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Major DNA Replication Initiation Sites in the *c-myc* Locus in Human Cells

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Abstract DNA replication initiation sites and initiation frequencies over 12.5 kb of the human *c-myc* locus, including 4.6 kb of new 5′ sequence, were determined based on short nascent DNA abundance measured by competitive polymerase chain reaction using 21 primer sets. In previous measurements, no comparative quantitation of nascent strand abundance was performed, and distinction of major from minor initiation sites was not feasible. Two major initiation sites were identified in this study. One predominant site has been located at ~0.5 kb upstream of exon 1 of the *c-myc* gene, and a second new major site is located in exon 2. The site in exon 2 has not been previously identified. In addition, there are other sites that may act as less frequently used initiation sites, some of which may correspond to sites in previous reports. Furthermore, a comparison of the abundance of DNA replication intermediates over this same region of the *c-myc* locus between HeLa and normal skin fibroblast (NSF) cells indicated that the relative distribution was very similar, but that nascent strand abundance in HeLa cells was approximately twice that in NSF relative to the abundance at the lamin B2 origin. This increased activity at initiation sites in the *c-myc* locus may mainly be influenced by regulators at higher levels in transformed cells like HeLa. J. Cell. Biochem. 78:442–457, 2000.

Key words: replication, origin, initiation; *c-myc*; human cells

Eukaryotic DNA is replicated in units called "replicons," each representing a DNA domain replicated from a single initiation site or initiation zone. In yeast, chromosomal replication origins coincide with short autonomously replicating sequence elements, which comprise easily unwound DNA and binding sites for protein complexes including the origin recognition complex (ORC) [for review, see Newlon, 1996]. In higher eukaryotic cells, DNA replication origins appear to be more complex. Replication may initiate nonrandomly at multiple sites within a replicon [Waltz et al., 1996; Kobayashi et al., 1998; Trivedi et al., 1998; DePamphilis, 1996; DePamphilis, 1999], and the activity of initiation sites is regulated during development [Walter and Newport, 1997]. At the embryonic stage, the distance between initiation sites is small, presumably to meet the need for rapid cellular division. In *Xenopus* embryonic cells, the average replicon size is ~7–12 kb [Mahbubani et al., 1992; Hyrien and Mechali, 1993]. In contrast, the average size of replicons in somatic mammalian cells is estimated to be ~50–300 kb, and the average number of replicons is estimated to be 10^4–10^6 per cell [Edenberg and Huberman, 1975; Hand, 1978; Martin, 1981].

Only a very small proportion of the total number of origins have been mapped on chromosomes [for review, see DePamphilis, 1999]. In the human *c-myc* locus, initiation of DNA replication has been studied principally within a small region of 2.4 kb upstream of exon 1 of the *c-myc* gene. A putative origin of human cellular DNA replication (*Ori*) was suggested to be located at approximately 2 kb upstream of the coding region of the *c-myc* gene [Iguchi-Ariga et al., 1988; McWhinney and Leffak, 1988, 1990; Dobbs et al., 1994]. An initiation zone centered ~1.5 kb upstream of exon 1 of the *c-myc* gene was located on chromosomal DNA in HeLa cells by

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polymerase chain reaction (PCR) [Vassilev and Johnson, 1990]. These sequences demonstrated autonomous replication activity when cloned into plasmids and transfected into HeLa cells [Iguchi-Ariga et al., 1988; McWhinney and Leffak, 1988, 1990; McWhinney et al., 1995], and in a cell-free replication system [Zannis-Hadjopoulos et al., 1994; Berberich et al., 1995; Ishimi et al., 1995]. Recently, a construct containing the 2.4-kb *c-myc* origin fragment transduced to an ectopic chromosomal site was shown to initiate replication and induce replication at flanking chromosomal sites, providing genetic evidence for replicator function of this *c-myc* DNA fragment [Malott and Leffak, 1999]. Non-random, multiple initiation sites within the 2.4 kb region located upstream of the *c-myc* gene were observed in plasmids in both in vitro and in vivo assays, as well as on the chromosome [Berberich et al., 1995; McWhinney et al., 1995; Waltz et al., 1996; Trivedi et al., 1998]. The majority of work above has been focused mainly on this 2.4 kb region, and limited information about DNA replication initiation has been available for the large *c-myc* locus. In previous measurements, no comparative quantitation of nascent strand abundance or origin activity was performed, and distinction of major from minor initiation sites was not feasible.

In our previous work, we found that the activity of the origin located at ~1.5 kb upstream of exon 1 of the *c-myc* locus in HeLa cells was approximately twofold that in normal skin fibroblast (NSF) cells [Tao et al., 1997]. In this study, to address whether the differential activities of the individual initiation sites in HeLa and NSF cells are caused by the alternative use among multiple initiation sites, we measured the origin activities at potential initiation sites distributed over 12.5 kb of the *c-myc* locus, including 4.6 kb of new 5′ sequence. Within the resolution of our assay, the two major initiation sites were detected among multiple initiation sites in both HeLa and NSF cells. Comparison of nascent strand abundance indicated that there was no significant difference in the pattern of initiation of DNA replication in the *c-myc* locus in HeLa and NSF cells. However, the origin activity at all initiation sites in HeLa cells was approximately twofold as high as those in NSF cells.

MATERIALS AND METHODS

Cell Cultures

Primary NSF (Biowhittaker), and HeLa cells (ATTC CCL 2.2) were grown in 5% CO_2 in 175 cm^2 tissue culture flasks and alpha-medium (Gibco) + 10% (vol/vol) fetal calf serum (Gibco). NSF and HeLa cells at 30%–50% confluence were harvested for the isolation of nascent DNA. For isolation of total genomic DNA, NSF cells were grown to confluence, and the cells were incubated in alpha-medium without serum, for 48 h to obtain a G0 population of cells, confirmed by fluorescence flow cytometry [Zannis-Hadjopoulos et al., 1988].

DNA Sequencing

pMYC/EX7 is a pUC18 plasmid carrying a ~7 kb EcoRI-XhoI fragment upstream of exon 1 of the *c-myc* gene (Fig. 1). pMYC/EX7 was amplified in *Escherichia coli*, isolated and purified with Qiagen Plasmid Maxi Kit. Sequence analysis was begun from immediately upstream of the 5′ end of the insert and from immediately downstream of the Hind III site. New primers for further sequence analysis were designed, avoiding repetitive sequences in the region of ~4.6 kb upstream from the 5′ terminal Hind III site of the GenBank accession number (AF176208). DNA sequence determination was performed with ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kits with AmpliTaq DNA Polymerase, and ABI 373 DNA sequencer (Perkin Elmer).

Construction of Unique Molecular Competitors

To rapidly and reliably measure the relative abundance of nascent DNA, we constructed unique molecules, each of which contained 10 or more competitors for competitive PCR (Fig. 2a). Each pair of PCR primers was designed to avoid repetitive sequence elements, and the sizes of PCR products were chosen to be ~400 bp to avoid amplification of Okazaki fragments. A single-strand DNA fragment containing 10 or more forward primers with an EcoRI restriction enzyme site at the 3′ ends was synthesized; double-stranded DNA fragments were amplified by PCR of the single-strand DNA fragments. Another double-stranded fragment containing 10 or more corresponding reverse primers with a HindIII site at one end was prepared in the same way. An unrelated double-stranded DNA with 5′-EcoRI and 3′-

