Autonomous replication of a DNA fragment containing the chromosomal replication origin of the human c-myc gene

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ABSTRACT

The c-myc genes of HeLa cells are preferentially replicated in the transcriptional direction, from chromosomal origin sequences which display cell type-specific activity. Using a run-off replication assay involving *in vitro* extension of replication forks initiated in intact HeLa cells, bidirectional replication was observed to begin within a 3.5 kb domain 5′ to the c-myc gene. To characterize the replication origin further a 2.4 HindIII-XhoI subfragment of the c-myc 5′ flanking DNA was cloned in a selectable vector and transfected into HeLa cells. The resulting pNeo.Myc-2.4 construct persisted as a circular extrachromosomal element for more than 300 cell generations under selection, with recovery of approximately 500–1000 times the mass of plasmid initially introduced into the cells. Extrachromosomal circular pNeo.Myc-2.4 monomer was reisolated in supercoiled form, along with oligomeric and miniplasmid variants which had been generated *in vivo*; however, chromosomally integrated copies of the plasmid were not detectable in cultures containing extrachromosomal pNeo.Myc-2.4. The recovered pNeo.Myc-2.4 plasmid was resistant to DpnI digestion and sensitive to MboI digestion. After transfection with pNeo.Myc-2.4 BrUdR pulse labeling of long-term or short-term cultures demonstrated that the plasmid replicated semiconservatively, under controls similar to those imposed on chromosome replication. Bisection of the pNeo.Myc-2.4 insert suggested that c-myc 5′ flanking DNA within 1.2 kb 5′ to promoter P1 was sufficient to confer autonomously replicating sequence activity on the plasmid vector in transient replication assays.

INTRODUCTION

Eucaryotic chromosomes are organized into multiple replicons whose size, number, and temporal order of replication vary in a cell type-specific and developmentally regulated manner (1, 2). As in yeast and with DNA viruses which replicate in minichromosome form in eucaryotic cells the initiation of bidirectional replication from chromosomal origin sites (3, 4) is likely to involve the binding of *trans*-acting factors to the replication origin sequences or to *cis*-acting regulatory elements (reviewed in 5, 6). Direct evidence for the existence of distinct non-viral DNA sites which function as replication origins *in vivo* is provided by the autonomously replicating sequence (ARS) elements of *Saccharomyces cerevisiae* (7–12) and the origin of replication of the extrachromosomal rDNA genes of *Tetrahymena* sp. (13, 14). Searches for DNA sequences with the potential to function as origins of replication in the genomes of higher eucaryotes have been predicated on several criteria. Using a novel method to extrude nascent DNAs which replicate very early in S phase (15) DNA fragments have been isolated which are able to replicate autonomously in CV-1 and HeLa cells (16). Based on the premise that ARS activity is inherent to chromosomal DNA sequences which function as origins of replication *in vivo*, murine (17) and human (18, 19) DNA fragments have been isolated based on their apparent ability to allow bacterial plasmids to replicate in eucaryotic cells. One concern with this approach is the necessity to demonstrate that the putative ARS plasmid does not persist due to cycles of chromosomal integration and excision. Thus, although a recent report that a DNA fragment derived from the 5′ flanking DNA of the human c-myc gene (18) allows autonomous plasmid replication in HL60 cells remains controversial, it is consistent with our earlier preliminary demonstration of the extrachromosomal persistence in HeLa cells of a plasmid containing c-myc flanking DNA (20).

DNA sequences involved in gene amplification during normal development or in cells subjected to drug selection have also been studied as model replication origins. Thus, Carroll et al. (29) have inferred the presence of a replication origin in a large autonomously replicating episome containing amplified CAD genes in hamster cells treated with PALA. In other systems, over-replication of the major chorion genes during Drosophila embryogenesis has been shown to initiate within localized regions of these gene clusters on the x chromosome and chromosome III (21–24). In hamster cells selected for resistance to methotrexate, regions ca. 40 kb and 60 kb 3′ to the amplified dihydrofolate reductase gene transcription start site have been shown to replicate early in S phase, before their 5′ and 3′ flanking sequences (25–28), suggesting that divergent replication initiates within these domains. The activity of these DHFR replication origins in wild-type diploid cells has recently been confirmed by Handeli et al. (59) by analysis of nucleosome segregation patterns within this domain.

The cell-type specificity of the replication polarity through the

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avian α-globin (30) and histone H5 genes (31) gives further evidence for the use of discrete origins of replication by eucaryotic cells. Moreover, in experiments analyzing the direction of replication through the c-myc gene a site within 4−5 kb 5′ to the c-myc gene was implicated as a potential chromosomal origin of replication in HeLa cells, while in the Burkitt lymphoma cell lines CA46 and ST486 the activity of this putative replication origin appeared to be suppressed (32). The present report confirms that bidirectional chromosome replication initiates within 3.5 kb 5′ to the c-myc gene in the majority of HeLa cells and that a 2.4 kb fragment of c-myc 5′ flanking DNA supports autonomous semiconservative replication of a selectable plasmid in HeLa cells.

