Nucleic Acids Research, 1996, Vol. 24, No. 10 **1887–1894**

DNA replication initiates non-randomly at multiple sites near the c-myc gene in HeLa cells

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Received January 24, 1996; Revised and Accepted March 30, 1996

ABSTRACT

The origin of replication of the c-myc gene in HeLa cells was previously identified at low resolution within 3.5 kb 5′ to the P_1 promoter, based on replication fork polarity and the location of DNA nascent strands. To define the initiation events in the c-myc origin at higher resolution the template bias of nascent DNAs in a 12 kb c-myc domain has been analyzed by hybridization to strand specific probes. Strong switches in the asymmetry of nascent strand template preference confirm that replication initiates non-randomly at multiple sites within 2.4 kb 5′ to the c-myc P_1 promoter, and at other sites over a region of 12 kb or more. The strongest template biases occur in the 2.4 kb region 5′ of the P_1 promoter, shown earlier to contain sequences which allow the autonomous semiconservative replication of c-myc plasmids. An asymmetric pyrimidine heptanucleotide consensus sequence has been identified which occurs 12 times in the c-myc origin zone, and whose polarity exactly correlates with the polarity of nascent strand synthesis.

INTRODUCTION

Molecular analyses of procaryotes, eukaryote viruses and yeast suggest that replication fork structure and enzymology have been conserved through evolution; nevertheless the expectation has not been realized that the replication origins of metazoan cells resemble in structure the compact origins of these simpler organisms (1–3). In complex eukaryotes distinct structural modules might idiotypically promote a single key step in initiation, e.g. DNA unwinding (4,5), followed by initiation at one or more candidate sites. Such a model may be easier to reconcile with the finding of diverse DNA sequences that promote autonomous plasmid replication with varying efficiency (6,7), and observations that replication can begin at numerous alternative sites within broad chromosomal zones (8–18). In this sense, the activation of one chromosomal site for replication initiation among many potential start sites may be opportunistic, and modulated by chromatin structure (19).

Previous studies of replication fork movement in the region of the human c-myc gene suggested the presence of an origin of bidirectional replication (OBR) within 3.5 kb 5′ to the c-myc promoter P_1 (9,20–23). PCR mapping of DNA nascent strands confirmed that replication frequently initiates within a zone of ~2 kb centered ~1.5 kb 5′ to the P_1 promoter (9). Plasmids containing all or specific portions of the c-myc 2.4 kb 5′ region display autonomous, semiconservative replication in transfected cells and in soluble cell extracts (21–25), and replication initiates selectively in the c-myc DNA insert of these plasmids (24,25; S.E.W., A.A.T., and M.L., in preparation).

In the prevailing model for a eukaryotic OBR, continuous leading strand synthesis begins at a single site on each template strand; on the lagging strand short nascent fragments (Okazaki pieces) are synthesized discontinuously on both sides of the OBR with 5′→3′ polarity pointing towards the origin. Convergent patterns of Okazaki piece synthesis have been interpreted as revealing the presence of OBRs near several rodent genes (15,27,28,38). To characterize replication initiation close to the human c-myc gene at increased resolution we have used nascent strand polarity mapping (27) to analyze the template specificity of short nascent strand synthesis through 10 fragments of the origin. The results suggest that replication begins at multiple sites in the c-myc 2.4 kb 5′-flanking DNA, which is part of an initiation zone of 12 kb or more. Within the initiation zone there are several switches of nascent strand template preference, and 12 occurrences of an asymmetric pyrimidine heptanucleotide consensus sequence, 5′-CTTTC(py)(py)-3′. The polarity of this consensus sequence correlates with the polarity of nascent strand synthesis at all 12 positions. In contrast to the prevailing model for the initiation of leading strand synthesis, we propose that there are multiple initiations of short nascent DNAs on the same template strand within the c-myc initiation zone, and that these short nascent strands are precursors of the leading strands which diverge from the origin region.

MATERIALS AND METHODS

Cell culture and preparation of probe DNA from permeabilized cells

HeLa cultures were grown in 15 cm dishes in DMEM with 10% newborn calf serum. Cells (~2×10^8) were synchronized by isoleucine deprivation (45 h) and released into complete medium containing 10 μg/ml aphidicolin (Sigma) for 12 h.

Synchronized cells were released into complete medium (without aphidicolin) for 30 min to allow entry into S phase and replication initiation at the c-myc origin. Synchrony was confirmed by BrdUrd labeling and flow microfluorometry. Cells were washed, permeabilized and labeled with [α-^{32}P]dATP and [α-^{32}P]TTP (27). For

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pulse-chase analysis, the sample was divided into two aliquots. One aliquot (pulse) was mixed with stop buffer (50 mM Tris, pH 7.8; 10 mM EDTA; 0.4 M NaCl; 0.6 % SDS; 0.2 mg/ml proteinase K). To the other aliquot (chase) unlabeled dATP and TTP were added to 0.1 mM. The chase tube was incubated for an additional 30 min and then mixed with stop buffer. DNA was isolated (27) and electrophoresed on 1.8% alkaline agarose gels (50 mM NaOH, 1 mM EDTA) alongside molecular weight size markers: 1 kb and 123 b ladders (BRL), and *Hae*III digested ϕX174 DNA. Gels were 20 × 20 cm and run at 30 V for 17 h. One lane was excised and cut into 1 cm blocks for scintillation counting. Okazaki piece sized DNA (40–300 nt) was eluted using an Elutrap apparatus (Schleicher & Schuell).