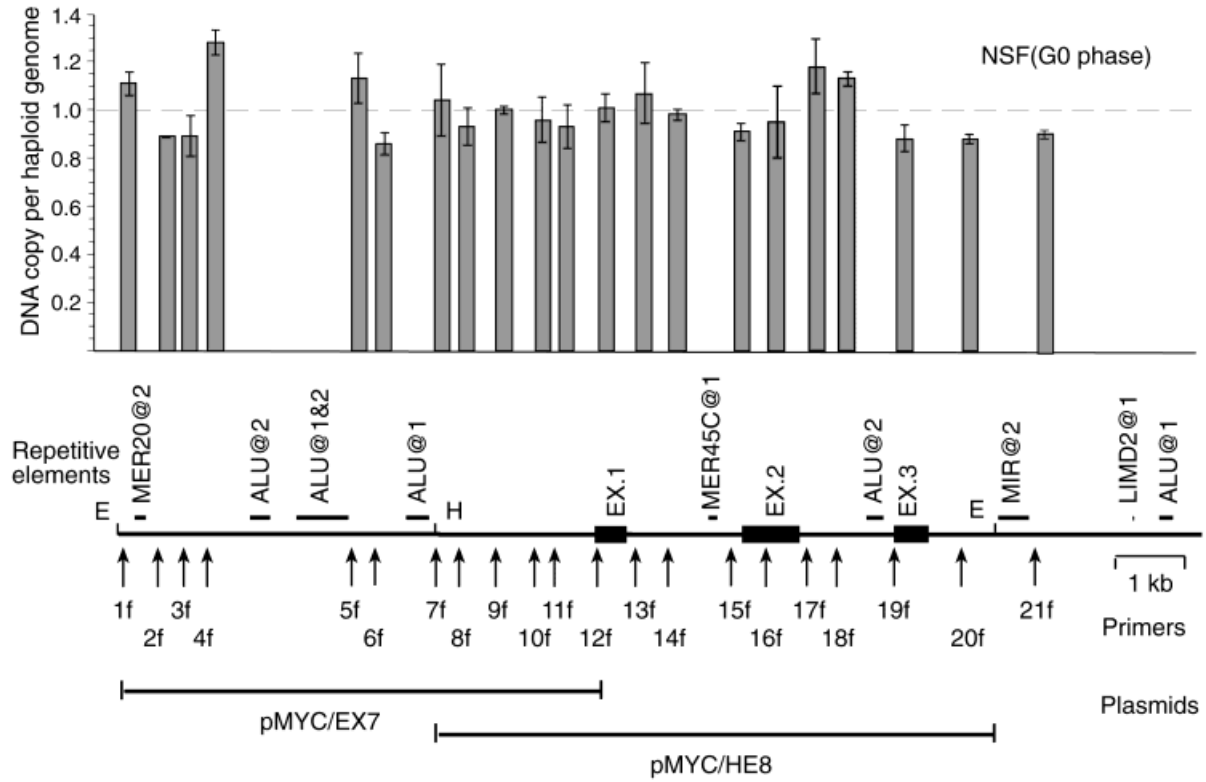

Fig. 1. DNA sequence analysis of the *c-myc* locus in human cells. The *c-myc* locus, the c-myc region sequences carried by plasmids pMYC/EX7 and pMYC/HE8, sequence analysis, and the primer set positions tested in this study. The 4.6 kb of sequence (from EcoRI to HindIII) upstream of the *c-myc* gene was determined. The final version of the sequence may be obtained from GenBank Accession Number AF176208. Arrows indicate the positions of the forward primers of the primer sets. The sequences of all primers are listed in Table 1. Measurement of copies per haploid genome at the sites to be tested in the *c-myc* locus. Total cellular DNA was extracted from NSF cells at G0 phase. The DNA abundance at each site was determined by competitive PCR. The average of five measurements of DNA abundance at position 9 was taken as a single copy per haploid genome [Lazo et al., 1989], and then the copy number from the abundance at each site was calculated. The average of the copy number per haploid genome and standard deviation was calculated from at least three separate measurements at each site.

HindIII restriction enzyme sites at the ends was prepared; the sequence of this double-stranded DNA was unrelated to any sequence in the region to be studied. The length of this double-stranded DNA fragment was adjusted such that each competitor, for example, with primer pair 1f and 1r, or with 2f and 2r, would generate an ~350-bp product, once the fragments were ligated to the arms containing forward and reverse primers, whereas those of the genomic targets were ~400 bp. The three double-stranded DNA fragments were codigested with EcoRI and HindIII, and the mixture was extracted with equal volumes of phenol:chloroform (1:1). DNA was precipitated and washed in ethanol. The codigested DNA fragments were ligated with T4 ligase. The unique molecules containing 10 or more competitors were amplified by PCR from the ligation mixture. The PCR products were run on 1% agarose gel; the unique molecular competitors were excised from the gel, eluted with Sephaglas BandPrep Kit (Pharmacia Biotech), and quantified with a spectrophotometer. The PCR amplification efficiencies on competitors and their targets were corrected, using the linear plasmids containing the sequences of the *c-myc* locus, and using total genomic DNA harvested at G0 phase.

Isolation of Nascent DNA

Nascent DNA samples were prepared with two different methods: a modified nascent strand extrusion method and a modified λ exonuclease digest method (Fig. 3a). The modified nascent strand extrusion method was based on the size selection of extruded nascent DNA, the details of which were described in a

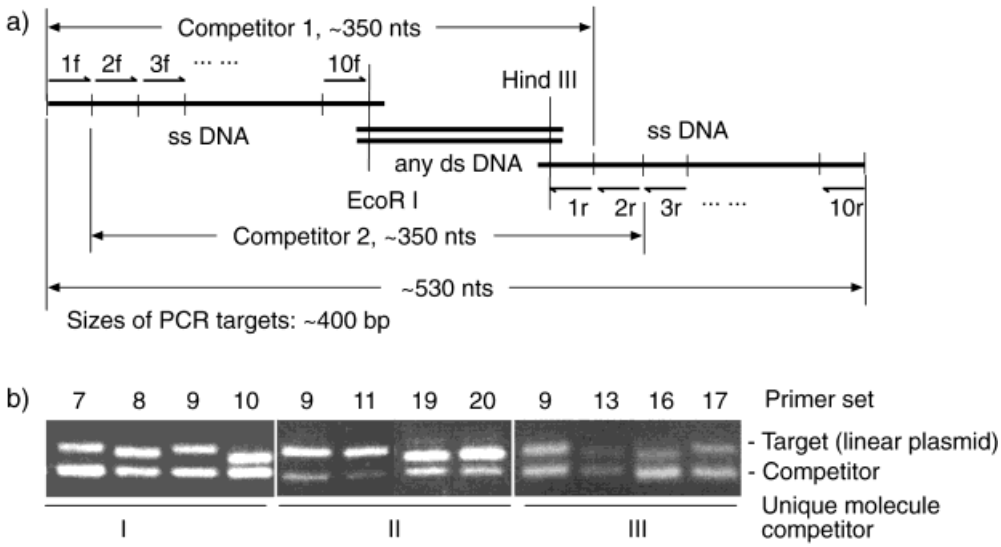

Fig. 2. Competitors on unique molecule constructs and PCR amplification efficiencies between targets and their competitors. **a:** Construction of unique molecule competitors. The purified construct was quantitated by spectrophotometer. The concentrations of the different constructs were corrected by the plasmid pMYC/EX7 and primer set 9, which was contained on each of the constructs. **b:** Variations of PCR amplification efficiencies between targets and their competitors. Each of the primer sets was tested at least three times.

previous publication [Tao et al., 1997]. The λ exonuclease digest method [Gerbi and Bielinsky, 1997; Kobayashi et al., 1998] was modified as follows: one 175 cm^2 flask of HeLa cells or four flasks of NSF cells at 30%–50% confluence were washed with 10 ml of ice cold phosphate-buffered saline buffer three times, and the cells were treated with 5 ml/flask of Hirt lysis buffer. Proteinase K was added to the lysate to a final concentration of 0.1 mg/ml, and incubated overnight at 37°C. The lysate was extracted with an equal volume of phenol:chloroform (1:1) once. Nucleic acids (DNA + RNA) were precipitated with 2 volumes of anhydrous ethanol in 50 ml plastic conical centrifuge tubes. The nucleic acids were spooled with a 1-ml-glass-pipette, whose tip was fused closed, washed in 70% ethanol (prepared with RNase-free water), and then placed into anhydrous ethanol again. The nucleic acids were air dried and dissolved in 200 μl of RNase-free water. The fresh nucleic acids (no older than 2–3 days) were repeatedly sheared by passage through a fine needle (26G 3/8) ~10 times. The nucleic acid concentration was estimated by spectrophotometer, and 1 μl of 1/10 dilution of sheared DNA was subjected to electrophoresis on 1% agarose gel. Phosphorylation of the 5′ DNA ends of ~20 μg of denatured total nucleic acid sample, together with 500 ng of linear pUC18 (dephosphorylated, as an internal control) was performed in 40 μl of total volume with 50 μM ATP + 10 U T4 polynucleotide kinase (New England Biolabs) for 30 min at 37°C. The mixture was heated for 3 min at 100°C to inactivate the kinase. The nucleic acid sample was digested with 12-15 U λ exonuclease (Gibco BRL Life Tech.) in 80 μl of total volume with 1x λ exonuclease reaction buffer [67 mM glycine-KOH (pH 8.8), 2.5 mM $MgCl_2$, 50 μg/ml bovine serum albumin] for 12 h at 37°C. The λ exonuclease was inactivated by heating for 10 min at 70°C. Nucleic acid samples before and after digestion with λ exonuclease, and after further digestion with RNase A (0.01μg/μl) were separated on 1% agarose gel. Typically, 5 μl of 1/50–1/200 dilution of λ exonuclease digest mixture could be directly used for PCR reactions.

