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Immortalization of Human WI38 Cells is Associated With Differential Activation of the c-myc Origins

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Abstract To study the possible relationships between origin activities and cellular processes leading to malignancy, we used an isogenic system of human embryo lung fibroblast cells WI38 and a SV40-transformed variant, WI38 VA13 2RA (WI38(SV40)). We found that the activities of all initiation sites at the c-myc locus were approximately two-fold as high in WI38(SV40) cells as in WI38 cells. Thus, higher initiation frequency of origins at certain loci is induced with cell immortalization, one of the steps in the multi-step process leading to malignancy. We measured the activities of the four c-myc promoters P0, P1, P2, and P3 with nuclear runon assay in the two cell lines in order to detect potential individual promoter changes that may be also associated with immortalization by SV40 virus. The results show that the activities of the promoters P0, P1, and P3 did not significantly change, but the activity of the major promoter P2 in WI38(SV40) cells was about 7.5- to 8.0-fold as high as that in WI38 cells. The increased activity of promoter P2, although ~600 bp downstream of one of the major DNA replication initiation sites, had no preferential influence on the major sites of origin activity. Since the distribution of nascent strand abundance was not significantly altered, binding of transcription factors does not seem to facilitate the assembly of pre-replication complex (pre-RC) or otherwise preferentially alter the activities of the DNA replication proteins at this major initiation site. J. Cell. Biochem. 82: 522–534, 2001.

Key words: replication origin; initiation; transcription promoter; *c-myc*; immortalization

Regulation of initiation of DNA replication is a critical event in cell proliferation. Initiation of DNA replication in higher eukaryotic cells is controlled by a number of regulators. The control can be at multiple levels through DNA sequences at or near origins, initiation proteins or other regulatory proteins, chromatin structure and nuclear organization [Zannis-Hadjopoulos and Price, 1998, 1999; DePamphilis, 1999]. These regulatory parameters of initiation of DNA replication are dramatically changed during the development of early embryonic cells. For example, in early embryonic *Drosophila* cells, linker histone H1 is absent from the chromatin and is first detected in cycle 7. H1 level dramatically increases after stage 7 (cycle 14), at which repression of replication origin activity is first detected [Ner and Travers, 1994; Sasaki et al., 1999]. In transformed cells, H1 level is lower than that in normal cells [Laitinen et al., 1995] and H1 is more phosphorylated in G1 phase by comparison to that in normal cells [Chadee et al., 1995]. Cell transformation and tumor progression have been suggested to be a return to the early stages of embryonic development. Therefore, comparison of initiation of DNA replication in transformed cells vs. normal somatic cells may provide an opportunity to better understand the regulatory mechanisms of initiation of DNA replication. Information on the features of DNA replication compared in immortal to normal cells is likely to suggest approaches to curb the growth of transformed cells.

In our previous studies, we measured the relative nascent DNA abundance at the c-myc locus in HeLa and normal skin fibroblast (NSF) cells, and thereby, determined the activities of all the DNA replication origins present at this locus. The results showed that the activities of all DNA replication origins at the c-myc locus were about two-fold as high in HeLa cells as in NSF cells and suggest that cell transformation may induce greater frequency of initiation of

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origins in certain loci [Tao et al., 1997, 2000]. However, the differential origin activities in HeLa and NSF cells might also be due to their being different cell types. To more closely investigate the possible relationships between origin activities and multiple steps leading to malignancy, in this study, we have used an isogenic system of human embryo lung fibroblast cells WI38, and their SV40-transformed variant, WI38 VA13 2RA (WI38(SV40)), thus eliminating the possible cell type effect on origin activities. Here again, we found increased activities of all origins at the c-myc locus in WI38 cells transformed by SV40 by comparison to their normal counterparts. Since WI38(SV40) cells are immortalized, but not malignant [Levenbook et al., 1985], we conclude that the higher origin activities are associated with cell immortalization, one of multi-steps leading to malignant transformation.

Cell immortalization allows cells indefinite life span, in which their telometric DNA sequences are maintained. Telomere maintenance is principally mediated by telomerase, which is absent in almost all primary somatic cells [Cerni, 2000]. However, telomerase activity alone is not sufficient for immortalization of human keratinocytes or mammary epithelial cells, for which inactivation of $p16^{INK4a}$/Rb pathway is also required [Kiyono et al., 1998]. Therefore, cell immortalization itself may be at least a two- or multi-step process. One step is inactivation of the Rb pathway. Later, the clones of premaliganant cells, that have begun to exhaust their endowment of telomeres, will activate their telomerase [Weinberg, 1998]. Recent studies showed that c-myc oncoprotein induces telomerase in both normal human mammary epithelial cells and normal human diploid fibroblasts [Wang et al., 1998], the C-MYC directly activates telomerase reverse transcriptase (TERT) promoter [Wu et al., 1999]. Overexpression of the C-MYC can also be achieved by the presence of the SV40 large T antigen [Luo et al., 1997; Marcu et al., 1997]. Therefore, it was particularly interesting to investigate the activities of transcription promoters and activities of replication origins at the c-myc locus in WI38 cells with and without SV40 transformation. It was possible that some additional information could be gained about the relationship between initiation of replication and transcription. In this study, we tested the activities of the four promoters (P0, P1, P2, and P3) of the c-myc gene in WI38 and WI38(SV40) to examine whether changes in the activities of individual promoters can be associated with the activities at specific initiation sites at the c-myc locus upon cell transformation. The results show that although 7.8 to 8.0-fold higher activity of the major promoter, P2, did occur, no preferential changes in activity of origins was observed at this locus in WI38 (SV40) cells.

MATERIALS AND METHODS

Cell Cultures

Human lung embryo fibroblast cells WI38 (ATCC CCL 75) and WI38 transformed with SV40 virus (WI38 VA13 2RA; ATCC CCL 75.1) were grown in 5% CO_2 in T175 or T75 cm^2 tissue culture flasks and alpha-medium (Gibco) $\pm$10% (v/v) fetal calf serum (Gibco). When the cells reached 30–50% confluence, they were used to isolate nuclei, total cellular RNA, or nascent DNA. For isolation of total genomic DNA, WI38 cells were grown to confluence, and the cells were incubated in alpha-medium without serum for another 48 h in order to obtain a G0 population of cells, confirmed by fluorescence flow cytometry [Zannis-Hadjopoulos et al., 1988].