Preparation of probe DNA from intact cells

Synchronized cells were washed with warmed medium, and complete medium containing 50 µM BrdUrd was added for 30 min. All manipulations of BrdUrd labeled DNA were performed under yellow light. Cells were washed with cold PBS, trypsinized and pelleted at low speed. The pellet was resuspended in 1 ml PBS and 1 ml 2× lysis buffer (Applied Biosystems) and mixed gently to minimize DNA shearing. DNA was purified on an ABI nucleic acid extractor and redissolved in TE (~500 µg DNA/ml). DNA (40–300 nt) was eluted from alkaline agarose gels using an Elutrap apparatus. Saturated alkaline CsCl (in 50 mM NaOH, 1 mM EDTA) was added to the DNA to a final refractive index of 1.408. Samples were centrifuged (Beckman Ti75 rotor) at 35 kr.p.m. for 72 h at 25°C. Gradients were fractionated from below into 300 µl aliquots. Fractions containing BrdUrd labeled DNA (density 1.82–1.85 g/ml) were pooled. The RI was adjusted to 1.4107 with alkaline CsCl and the DNA was recentrifuged, fractionated and pooled as above. DNA was precipitated, dissolved in TE and labeled with [α-^{32}P]dCTP using a random primer extension kit (Boehringer Mannheim). Control experiments in which λDNA digested with *Hin*dIII was incubated for >1 week with alkaline CsCl showed no significant degradation of the DNA (<1 break/55–60 kb).

Transcription and hybridization

Strand-specific RNAs were transcribed from c-myc DNAs cloned in pGem3zf(–) (Promega). Nucleotides are numbered from the *Hin*dIII site (nt +1) located ~2300 bp 5′ to the c-myc P_1 promoter. The names of the clones, their boundaries, and the polymerase used to transcribe the upper strand of each clone are as follows: pGemEP, nt –4514 to –3914 (SP6); pGemA, nt 1–210 (T7); pGemB, nt 211–498 (T7); pGemC, nt 499–930 (SP6); pGemD, nt 931–1271 (T7); pGemE, nt 1272–1611 (T7); pGemF, nt 1612–1920 (SP6); pGemE/F, nt 1272–1920 (T7); pGemG, nt 1921–2227 (SP6); pGemH, nt 2228–2395 (SP6); pGemSEW, nt 7293–8092 (SP6). Transcription of strand-specific RNAs from templates linearized with *Eco*RI or *Hin*dIII was performed with SP6 or T7 RNA polymerases using an *in vitro* transcription kit (Ambion) according to the manufacturer's instructions. Each reaction contained 1 µl Inhibit ACE (RNase inhibitor; 5 prime→3 prime). Reaction products were digested to completion with RNase-free DNase.

RNA was electrophoresed on denaturing agarose gels (1%) in MOPS buffer (20 mM MOPS, pH 7.0, 8 mM Na-acetate, 1 mM EDTA) with 2.2 M formaldehyde (29). RNA was transferred overnight by capillary action and fixed to Hybond N$^+$ membranes (Amersham) by UV crosslinking. The correct size RNA transcripts

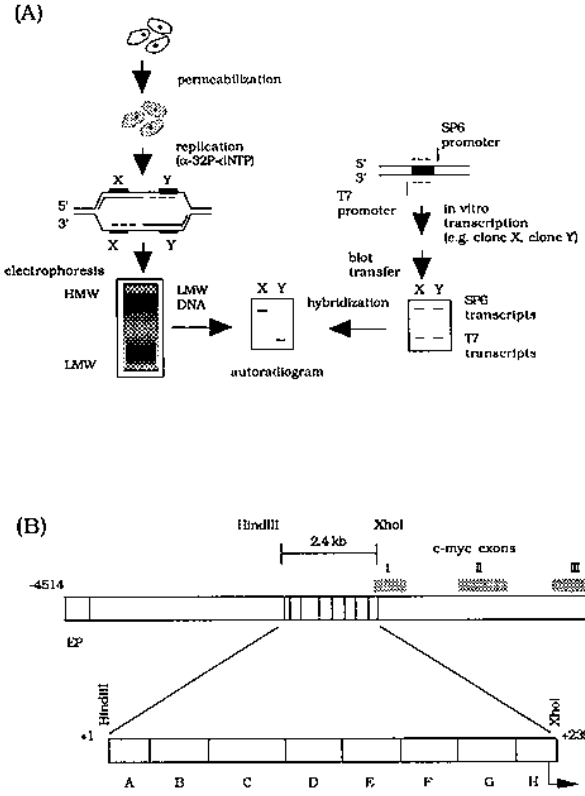

Figure 1. Mapping of an OBR. (**A**) Synchronized cells are permeabilized and allowed to replicate DNA briefly in a replication cocktail including [α-^{32}P]dNTPs, to label Okazaki pieces and DNA leading strands. Total cellular DNA is resolved on alkaline agarose and the low molecular weight fraction containing ^{32}P-Okazaki pieces is isolated and hybridized to RNAs transcribed from separate strands of specific DNA regions (e.g. clone X and clone Y) by SP6 or T7 RNA polymerases. Bias in the hybridization pattern indicates the template for nascent strand synthesis. A switch in the asymmetry of hybridization which indicates the presence of convergently synthesized Okazaki pieces is interpreted to define an OBR. (**B**) Map of the human c-myc locus. The locations of the cloned c-myc DNA fragments used in this study are shown. Restriction fragments (e.g. A) from the c-myc locus were cloned into pGem-3Zf(–) and linearized at either *Eco*RI or *Hin*dIII restriction sites in the polylinker for synthesis of strand-specific RNAs by T7 or SP6 RNA polymerases. The boundaries of transcribed fragments are given in Materials and Methods.

were excised and used in hybridization reactions. Alternatively, the *in vitro* transcription products were directly applied in slots to Hybond N$^+$ membranes. Hybridization of probes (5–10 ml; 1–5 × 10^6 c.p.m.) labeled in permeabilized cells was in 1% BSA, 1 mM EDTA, 0.5 M Na-phosphate, pH 7.2, 7% SDS (30) for 16–20 h at 65°C. Final stringency washes were in 0.2× SSC, 0.2% SDS (65°C, 30 min). Hybridization of random-primed probes was in 50% formamide, 6× SSC (0.9 M NaCl, 0.09 M Na-citrate, pH 7.4), 0.5% SDS, 100 µg/ml sonicated salmon sperm DNA, for 18–20 h at 42°C. Final stringency washes were in 0.1× SSC, 1% SDS (65°C, 30 min). Filters were exposed to X-ray film at –80°C with intensifying screens.