MATERIALS AND METHODS

In vitro replication

Run-off replication was carried out essentially as described previously (30−32) using HeLa cells in logarithmic growth. The data of Figure 2 were obtained by fractionating identical CsCl gradients containing DNA substituted with bromodeoxyuridine *in vivo* or *in vitro*; within each gradient the five fractions spanning the density range 1.765−1.785 g/ml were pooled.

Transfection of plasmid pNeo.Myc-2.4

A 12.5 kb EcoRI fragment containing the human c-myc gene was generously provided as a lambda phage clone by P. Leder. The 8 kb HindIII/EcoRI c-myc fragment was subcloned in pBR322 and the 2.7 kb subfragment extending from the vector BamHI site to the XhoI site in exon 1 of the c-myc gene was cloned between the BamHI and SalI sites in the vector pdMMTneo(302−3) [33, kindly provided by P. Howley] to produce pNeo.Myc-2.4. The plasmid pNeo.Myc-1.2 was constructed by deletion of the 1.5 kb BamHI/SstII fragment of pNeo.Myc-2.4 (containing the 5′ most 1.2 kb of c-myc DNA), blunting of the resulting ends with mung bean nuclease and Klenow polymerase, and religation. Two micrograms of plasmid (with or without 30 μg of carrier salmon sperm DNA) were introduced into HeLa cells by calcium phosphate coprecipitation (34); transfections by electroporation used 10−20 μg of plasmid DNA. After four hours the transfection medium was replaced by fresh medium and the cells incubated for an additional 24 hours. The antibiotic G418 was applied at 500 μg/ml (35) and the cells maintained under selection. Transformation efficiency is reported as foci/5×10^6cells/plate at 10−12 days after G418 selection was applied. Plasmid uptake was estimated by blot hybridization of total cellular DNA isolated from cells washed in SSC (150 mM NaCl, 15 mM NaCitrate, pH 7.4), or from nuclei isolated in RSB/NP-40 (15 mM NaCl, 10 mM Tris-Cl, pH 7.4, 3 mM $MgCl_2$, 0.5% NP-40). Long-term transfected cultures used in these experiments were harvested at 120−450 cell generations after transfection. Short-term cultures were used in transient replication assays within 2−9 days after transfection and were not subjected to G418 selection.

The extent of plasmid replication vs. chromosome replication was quantitated after DNAs from transfected, BrUdR labeled cell cultures were centrifuged, fractionated, and electrophoresed. The percentages of chromosomal and plasmid DNAs density labeled were measured by laser densitometry of negative photographs of ethidium bromide stained gels and scanning of autoradiograms after blot hybridization (30). Care was taken to ensure linear film response.

Blot hybridization

DNA was subjected to electrophoresis on 0.8% agarose gels and was transferred to nitrocellulose or Nytran filters (Schleicher and Schuell) (36, 37). Filters were hybridized to a neo^r probe (the BamHI/BglII fragment of pNeo.Myc-2.4) or to a c-myc probe (the PstI/PstI fragment internal to the c-myc insert of pNeo.Myc-2.4) labeled with α-^{32}P-dCTP by the procedure of James and Leffak (38) or using a random primer labeling kit (Boehringer Mannheim). The data of Figure 2 were obtained by hybridization to two DNA probes (a 4.6 kb EcoRI/HindIII fragment and the 2.4 kb HindIII/XhoI fragment) which together span the 7 kb of DNA immediately 5′ to the c-myc gene, in the presence of human genomic DNA as competitor. Hybridization was performed in 50% formamide under standard conditions (36) with final stringency washes at 65°C in 0.1×SSC, 0.1% SDS. Filters were exposed to Kodak XAR film at −80°C with intensifying screens.

Isopycnic centrifugation and isolation of plasmid DNA from HeLa cells

Low molecular weight DNA was extracted by the method of Hirt (39); mitochondrial DNA was quantitatively recovered in the supernatants of these preparations. Plasmid DNA was purified from RNA and contaminating chromosomal DNA by centrifugation of cleared bacterial cell lysates, total cellular DNA, or from Hirt supernatant DNA on CsCl/ethidium bromide (CsCl/EtBr) gradients. Plasmid was recovered by side puncture of the tube and withdrawal of the plasmid region of the gradient and ca. 20% of the tube volume below it, avoiding contamination by RNA. Ethidium bromide was removed by repeated isopropanol extraction (36) and BrUdR substituted and unsubstituted DNAs were resolved on native or denaturing CsCl gradients as described by Epner et al. (40). DNA was centrifuged in 5.9 M CsCl, 28 mM NaCl, 7 mM Na_2EDTA (pH 7.5) at 35,000 rpm for 60−72 hr in a Beckman Ti75 or Ti50 rotor at 22°C. Alternatively, DNA was centrifuged in 6.5 M CsCl, 0.1 M NaOH, 10 mM EDTA at 39,000 rpm for 72 hr in a Beckman Ti75 or Ti50 rotor at 22°C. In Figures 5 and 7, less than 15% of the DNA signal appeared in fractions lighter than the lightest fraction shown; no signal banded denser than the densest fraction shown. In all other isopycnic centrifugation experiments in which the central portion of the gradient is shown, the fractions presented contained greater than 90−95% of the signal above background for the entire gradient. DNA recovered from the gradient was dialyzed against 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, concentrated with butanol, and ethanol precipitated. To test for plasmid integration, chromosomal DNA was purified from a low speed Hirt pellet preparation by two cycles of CsCl/EtBr centrifugation.