Western Blot of Large T Antigen Expression

Cells at 30–50% confluence in T75 cm^2 cell culture flasks were washed twice in ice-cold PBS, harvested and the cell pellets were stored at -70°C. The cell pellet obtained from one T75 cm^2 flask was resuspended in 100 μl 0.1 M Tris (pH 8.0), frozen on dry ice for a few minutes, thawed at 37°C and vortexed briefly. Freeze and thaw cycles were repeated twice after which the nuclei were pelleted by centrifugation in a microfuge at 14,000 rpm at 4°C for 5 min. The nuclear pellet was resuspended in 15 μl of 0.1 M Tris (pH 8.0) and 15 μl of 2X protein SDS–PAGE loading buffer (100 mM Tris Cl (pH 6.8)-200 mM dithiothreitol-4% SDS-0.2 % bromophenol blue-20% glycerol) was added. The sample was boiled for 10 min, cooled on ice, and spun down briefly. 1, 5, and 10 μl of the samples were loaded on 10% SDS–PAGE gel, and after electrophoresis the proteins were transferred onto Immobilon-P membrane (MILLIPORE). SDS–PAGE and protein immobilization were performed as described by [Sambrook et al., 1989]. The

membrane was soaked in PBS-0.5% Tween 20-5% milk (Instant Skim Milk Powder, Carnation, Nestlé, Ontario, Canada) at 4°C for 4 h or overnight, and then in PBS-0.1% Tween 20 at room temperature (RT) for 10 min. The membrane was incubated with a 1/666 dilution of mouse monoclonal antibody against SV40 T Ag (sc-148, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS-0.1% Tween 20-1% milk at RT for 45 min. The membrane was washed in PBS-0.1% Tween 20 at RT for 10 min, then in PBS-0.1% Tween 20-1% milk at RT for 10 min, then incubated with 1/3,000 dilution of anti-mouse IgG-HRP (Santa Cruz Biotechnology) in PBS-0.1% Tween 20-1% milk at RT for 35 min, and finally washed twice in PBS-0.1% Tween 20 at RT for 5 min. The chemiluminescence reaction was performed with Western Blot Chemiluminescence Reagent (RENAISSANCE®, NEN Life Science), and developed on REFLECTION Autoradiography Film (NEN Life Science). After the Western blotting analysis, the membrane was stained in commassie brilliant blue R250 staining solution.

Determination of c-myc Transcript Levels by RT-PCR

Each of the total cellular RNA samples were prepared from cells in one 30–50% confluent T75 cm^2 flask with TRIzol reagent. The RNA samples were quantified by spectrophotometer. Approximately 200 ng of each of the RNA samples from WI38 and WI38 (SV40) cells was added into 10 μl of RNA buffer (1× MOPS, 4.5% fomaldehyde, and 50% formamide), subjected to electrophoresis on an 1.5% agarose gel and stained with ethidium bromide to check whether the RNA samples were degraded (data not shown). Approximately 1 ng of RNA prepared from WI38 and WI38(SV40) was amplified by RT-PCR (GeneAmp® Thermostable rTth Reverse Transcriptase RNA PCR Kit, PERKIN ELMER). Primers: 2F and 2R were used for amplifying the c-myc (GenBank Accession No. J00120 and Locus: HUMMYCC) transcripts (see their locations on Fig. 5A and Table I); primers 1F and 1R were used for the human β-actin gene (LOCUS HUMACCYBB, Accession No. M10277) transcripts; Primers 9f and 9r2 are located in a c-myc origin region (see Fig. 5A and Table I), which is also a non-transcribed region. Fragment 9c is a 424 bp double-stranded DNA fragment, a competitor which can be amplified with primers 9f and 9r2 (see Fig. 5B).

TABLE I. Primers (5′- > 3′) Used for RT-PCR

Primer	Sequence	Position
2F	AGGGAGATCCGGAGCGAATA	2591–2610
2R	AGCAGCTGCAAGGAGAGCCT	2826–2845
1F	TCCTGTGGCATCCACGAAACT	2571–2591
1R	GAAGCATTTGCGGTGGACGAT	2977–2997
9f	TTGTGAGTCAGTGAACTAGG	947–966
9r2	CCACACGGAGTTCCCAATT	1398–1416

Primer set 2F-2R was used for measurements of relative stable transcript level of the c-myc gene in WI38 and WI38(SV40) cells. The position numbers of primer sets 2F-2R and 9f-9r2 are from GenBank Locus HUMMYCC (Accession No. J00120). Primer set 1F-1R was used for determination of relative stable transcript levels of the β-actin gene in WI38 and WI38(SV40) cells. The position numbers of primer set 1F-1R are from GenBank Locus HUMACCYBB (Accession No. M10277).

The quantitative RT-PCR conditions were the same as described above, but after reverse transcription reactions, a series of amounts of the 299 bp DNA competitors were added into the RT-PCR reaction tubes. Importantly, the RNA samples from WI38 and WI38(SV40) were tested by RT-PCR at the same time and under the same conditions. The double-stranded DNA competitors for 2F-2R and 1F-1R were prepared as described in our previous work [Tao et al., 1997]. The RT-PCR products were resolved by electrophoresis on 2% agarose gel, stained with ethidium bromide, and recorded on film. The bands on the film were scanned and quantified with BioImage (Millipore) hardware and software. The ratios of amplified competitors and targets are plotted against added competitors; the reverse transcribed cDNA is equal to the added competitors when the ratio is 1. The actin RNA transcripts from WI38 and WI38(SV40) cells were also tested (data not shown), and then the c-myc transcription levels were normalized against the actin transcript levels.