These results are from four independent RNA transcription reactions, six independent labeling reactions of permeabilized cells, and four independent *in vivo* labelings. Hybridization signals were quantitated as the average of three two-dimensional densitometric scans across each RNA band or slot. Relative signal intensities were

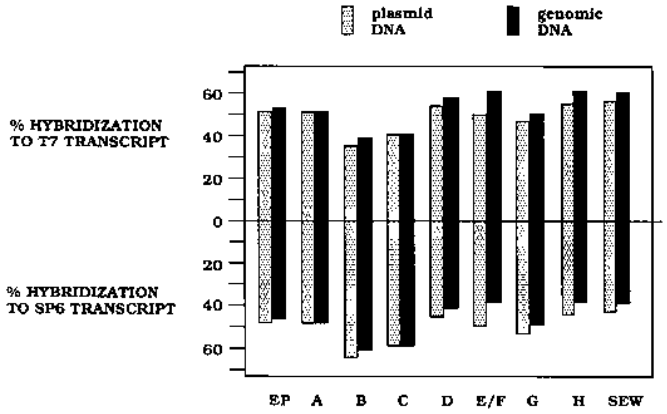

Figure 2. Control hybridizations. RNA transcripts were electrophoresed on agarose gels and transferred to nylon filters. The immobilized RNAs were first hybridized with HeLa DNA labeled by random primer extension, then stripped and rehybridized to cloned c-myc DNA (sequences nt –4514 to nt +8092) labeled by random primer extension. The relative hybridization to the T7 and SP6 transcripts in each pair was normalized to a total of 100%. Data are shown for hybridization of genomic DNA (solid bars) and plasmid DNA (stippled bars).

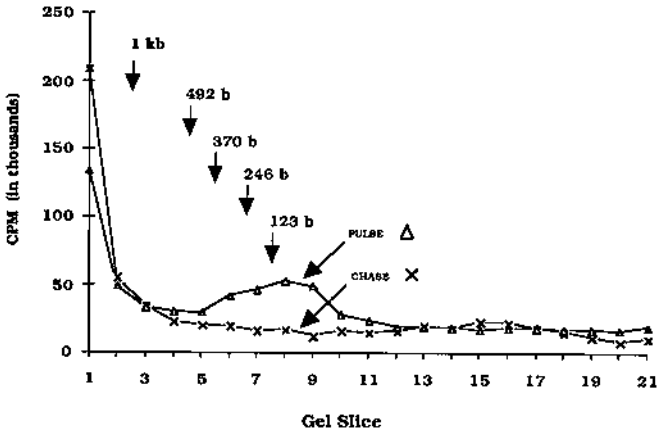

Figure 3. Analysis of short nascent strands synthesized in permeabilized HeLa cells. Synchronized HeLa cells were released into S phase and permeabilized (Materials and Methods). Cells were incubated with [^{32}P]dNTPs for 1.5 min (pulse), or incubated for 1.5 min with [^{32}P]dNTPs followed by 30 min with excess unlabeled dNTPs (chase). DNA was purified, heat denatured and fractionated in alkaline agarose gels alongside low molecular weight DNA markers. Gel lanes were neutralized, sliced into equal fractions, and radioactivity was quantitated by liquid scintillation spectrometry.

confirmed by quantitation of Cerenkov radiation. Results using RNAs transferred from agarose gels (Northern blot) or applied directly to membrane filters (slot blot) were qualitatively and quantitatively consistent.

RESULTS

Experimental design

The identification of a chromosomal OBR by analysis of Okazaki piece polarity is based on a model of the eukaryotic replication fork in which the leading strand template is replicated processively away from the origin, while the lagging strand is replicated toward the origin by the discontinuous synthesis of 40–300 nt transient intermediates (Okazaki pieces) (27). Based on this model, the assay used to determine DNA nascent strand polarity is summarized in Figure 1. Cells are synchronized at the G1/S boundary and briefly released into S phase. The cells are permeabilized and allowed to continue replication for 1.5 min in the presence of radiolabeled deoxyribonucleotides, to label short and long nascent strands. The radiolabeled short nascent fragments (40–300 nt) are isolated by denaturing electrophoresis, electro-eluted, and hybridized to strand-specific RNAs transcribed from the regions under study (X and Y in Fig. 1). Okazaki pieces synthesized from one strand will hybridize asymmetrically to the pair of strand-specific transcripts. From this bias the polarity of Okazaki piece synthesis can be determined, and the transition from discontinuous to continuous DNA synthesis can be used to locate an OBR (15,27,28).

Subclones from the c-myc 2.4 kb *Hin*dIII–*Xho*I ARS fragment previously suggested to contain the c-myc OBR (9,20–25) were constructed in pGem-3Zf(–), to allow synthesis of strand-specific RNAs by T7 or SP6 RNA polymerases. Eight subclones (labeled A–H, Fig. 1) were made from sequences contiguous in the *Hin*dIII–*Xho*I fragment; two additional subclones containing sequences further 5′ and 3′ (EP and SEW) were also prepared.

The strand-specific RNA transcripts were electrophoresed on agarose gels and transferred to nylon filters. To confirm that each transcription reaction produced RNA predominantly from the expected template strand, the complete set of 20 filters was probed with each of the individual RNA transcripts, and transcripts of vector sequences, synthesized in the presence of [α-^{32}P]UTP. For each transcript, a strong (30- to 50-fold) bias of hybridization to the expected complementary strand relative to the non-complementary strand was seen, indicating that only the expected strand had been used as template (not shown). Each pair of c-myc transcripts was also hybridized to genomic Southern blots to confirm the absence of repetitive sequence hybridization.

The immobilized RNAs were further tested for repetitive sequences or strand hybridization bias by probing sequentially with HeLa genomic DNA and cloned c-myc DNA ^{32}P-labeled by random primer extension (Fig. 2). The relative hybridization to each transcript was similar for the c-myc DNA and genomic DNA probes, confirming that there was no large strand hybridization bias or significant contribution of repetitive sequence hybridization. In each experiment, following hybridization of the radiolabeled nascent DNA fragments to the set of RNA transcripts the filters were stripped and sequentially rehybridized to the genomic DNA and plasmid probes, to normalize for differences in RNA loading. The relative hybridization of the RNAs to the genomic DNA and plasmid probes was consistently equivalent.