Enzyme digestion

Low molecular weight plasmid DNA was isolated as described above and DNA precipitated in 70% ethanol. Restriction enzymes and DNA gyrase were purchased from New England Biolabs or Bethesda Research Laboratories and used in the buffers specified by the supplier. We found it important to titrate the DpnI and MboI digestions carefully so as to avoid incomplete cleavages or nonspecific overdigestion (41) with these enzymes. Therefore, supercoiled monomer plasmid recovered from HeLa cells by Hirt (39) extraction, shearing of the Hirt supernatant, and two cycles of CsCl/EtBr centrifugation was mixed with an excess of pUC18 and digested with a 4-fold excess of DpnI required to yield

complete digestion of the internal reference plasmid (10U/μg pUC18) or a 4-fold excess of MboI required to digest the contaminating chromosomal DNA to completion. The blot shown in Figure 10 was probed with the plasmid pRSV.Neo (42). To analyze the structure of the high molecular weight plasmid forms a DNA preparation enriched for plasmid oligomer by CsCl/EtBr centrifugation of a Hirt pellet preparation was digested with EcoRl, which cuts once in the monomer 9.2 kb pNeo.Myc-2.4 DNA.

Cell Culture

HeLa cells were cultured in Dulbecco's modified Eagle's minimal medium (GIBCO) containing 5−10% fetal calf serum, 40 μg/ml gentamicin, and repeatedly tested for mycoplasma. The cells were seeded the day before transfection at a density of 5×10^5 cells per 9 cm plate. G418 selection was applied twenty-four hours after transfection. The apparent cell doubling time for transfected cultures grown in G418 was in the range of 24−30 hours. In density substitution experiments, cells were labeled with 5−100 μM bromodeoxyuridine. This range of BrUdR concentrations represents a compromise between obtaining the maximal density shift of replicated DNAs and a cytotoxic effect on the cell cultures. As discussed in the text, labeling at the highest concentrations of BrUdR occasionally led to partial inhibition of replication of plasmid and genomic DNA.

A preliminary report of this work, including the data shown in Figure 4, was presented at the 1987 Cold Spring Harbor Laboratory meeting on Eucaryotic DNA replication. (20)

RESULTS

Bidirectional replication from a chromosomal domain

The *in vivo* direction of replication through a DNA segment can be deduced after allowing replication forks initiated in intact cells to complete replication in an *in vitro* nuclear replication reaction in the presence of a labeled DNA precursor (30). In the vicinity of a replication origin, and in the absence of other barriers to replication fork movement, a graded incorporation of the DNA label results, with DNA fragments distal to the replication origin incorporating relatively more of the label than origin-proximal fragments. We have previously used the dense nucleotide analogue bromodeoxyuridine triphosphate (BrdUTP) in this type of experiment to study the replication polarity of the avian α-globin genes (30; S. Berberich and M.L., in preparation), the avian histone H5 genes (31) and the human c-myc genes (32). A similar strategy was used recently to examine the direction of replication through the DHFR locus of CHOC 400 cells (27).

When HeLa nuclei were isolated from an asynchronous cell culture and incubated in a replication cocktail containing BrdUTP an approximate two-fold increase in the density labeling of c-myc intron 1 relative to exon 1 was observed (32). To test for the presence of the c-myc replication origin by the criterion of the initiation of bidirectional replication, HeLa nuclei were density labeled in vitro as above, and the DNA was purified, digested to completion with PstI, and banded on a neutral CsCl gradient. DNA isolated from HeLa cells labeled for 6 hours with BrUdR in vivo and digested with PstI was centrifuged in parallel to provide a reference for comparison of the stoichiometry of the in vitro density labeled c-myc fragments. To avoid contamination with the large amount of light DNA present in the in vitro replicated sample, DNA was isolated from fractions on the heavier side of the BrUdR-substituted DNA distribution (1.765−1.785 g/ml density). DNAs replicated in vivo or in vitro were electrophoresed on the same gel and blot hybridized. PstI digestion produces five fragments (0.4, 1.3, 1.7, 2.4 and 2.7 kb) from the 5′ flanking DNA of the c-myc gene (Figure 1). In the in vivo density labeled DNA the large fragments show decreasing signals with increasing molecular weight (Figure 2). We attribute this effect to the facts that higher molecular weight species form narrower bands on isopycnic gradients and that the dense DNA fraction in these experiments is taken from the heavier side of the distribution of BrUdR-substituted DNA, although we have not eliminated the possibility that the base composition of these restriction fragments skews their distributions. Nevertheless, when in vitro replicated DNA is isolated from the same density fractions we observe a strong enrichment for the 2.7 kb fragment (containing exon 1 and a portion of intron 1) in dense DNA relative to the 1.7 kb fragment which is located immediately 5′ to it, consistent with our previous report (32) that the c-myc gene is replicated in the transcriptional direction in HeLa cells. Conversely, the 5′-most 2.4 kb fragment is enriched relative to its 3′ neighboring 1.3 kb fragment, indicating that replication proceeds in the opposite direction, away from the c-myc gene, in the farther 5′ flanking region. Therefore, bidirectional replication initiates within ca. 3.5 kb 5′ to the c-myc gene. The sensitivity of the in vitro replication assay was not sufficient to localize the origin of replication further to the 0.4 kb, 1.3 kb, or 1.7 kb PstI fragment. However, as shown below, the 1.3 kb fragment was not essential to impart autonomously replicating activity to a nonreplicating plasmid vector.