Determination of the c-myc Promoter Activities by Nuclear Runon Assay

The nuclear runon assay was performed essentially as described by [Schubeler et al., 1996]. In this study, 5×10^6 cells at 30–50% confluence were washed in 10 ml of ice-cold PBS, and detached using 5 ml of trypsin solution. The cells were centrifuged at 200*g* for 5 min at RT, then washed in 10 ml of ice-cold PBS. The cells were placed in 5 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP_{40}) for 3 min on ice, and the nuclei were obtained by centrifugation at 500*g* for 5 min at 4°C. The nuclei were washed four times in 5 ml lysis buffer. The nuclear pellet was suspended

in 100 μl nuclear freezing buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) and transferred to microtubes. The nuclei were either used directly, frozen in liquid nitrogen or stored at −70°C. If frozen, nuclei were thawed on ice before use. The nuclei were mixed with 30 μl of 5× runon buffer (25 mM Tris-HCl, pH 8, 12.5 mM $MgCl_2$, 750 mM KCl, 1.25 mM each of ATP, GTP, and CTP and 100 μCi (10 μl) -^{32}P-UTP 3,000 Ci/mmol), and then sarkosyl was added to a final concentration of 0.6%. The mixture was incubated for 30 min at 30°C, and then 15 μl DNaseI (Promega; 1 U/μl) was added and incubated for another 15 min. One ml of Trizol was added to the reaction mixture and shaken at 1,000 revolutions/min until a homogeneous suspension was obtained (2 min). Three hundred microliter of $CHCl_3$ was added to the mixture and shaken for another 5 min. After centrifugation (12,000*g*, 15 min, 4°C), the clear aqueous upper phase was transferred to a fresh microtube containing 500 μl of isopropanol, and kept for 5 min at RT. RNA was precipitated by centrifugation at 12, 000*g* at 4°C for 15 min. The RNA pellet was washed twice with 75% ethanol, dried at RT, dissolved in 100 μl of DEPC water (5 min at 60°C) and then kept on ice. One microliter of the RNA was taken to measure the cpm (typically $1–2 \times 10^5$ counts/min). RNA sample (1×10^5 cpm) was run on 1% agarose gel, and the wet gel was exposed overnight to view the radiolabeled RNA. The wet gel was also stained with ethidium bromide to view the total RNA. The RNA was either used directly for hybridization or stored at −70°C. Commercially made single-stranded oligonucleotide (0.5 μg; see Table II) was applied to GeneScreen plus membrane (Dupont) using a slot blot apparatus. The blot was prehybridized at 60°C overnight in 1% SDS, 10% dextran sulfate, 1.4 M NaCl, 500 μg/ml each of herring sperm DNA and yeast tRNA. The prehybridized blot was preincubated for 10 min with 500 U RNAsin (Promega) plus 40 mM dithiothreitol (DTT). The runon sample of RNA (equivalent to 3×10^6 counts/min) was added and hybridized for 72 h at RT. The blot was washed in 2× SSC twice at RT for 5 min; in 2× SSC, 1% SDS at 65°C for 15 min; in 2× SSC at RT for 5 min; in 2× SSC, 10 μg/ml RNase A at 37°C for 10 min to degrade non-hybridized ss RNA; in 2× SSC at RT for 5 min; in 0.1× SSC three times at RT for 5 min. The membrane was dried at RT and exposed to a FUJI Imaging Plate. The nuclear runon signals were visualized and quantitated with FUJI film Scanner software (BAS 2000).

Determination of Origin Activities at the c-myc Locus by Quantitative PCR

Isolation of short nascent DNA, quantitative PCR conditions, primers, competitors, determination, and normalization of short nascent DNA abundance are described elsewhere [Tao et al., 2000].

RESULTS

Large T Antigen Expressed in WI38(SV40) Cells

Large tumor (T) antigen (T-Ag) of SV40 virus is an origin recognition protein for viral replication and also possesses intrinsic DNA helicase and ATPase activities. T-Ag is an oncoprotein. It binds to Rb/E2F complexes, resulting in the release and activation of the transcription activator E2F, whose targets are essential for

TABLE II. Single Stranded Probes (5′- > 3′) Used for Nuclear Runon Assay

Probe	Sequence	Position
pr0	TTAGTGTAGATAGGGAGGAATGATAG AGGCATAAGGAGGAAAACGATGCC	1761–1810
pr1	CGTTCTTTTTCCCGCCAAGCCTCTGAG AAGCCCTGCCCTTCTCGAGGCAG	2390–2439
pr2	CTGCTATGGGCAAAGTTTCGTGGATG CGGCAAGGGTTGCGGACCGCTGGC	2652–2701
pr3	GGTGAGGAAAACAATTTGCCAAAAT CCAAGGCACAAAGTTTTGCGCCACC	4111–4160
pra	CGAGGCCTCGGCAGCTGGAAGCGG GGCCAGCCGGGGTCGGGGGGCGCAGG	583–632

Single stranded probes pr0, pr1, pr2, and pr3 were used for measuring the activities of promoters P0, P1, P2, and P3 of the c-myc gene in WI38 and WI38(SV40) cells in the nuclear runon assays. The location numbers of these probes are from GenBank Locus HUMMYCC (Accession No. J00120). Probe prA was used for measuring the signals form γ-actin in WI38 and WI38(SV40) cells and for the normalization of signals from P0, P1, P2, and P3 of the c-myc gene. The position numbers of the prA are from GenBank Locus HUMACTGA (Accession No. M19283).

cell cycle G1/S transition. T-Ag also interacts with the tumor supressor p53, interfering with regulatory pathways mediated by it [Hassell and Brinton, 1996]. In this study, we used an isogenic system of human embryo lung fibroblast cell WI38 and its SV40-transformed variant, WI38 VA13 2RA (WI38(SV40)), to study the possible relationships between origin activities and cell immortalization and transformation. We also hoped to determine an effect of transcription on replication at the c-myc locus. The expression of large T antigen in WI38(SV40) cells was confirmed by Western blot, using antibody against the SV40 large T antigen (Fig. 1). A 94 kDa band corresponding to the SV40 large T antigen oncoprotein was detected in WI38(SV40) nuclear extract (Fig. 1B; lanes 2, 3, and 4), while it was not detected in the same amount of nuclear extract from WI38 cells (Fig. 1A and B; lanes 6, 7, and 8).