Isolation of Parental DNA

For preparation of total genomic parental DNA from G0 NSF cells, the procedure was the same as that described above, but it was concluded after the DNA sample was sheared with a needle, and before phosphorylation.

Visualization of Nascent DNA Population

The λ exonuclease and RNase A digested nascent DNA samples were run for 24 h at 4°C

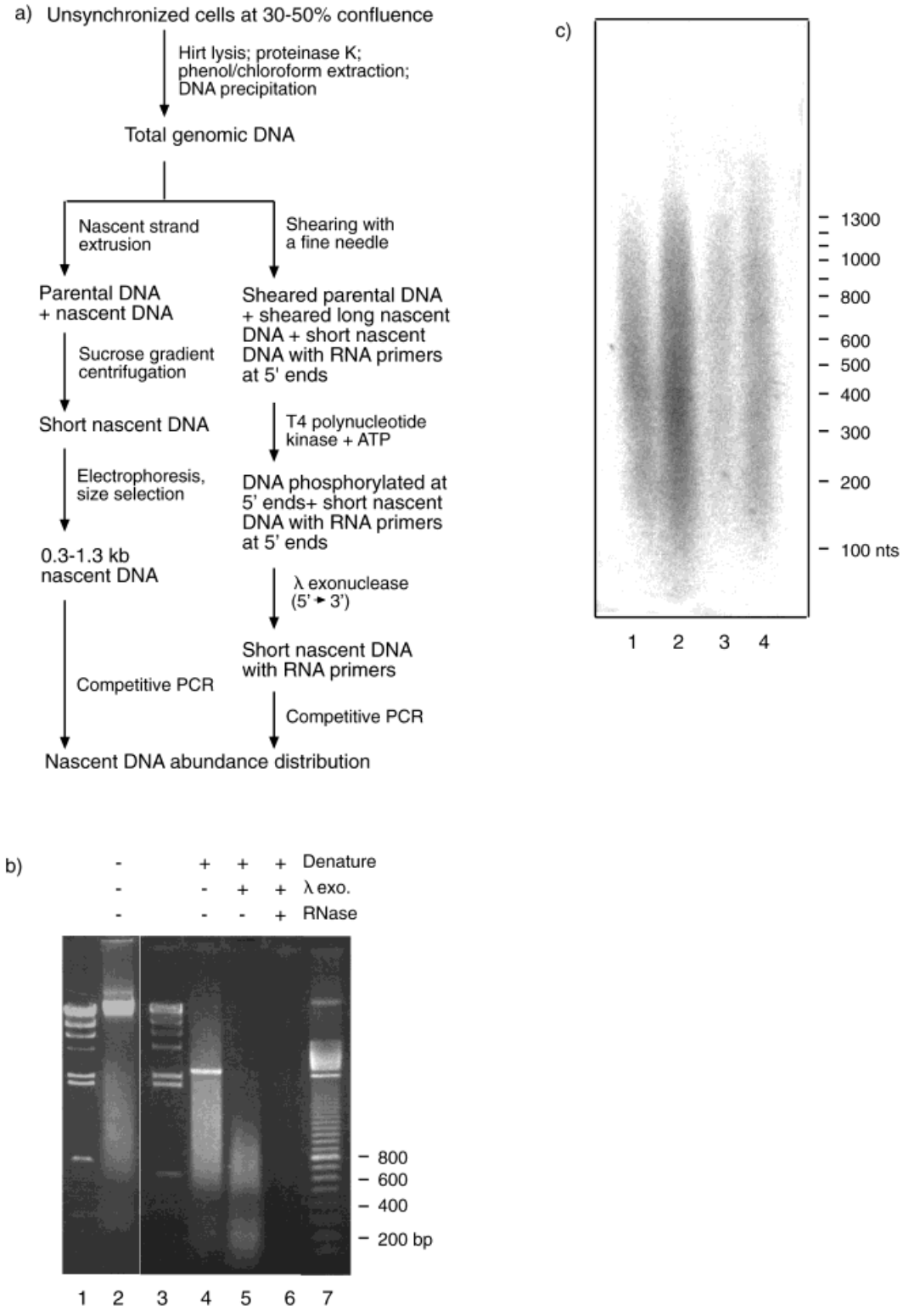

Fig. 3. Nascent DNA preparations. **a:** Strategies of nascent DNA preparations. **b:** Nascent DNA preparation with λ exonuclease digestion. **Lane 2:** ~500 ng of total cellular (DNA + RNA) was sheared with a fine needle (26G 3/8); **lane 4:** ~1,000 ng of denatured total cellular (DNA + RNA); the single band was the internal control linear pUC18 (~40 ng); **lane 5:** sheared total cellular (DNA + RNA) and plasmid DNA digested with λ exonuclease; **lane 6:** the nascent DNA sample treated with RNase A; **lanes 1 and 3:** Hind III digested λphage DNA marker; lane 7: 100-bp ladder DNA marker. The nucleic acids were separated on 1% agarose native gel. **c:** Visualization of λ exonuclease treated nascent DNA samples by Southern Blot assay of a 1.2% alkaline agarose gel. **Lanes 1 and 2:** 2 μl, 4 μl of 80 μl of nascent DNA isolated from 20 μg of total nucleic acids of NSF cells, respectively; **lanes 3 and 4:** 2 μl, 4 μl of 80 μl of nascent DNA isolated from 20 μg of total nucleic acids of HeLa cells, respectively.

on alkaline 1.2% agarose gel in 50 mM NaOH and 1 mM EDTA. The gel was neutralized in 1x TBE buffer, and molecular markers were stained with ethidium bromide. The gel with nascent DNA was agitated for 10 min in 0.25 N HCl. The nascent DNA was denatured by soaking the gel in 0.4 M NaOH for 30 min. The denatured DNA was vacuum-transferred onto a membrane (GeneScreen Plus, Dupont) for 90 min at 50–60 mbar (Vacu Gene Pump, Pharmacia LKB). The membrane was washed in excess 2x SSC for 1–2 min, and then air dried. Before prehybridization, the membrane was briefly wetted in 2x SSC. Probes were prepared by nick translation (Nick Translation Kit, Gibco BRL) from 500 ng of sheared total genomic DNA of G0 NSF cells, and were purified through a Nick Column (Sephadex G-50, Pharmacia Biotech.). The membrane was prehybridized for 30 min at 42°C in 10 ml of prehybridization buffer [1.0% sodium dodecyl sulfate (SDS), 2x SSC, 10% dextran sulphate, 50% deionized formamide]. One milliliter of probe solution ($\sim 10^7$ cpm and 500 μg of sheared herring sperm DNA) was added to the prehybridization solution, and the blot was hybridized overnight at 42°C. The membrane was washed in excess 2x SSC for 10 min at room temperature, then washed twice for 20 min at 42°C in 2x SSC, 1.0% SDS. Finally, the membrane was washed twice for 20 min at 42°C in 0.2x SSC, 1.0% SDS; the blot was air dried and exposed onto Fuji Image Plate for 1–6 h. The hybridization signals were visualized with Fujix Bio-Imaging Analyzer (BAS 2000, Fuji Photo Film Co. Ltd).

Competitive PCR

PCR conditions were as described previously [Tao et al., 1997]. The primer sequences and their locations are listed in Table I. The abundance (DNA molecular copies) characterized by primer set 11 was estimated by competitive PCR in 5 μl of a series of 10-fold scalar dilutions of nascent DNA samples [Kumar et al., 1996]. The dilution of nascent DNA sample selected and used for the measurements of nascent DNA abundance over the *c-myc* locus was that providing several thousands of copies for the PCR characterized by primer set 11. The relative nascent DNA abundance distribution is indicated by the molecular copies of nascent DNA strands at each site in 5 μl of the selected dilution of the nascent DNA sample.