Labeling of short nascent DNAs

Among the characteristics used to identify Okazaki pieces are their size (40–300 nt), rapid labeling, transient nature, and maturation to high molecular weight DNA (27,28). When permeabilized cells were pulsed with [^{32}P]dNTPs for 1.5 min, two major peaks of rapidly labeled DNA could be resolved by denaturing gel electrophoresis (Fig. 3); high molecular weight (≥ 1–2 kb) nascent DNA located near the gel origin, and a broad peak (~40–300 nt) of low molecular weight DNA. When the 1.5 min [^{32}P]dNTP pulse was followed by a 30 min chase in the presence of excess unlabeled dNTPs, virtually all of the radioactivity incorporated into the low

molecular weight nascent strands during the pulse was recovered in the high molecular weight DNA.

Short nascent strand polarity in the c-myc locus

To map the polarity of synthesis of short nascent DNAs in the c-myc origin, pulse labeled 40–300 nt nascent DNA fragments were hybridized to the strand-specific transcripts of eight contiguous sequences comprising the 2.4 kb c-myc *Hin*dIII–*Xho*I origin fragment (Fig. 1, A–H), and two additional sets of transcripts further 5′ and 3′ to the c-myc promoter (Fig. 1, EP and SEW, respectively), spanning a total of ~12 kb. In Figure 4 the upper row of Northern blots contain RNAs whose 5′→3′ polarity points rightward; the lower row contains RNAs whose 5′→3′ polarity points leftward. In the EP region, the strong bias of hybridization indicated that the presumptive Okazaki pieces were synthesized 5′→3′ in the rightward direction, while the fragments which hybridize to the region A RNAs were synthesized 5′→3′ in the leftward direction. According to the conventional OBR model the signals from these converging Okazaki pieces would suggest that a chromosomal OBR is located between the EP and A sequences.

The short nascent DNAs which hybridized to the region B RNAs were synthesized 5′→3′ in the rightward direction, while the fragments which hybridized to the region C RNAs were synthesized 5′→3′ in the leftward direction. Similarly, convergently synthesized short nascent fragments hybridized to the region D and E (or EF) RNAs. The bias of hybridization to the RNAs transcribed from the G, H and SEW clones indicated that the fragments hybridizing in these regions were synthesized 5′→3′ in the rightward direction.

To normalize for variation in the binding and hybridization of the immobilized strand-specific RNAs, the filters were stripped of labeled fragments synthesized in the permeabilized cells and rehybridized to probes generated from cloned double stranded

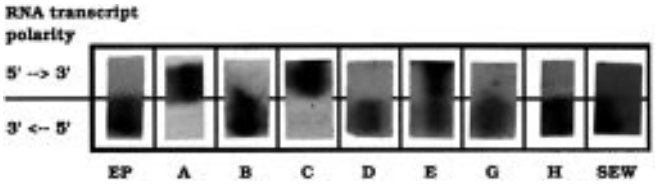

Figure 4. Hybridization of short nascent strand DNAs to strand-specific RNAs from the c-myc locus. Strand-specific RNAs were transcribed from the indicated c-myc DNA segments, electrophoresed and transferred to nylon filters. Radiolabeled short nascent strands were isolated as described (Materials and Methods) and hybridized to the immobilized RNAs. The 5′→3′ polarity of the RNAs is from left to right for the RNAs in the upper row, and right to left for RNAs in the lower row. In this and other experiments, hybridization to either upper or lower strand RNAs from fragment F was below detectable limits.

c-myc DNA containing the 12 kb region between and including the EP and SEW sequences. The hybridization of the radiolabeled cellular nascent fragment DNA and the control DNA probes are quantitated in Table 1 for the experiment of Figure 4. The data from six independent experiments are averaged in the bar graph of Figure 5A. The hybridization signal biases observed in the c-myc origin are quantitatively comparable with those observed at other OBRs (15,27,28,31,38). The asymmetries of hybridization of the nascent DNA fragments were strongest in the B, C, D and E (or E/F) regions. Quantitation of the hybridization biases suggests that in the region between probes B and E (or E/F) the same asymmetric pattern of synthesis of short nascent strands occurs in >80% of the labeled chromosomes in the population. Hence, these data argue that there are multiple initiations on each template strand in each cell.

Multiple switches in nascent strand template polarity in the region between probes EP and E imply that replication initiates non-randomly at several sites within this region. However, the pattern of short nascent fragments is difficult to reconcile with the prevailing OBR model. For example, convergent synthesis of

Table 1. The hybridization fo short nascent strands or control probes to upper (U) or lower (L) strand c-myc RNAs was quantitated by densitometry (Materials and Methods)

RNA	NASCENT		CONTROL		BIAS (d)		NASCENT		CONTROL		BIAS (d)	
	PROBE (a)		PROBE (c)				PROBE (b)		PROBE (c)			
	U	L	U	L	U/L	L/U	U	L	U	L	U/L	L/U
EP	0.2	0.8	0.7	0.3		11.4	0.8	0.2	0.7	0.3	2.1	
A	0.8	0.2	0.5	0.5	5.7		0.4	0.6	0.7	0.3		2.9
B	0.1	0.9	0.4	0.6		3.5	0.7	0.3	0.4	0.6	3.5	
C	0.9	0.1	0.7	0.3	3.2		0.2	0.8	0.5	0.5		3.5
D	0.2	0.8	0.4	0.6		3.0	0.8	0.2	0.6	0.4	3.1	
E	0.7	0.3	0.2	0.8	6.4		0.3	0.7	0.7	0.3		5.8
G	0.3	0.7	0.4	0.6		1.9	0.6	0.4	0.4	0.6	2.2	
H	0.1	0.9	0.2	0.8		5.6	0.8	0.2	0.7	0.3	2.3	
SEW	0.2	0.8	0.4	0.6		3.2	0.8	0.2	0.7	0.3	1.8	

Values are expressed as the fraction of the total hybridization to each pair of complementary transcripts, rounded to one decimal place.