Extrachromosomal forms of a c-myc plasmid

The 2.4 kb HindIII-XhoI fragment immediately 5′ to the human c-myc gene was cloned in the vector pdMMTneo(302-3) (Figure 1; 33), which contains the bacterial neomycin phosphotransferase (neo^r) gene. This construct, pNeo.Myc-2.4, was introduced into HeLa cells by calcium phosphate coprecipitation and selection with G418 was applied after 24 hours. The input plasmid contained both supercoiled monomer and oligomer forms (Figure 3, lanes 2, 5). Approximately 175 cell doublings after transfection low molecular weight DNA was isolated by Hirt extraction and enriched for circular DNA by CsCl/EtBr centrifugation. The undigested DNA displayed a complex mixture of monomer and oligomer forms, as well as the presence of in vivo-generated miniplasmid (Figure 3, lanes 3, 6). In other experiments, pre-digestion of the DNA preparations with SalI (which does not cleave monomer pNeo.Myc-2.4) did not alter the pattern of recovered DNA. In more than forty repeats of this type of experiment in which monomer plasmid was consistently recovered the relative amounts of miniplasmid and oligomer were quite variable between cultures and depending on the method of transfection. In general, the miniplasmid and oligomer forms were apparent over the first 2−9 months after transfection, but their amounts subsequently decreased with time. The DNAs identified as monomer and miniplasmid (Figure 3, lanes 3, 6) can be relaxed by the topoisomerase II activity of DNA gyrase (20), demonstrating that these plasmid species are present in the HeLa cell culture as circular supercoiled extrachromosomal DNA molecules.

The structure of the oligomer DNA recovered from long-term cultures was not the same as that of the high molecular weight input DNA. Oligomer was separated from the HeLa chromosomal DNA of a Hirt pellet preparation by CsCl/EtBr