TABLE I. Primers (5′ → 3′) Used for PCR Amplifications[a]

Primer	Primer sequence	Location
1f:	TAACAGCCATAGGTAGTGG	147-165
1r:	GGTATCTCATGACCTCCAA	544-562
2f:	AAACAACCAAGGGTGAGCTA	637-656
2r:	CAACCCTGCCTTACTTTGTA	998-1017
3f:	GGAACCATTTGTCCACTCTA	973-992
3r:	CGGCATCACTAATTCATAGG	1329-1348
4f:	TTCACTGCCGTTTTTGTTATC	1307-1325
4r:	TACTGAGTTGGTCCTCTAGT	1687-1706
5f:	TCAGCCATTTTTCAACCCAG	3395-3415
5r:	AACCCTCAACAACAAACAAAC	3773-3793
6f:	ATGAGGTCAAGCTGGACCT	3733-3751
6r:	GAAGGGGTATTTGAGATCTC	4109-4128
7f:	AGCTTGTTTGGCCGTTTTAG	1-20
7r:	GCGTTCAGGTTTGCGAAAG	389-407
8f:	GATGATCTCTGCTGCCAGT	351-369
8r:	TCTTCTCATCCTTGGTCCC	725-743
9f:	TTGTGAGTCAGTGAACTAGGb	947-966
9r:	CGCCGGTTTGCAACAGTC	1334-1351
10f:	TACAGACTGGCAGAGAGCA	1442-1460
10r:	CGTGGGATGTTAGTGTAGA	1801-1819
11f:	ATACGTGGCAATGCGTTGCT	1721-1740
11r:	GAGCCGCATGAATTAACTAC	2105-2124
12f:	TTCTCAGAGGCTTGGCGG	2411-2428
12r:	GCCTTTCAGAGAAGCGGG	2812-2829
13f:	ATGCTGAGATGAGTCGAATG	2930-2950
13r:	CAAGTCAGCGGCTGCGGA	3312-3329
14f:	CCGGGAGGGCATTTAAATTT	3339-3358
14r:	CCAAGGGTAGCAGCTGTTC	3715-3733
15f:	CAAGCCGCTGGTTCACTAA	4279-4297
15r:	AGCAGCTCGAATTTCTTCCA	4668-4687
16f:	GAGCTTCTCCACGGCCGA	4793-4810
16r:	GTCGAGGAGAGCAGAGAAT	5182-5200
17f:	TAACGGGCCACTCTTATTAG	5351-5370
17r:	CTGCCTAGCCTCTAAACAC	5725-5744
18f:	CTGGGATCTTCTCAGCCTA	5801-5819
18r:	ACCCTTACTCCTCTCACCA	6188-6206
19f:	TCCAGAGACCTTTCTAACGT	6541-6560
19r:	GGGCTGGTGCATTTTCGG	6913-6930
20f:	CTGGCAAATATATCATTGAGC	7662-7682
20r:	GACTGGCACTGGTTAACTAA	8037-8056
21f:	TCTCAACATCTAAGCCTGGT	8700-8719
21r:	GAACAGCAATAGCATCCTTC	9086-9105
L48f2:	ACTTTCATTTCAGAGATTCGG	3850-3870
L48r2:	GCGTCTATACAGCGTGTTG	4222-4240

[a]Primer sets 1–21 were used for measurements of nascent DNA abundance in the *c-myc* locus. The location numbers of primer sets 1–6 are from GenBank Accession Number AF176208. The location numbers of primer sets 7–21 are from GenBank Locus HUMMYCC (Accession Number J00120).

RESULTS

Sequence Analysis of the *c-myc* Locus

A contig sequence of 4,638 nucleotides from the EcoRI to Hind III site to the human *c-myc* gene (Fig. 1) was obtained from the alignment of overlapping sequences; each sequence was obtained from at least three independent de-

terminations (GenBank accession number: AF176208). An Internet Server (http://www.girinst.org) localized the repetitive sequences. Five *Alu* sequences as well as other repetitive elements such as MER, MIR, and LIMD2 [Kariya et al., 1987; Donehower et al., 1989; Kaplan et al., 1991; Smith et al., 1995; Smith and Riggs, 1995; Schmid, 1998], are present in the 15.5-kb *c-myc* locus shown in Figure 1.

Unique Molecular Competitors

To facilitate the determination of nascent DNA abundance using a great number of competitors, we prepared unique molecular constructs, each of which contained 10 or more PCR competitors (Fig. 2a). Primer sites were chosen to avoid repetitive elements, as indicated in Fig. 1 (arrows) and Table 1. The PCR products of all of the primer sets include no repetitive elements, except for primer pair 1 that spanned a MER20@2 repetitive element. Primer sets 2, 14, 15, 20, and 21 are near repetitive elements MER20 @2, MER45C@1, and MIR@2, respectively, and primer sets 5, 6, 7, 18, and 19 are close to *Alu* repetitive elements. Three unique molecular constructs I, II, and III were created for this study (Fig. 2b). The competitor constructs I, II, and III were purified from agarose gels, and their concentrations (copies per microliter) were determined by spectrophotometric measurements. We next determined the relative PCR amplification efficiencies of the primer sets for the genomic targets and competitors. Equimolar amounts of the unique molecular constructs I, or II, or III and plasmid pMYC/EX7 or pMYC/HE8 (Fig. 1) were amplified with each set of primers. The results (Fig. 2b) indicate that the PCR amplification efficiencies between targets and competitors varied with different pairs of primers, although the primer sequences on target and competitor were identical. Therefore, it is necessary to correct those PCR amplification efficiencies between target and competitor for each primer set. To correctly determine the relative DNA abundance with those primer sets, any primer set may be chosen as a reference standard. Here, primer set 9 was taken as a reference standard, and thus the correction factor for primer set 9 was assigned as 1.00; the other primer sets were then normalized to primer set 9. The correction factors for all the primer sets ranged from 0.46 ± 0.08 to 2.43 ± 0.18 (mean ± SD). For example, the correction factor of primer set 7 is 1.36 ± 0.16, and the final relative abundance was determined by multiplying the copy number, determined by the competitor of one of the unique molecular constructs, by the efficiency correction factor of 1.36. Constructs I, II, and III all contain primer set 9; therefore, primer set 9 was used for correction of the variation in amplification efficiencies of targets and their competitors of all the other primer sets.

To confirm the accuracy of the quantitation, total DNA prepared from NSF cells at G0 phase was used to measure the genomic DNA abundance at the locations of each of the primer sets, and the copy number per haploid genome at all of the testing sites was calculated. The result (Fig. 1) indicates that the copy number at each of the sites was consistent with an overall average of 1.00 copy/haploid genome with a standard deviation of 0.117. Therefore, it is possible to measure DNA abundance reliably with these competitors. Figure 4 shows examples in which the number of DNA strands was determined by competitive PCR using primer set 9 and a total genomic DNA sample from G0 NSF cells (Fig. 4a) or a preparation of nascent DNA from NSF cells (Fig. 4b).