(a) Intrinsically labeled short nascent strands (from Fig.4)

(b) Short nascent strand complementary DNA probe (from Fig.6)

(c) Random primer labeled, double stranded cloned c-myc DNA (sequences complementary to all transcripts nt –4514 to nt +8092)

(d) Hybridization bias, calculated as the ratio of hybridization of the short nascent strand probe to the control probe. U/L values > 1 indicate preferential hybridization to the lower strand RNA.

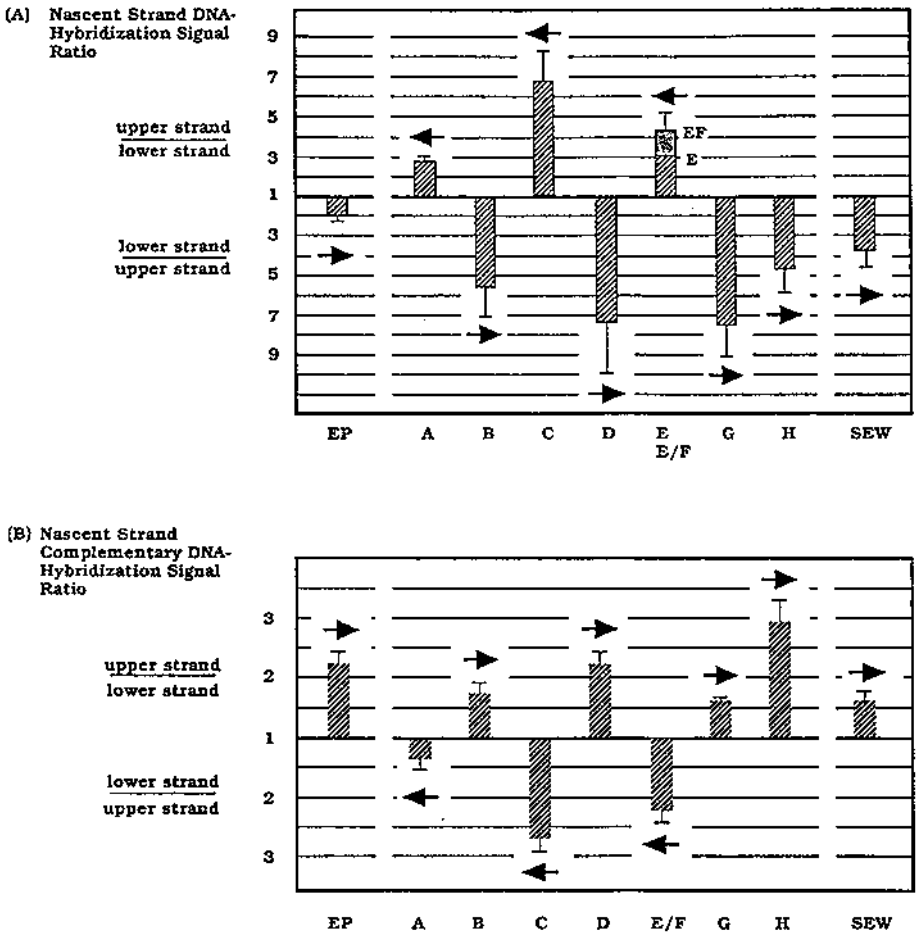

Figure 5. Polarity of short nascent strand synthesis in the c-myc locus of HeLa cells. (**A**) The hybridization signals for radiolabeled short nascent strands synthesized in permeabilized HeLa cells were quantitated by densitometry. The filters were then stripped and rehybridized to double stranded cloned c-myc DNA labeled by random primer extension. The ratios of nascent strand hybridization to the RNAs transcribed from DNA segments A–H, EP and SEW were calculated from six independent experiments, normalized against the hybridization signals of the control DNA to the same RNA blots, and the average values are plotted. Bars indicate standard errors of the means. In three experiments RNAs transcribed from the pGemE/F clone were used instead of pGemE RNAs. Solid arrows indicate the deduced direction of short nascent strand synthesis. (**B**) Summary of nascent strand complement labeling. BrdUrd-labeled short nascent strand DNA was isolated from intact, synchronized cells. ^{32}P-labeled complementary strands were synthesized by random primer extension. The hybridization signals for the nascent strand complementary DNAs were quantitated by densitometry. The filters were stripped and rehybridized to double stranded cloned c-myc DNA labeled by random primer extension. The ratio of the signals after hybridization to the RNAs transcribed from DNA segments A–H, EP and SEW were calculated from four independent experiments, normalized against the hybridization signals of the control DNA, and the average values are plotted. RNAs transcribed from the pGemE/F clone were used instead of pGemE RNAs. Solid arrows indicate the deduced direction of short nascent strand synthesis.

short nascent fragments occurs preferentially on the lower strand template of region B and the upper strand template of region C, but leading strand synthesis does not appear to begin on the lower strand template of region C, since short nascent strands are observed on the lower strand templates of regions D and G.

Nascent fragment complementary strand labeling

To confirm the polarity of short nascent strand synthesis in the c-myc locus without cell permeabilization, cultures were synchronized by isoleucine deprivation and aphidicolin block, and labeled during release from the drug into complete medium containing BrdUrd (Materials and Methods). Total cellular DNA was size fractionated by denaturing gel electrophoresis and DNA of 40–300 nt was eluted. Nascent DNA was partially purified from broken fragments of unreplicated DNA by alkaline CsCl centrifugation. The density labeled, low molecular weight DNAs were radiolabeled by random primer extension and hybridized to the immobilized RNAs described above.

