

The studies were performed using cultured mouse Pre-B cells that harbor a 4-hydroxytamoxifen- (4HT) responsive Myc-ER^{TM} construct (Littlewood *et al*., 1995). The culture and activation of these cells is described (Kuschak *et al*., 1999).

EMSA

Electromobility shift assays (EMSA) were performed as described (Mai and Jalava, 1994). Briefly, DNA/protein interactions of four oligonucleotide sequences from the *R2* gene were tested (Figure 3a). The sequences of the oligonucleotides tested were as follows: R2-2: 5′-AAGTCATGCATGTGAACAGTAGAG-3′; R2-3: 5′-GAGCCCCACGGTGACCTTGAACG-3′; R2-4: 5′-TAGGGTTTACACTGTGACTTTGCC-3′; R2-5: 5′-ATAGAGCAGGTGAGTGACTGCGTGC-3′. The E-box motifs are underlined. Positive and negative control oligonucleotides *DHFRI* and *DHFRm*, respectively, are described in (Mai and Jalava, 1994). The antibodies used were as follows: pan-Myc Clone M-911-94 antibody (Genosys). Anti-c-Fos (Clone (4)-G), anti-USF-1 (Clone 20), and anti-Mxi-1 (Clone 17) were from Santa Cruz Biotechnology, Inc (Santa Cruz, USA). EMSA assays were also conducted using GST-Myc and GST-Max fusion proteins, as described (Mai and Mårtensson, 1995).

Neutral–neutral 2 dimensional gel electrophoresis

Neutral–neutral 2-dimensional (2D) gel electrophoresis was performed as follows: Genomic DNA was isolated from synchronized (Wang *et al*., 1998), non-activated and 4-HT-activated cells by standard procedures. One hundred μg of each DNA sample was digested overnight at 37°C with *Hin*dIII (Roche Diagnostics, Laval, Québec, Canada) and roto-evaporated to a 40 μL volume. The samples were electrophoresed in two dimensions under neutral–neutral conditions (Dijkwel *et al*., 1991). The 2D gels were washed and blotted (Kuschak *et al*., 1999). The following probes were used: A 1487 base pairs (bp) *Pst*I *R2* cDNA (Thelander and Berg, 1986) fragment, *R2* oligonucleotide 20-mers corresponding to regions of the *R2* gene containing E-box motifs, and two *Eco*RI *cyclin C* (*CCNC*) fragments (400 and 500 bp) (Li *et al*., 1996). These were labeled by random priming (Kuschak *et al*., 1999). Southern blots probed with the *R2* and *CCNC* cDNAs were hybridized as described (Kuschak *et al*., 1999). Oligonucleotide-probed membranes were hybridized as described (Mai *et al*., 1996a) and washed at 42°C.

Bromodeoxyuridine incorporation assays

All bromodeoxyuridine (BrdU) incorporation assays were conducted in synchronized (Wang *et al*., 1998) Pre-B− and Pre-B+ cells (Mai *et al*., 1999; Kuschak *et al*., 1999). BrdU incorporation and DNA replication was assessed during synchronization and at 0, 24, 48 and 72 h after seeding into complete medium and following mock-treatment (95% ethanol) or c-Myc activation (4-HT treatment). Cells were incubated in 10 μM BrdU (Sigma-Aldrich Chemical Company, Winston, ON, Canada) for 15 min. BrdU incorporation into replicating DNA was assayed by quantitative fluorescent immunostaining using a mouse monoclonal anti-BrdU antibody (Becton Dickinson, San Jose, USA). Over 100 cells were counted in each experiment. Relative BrdU intensity levels for each set of cells were tested for significance using a Kruskal-Wallis non-parametric test. Chromosome 12-specific BrdU uptake was measured in metaphase spreads of synchronized Pre-B− and Pre-B+ cells following a 30 min 10 μM BrdU pulse at 24 h. Chromosomes were prepared as described (Mai, 1994) following a 24 h incubation in fresh medium. BrdU was visualized as described (Leonhardt *et al*., 1992), with modifications. Metaphase chromosomes from BrdU-incubated cells were banded as described (Wiener and Mai, 2000), visualized (Zeiss Axiophot microscope, Carl Zeiss Canada Ltd.), and images were acquired (Photometrics CH250/a CCD camera). The chromosomes were subsequently stained for BrdU incorporation and visualized using fluorescein isothiocyanate- (FITC) conjugated anti-BrdU (Becton Dickinson), followed by signal amplification with rabbit anti-FITC (Cambio, Cambridge, UK) (25 ng μL^{-1}) and FITC-conjugated goat anti-rabbit IgG (H and L) (Pierce, Rockford, USA) (25 ng μL^{1}). DAPI and BrdU signal intensities and lengths along chromosomes 12 were measured (IPLab software, Scanalytics, Fairfax, VA, USA). Statistical significance of BrdU signal intensity and signal length differences between Pre-B− and Pre-B+ chromosomes 12 were compared using a Kruskal-Wallis non-parametric test. The Kruskal-Wallis non-parametric analysis

Pre-B+ chromosomes 12 (band A) are significant ($P=0.0725$). Also, the $\log_{10}$ of the difference ratios of the lengths between the Pre-B− and Pre-B+ chromosome 12 chromatids are significant ($P=0.0133$). (**c**) Scheme of c-Myc-dependent illegitimate *R2* replication profiles as determined by BrdU uptake into band A of chromosome 12. The scheme summarizes c-Myc-dependent alterations in BrdU uptake into band A of mouse chromosome 12 where the *R2* gene (arrow) is located. (A) represents BrdU incorporation found in Pre-B− chromosome 12. (B–F) represent a spectrum of possibilities for BrdU incorporation that can be found in Pre-B+ chromosomes 12. (B) indicates the deregulation of c-Myc may have no effect on the allelic uptake of BrdU. (C) shows that c-Myc deregulation can result in equal uptake of BrdU into both alleles. (D) depicts the enhanced length of BrdU incorporation into the single active allele. (E) illustrates the enhanced length of BrdU uptake into both alleles, one showing higher incorporation. (F) shows the enhanced BrdU signal length into both chromosome 12 alleles. (C–F) were found in Pre-B+ cells

of variance is used to assess the significance among multiple independent groups of data that have a non-Gaussian and highly variable distribution (Statsoft Electronic Statistics Textbook Search Page/Non-parametric Statistics).

Mitotic indices

Mitotic indices of synchronized (Wang *et al*., 1998) Pre-B− and Pre-B+ cells were assessed 24 h after mock or 4-HT activation. Cells were cytospun onto microscope slides, stained with 4′6′ diamidino-2-phenylindole (DAPI) (1 μg mL^{1}), analysed on a Carl Zeiss Axioplan 2 (Carl Zeiss Canada, Ltd.) microscope (at 40 × magnification), and images were captured on a Sony XC75 CCD camera. Mitotic cells were counted from among a total of 800 cells in each group.

Acknowledgments
The authors thank Mrs Mary Cheang (Biostatistical Consulting Unit, Department of Community Health Services, Faculty of Medicine, University of Manitoba) for statistical analysis. We thank our colleagues, Drs A Begleiter, GG Hicks, T Fest and JF Mushinski, for critical reading of this manuscript. TI Kuschak was supported by a GH Sellers Graduate studentship awarded through CancerCare Manitoba. S Mai and JA Wright were funded through the National Science and Engineering Research Council of Canada (S Mai NSERC grant # 311-3135-03; JA Wright NSERC grant # 311-1735-05), and the Medical Research Council (JA Wright MRC grant # 321-3119-70, S Mai CIHR grant # 321-3135-19).

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