Nascent DNA Preparation and Quality Assurance

In recent years, we and others have reported simplified methods for nascent DNA preparation [Kumar et al., 1996; Tao et al., 1997], which avoid the risk of contamination and strand breaking that attend antibody purification of bromodeoxyuridine (BrdU)-labeled nascent DNA [Vassilev and Johnson, 1990]. These methods were mainly based on size selection of nascent DNA strains by sucrose gradient centrifugation or by gel electrophoresis, omitting BrdU labeling. In our approach, nascent DNA strands were extruded from replication bubbles, and the small nascent DNA strands were isolated by sucrose gradient centrifugation. Previously, this method was shown to yield good-quality nascent DNA like that of the BrdU labeling approaches [Tao et al., 1997]. We also used this simplified strategy for the preparation of nascent DNA in this study (Fig. 3a). The size range of the nascent DNA sample was 0.3–1.3 kb, and the peak of the nascent DNA size distribution was 0.6–0.7 kb, as previously published [Tao et al., 1997]. The ratio of

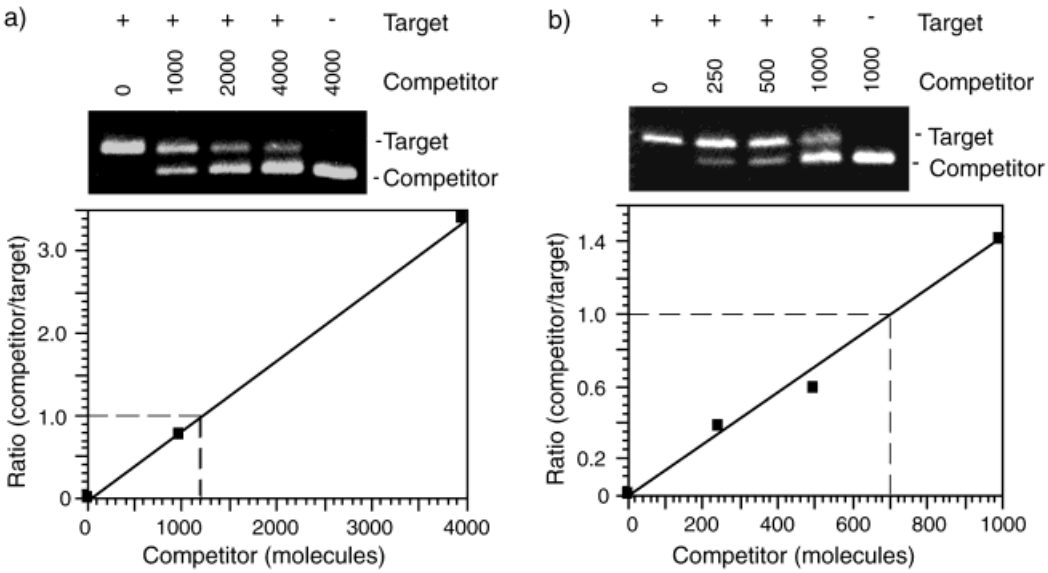

Fig. 4. Competitive PCR measurement. The number of target DNA strands characterized by primer set 9 was determined in (**a**) a total genomic DNA sample from G0 NSF cells and (**b**) a λ exonuclease digested nascent DNA sample from NSF cells at 30%–50% confluence. Target DNA in the same volume of DNA sample and different amounts of competitor were coamplified with Taq polymerase. The amplified products of targets and competitors were resolved on agarose gel. The bands of amplified targets and competitors were scanned and quantified using Bio Image (Millipore) hardware and software. The ratio of competitors and targets was plotted against competitors. The linear curve represents the best-fitted line (linear regression) for the data. When the ratio is 1, the number of the targets is equal to that of the competitors. In these examples, 1,200 target DNA strands were measured as present in the genomic DNA sample from (a) G0 NSF cells and 700 nascent DNA strands in a nascent DNA sample prepared from NSF cells at 30%–50% confluence.

the highest abundance to the lowest was close to 10 (Fig. 5a,b), which is similar to that obtained by other methods [Kumar et al., 1996].

Recently, Gerbi and Bielinsky [1997, 1998, 1999] used λ exonuclease in preparation of nascent DNA, and successfully mapped the DNA replication initiation points on the SV40 viral genome and on a yeast chromosome. The key point of this nascent DNA preparation strategy is that 5′ phosphorylated parental and broken DNA strands can be recognized and digested by λ exonuclease, but the nascent DNA strands with 5′ RNA primers are resistant to this enzyme. The majority of newly synthesized DNA contains 5′ RNA primers, except those nascent DNA from which the RNA primers were removed immediately before the newly synthesized Okazaki fragments were ligated. Any RNA primer loss before phosphorylation will result in the digestion of nascent DNA by λ exonuclease. Therefore, the nascent DNA strands in the total cellular DNA population still contains 5′ RNA primers if RNA degradation by enzymatic and chemical hydrolysis is avoided during the preparation procedures. To maximize the yields of nascent DNA treated with λ exonuclease, care was taken to avoid RNA degradation before the phosphorylation. The procedure for a modified nascent DNA preparation with λ exonuclease digestion is indicated in Fig. 3a. Total cellular DNA (together with total RNA) was isolated and then was sheared with a fine needle. In this manner, parental DNA and the longer nascent DNA was sheared, but short nascent DNA was unlikely to be sheared. The average size of the sheared DNA was approximately 20 kb, which was seen as a band on agarose gel; the smear of lower molecular weight was an RNA population (Fig. 3b, lane 2). The sheared DNA without RNA primers was removed by digestion with λ exonuclease, leaving RNA and RNA-primed nascent DNA. Therefore, the nascent DNA sample would ideally contain only shorter nascent DNA strands. The nascent DNA might also contain Okazaki fragments with an average size of 250 nucleotides, which, however, should not be amplified, because the PCR product sizes were ~400 bp.

Phosphorylation and the λ exonuclease digestion were monitored by an internal control of 500 ng of dephosphorylated linear pUC18