Labeling of the complementary strands of the nascent fragments should result in hybridization biases opposite to those observed when the short nascent fragments were intrinsically labeled in permeabilized cells. As shown in Figure 6, in each case the hybridization asymmetry of the labeled nascent fragment complementary strands was opposite to that observed with the intrinsically labeled nascent fragment DNA. Hence, the polarity of fragment synthesis deduced from these data is the same as that deduced from the hybridization of the intrinsically labeled fragments. The hybridization of the radiolabeled nascent fragment complementary strand DNA, and the control c-myc DNA, are quantitated in Table 1 for this experiment. The hybridization data from four independent experiments are averaged in the graph of Figure 5B. The hybridization asymmetries observed in these trials were approximately half those observed when the nascent fragment strands were

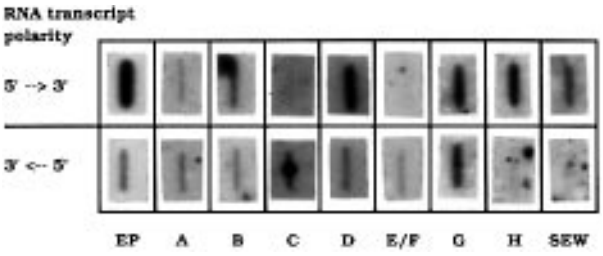

Figure 6. Hybridization of short nascent strand complementary DNA to strand-specific RNAs from the c-myc locus. Strand-specific RNAs were transcribed from the indicated c-myc DNA segments and applied directly to nylon membrane filters. Synchronized, intact HeLa cells were briefly labeled with BrdUrd and low molecular weight fragments (40–300 nt) were isolated from alkaline agarose. Nascent BrdUrd-labeled DNA was purified by alkaline CsCl centrifugation, labeled by random primer extension, and hybridized to the immobilized RNAs. The 5′→3′ polarity of the RNAs is from left to right for the RNAs in the top row, and right to left for RNAs in the bottom row.

labeled directly (Table 1), presumably because of the symmetrical hybridization of contaminating fragments derived from non-BrdUrd labeled DNA. Nevertheless, the data show three nascent strand template switches, in the regions corresponding to probes EP and A; B and C; D and E/F.

As a third variation on this protocol, the low molecular weight BrdUrd-substituted DNA was radiolabeled with [γ-^{32}P]ATP and polynucleotide kinase, and used to probe the immobilized RNAs. The hybridization biases of this end-labeled probe fraction were qualitatively the same as those obtained with the intrinsically radiolabeled fragments (data not shown). Thus, in all three methods utilized to map nascent fragment template polarity, the results consistently suggested the existence of multiple initiation sites in the chromosomal c-myc origin.

DISCUSSION

Two different methods for the isolation of short nascent DNA strands indicate that replication begins at multiple sites on each template strand of the c-myc replication origin. Quantitation of the signal intensity of the short nascent fragments synthesized in permeabilized cells revealed hybridization biases of 4- to 6-fold to strand specific RNAs representing the central zone of the 2.4 kb c-myc 5′-flanking DNA (probes B, C, D and E/F). By comparison with the hybridization of strand specific transcripts to the same RNA probes, this data suggests that synthesis initiated at all of the same sites in >80–85% of the labeled chromosomes. The asymmetry of synthesis of the shortest nascent strands (e.g. ~40 nt) may be higher *in vivo* than the observed bias of 4- to 6-fold, but the observed bias may be decreased by nascent fragments large enough (e.g. 300 nt) to straddle neighboring RNA transcripts. It is unlikely that the short nascent strands arise by fragmentation of long nascent strands, since this would not account for the observed switches in template polarity. The shortest nascent strands may correspond to the ~34 nt DNA primers (32–34) or the initiator DNAs identified during SV40 DNA replication (35). These DNA primers are synthesized by pol α-primase and are extended by pol δ or ε to form mature Okazaki pieces after removal of the initiator RNA by FEN-1/RNase H (34–36) and ligation. Waga and Stillman (35) have suggested that the same mechanism is used for the synthesis of lagging strand Okazaki fragments and of initiator Okazaki fragments near the replication origin, which will become DNA leading strands.

The pattern of synthesis of short nascent c-myc DNA fragments is difficult to reconcile with the conventional OBR model. For example, convergent synthesis of short nascent fragments occurs preferentially on the lower strand template of region B and the upper strand template of region C, but leading strand synthesis does not begin on the lower strand template of region C, since short nascent strands are observed on the lower strand templates of regions D and G.

We propose that the short nascent DNAs which hybridize asymmetrically to the c-myc origin region are not lagging strand Okazaki pieces, but leading strand DNA primers which form nascent leading strand DNA. In the leading strand primer model of Figure 7, unwinding of the origin begins at the c-myc DUE (5,25), and synthesis by pol α-primase initiates at multiple preferred sites on each template strand. Maturation and fusion of the leading strand primers results in the formation of DNA leading strands which move bidirectionally, as observed during run-off replication labeling of the c-myc locus (20–22). Some of the leading strand primers could be displaced from the template by the processive leading strand initiated upstream, however, we do not favor the idea that the synthesis of a significant fraction of short nascent strands is aborted, since PCR amplification of size fractionated nascent strands shows that short nascent DNAs (≤400 nt) initiated in regions EP, A, D, SEW, and ~1.5 kb 3′ to region H, mature to >5–10 kb (A.A.T., S.E.W. and M.L., in preparation).

One possible reason for the asymmetry of DNA primer synthesis on the two template strands is that there is a sequence preference for initiation by pol α-primase. Eukaryotic pol α-primase is thought to initiate preferentially at pyrimidine-rich sequences. Examination of the available nucleotide sequence data reveals that the asymmetric pyrimidine heptanucleotide consensus sequence 5′-CTTTC(py)(py)-3′ occurs 12 times in the regions assayed using c-myc strand specific RNA probes. In every instance, the 5′→3′ polarity of the consensus sequence is complementary to the direction of nascent strand synthesis deduced in the present experiments. In recent work, Harrington and Perrino (37) used purified calf thymus pol α-primase to replicate a pBluescript plasmid constructed to contain an open helix replication bubble. In each of the three preferential primase initiation sites characterized the sequence 5′-CTTT-3′ was found. Strikingly, one of the iRNAs began with the sequence 5′-(G)GAAGAAAG-3′, which contains the entire complement to the c-myc pyrimidine heptanucleotide consensus. It is possible, therefore, that the heptanucleotide 5′-CTTTC(py)(py)-3′ is part of an initiation consensus sequence in the c-myc origin.