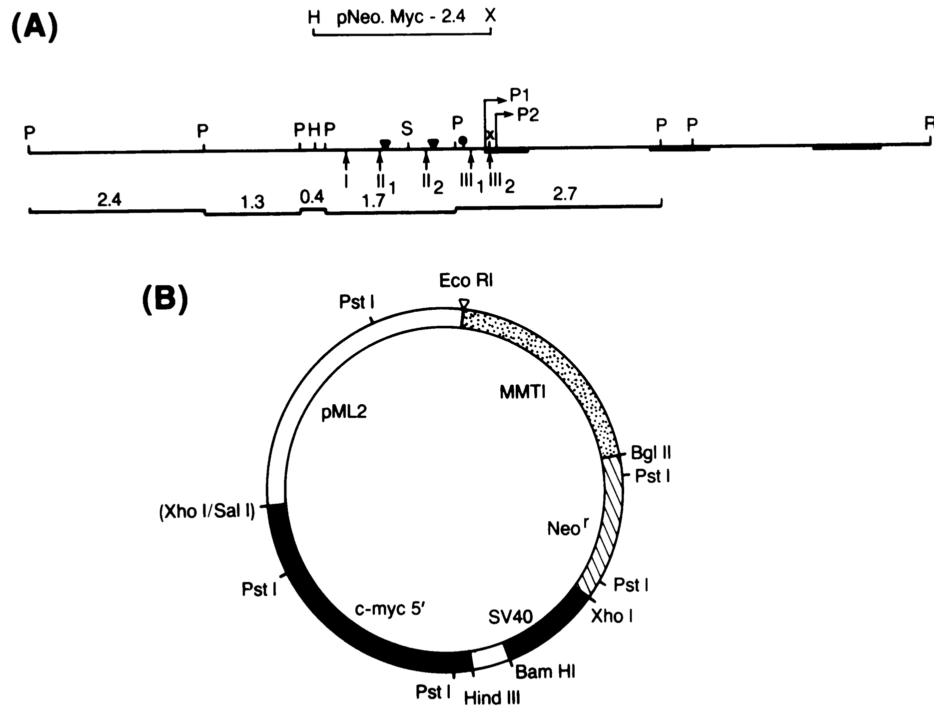

Figure 1. Restriction maps. (A) Map of the chromosomal c-myc domain. Solid boxes, c-myc exons; thin line, c-myc introns and flanking DNA. P1, P2, c-myc promoters. Filled triangles, NF-1 binding sites (50); filled circle, bovine papilloma virus PMS1-related sequence. Roman numerals, DNaseI hypersensitive sites (50, 51). Jointed line, PstI fragments referred to in text. Overline, c-myc DNA insert of plasmid pNeo.Myc-2.4. H, HindIII; R, EcoRI; P, PstI; S, SstII; X, XhoI. (B) Plasmid pNeo.Myc-2.4. Stippled bar, mouse metallothionein 5′ flanking DNA and promoter region; hatched bar, aminoglycoside phosphotransferase gene; checkered bar, SV40 early region splicing and 3′ processing signals; open bar, pML2 DNA; solid bar, c-myc DNA. Non-c-myc DNA is derived from the vector pdMMTneo(302-3) (33). The site denoted EcoRI represents an inverted duplication of the 29 bp EcoRI/HindIII fragment of pML2.

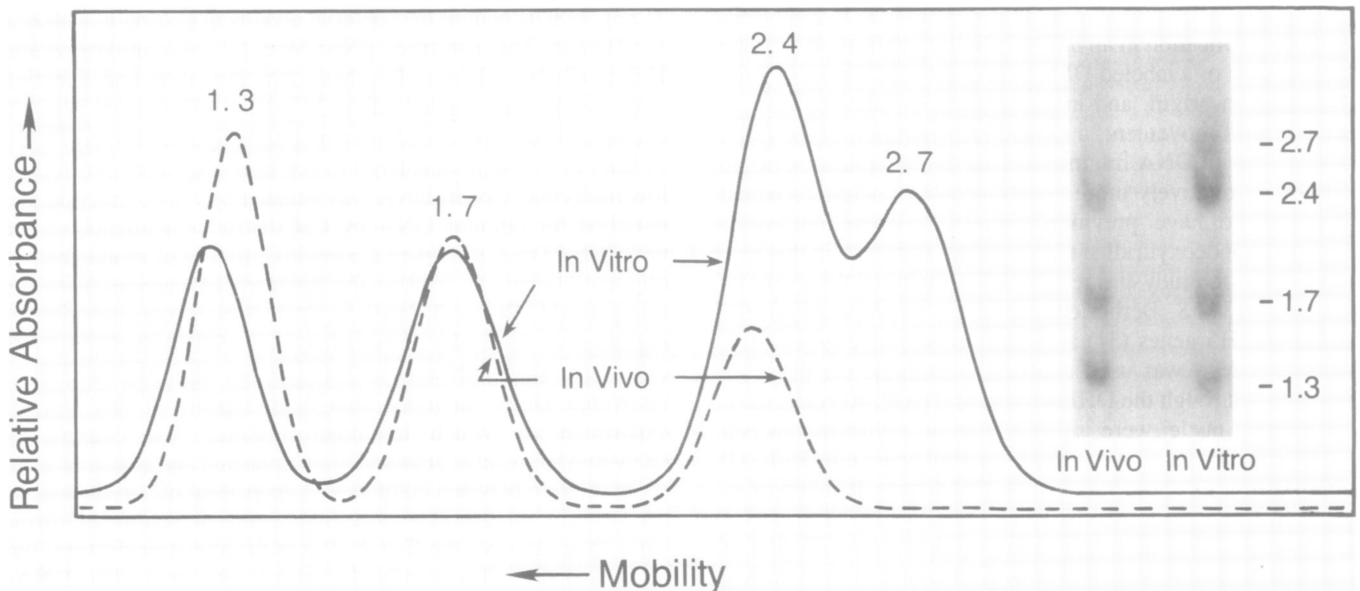

Figure 2. *In vitro* run-off replication in HeLa nuclei. HeLa nuclei were isolated from an asynchronous cell culture and submitted to the IVR reaction in the absence of exogenous restriction enzyme. The DNA was digested to completion with PstI, banded on neutral CsCl in parallel with DNA from cells density labeled with BrUdR *in vivo* (6 hours), electrophoresed on the same gel and blot hybridized to probes spanning the 5′ c-myc sequences and flanking DNA (Materials and Methods). Inset: autoradiogram of the *in vivo* replicated (left) and *in vitro* replicated (right) dense DNAs. Size markers are in kilobase pairs. The scans compare the hybridization pattern of the dense *in vitro* (solid line) and in *vivo* (broken line) labeled DNAs after normalization of the 1.7 kb band signals.

centrifugation and digested with EcoRI. Whereas digestion of the input DNA with EcoRI generates only a single 9.2 kb linear band EcoRI digestion of the oligomer produces two major bands of approximately 3.8 kb and 14 kb which hybridize with the neo^r probe (20). While the structure of the oligomer DNA has not been characterized further, the high molecular weight