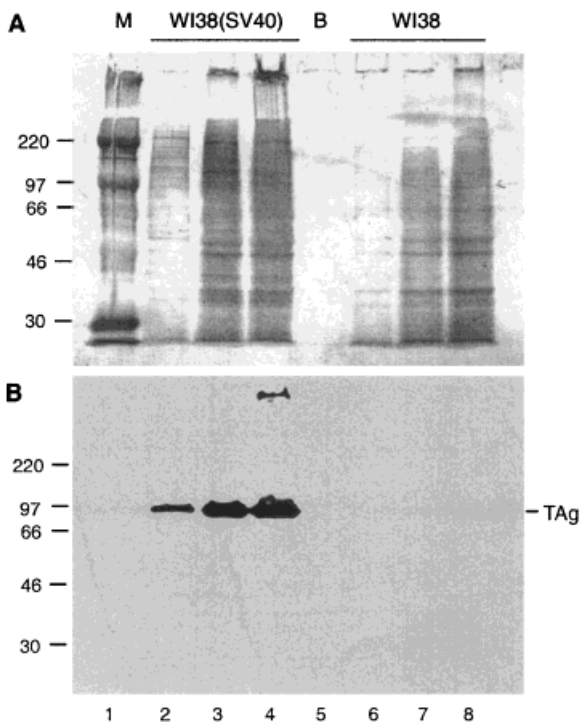

Fig. 1. Expression of SV40 large T antigen in WI38 and WI38(SV40) cells. **A:** Total nuclear proteins of WI38(SV40) or WI38 cells. Thirty microliter of total nuclear proteins were made from $\sim 10^5$ WI38(SV40) or WI38 cells. 1, 5, and 10 μl total nuclear protein samples of WI38(SV40) or WI38 cells were loaded in lanes 2, 6; 3, 7, and 4, 8 respectively. **Lane 1:** Molecular marker; **lane 5:** Blank. The concentrations of total nuclear proteins of WI38(SV40) and WI38 were approximately the same. **B:** Visualization of SV40 large T antigen of WI38(SV40) cells by western blot. Expression of large T antigen (94 kDa) was detected in WI38(SV40), but not in WI38 cells.

Activities of Origins at the c-myc Locus of WI38 and WI38(SV40) Cells

Short nascent DNA abundance distributions at a specific locus can be measured by competitive PCR. The locations with peak abundance of nascent chains indicate the site of initiation of DNA replication. Initiation sites co-localize with replicators, which are genetically defined sequences functioning as replication origins, in simple eukaryotic genomes [DePamphilis, 1999]. In mammalian cells, the characteristics of replicators have not been identified. However, short nascent DNA abundance at an initiation site represents the activity of the replicator. To measure the origin activities at the c-myc locus of WI38 and WI38(SV40) cells, we first isolated short nascent DNA samples of average size from 400 to 600 bases, using a modified λ exonuclease treatment [Tao et al., 2000]. Nascent DNA strands carry 5′ RNA primers, which are resistant to λ exonuclease digestion. In this manner, the short nascent DNA strands can be purified from a short (fragmented) DNA population. The short nascent DNA abundance over 12.5 kb of the c-myc locus in WI38 and WI38(SV40) was quantitatively determined by competitive PCR using 21 sets of primers, whose locations do not lie within repetitive elements (see Fig. 2C) [Tao et al., 2000]. Figure 3 shows the data for competitive PCR measurements with primer set L48f2-L48r2 (described in Tao et al., 2000) at the origin in the lamin B2 locus in WI38 and WI38(SV40) cells (Fig. 3 A–D); the abundance at the origin in lamin B2 was used for the normalization of the origin activities signals (see below). Our results show that 35–37 PCR cycles avoids formation of heteroduplex bands of amplified targets and competitors, as observed on electrophoretic gels when 50 PCR cycles were used [Kobayashi et al., 1998]. The short nascent DNA abundance levels at the c-myc locus in WI38 and WI38(SV40) cells are shown in Figure 2A and B. The ratios of the highest to lowest abundance are ~13 in a nascent DNA sample from WI38 (Fig. 2A) and about 10 from WI38(SV40) (Fig. 2B), affirming the quality of the nascent DNA samples. The ratio of signals from the initiation site to distant sites in non-initiation regions is usually ≥ 10 [DePamphilis, 1997].

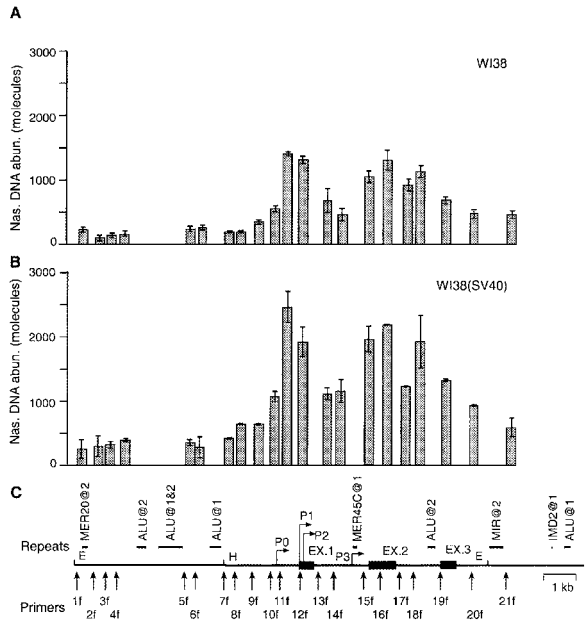

Fig. 2. Short nascent DNA abundance distribution in the c-myc locus of WI38 and WI38(SV40) cells. **A:** Nascent DNA sample prepared with λ exonuclease digestion from WI38 cells. **B:** Nascent DNA samples prepared with λ exonuclease digestion from WI38(SV40) cells. **C:** The c-myc locus, transcription promoters, repeat elements, and primer sets used for PCR. The nascent DNA abundance at each position in each nascent DNA sample was measured by competitive PCR at least three times. The abundance levels are indicated as the averages (±S.D.). At least two different nascent DNA samples were prepared from each cell line. We show the result from one nascent DNA sample here. No significant variations from this distribution were seen among the other samples.

From the distribution of short nascent DNA abundance, we can identify the peaks of abundance for short nascent DNA, which indicate the locations of the DNA replication initiation sites. A major initiation site was observed at ~0.5 kb upstream of exon 1. This major replication initiation site lies in the transcription promoter region in both cell lines (see Fig. 2C), at ~0.2 kb downstream of the P0 promoter, and ~0.5 and ~0.6 kb upstream of the P1 and P2 promoters respectively. P2 is the main transcription promoter for the c-myc gene [Spencer and Groudine, 1991]. A second major replication initiation site was found in exon 2 in both WI38 and WI38(SV40) cell lines, ~1 kb downstream of the transcription promoter P3 (see Fig. 2C). No significant replication initiation signals were observed in the region upstream of the HindII site of the c-myc locus in either WI38 or WI38(SV40) cells. DNA replication initiates at the major initiation sites in an initiation zone from restriction enzyme sites Hind III to EcoRI (Fig. 2C) at the c-myc locus in both cell lines.