Four additional copies of the heptanucleotide consensus sequence are present between probes H and SEW (Fig. 7), in a region where we have not yet examined the polarity of short nascent strands. In the leading strand primer model, the 5′-end of the leading strand (the most 5′ DNA primer) is outside the 3′-end of the DNA primer on the opposite strand, otherwise there would be short nascent DNAs on both strands. Since the SEW probe detects preferential synthesis of short nascent DNAs on the lower strand template, another leading strand DNA primer is predicted to initiate to the right of the SEW probe, possibly at another copy of the heptanucleotide consensus sequence. Thus, the boundaries of the c-myc initiation zone are not yet defined.

The model of an extended c-myc replication initiation zone is compatible with PCR mapping of DNA nascent strands and two-dimensional electrophoretic analyses of other origins, that have implicated regions of up to 55 kb as replication initiation domains

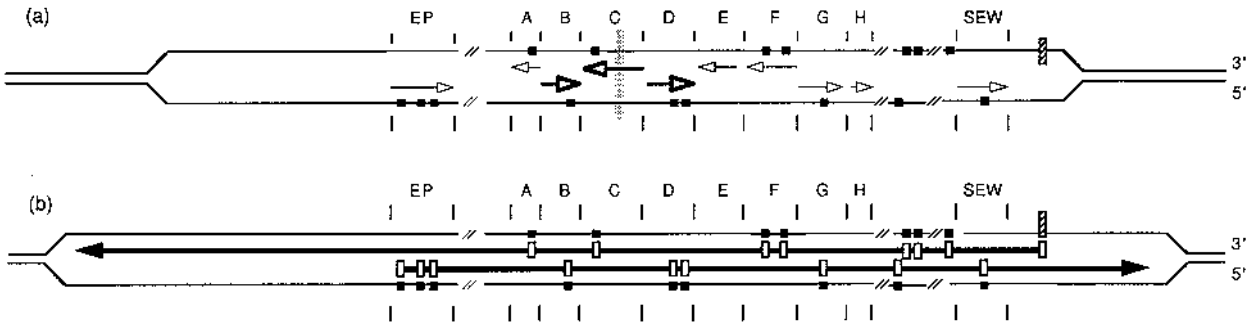

Figure 7. A leading strand primer model for replication initiation at the c-myc origin. (**a**) The c-myc origin opens at the DNA unwinding element (DUE, stippled rectangle). Multiple short nascent strands are synthesized by pol α-primase beginning at or near the heptanucleotide consensus sequence CTTTC(py)(py) (solid rectangles). All occurrences of the consensus sequence in the region between EP and SEW (inclusive) are shown. The hatched rectangle denotes a predicted initiation consensus sequence at an unspecified distance 3′ to clone SEW. The polarity of synthesis is indicated by the open arrows. Regions B, C and D (bold arrows) show the strongest template asymmetry (from Fig. 5). (**b**) The DNA primers (open rectangles) synthesized by pol α-primase are extended by pol δ or ε and fused together by FEN-1/RNaseH and DNA ligase to form long leading strands (thick arrows).

(8–18). That replication can begin at multiple sites over an extended region of the c-myc locus can be reconciled with the Okazaki piece mapping of other OBRs in which localized (27) or extended (15,28) replication initiation zones were inferred. In those experiments multiple closely-spaced template strand switches may not have been revealed because strand specific probes were used which were longer, or separated by greater chromosomal distances, than the RNAs used here. In the present experiments, the hybridization biases would presumably have been less had larger clones been used which contained heptanucleotide consensus sequences on opposite strands. In other work, we have re-examined the chromosomal c-myc origin using a higher resolution modification of the technique for PCR mediated mapping of DNA nascent strands (9,14,26). These PCR mapping results agree in detail with the conclusion that replication initiates at multiple non-random sites in an extended c-myc origin domain (A.A.T., S.E.W., and M.L., submitted).

Two dominant template strand switches for nascent strand synthesis occur between 1900 (probe B) and 400 nt (probe F) upstream of the c-myc P_1 promoter. Using purified replication proteins and supercoiled c-myc plasmid DNA, Ishimi *et al.* (24) recently reported that the earliest replicated plasmid restriction fragments were in the 800 bp region between 1730 and 930 nt upstream of the c-myc P_1 promoter. Berberich *et al.* (25) corroborated these findings with human cell extracts, using early fragment labeling, two-dimensional gel electrophoresis, and electron microscopy. In the latter work, nascent strand template analysis revealed two dominant template strand switches between 1900 and 400 nt upstream of the c-myc P_1 promoter (25). The same polarity of short nascent strand synthesis is seen in this region *in vitro* and in HeLa cells for templates which contain the heptanucleotide consensus sequence, i.e. B, C, D, F and G. These data suggest that the same or similar sites are used for initiation at the c-myc origin *in vitro* and in the chromosome. The short nascent strands which hybridize to regions A and H have opposite polarity *in vitro* and *in vivo*. Lagging strand Okazaki fragments may be synthesized *in vitro* in regions A and H due to less extensive unwinding of the c-myc origin, or because of the absence of the unique *in vivo* chromatin structure of the origin (39,40).

Plasmids containing the 2.4 kb *Hin*dIII–*Xho*I c-myc restriction fragment, or c-myc DNA segments A–C and E–H, display ARS activity in HeLa cells (21–23). PCR mapping of replication initiation in ARS plasmids containing the c-myc *Hin*dIII–*Xho*I restriction fragment reveal that replication initiates non-randomly within a 3 kb zone centered over the c-myc DNA insert (S.E.W., A.A.T. and M.L., in preparation). Taken together, these data imply that the 5′-flanking DNA of the human c-myc gene contains multiple elements which are used preferentially for replication initiation in intact chromosomes, in transfected plasmids, and in plasmids replicated *in vitro*.