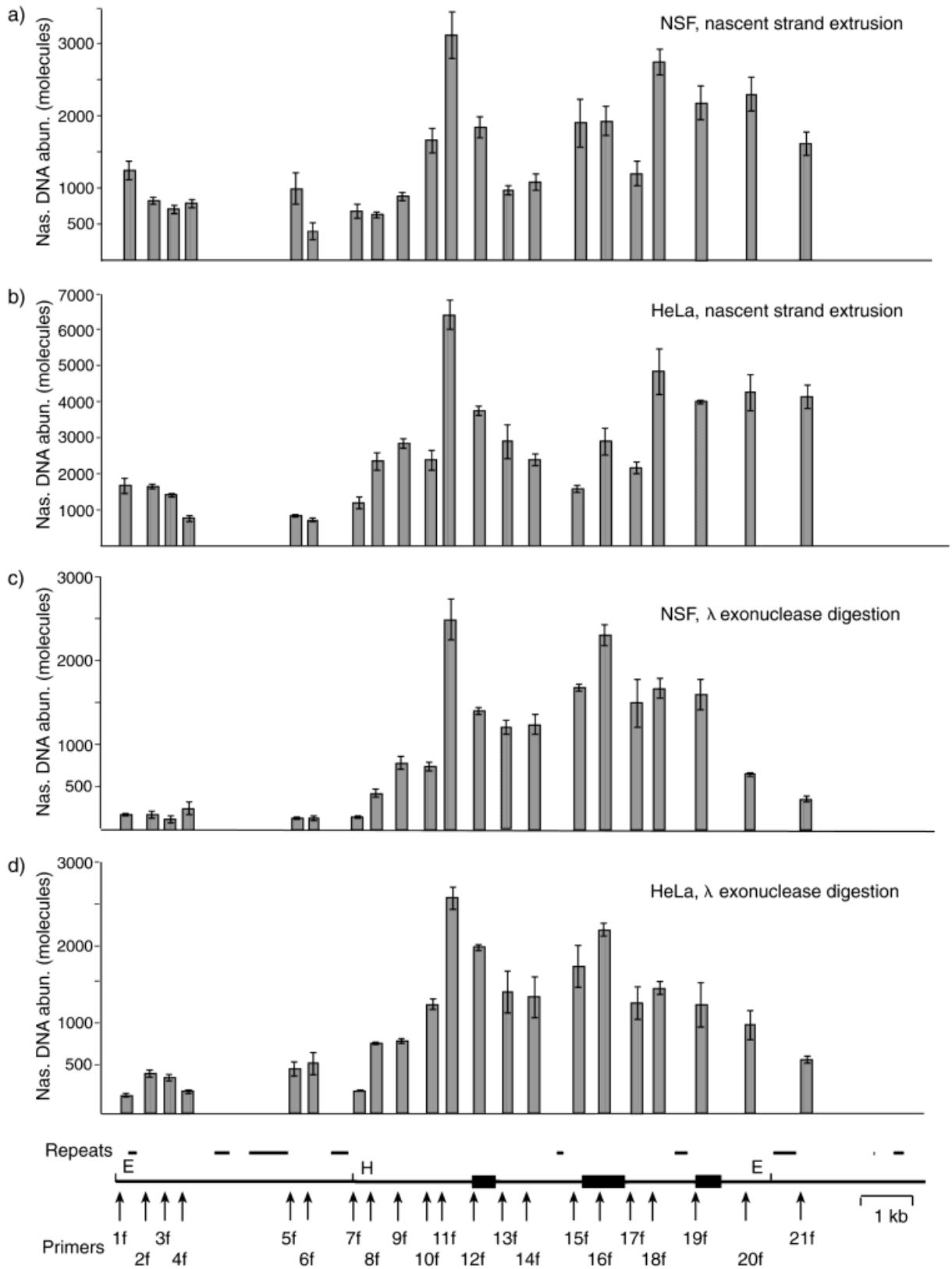

Fig. 5. Nascent DNA abundance at *c-myc* locus in nascent DNA samples prepared with nascent strand extrusion and with λ exonuclease digestion from NSF and HeLa cells. **a:** Nascent DNA sample prepared with nascent strand extrusion from NSF. **b:** Nascent DNA sample prepared with nascent strand extrusion from HeLa. **c:** Nascent DNA sample prepared with λ exonuclease digestion from NSF. **d:** Nascent DNA sample prepared with λ exonuclease digestion from HeLa. The nascent DNA abundance at each position in each nascent DNA sample was measured by competitive PCR at least three times. Those with apparent peak abundance were tested four or five times. The abundance levels are indicted as the averages (±SD). At least two nascent DNA samples were prepared with the same method from each cell type. We show the result from one nascent DNA sample here. No significant variations from this distribution were seen in the other samples.

[Kobayashi et al., 1998] (Fig. 3b, lane 4). Under the conditions used, the plasmid DNA was completely phosphorylated, and digested by λexonuclease (Fig. 3b, lane 4 versus lane 5), and residual RNA (Fig. 3b, lane 5) was removed by RNase A digestion (Fig. 3b, lane 6). The nascent DNA samples were visualized by Southern blot, using probes made from total genomic parental DNA (Fig. 3c). The size distribution of these nascent strands corresponded to the size distribution of nascent strands prepared by the nascent strand extrusion method. The majority of DNA strands in the nascent DNA samples were distributed from 400 to 600 nts (Fig. 3c). Nascent DNA strands over 1,300 nts were 2%–7% of all nascent DNA populations. In brief, there was no significant contamination of parental DNA in the nascent DNA sample.

Nascent Strand Abundance Across 12.5 kb of the *c-myc* Locus

We first used the nascent DNA samples prepared by nascent strand extrusion to measure nascent DNA abundance from NSF and HeLa cells in the *c-myc* locus. The distributions of the nascent DNA abundance over ~12.5 kb of the *c-myc* locus in the two cell types, as measured by quantitative competitive PCR, are shown in Figure 5a,b. In both cell types, the highest abundance was located at the position of primer set 11. In addition, several other sites (e.g., Fig. 5a, primer sets 15, 16, 18, 19, and 20; Fig 5b, primer sets 18, 19, 20, and 21) gave evidence of apparently high abundance, possibly indicative of other initiation sites in this region. In accordance with previous studies using abundance of nascent DNA strand measurements [Kumar et al., 1996], the ratio of highest to lowest abundance in the region to be studied is given as indicative of quality of preparation and signal to noise estimates. The ratio of highest abundance at primer set 11 to the lowest abundance at primer set 6 were 8.0 and 9.5 for NSF and HeLa, respectively.

To reduce the potential of broken, nonreplicative DNA strands contributing significantly to these results, we also used nascent DNA samples prepared by the λ exonuclease digestion procedure [Gerbi and Bielinsky, 1997; Kobayashi et al., 1998], which was greatly simplified in this study (Fig. 5c,d). (Fig. 4b shows an example of quantitative competitive PCR analysis using nascent DNA samples prepared using λ exonuclease digestion.) The results from nascent DNA samples prepared by the two different methods were consistent, except that the nascent DNA abundance was lower at primer sets 1, 20, and 21 in nascent DNA samples prepared with λ exonuclease digestion from both cell types. Repeatedly, the highest nascent strand abundance was localized at primer set 11 in both cell types. The ratios of the highest abundance at primer set 11 to the lowest abundance at primer set 7 was 16.7 in NSF and 9.8 in HeLa. An apparent peak of abundance was also revealed at primer set 16, whereas very low abundance at primer sets 5, 6, and 7 in both cell types was again observed (Fig. 5c,d). In the nascent DNA samples treated with λ exonuclease, the abundance at primer sets 20 and 21 was significantly lower, suggesting that these primer sets were not indicative of initiation sites. No initiation event at positions corresponding to primer sets 1 to 7 in NSF cells was indicated.

The results from the two different methods indicated that λ exonuclease digestion treatment may be a better way to prepare nascent DNA sample. The nascent strand extrusion method was mainly based on size selection. However, the nascent DNA may be contaminated with small DNA fragments from nonreplicative regions, such as the region with primer sets 1, 20, and 21, which contain repetitive elements. If contaminating repetitive fragments have the same flanking sequences as present in primer sets 1, 20, or 21, then the small fragments from these particular regions may contribute to the nascent DNA abundance measured with those primer sets. Fortunately, this kind of problem can be greatly reduced by λ exonuclease digestion. Therefore, we have analyzed mainly the nascent DNA abundance distribution data obtained by the λ exonuclease digestion method.

It is also true that during the λ exonuclease digestion treatment, DNA fragments from nonreplicative regions might not be completely eliminated if not all 5′ ends of DNA fragments without RNA primers were phosphorylated. Such DNA fragments, however, would not be enriched for any specific locus. In addition, there is also the possibility that there are a few longer nascent DNA fragments still retaining RNA primers at their 5′ ends. However, we have also done size selection of 300 nts-1.3 kb after digestion with λ-exonuclease, and the rel-

ative abundance of nascent DNA detected using different primer sets was unchanged. These limitations of λ exonuclease digestion treatment may contribute to abundance levels at the primer sets 1–7 in Fig 5c and at primer sets 1, 4, and 7 in Figure 5d, but the contributions are not likely to be meaningful.

Two Major Replication Initiation Sites in the *c-myc* Locus

The abundance of defined markers within the origin region is greatest at the sites where DNA replication begins [Giacca et al., 1997]. Kobayashi et al. determined the DNA replication initiation frequencies according to distribution of short nascent DNA strand abundance [Kobayashi et al., 1998]. The highest peak of nascent DNA abundance was at the position characterized by primer set 11, and the second peak was characterized by primer set 16. Therefore, the position at ~0.5 kb upstream of exon 1 is now defined as a major initiation site in the *c-myc* locus, which was mapped with nascent DNA samples prepared by two different methods from both cell types (Fig. 5a–d). The second new major initiation site, with 85%–90% as much nascent strand abundance (initiation frequency) as the first major site, is in exon 2, mapped in both cell types with the λ exonuclease digestion method (Fig. 5c,d). Waltz et al. [1996] confirmed that DNA replication initiates nonrandomly at multiple sites near the *c-myc* gene in HeLa cells by nascent strand polarity mapping. There were three initiation sites in the 2.4-kb region upstream from exon 1, located approximately at 1–498, 499–1,271, and 1,612–2,227 (GenBank Locus: HUMMYCC). The 1,612–2,227 site corresponds to one of our major initiation sites at ~0.5 kb upstream of exon 1.

Vassilev and Johnson [1990] mapped an initiation zone centered at ~1.5 kb upstream of exon 1 in the *c-myc* locus in HeLa cells by measuring the nascent strands lengths using PCR (Fig. 6c, line 3). This initiation site has also been predicted by others (Fig. 6c, line 2 [Waltz et al., 1996]). The region centered at ~1.5 kb upstream of exon 1 is defined by our primer set 9. For nascent strand DNA prepared by nascent strand extrusion from HeLa cells (Fig. 5b) or prepared by λ exonuclease digestion from NSF cells (Fig. 5c), a small peak of abundance was observed with primer set 9. For nascent strand DNA prepared by nascent strand extrusion from NSF cells or prepared by λ exonuclease digestion from HeLa cells, no such peak was observed (Fig. 5a,d). To the extent that this position corresponds to an initiation site, it is a minor initiation site (Fig. 5b,c) that was not detected in some nascent DNA samples (Fig. 5a,d) because of a lower initiation frequency. There may be other possible initiation sites, but with lower frequencies in this region, for example, at position of primer set 18 (Fig. 5c,d).