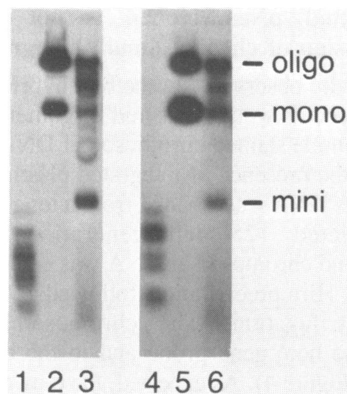

Figure 3. Long-term retention of c-myc DNA in extrachromosomal plasmids. DNA was isolated by CsCl/EtBr centrifugation of Hirt supernatant DNA from HeLa cells 175 cell doublings post-transfection. Gradients were fractionated based on the banding of pNeo.Myc-2.4 marker run on a parallel gradient. Lanes 1 and 4, DNA isolated from transfected HeLa cells, digested with Sau3AI. Lanes 2 and 5, undigested marker pNeo.Myc-2.4 (untransfected). Lanes 3 and 6, undigested DNA isolated from transfected HeLa cells (as used in lanes 1 and 4). Lanes 1, 2 and 3 were probed with the neo^r probe. After the filter was stripped twice with boiling water no remaining signal was detected with a seven-day film exposure. The blot was rehybridized to the c-myc probe (lanes 4, 5, and 6) and exposed to film for two days.

oligomer species is resistant to disaggregation by topoisomerase II and bands below chromosomal DNA on CsCl/EtBr gradients. These observations suggest that the oligomers exist as large, non-interlocked, circular DNA molecules (20).

Because the pNeo.Myc-2.4 plasmid was susceptible to in vivo recombination and deletion we determined whether each of the plasmid forms retained c-myc DNA sequences after 175 cell doublings. As shown in Figure 3, while neo^r and c-myc probes detected different Sau3AI restriction fragments in this DNA, both probes hybridized to the pNeo.Myc-2.4 miniplasmid, monomer, and oligomer. Additional faint bands detected by the neo^r probe are less clearly visualized by the c-myc probe in this photograph but are visible in the original autoradiogram.

To date, mixed HeLa cell cultures containing pNeo.Myc-2.4 have been maintained under G418 selection for more than 300 cell generations, and more than 500–1000 times the mass of plasmid DNA estimated by blot hybridization to have been taken into nuclei during the initial transfection has been recovered. In contrast, in more than ten independent experiments HeLa cells transfected with the parent vector and selected for G418 resistance did not retain the pdMMTneo(302-3) plasmid as an extrachromosomal element beyond 19–25 days. A series of experiments designed to quantitate transformation efficiency showed that the putative ARS plasmid pNeo.Myc-2.4 was only 2–3 fold more effective at transforming HeLa cells to G418 resistance than its parent vector pdMMTneo(302-3) (Table 1). While this result contrasts with the approximate thousand-fold enhancement of transformation frequency associated with some yeast ARS elements (8, 11, 12), the transformation efficiencies of the pNeo.Myc-2.4 and pdMMTneo(302-3) plasmids are closely similar to the efficiencies of transformation to G418 resistance reported for mouse cells transfected with the plasmids pdBPV-MMTneo(342–12) and pMMTneo(302-3) (33) and are consistent with previous observations that the bovine papilloma virus plasmid maintenance sequence PMS-1 had limited effect on the efficiency of transformation to G418 resistance of a linked neo^r gene (43). As was suggested to explain the slight increase

Table 1. G418 Transformation Efficiency[a]

	1	2
pdMMTneo(302-3)	29	40
pNeo.Myc-2.4	74	117

[a]G418-resistant foci/plate (Methods).

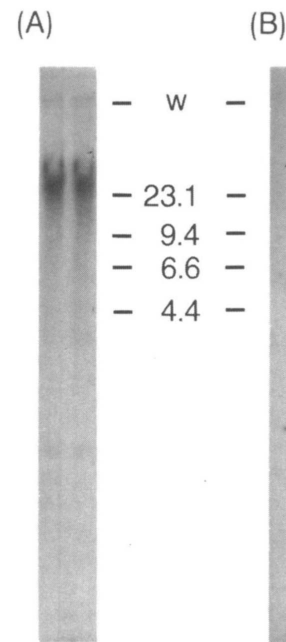

Figure 4. Absence of chromosomally integrated pNeo.Myc-2.4. High molecular weight chromosomal DNA was isolated from HeLa cells 125 cell doublings post-transfection and duplicate samples were hybridized to the PstI-PstI c-myc fragment of pNeo.Myc-2.4 (panel A), and to the BglII-BamHI fragment neo^r probe (panel B). W, sample wells. Not shown is the marker lane containing 2–5 pg of pNeo.Myc-2.4 plasmid. The exposures in (A) and (B) displayed equivalent intensities of marker hybridization.