Loss of the nascent DNA strands in the preparation of nascent DNA could contribute to variation in the abundance. Therefore, to compare the activities of an origin in different cell types or cell lines, an internal control is needed for measuring the nascent DNA abundance in the aliquots of nascent DNA samples from the cell lines. We had previously taken the

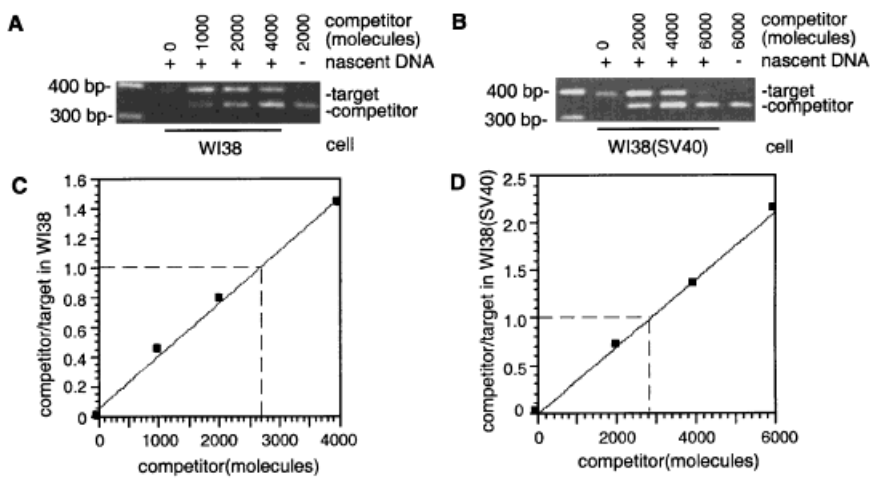

Fig. 3. Determination of nascent DNA abundance by competitive PCR. **A** and **B:** Determination of nascent DNA abundance associated with the origin at the lamin B2 locus of WI38 and WI38(SV40) cells with primer set L48f2-L48r2. An aliquot of nascent DNA sample from WI38 or WI38(SV40) cells treated with λ exonuclease digestion [Tao et al., 2000] was coamplified with a series of concentrations of competitors by PCR and resolved on 2% agarose gel. **C** and **D:** The ratios of band intensities of competitors to targets are plotted against the concentrations of competitors. The concentrations of competitor and target are equal when the ratio is 1. In these two examples, the copy number of the sequence amplified by primer set L48f2-L48r2 is 2700 in an aliquot of nascent DNA sample from WI38 cells and 2800 in that from WI38(SV40) cells. The measurement of a nascent DNA sample was repeated at least three times.

signals nearby the β-globin origin [Tao et al., 1997] or the lamin B2 origin [Tao et al., 2000] as internal controls. Here again, we used the β-globin and lamin B2 loci as internal standards for comparison of the activities of origins in the c-myc locus in WI38 and WI38(SV40). We first confirmed by quantitative PCR that the genomic copy numbers in the c-myc locus relative to those in the lamin B2 locus and the β-globin locus were the same in both WI38 and WI38(SV40) cell lines. The ratios of copy numbers at the c-myc locus in WI38(SV40) to those in WI38 cell lines determined by using primer set 11 were 1.3±0.1 (mean±S.D.; relative to the lamin B2 locus) and 1.1±0.1 (relative to the β-globin locus) (data not shown). This confirms that the copy numbers of the c-myc locus per haploid genome in WI38 and in WI38(SV40) cell lines (relative to the internal standards) can be viewed as identical. The nascent DNA abundance at the c-myc locus, normalized to that at lamin B2, in nascent DNA samples prepared with λ exonuclease treatments from WI38 and WI38(SV40) cells are shown in Figure 4. The normalized short nascent DNA abundance at the peak is indicative of origin activity. Thus, we are able to compare the origin activities in a certain locus in different cell lines. Figure 4 shows that origin activities in the initiation zone of the c-myc locus in WI38(SV40) cells are 1.8-fold at primer set 11 and 16 and 1.7-fold at primer set 18 as high as those in WI38. It should be emphasized that differences in normalized abundance levels indicate differences at the c-myc locus relative to those at lamin B2 in the two cell lines. If the short nascent DNA abundance measurements

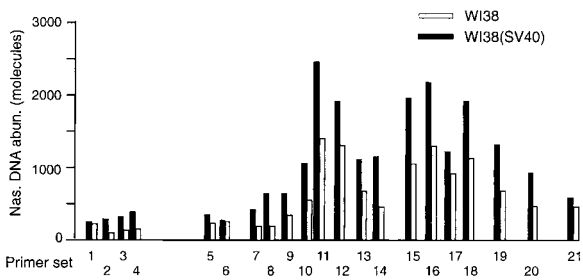

Fig. 4. Comparison of origin activities over the c-myc locus between WI38 and WI38(SV40) cells. The nascent DNA abundance levels at the c-myc locus (see Fig. 2) were normalized to the nascent DNA abundance at the origin associated with lamin B2 (see Fig. 3A–D). The nascent DNA samples were prepared with λ exonuclease digestion from WI38 (white bars) and WI38(SV40) (black bars). The normalized peak abundance represents the origin activity at the initiation site.

shown in Figure 2 are normalized to the origin activities at the β-globin locus (data not shown), differences of origin activities over all the initiation zone in WI38(SV40) vs. WI38 cells are 1.7-fold at primer set 11 and 16 and 1.6-fold at primer set 18, similar to those observed when the lamin B2 locus was used as an internal standard. These results indicate that the distribution of short nascent DNA abundance at the c-myc locus is not changed in the two cell lines, but that the activities of the origins are approximately two fold as high, relative to those of lamin B2, in WI38(SV40) cells as that in WI38 cells.

Promoter Activities in the c-myc Locus in WI38 and WI38(SV40) Cells

Transcription activity may correlate with DNA replication. It has been shown that the immunoglobulin heavy-chain (Igh) enhancer (Eμ) is near or in an initiation zone of chromosomal DNA replication. The Eμ is required for tissue-specific transcription of Igh. The Eμ-associated origin may be more active in B lymphocytes than fibroblasts [Ariizumi et al., 1993]. Overexpressed c-myc oncoprotein is frequently observed in immortal and malignant cells [Spencer and Groudine, 1991]. Therefore, there may be a possible correlation between the increased replication origin activities and increased transcription activity at the c-myc locus in immortal and malignant cells. To assess this, we first quantitatively measured the stable c-myc transcript levels in WI38 and WI38(SV40) by RT-PCR. Primer set 2F-2R, located immediately downstream of promoter P2 (Fig. 5A), was used to quantify the c-myc transcripts by RT-PCR; this set of primers was chosen because 75–90% transcription may be initiated by P2 [Spencer and Groudine, 1991]. A negative control was chosen using primer set 9f-9r2 (Fig. 5A; Table I), which is upstream of the c-myc promoters. For normalization using an internal standard, primer set 1F-1R located in an exon of the human housekeeping gene β-actin (Table I) was used (see Materials and Methods section). Total RNA samples prepared from WI38 or WI38(SV40) cells were amplified by RT-PCR with primer set 2F-2R or 1F-1R, and a single product was resolved on agarose gel (Fig. 5B). No product was detected with primer set 9f-9r2, indicating no significant DNA contamination in the total RNA samples (Fig. 5B). RT-PCR amplification contains two steps — (1)