ACKNOWLEDGEMENTS

The authors sincerely thank Dr G. Wahl (Salk Institute) and the members of the Wahl group for teaching S.E.W. the Okazaki piece mapping technique and allowing her to perform several c-myc origin mapping experiments in their laboratory. We are also grateful to the anonymous reviewers whose encouragement resulted in the model shown in Figure 7. We thank Steven Berberich, John Turchi and Marc Wold for their thoughtful discussions. S.E.W. and A.A.T. acknowledge the support of the WSU Biomedical Sciences PhD Program. This work was supported by Public Health Service grant GM53819.

REFERENCES

1 Coverley, D. and Laskey, R.A. (1994) *Annu. Rev. Biochem.* **63**, 745–776.
2 DePamphilis, M.L. (1993) *Annu. Rev. Biochem.* **62**, 29–63.
3 Stillman, B. (1994) *J. Biol. Chem.* **269**, 7047–7050.
4 Natale, D.A., Schubert, A.E., and Kowalski, D. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 2654–2658.
5 Dobbs, D.L., Shaiu, W.L., and Benbow, R.M. (1994) *Nucleic Acids Res.* **22**, 2479–2489.
6 Krysan, P.J., Haase, S.B., and Calos, M.P. (1989) *Mol. Cell. Biol.* **9**, 1026–1033.
7 Landry S., and Zannis-Hadjopoulos, M. (1991) *Biochim. Biophys. Acta* **1088**, 234–244.
8 Heck, M. and Spradling, A.C. (1990) *J. Cell Biol.* **110**, 903–914.
9 Vassilev, L. and Johnson, E. (1990) *Mol. Cell. Biol.***10**, 4899–4904.
10 Kitsberg D., Selig S., Keshet I., and Cedar H. (1993) *Nature* **366**, 588–590.
11 Shinomiya, T. and Ina, S. (1991) *Nucleic Acids Res.* **19**, 3935–3941.
12 Gale, J.M., Tobey, R.A., and D'Anna, J.A. (1992) *J. Mol. Biol.* **224**, 343–358.
13 Liang, C., Spitzer, J.D., Smith, H.S., and Gerbi, S.A. (1993) *Genes Dev.* **7**, 1072–1084.
14 Virta-Pearlman, V.J., Gunaratne, P.H., and Chinault, A.C. (1993) *Mol. Cell. Biol.* **13**, 5931–5942.
15 Tasheva, E.S. and Roufa, D.J. (1994) *Mol. Cell. Biol.* **14**, 5628–5635.

16 Little, R.D., Platt, T.H., and Schildkraut, C.L. (1993) *Mol. Cell. Biol.* **13**, 6600–6613.
17 Dijkwel, P.A., Vaughn, J.P., and Hamlin, J.L. (1994) *Nucleic Acids Res.* **22**, 4989–4996.
18 Wu, C., Zannis-Hadjopoulos, M., and Price, G. (1993) *Biochim. Biophys. Acta* **1174**, 258–266.
19 Gilbert, D.M., Miyazawa, H., Nallaseth, F.S., Ortega, J.M., Blow, J., and DePamphilis, M.L. (1993) *Cold Spring Harbor Symp. Quant. Biol.* **58**, 475–485.
20 Leffak, M. and James, C.D. (1989) *Mol. Cell. Biol.* **9**, 586–593.
21 McWhinney, C. and Leffak, M. (1990) *Nucleic Acids Res.* **18**, 1233–1242.
22 McWhinney, C., and Leffak, M. (1988) In B. Stillman and T. J. Kelly, (eds) *Cancer Cells 6*, CSH Laboratory Press, N. Y. pp 467–471.
23 McWhinney, C., Waltz, S.E., and Leffak, M. (1995) *DNA Cell Biol.* **14**, 565–579.
24 Ishimi, Y., Matsumoto, K., and Ohba, R. (1994) *Mol. Cell. Biol.* **14**, 6489–6496.
25 Berberich, S., Trivedi, A., Daniel, D.C., Johnson, E.M., and Leffak, M. (1995) *J. Mol. Biol.* **245**, 92–109.
26 Vassilev, L. and Johnson, E.M. (1990) *Nucleic Acids Res.* **17**, 7963–7705.
27 Burhans, W.C., Vassilev, L., Caddle, M.S., Heintz, N.H., and DePamphilis, M.L. (1990) *Cell* **62**, 955–965.
28 Carroll, S.M., DeRose, M.L., Kolman, J.L., Nonet, G.H., Kelly, R.E., and Wahl, G.M. (1993) *Mol. Cell. Biol.* **13**, 2971–2981.
29 Sambrook, J., Fritsch, E., and Maniatis, T. (1989) *Molecular Cloning; A Laboratory Manual.* 2nd Ed., Cold Spring Harbor Press, New York.
30 Church, G.M. and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1991–1995.
31 Hay, R.T. and DePamphilis, M.L. (1982) *Cell* **28**, 767–779.
32 Nethanel, T., Reisfeld, S., Dinter-Gottlieb, G., and Kaufmann, G. (1988) *J. Virol.* **62**, 2867–2873.
33 Nethanel T., and Kaufmann G. (1990) *J. Virol.* **64**, 5912–5918.
34 Nethanel, T., Zlotkin T., and Kaufmann, G. (1992) *J. Virol.* **66**, 6634–6640.
35 Waga, S., and Stillman, B. (1994) *Nature* **369**, 207–212.
36 Turchi, J.J., Huang, L., Murante, R.S., Kim, Y., and Bambara, R.A. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 9803–9807.
37 Harrington, C., and Perrino, F. (1995) *Nucleic Acids Res.* **23**, 1003–1009.
38 Kelly, R.E., DeRose, M., Draper, B., and Wahl, G.M. (1995) *Mol. Cell. Biol.* **15**, 4136–4148.
39 Kumar, S., and Leffak, M. (1989) *Nucleic Acids Res.* **17**, 2819–2833.
40 Kumar, S., and Leffak, M. (1991) *J. Mol. Biol.* **222**, 45–57.