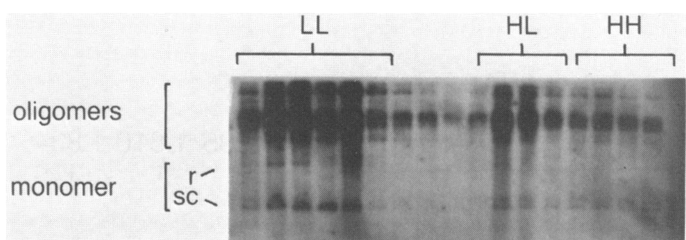

Figure 5. Eighteen hour BrUdR density labeling of pNeo.Myc-2.4; neutral CsCl banding. HeLa cells 125 cell doublings post-transfection were labeled overnight with 100 μM BrUdR. Plasmid DNA was purified and banded on neutral CsCl. Aliquots were collected from the top to the bottom of the gradient and were electrophoresed and blot hybridized to the neo^r probe. Only plasmid monomer and oligomer were seen in this subculture. The central 17 fractions of the gradient are shown, with the top of the gradient at the left, and the banding positions of light-light (LL), heavy-light (HL) marker DNAs run in parallel and the predicted banding position of heavy-heavy (HH) DNA indicated.

in the transformation efficiency of pdBPV-MMTneo(342-12) (33), the increase in transformation efficiency of pNeo.Myc-2.4 may be due to the presence of a *cis*-acting enhancer in the c-myc DNA, or to the fact that the selective marker resides on a stable replicon.

Subcultures of HeLa cells 120 generations post-transfection with pNeo.Myc-2.4 were shifted to growth in medium containing no G418 to examine whether the plasmid was stably maintained. In the absence of drug selection the pNeo.Myc-2.4 extrachromosomal DNA showed decreased stability and was lost at a rate of approximately 5% per cell generation.

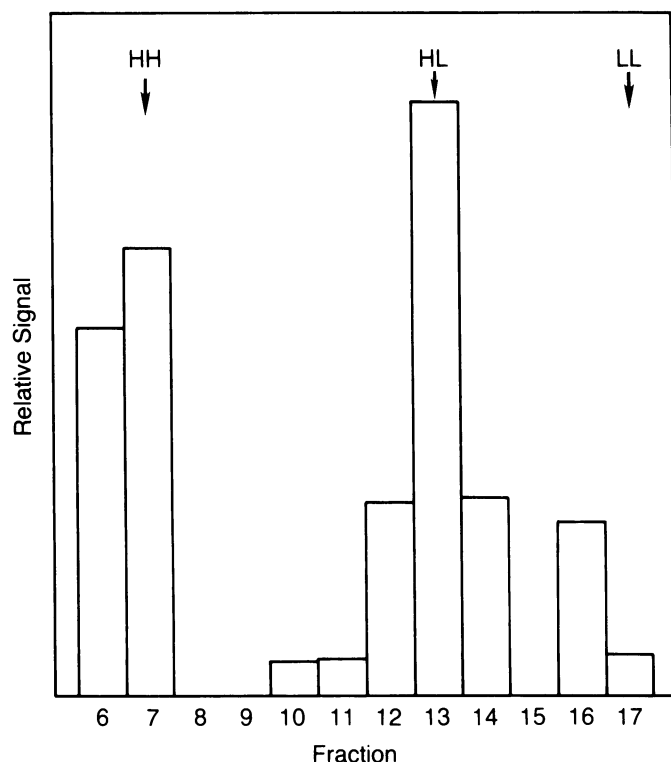

Figure 6. Forty-eight hour BrUdR density labeling of pNeo.Myc-2.4; neutral CsCl banding. HeLa cultures 350 cell doublings post-transfection were labeled with 5 μM BrUdR for 48 hours and DNA was banded on neutral CsCl. To allow estimation of the relative amounts of replicated and unreplicated DNAs aliquots taken across this gradient were slot-blotted and hybridized to the neo^r probe. The top of the gradient is at the right.

Extrachromosomal pNeo.Myc-2.4 is not generated by continuous excision of chromosomally integrated plasmid

We have repeatedly observed that the blot hybridization pattern of the high molecular weight plasmid oligomer is offset from the ethidium bromide stained chromosomal DNA. However, to test directly for the presence of integrated plasmid sequences at single copy levels DNA was isolated from a long-term HeLa cell culture transfected 125 cell generations earlier with pNeo.Myc-2.4 and chromosomal DNA was extensively purified by a low speed Hirt precipitation followed by two cycles of CsCl/EtBr centrifugation. The chromosomal DNA was hybridized to the neo^r gene probe and to the c-myc probe of pNeo.Myc-2.4 (Figure 4). As expected, the c-myc probe detected hybridizable DNA, most probably the germ-line c-myc sequences, at single copy intensity (Figure 4A). Virtually no hybridization was observed to contaminating low molecular weight plasmid DNA, attesting to the purity of the chromosomal DNA preparation. Most importantly, no hybridization above background was seen to the neo^r probe (Figure 4B), even on 5- to 10-fold overexposure of the film, demonstrating that the G418 resistance was not propagated in these cells as a result of continuous excision of the pNeo.Myc-2.4 plasmid from chromosomal DNA. In only one experiment to date has a long-term culture transfected with pNeo.Myc-2.4 and resistant to G418 selection shown apparent chromosomal integration of the plasmid DNA into high molecular DNA which pelleted during Hirt extraction, banded as relaxed DNA on CsCl/EtBr and co-electrophoresed with chromosomal DNA. In this culture, no extrachromosomal pNeo.Myc-2.4 was detected. The extremely low frequency of detectable chromosomal integration of pNeo.Myc-2.4 sequences is reminiscent of the low frequency at which plasmids containing the bovine papilloma virus plasmid maintenance sequence PMS-1 are found integrated into the chromosomes of permissive cells (43).