a reverse transcription reaction that converts RNA into cDNA and (2) a polymerase chain reaction that amplifies the cDNA. Double-stranded DNA competitors were used to compete the cDNA in our quantitative RT-PCR assays (Fig. 5C). The total stable c-myc RNA levels were measured from the cDNA produced from the total RNA (Fig. 5 C–E). Normalized to that of the internal standard β-actin (data not shown), the total c-myc RNA transcript level in WI38(SV40) cells was ~2.0±0.2 -fold as high as that in WI38 cells.

Since c-myc expression is regulated at different levels, such as initiation of transcription, RNA elongation, RNA splicing, RNA degradation, protein degradation etc. [Spencer and Groudine, 1991; Potter and Marcu, 1997], the stable RNA transcript level may not be indicative of the promoter transcription activity. Therefore, we further assessed the activities of the c-myc promoters P0, P1, P2, and P3 (see Fig. 6A) in WI38 and WI38(SV40) cells, using a nuclear runon assay. Four 50-nucleotide single-stranded DNA probes (pr0, pr1, pr2, and pr3) were chosen immediately after the four promoters P0, P1, P2, and P3, respectively, equivalent to the probe positions chosen by Luo et al. [1997] to measure the activities of the c-myc promoters in mouse cells (Fig. 6A). Nuclear runon signals indicate the activities of the promoters. The data (Fig. 6) show that the major c-myc promoter used in both cell lines was P2, which was responsible for ~60% of the transcription activity in WI38 and ~90% transcription activity in WI38(SV40) by comparison to the other promoter. Significant alternate usage of the

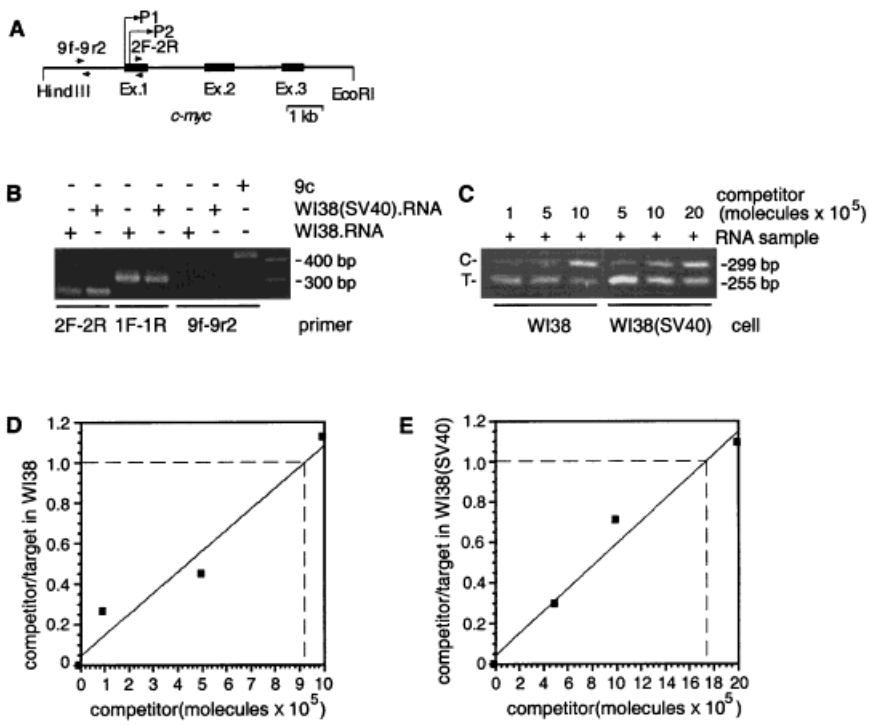

Fig. 5. Determination of stable RNA transcripts of WI38 and WI38(SV40) by RT-PCR. **A:** The c-myc locus, promoters P1 and P2, and primer sets used in RT-PCR (see sequences and position numbers in Table I). **B:** The RNA samples of WI38 and WI38(SV40) cells were amplified by RT-PCR. A single product can be seen in RT-PCR amplification of an aliquot of total RNA sample from WI38 or WI38(SV40) cells with primer set 2F-2R (for c-myc) or 1F-1R (for β-actin). Primer set 9f-9r2 identifies a non-transcribed region upstream of the c-myc exon 1; no product after RT-PCR amplification of the RNA samples with the primer set 9f-9r2 was observed. This result serves as a negative control for the RNA samples, indicating that there is no significant DNA contamination in the RNA samples. 9c is a DNA fragment, which can be amplified with primer set 9f-9r2; this single product serves as a positive control for the negative results with primer set 9f-9r2. **C:** Quantitative RT-PCR. cDNA was prepared from RNA samples from WI38 and WI38(SV40) cells by reverse transcriptase using primer 2R; a series of concentrations of double-stranded DNA competitors were added to each cDNA sample generated by the first step of the RT-PCR, and then competitive PCR amplification was preformed with primer set 2F-2R. 'C': competitor; 'T': target. **D–E:** The ratios of competitor product intensity signals to the target product intensity signals were plotted against the competitor concentrations. The concentrations of target and competitor is equal, when the ratio is 1. The relative cDNA concentration represents the relative abundance of the total RNA targets.

c-myc promoters in the two human cell lines was not seen, unlike that observed in murine cell lines, in which the large T antigen was induced by temperature shift, resulting in significant usage of promoters P1 and P3 [Luo et al., 1997]. The runon signals were normalized to those of housekeeping gene γ-actin, and the promoter P2 activity in WI38(SV40) cells was about 7.5–8.0-fold as high as that in WI38 cells, but the activities of the other promoters, P0, P1, and P3, had no significant change, maintaining a low level of transcription activity (Fig. 6B). In considering the coupling of transcription and replication, while all replication origins approximately double their activities, only promoter P2 significantly increases its activity at the c-myc locus in WI38(SV40) cells in comparison with those in WI38 cells. This suggests that the significantly increased activity of promoter P2 had no preferential influence on the activity of individual replication origins.