Higher Activities of Origins in the 12.5-kb of *c-myc* Locus in HeLa and NSF Cells but With Similar Initiation Frequency Pattern

We previously reported that the abundance of nascent DNA near the *c-myc* replication origin was increased in transformed cells (HeLa) approximately twofold versus normal cells (NSF) [Tao et al., 1997]. Here, we have quantitatively compared the detailed distribution patterns of nascent DNA abundance over the 12.5 kb of *c-myc* locus in NSF and HeLa cells. We again used an internal standard to normalize the origin activities measured. Any variation in nascent DNA preparation was minimized by using an internal standard of nascent DNA abundance measured at another origin.

The *c-myc* locus, located on chromosome 8, is a single copy per haploid genome in HeLa cells. The ratio of chromosome 8 DNA to total DNA of aneuploid HeLa is similar to that of normal cells [Popescu et al., 1987; Lazo et al., 1989]. Our quantitative PCR data showed that the ratio of copy numbers at the *c-myc* locus (relative to the lamin B2 locus) in HeLa and NSF cells was 1.22 ± 0.07, which confirmed that the copy numbers of the *c-myc* locus per haploid genome in HeLa and in NSF cells (relative to lamin B2 locus) can be viewed as identical. We chose the activities of the origin associated with lamin B2 to normalize the measurements of nascent DNA abundance in the *c-myc* locus. Lamin B2 origin lies in a constitutively expressed gene domain, coding for a housekeeping protein [Biamonti et al., 1992; Kumar et al., 1996]. This origin use has been observed in proliferating human cells: IMR90 (primary human embryonic lung fibroblasts), SKNMC, HeLa, IMR32, HL-60, SKNBE [Kumar et al., 1996], and NSF (this study). Finally, as will be demonstrated, the internal standard sequence associated with the lamin B2 origin gives similar ratios of normalized nascent DNA abun-

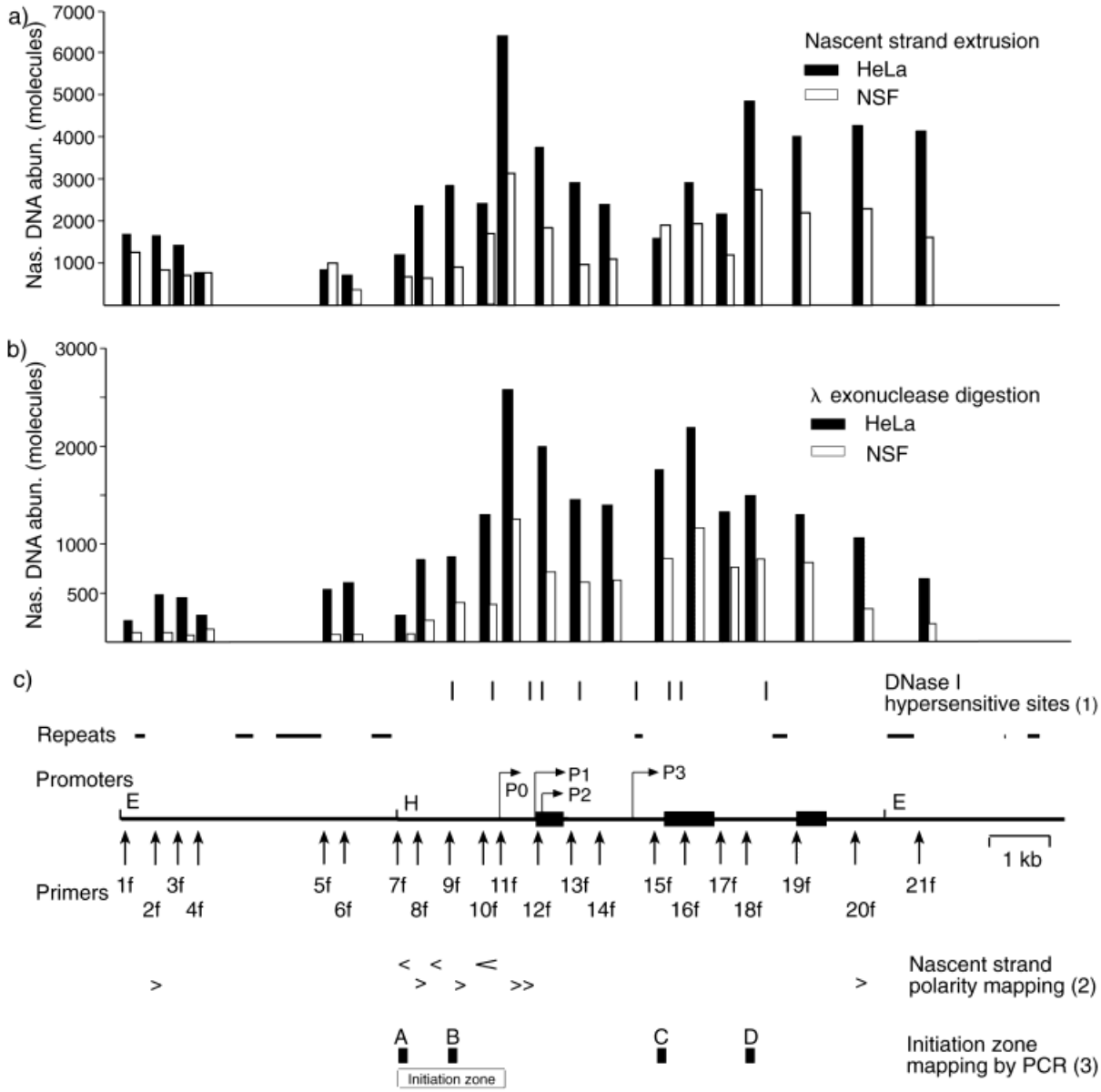

Fig. 6. Comparisons of origin activities over the **c-myc** locus in NSF and in HeLa. The nascent DNA abundance levels at the *c-myc* locus were normalized to the nascent DNA abundance at the origin associated with lamin B2. **a:** Normalized nascent DNA abundance levels in Figure 4a and b, which were the nascent DNA samples prepared with nascent strand extrusion method from NSF (white bars) and HeLa (black bars) cells. **b:** Normalized nascent DNA abundance levels in Fig. 5c and d, which were the nascent DNA samples prepared with λ exonuclease digestion from NSF (white bars) and HeLa (black bars) cells. **c:** Line 1: DNase I hypersensitive sites in the *c-myc* locus [Mautner et al., 1995]. Line 2: The direction of replication forks determined by nascent strand polarity mapping [Waltz et al., 1996]. < or > indicates the left or right direction of a DNA leading strand; bidirectional replication origins are located between < and >. Line 3: The DNA replication initiation zone mapped by initiation zone mapping using PCR with primer sets A, B, C, and D [Vassilev and Johnson, 1990]. P0, P1, P2, and P3 are four promoters of the *c-myc* gene [Mautner et al., 1995].

dance in the *c-myc* locus in HeLa and NSF cells as using sequence associated with human β-globin gene [Tao et al., 1997].

Primers L48f2 and L48r2 (Table I) were chosen to measure nascent DNA abundance at origin lamin B2 in nascent DNA samples prepared from NSF and HeLa with the two different methods. Normalized to the nascent DNA abundance at the origin lamin B2 as an internal standard, the nascent DNA abundance dis-

tribution across the *c-myc* locus in NSF and HeLa can be compared (Fig. 6a,b). Normalized abundance values over most of the region, e.g., at positions indicated by primer sets 9, 11, 16, and 18 in nascent DNA samples prepared with nascent strand extrusion from HeLa, were 3.2-, 2.1-, 1.5-, and 1.9-fold higher than those from NSF, respectively. Similarly, normalized abundance values at primer sets 9, 11, 16, and 18 in nascent DNA samples prepared with λ exonuclease digestion from HeLa were 2.1-, 2.0-, 1.7- and 2.1-fold higher than those from NSF, respectively. These data are consistent with our previous results, in which an approximately twofold as high abundance for a single primer set 9 in the *c-myc* locus in HeLa versus NSF cells was detected [Tao et al., 1997]. The data indicate that multiple initiation sites appear coordinately more active (across the entire locus) in HeLa cells than in NSF cells.