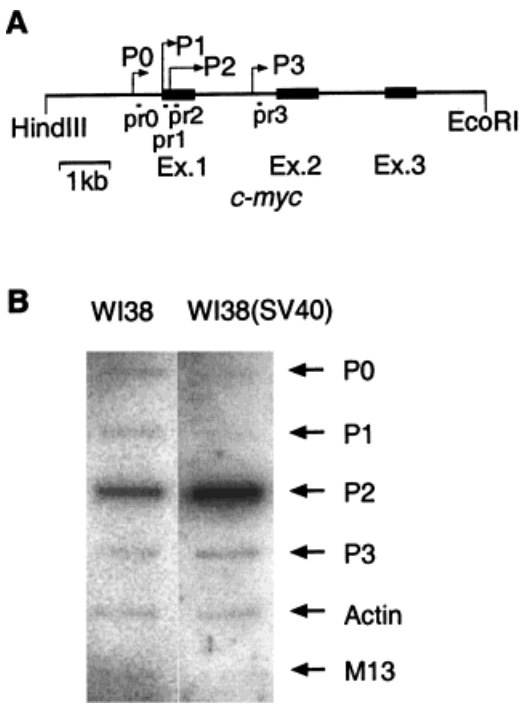

Fig. 6. Nuclear runon assay. **A:** The c-myc locus; promoters P0, P1, P2, and P3; and single stranded probes pr0, pr1, pr2, and pr3 (see sequences and position numbers in Table II). **B:** Nuclear runon assay. Probe prA (see sequence and position numbers in Table II) is used for normalization of the signals from promoters P0, P1, P2, and P3. Single-stranded M13 DNA plasmid served as negative controls. The normalized signals from the promoters represent the promoter activities. P2 was the principal promoter used in the c-myc gene transcription in both cell lines, but P2 activity in WI38(SV40) cells was 7.5- to 8.0-fold higher than in WI38 cells.

DISCUSSION

Origin Activity and Cell Immortalization and Malignancy

Our previous studies indicated that the activities of all DNA replication origins over 12.5-kb region in the human c-myc locus were about two-fold as high in malignant epithelial HeLa cells as in normal human skin fibroblast (NSF) cells, suggesting that cell transformation may induce greater frequency of initiation of origins at certain loci [Tao et al., 1997, 2000]. However, the differential origin activities in HeLa and NSF cells might also have been due to differences between cell types. Here, we used the isogenic cell lines from WI38 with and without SV40 transformation, and again found higher origin activities in transformed cells, eliminating the possible reason caused by cell types. Although the c-myc locus is amplified in some transformed cells [Spencer and Groudine, 1991], here, we confirmed that there are identical genomic copy numbers of the c-myc locus in both WI38 and WI38(SV40). Therefore, the increased origin activities in the c-myc locus in WI38(SV40) are due to the cell transformation by SV40 virus.

Upon transformation, a greater population of cells can be observed in S phase compared to the non-transformed counterparts. Therefore, it might be argued that the observed differences in the replication activities at the c-myc locus might be because the transformed population at the time of DNA isolation had more cells in S phase, thus leading to greater recovery of nascent DNA from the transformed population vs. the normal population. Such potential problems were eliminated by the use of internal controls, such as the activities of origins associated with lamin B2 and β-globin.

We chose lamin B2 origin as an applicable internal reference for our comparison of differential origin activities in cell lines, because: (1) the lamin B2 origin lies in a constitutively expressed gene domain, coding for a housekeeping protein [Biamonti et al., 1992; Kumar et al., 1996], (2) usage of lamin B2 origin has been observed in a number of different proliferating human cells [Kumar et al., 1996; Tao et al., 2000; this study], (3) it is unlikely that the activity of lamin B2 origin would decrease with cell transformation, since the number of active origins increase in transformed cells [Martin and Oppenheim, 1977; Oppenheim and Martin,

1978], (4) although the lamin B2 origin activity may also increase with cell transformation, this would indicate even greater effects at the c-myc locus in immortalized WI38(SV40) cells, and (5) some origins appear unaffected with cell transformation. In our previous work [Tao et al., 2000], the ratio of activity of c-myc in HeLa vs. NSF cells relative to the origins associated with β-globin, lamin B2, or origin 343 [Wu et al., 1993a,b] was ~2. There were apparently no changes in activity among the sites, suggesting that the lamin B2 origin was an approximately representative of many other less affected or unaffected origins.

WI38(SV40), immortalized by SV40 virus, is a transformed cell line. WI38(SV40) does not cause tumors in animals, although it behaves as if it were neoplastic in a muscle organ culture (MOC) and forms colonies in soft agarose (SA) [Levenbook et al., 1985]. Large T antigen of SV40 binds tumor suppressor Rb, interfering with formation of E2F/Rb complex [Hassell and Brinton, 1996]. This free E2F may then activate its target genes, e.g., c-myc, which has several E2F binding sites in its promoter region. E2F and other regulatory factors jointly enhance the expression of the c-myc [Marcu et al., 1997]. Overexpressed C-MYC oncoprotein may directly activate telomerase reverse transcriptrase (TERT), a core component of the telomerase complex, which is responsible for telomere maintenance in a majority of immortal and tumor cells [Kiyono et al., 1998; Wu et al., 1999; Cerni, 2000]. If the increased promoter activities and increased replication origin activities of replication at the c-myc locus are directly correlated or both are regulated by higher order common regulators (see below Discussion), then it might be suggested as a mechanism of a direct association of the increased origin activity at the c-myc locus and cell immortalization. On the other hand, one early step of immortalization is inactivation of Rb pathway or p53 pathway, which bypasses senescence crisis [Weinberg, 1998; Cerni, 2000]. Inactivation of one of these pathways may be responsible for global increases of activities of replication origins at chromosomes in premalignant cells, in which the chromatin structure and nuclear components have changed. Therefore, the increased activities of replication origins can occur at a cell immortalization stage, before further progression to cell malignancy. The results from our series of studies in NSF (normal), WI38 (normal), WI38(SV40) (immortal), and HeLa (malignant) cells support this interpretation. This also suggests that the frequent observation of a 2-fold activity as opposed to 3-, 4-fold, or something else is indicative of some fundamental process of limitation imposed upon the immortalization to malignant tumor transformation process

Replication Origin Activities and Transcription Promoter Activities at the Human c-myc Locus

There seems to be a temporal correlation between replication and transcription in mammalian cells. Origins in regions with constitutively expressed or transcribed genes are usually activated in early S phase, but loci with inactive genes are replicated later in S phase [Simon and Cedar, 1996; Ermakova et al., 1999]. The mechanism(s) of coupling of transcription and replication in mammalian cells remain unclear. Here, we analyzed increased activity of promoter P2 and increased activities of origins at the c-myc locus in WI38(SV40) cells in comparison with those of WI38 cells.

The expression of the c-myc is closely correlated with cell proliferation [Spencer and Groudine, 1991; Obaya et al., 1999]. The RNA levels of the c-myc are low in quiescent cells, but are rapidly induced after stimulation with a variety of mitogens [Kelly et al., 1983; Campisi et al., 1984; Blanchard et al., 1985; Reed et al., 1985]. Peak levels of the c-myc expression are usually reached at ~2–4 h after stimulation, and then fall to a low constitutive level throughout the remaining cell cycle [Persson et al., 1985; Rabbitts et al., 1985]. Transcription of c-myc is strongly increased in some transformed cells. It has been suggested that either loss of the repressor for transcription [Dufort and Nepveu, 1994] or loss of the block for RNA elongation [Krumm et al., 1992; Strobl and Eick, 1992; Wolf et al., 1995] contributes to the increased expression of the c-myc gene. The endogenous c-myc locus in human cells is replicated in very early S phase [Calza et al., 1984; Selig et al., 1992]. If one assumes that the mechanism of the initiation of DNA replication in mammalian cells resembles those in budding yeast and *Xenopus* [Leatherwood, 1998; DePamphilis, 1999], the cells must start to assemble the prereplication complex (pre-RC) at origins in early G1 phase, when the c-myc transcription is most active. Although transcription of the c-myc gene

begins a few hours earlier than the replication of the c-myc locus, the preparation for initiation of DNA replication and transcription at the c-myc locus may temporally overlap, allowing for a functional interaction between the transcription factors and the pre-RC at the c-myc locus. The c-myc promoter P2 is very near (~ 600 bp) to the first major replication initiation site in the locus (Figs. 2 and 5). It is likely that an increased promoter P2 activity might affect the activity of the origin at the major initiation site nearest to it. However, our data show that 7.5–8.0-fold increased P2 promoter activity did not change the relative relationship of the activities of all initiation sites at the c-myc locus in WI38(SV40) cells. That is, the increased activity of promoter P2 had no preferential influence on the early step of initiation of DNA replication throughout the c-myc locus. Thus, binding of transcription factors at this locus does not preferentially facilitate the assembly of the pre-replication complex or preferentially alter the activities of the DNA replication proteins at specific initiation sites.

Negative supercoiling is facilitative for DNA replication. Transcription initiation and elongation may induce DNA negative supercoiling, facilitating the initiation of chromosomal DNA replication over a large region. Ohba et al. [1996] demonstrated an induction of DNA replication by transcription in an in vitro system. A plasmid, pHMYCT7F, was constructed, containing a combination of a replication initiation zone from the region upstream of the human c-myc gene (GenBank Accession No. J00120 and Locus: HUMMYCC, Position 1 to 2845 (HindIII to PvuII) and promoter T7. Relaxed plasmid was used for measuring the induction of initiation of DNA replication by transcription initiated from promoter T7. DNA replication was induced by initiation of transcription and RNA elongation, most likely by the introduction of negative supercoiling into this region [Ohba et al., 1996]. However, since the peak for transcription for c-myc is a few hours earlier than the replication of the c-myc locus in cells, it is unlikely that the negative supercoiling caused by transcription would be maintained for several hours, thereby influencing DNA replication that follows at this locus.

Increased transcriptional activity by P2 promoter also occurs with the increases in activities of origins across the entire c-myc locus. It is therefore also possible that the coupling of transcription and replication may be affected through higher order regulations associated with nuclear and nucleosomal structures. Nuclear attachment is required for efficient transcription and replication [Bode et al., 1996; DePamphilis, 1998]. Increasing data support a model in which DNA and RNA polymerases are immobile [Cook, 1999]. In this model, DNA and RNA polymerases are attached to larger structures on the nuclear matrix, through which DNA templates are reeled, extruding newly made nucleic acids. Furthermore, DNA polymerases are immobilized in a 'replication factory', where ~40 active replication forks on different DNA templates in early S phase in human cells can be observed. [Hozak et al., 1993; Cook, 1999]. Active RNA polymerase, like active DNA polymerases, appear concentrated in 'transcription factories' [Cook, 1999]. One 'transcription factory' contains ~30 active polymerases with associated transcripts in HeLa cells [Jackson et al., 1998]. Based on the functional dynamics of nuclear architecture, Berezney and Wei [1998] showed that replication and transcription sites are spatially and temporally clustered into separated higher-order domains or 'zones' in a nucleus. These large replication and transcription regions are dynamic and 're-zoned' during the cell cycle. Large regions of chromatin can be recruited for replication or transcription at a specific time and be switched at a later time (e.g., 1 h later) into another type of zone [Wei et al., 1998]. The dynamic three-dimensional networks of transcription and replication may reflect coordination of transcription and replication programs in the cell nucleus [Berezney and Wei, 1998]. One can delete the promoter to eliminate transcription of a given unit, or delete a transcription factor binding site to manipulate the activities of transcription factors. These changes, however, are unlikely to change the transcription unit organization on the nuclear matrix or to affect DNA replication in the 'replication factory' switched from the 'transcription factory'. Our observations of increased activity of all origins may also be explained by the 're-zone' model [Wei et al., 1998]. That is, the 'replication zone' containing the c-myc locus may be switched from the 'transcription zone'. This 'transcription zone' with increased c-myc transcriptional activity may transmit or carry some active feature of the chromatin structure or its active environ-

ment into the 'replication zone', resulting in global increases in activity of origins at the c-myc locus.

Nuclear attachment of chromatin DNA can mediate chromatin remodeling. Chromatin configuration can be locally modified at specific sites or at certain domains [Schubeler et al., 1996; Pemov et al., 1998]. Alternate chromatin structure can occur generally and non-specifically, and both transcription and replication may be affected by common regulatory factors [Zhao et al., 1993]. Non-specific nucleosomal structure remodeling may be responsible for the increased activities of replication origins in WI38(SV40) cells observed in this study.

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