DISCUSSION

Major Initiation Sites in the Replication Initiation Zone in the Human *c-myc* Locus

In previous studies, a limited number of unevenly distributed probes were used to map the initiation sites at the *c-myc* locus [Vassilev and Johnson, 1990; Trivedi et al. 1998]. Origin activities at the potential initiation sites in the *c-myc* locus were not quantitated [Waltz et al., 1996; Trivedi et al., 1998]. In this study, it was possible to view the replication initiation features by using 21 sets of primers covering most of the 12.5-kb region of the *c-myc* locus, including 4.6 kb of the new 5′ sequence. Two major initiation sites were observed in this more detailed initiation map, one ~0.5 kb upstream of exon 1 and a second in exon 2. The initiation site at ~0.5 kb upstream of exon 1 had also been previously suggested by nascent strand polarity mapping [Waltz et al., 1996]; however, we are able to identify it as one of two major initiation sites among many potential initiation sites. Furthermore, the fragment containing this site had been demonstrated to possess autonomous replication activity [McWhinney et al., 1995]. The second major initiation site in exon 2 that is observed in this study is novel. Using two different methods of preparation of short nascent DNA, the two major initiation sites were present in both HeLa and NSF cells, further supporting a conclusion that the major initiation sites at the *c-myc* locus are nonrandom. Only one major initiation site is located upstream of exon 1, and it is not located in the initiation zone mapped by Vassilev and Johnson [1990], but is located nearby the zone in the 3′ flanking region. However, Vassilev and Johnson [1990] only used four sets of primers (Fig. 6c); none of them are located at the major initiation sites identified in this study. According to the nascent DNA abundance distribution in our studies, it can be seen that there appeared to be no significant initiation event in the upstream region spanned by the primer sets 1–7 (Fig. 5c,d). However, the nascent DNA abundance downstream from primer set 7 (near the restriction enzyme HindIII site) was lower than that at the two major initiation sites, but higher than the levels in primer sets 1–7 in both cell types. There may be some other nondominant initiation sites in this region; a few such sites in the 2.4-kb region upstream of exon 1 have been described (Fig. 6c, line 2 [Waltz et al., 1996]). Chromatin structure can inhibit both promoter and origin activities by blocking access of initiation factors to specific DNA sites [for review, see DePamphilis, 1999]. It should be noted that the two major initiation sites are close to DNase I hypersensitive sites (Fig. 6c, line 1). Furthermore, both major initiation sites are located in proximity to the four promoters P0, P1, P2 and P3 (Fig. 6c).

Identification of the major initiation sites is important to future studies of replication initiation and its regulation in higher eukaryotic cells. A DNA replication initiation zone may consist of one or more higher frequency initiation sites. It has been proposed that the lower frequency sites may represent other accessible initiation sites and may be related to structural features like DNA unwinding elements (DUEs), and the higher abundance sites may be associated with strong ORC binding sites adjacent to a DUE [review, DePamphilis, 1999].

Origin Activities in Transformed Cells

Our previous work [Tao et al., 1997] showed that the origin activities, determined by the nascent DNA abundance at the same site as characterized by primer set 9 in this study, was about twice as high in HeLa cells as in NSF. Here, the results shown in Figure 6a,b indicate that the nascent strand abundance at each of the multiple initiation sites at the *c-myc* locus in HeLa cells was about twice as high as those

in NSF. One of the possible reasons for the differential origin activities might be the alternation of the activities of the multiple initiation sites at this *c-myc* locus, which would be indicated by significant, nonuniform changes in the nascent DNA abundance distributions between HeLa cells and NSF cells. Therefore, we determined the nascent DNA abundance at each site over the entire region. There was no significant alternation in the overall distribution pattern of the nascent DNA abundance in these two cell types. This result excludes the possibility that there are changes in the initiation frequencies at some initiation sites relative to others in this region.

The consistent, approximate twofold difference in nascent strand abundance detected at all sites in the *c-myc* locus in NSF and HeLa cells suggests that the multiple initiation sites in the *c-myc* locus may be coordinated by common regulatory elements. In this regard, it is of interest that recent results have shown that the *c-myc* 2.4-kb HindIII/XhoI fragment is capable of inducing the initiation of replication at multiple sites in the flanking chromosomal sequences when integrated at an ectopic location [Malott and Leffak, 1999].

The human *c-myc* locus is neither rearranged nor amplified in HeLa cells [Lazo et al., 1989]. We propose here that at least two sorts of cellular behaviors may be reflected by differential origin activities. One is that some origins are fired more than once; however, the repeated initiations would have to be aborted to correctly replicate the whole genome for a cell. Another possible reason for the differential activities may be that this initiation region containing multiple initiation sites may not always be used in some somatic cells like NSF, but are used in a higher percentage of HeLa cells. The DNA in the *c-myc* region of NSF cells could be replicated by origins flanking the *c-myc* region.

These two possibilities require different regulatory parameters: 1) If the amounts of initiation machinery proteins, such as ORC proteins or replication licensing factor (RLF), are expressed more in certain cells, the initiation frequencies of the origins in these cells may be increased. However, the experimental results indicated that at least RLF does not specify replication initiation sites in higher eukaryotes [Wu and Gilbert, 1997]. 2) Increased effects of the trans-acting regulatory factors, such as transcription factors, may facilitate the origin initiation activities. A general phenomenon has been observed, namely, that an origin is fired earlier in S phase if it is located in the transcribed region of an active gene [Michaelson et al., 1997]. It has also been reported that DNA replication was induced by transcription in the region upstream of the human *c-myc* gene in a model replication system in vitro [Ohba et al., 1996]. On the other hand, transcription of either the *DHFR* or *2BE2121* genes did not correlate with the origin decision point (ODP), and deletion of the *DHFR* gene promoter had no effect on the frequency of initiation at *ori*-β [DePamphilis et al., 1998]. Moreover, E2F-dependent transcription of the *DHFR* gene is not linked to initiation of DNA replication at *ori*-β [Illenye et al., 1998]. We observed that the *c-myc* transcript level, determined by reverse transcriptase-PCR, in HeLa cells was about twice as high as that in NSF cells (data not shown), but we have no evidence yet to answer whether there may be a functional association of the levels of transcription and replication in the *c-myc* locus. Recently, Wei et al. [1998] observed segregation of transcription and replication sites into higher order domains. They found that the individual sites of DNA replication and transcription are predominately, spatially distinct during all periods of the S phase; more than 95% of the replication sites activated early in the S phase do not coincide with transcription sites. They suggested that the interdigitation of replication and transcription domains in three dimensions might be an indication of dynamic cross-talk between the replication and transcription domains or temporal transitions from one functional state to the other. 3) The apparent association of DNA replication and gene transcription may be affected by regulatory factors at higher levels, such as those factors controlling chromatin structures and nuclear organization. It has been suggested that the difference in replication of the IgH locus in different transcriptional states (in pre-B and non-B cells) may provide a model for studying how changes in replication organization affect DNA loops associated with the nuclear matrix [Ermakova et al., 1998, 1999]. Chromatin structure generally represses DNA replication and gene transcription [Aladjem et al., 1995; Andrin and Spencer, 1994]. Histone H1 can reduce the frequency of initiation in *Xenopus* egg extract by limiting the assembly of prerep-

lication complexes on sperm chromatin [Lu et al., 1998]. H1 levels are lower in transformed cells, and more phosphorylated H1 is found in the G1 phase of transformed cells [Laitinen et al., 1995], resulting in less condensation of the chromatin structures. Therefore, the relaxed chromatin structures may contribute to increased origin use in transformed cells. Some nuclear structure is required for the metazoan DNA replication [Blow and Sleeman, 1990], wherein origins are activated on the nuclear matrix [Hozak et al., 1993]. However, a recent study concluded that although nucleoskeleton attachments may facilitate either the assembly or activity of replication forks in metazoan nuclei, they do not appear to be involved in the initiation of DNA replication [Ortega and DePamphilis, 1998]. It has been observed that alternation in nuclear structure size, shape, and organization accompany cell malignancy [Kamel et al., 1990; Underwood, 1990]. Tumor cells express specific nuclear matrix proteins [Keesee et al., 1996, 1998], which are normally expressed only at earlier developmental stage [Bidwell et al., 1994]. Thus, alternate matrix compositions and structures may facilitate DNA replication in transformed cells.

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