Replication of pNeo.Myc-2.4

Overnight labeling of long-term transfected HeLa cultures with 100 μM BrUdR resulted in the density labeling of 25–30% of

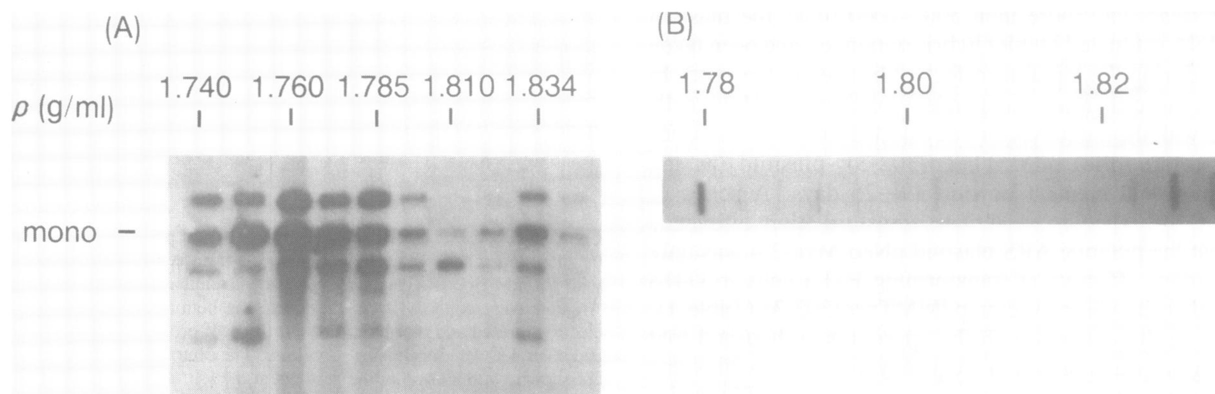

Figure 7. BrUdR density labeling of pNeo.Myc-2.4; alkaline CsCl banding. (A) HeLa cells 125 cell doublings post-transfection were labeled with 100 μM BrUdR for 24 hours. Plasmid DNA was purified, digested with EcoRI, denatured and banded on alkaline CsCl. Aliquots (0.3 ml) were collected from the top to the bottom of the gradient and were analyzed by neutral gel electrophoresis (central 10 of 38 fractions shown) and hybridization to an asymmetrically labeled probe complementary to the sense strand of the neo^r gene. The DNA identified as monomer had the same mobility as linearized marker pNeo.Myc-2.4 on this gel. The remaining bands derive from the oligomer and miniplasmid forms of pNeo.Myc-2.4 based on comparison with the mobility of EcoRI digested pNeo.Myc-2.4 DNAs on native gels. (B) HeLa cells 140 cell doublings after transfection were labeled with 65 μM BrUdR for 48 hours, and DNA was treated as in (A). Aliquots (0.4 ml) of an alkaline CsCl gradient (central 14 of 28 fractions shown) were analyzed by slot blot hybridization to the neo^r sense strand probe. In (A) and (B) the top of the gradient is at the left.

the pNeo.Myc-2.4 plasmid DNA (Figure 5). The heavy-light DNA recovered from these gradients banded between 1.74–1.78 g/ml on each of two successive recentrifugations. The low percentage of DNA density labeled at this BrUdR concentration reflects similar toxic effects on the replication of both plasmid and chromosomal DNAs as only 40–50% of the cellular DNA visualized by ethidium bromide staining appeared in the heavy-light fractions (not shown). Close inspection of the density labeled DNA in Figure 5 reveals a second band migrating slightly slower than the supercoiled pNeo.Myc-2.4 monomer. The electrophoretic mobility of this band suggests that topoisomers of pNeo.Myc-2.4 may exist which correspond to the underwound 'U' form plasmid replication intermediate described by Dean et al. (44).

When long-term transfected cultures were labeled for 48 hours with 5 μM BrUdR approximately equal amounts of heavy-light and heavy-heavy pNeo.Myc-2.4 plasmid were recovered, with only a small residual of unreplicated DNA (Figure 6). These data imply that the heavy-light DNA contains one uniformly dense DNA strand which is synthesized semiconservatively and serves as the precursor to heavy-heavy DNA. Consistent with this expectation, uniformly dense DNA single strands were released from each of the plasmid forms upon alkaline CsCl centrifugation of the DNA labeled overnight at 100 μM BrUdR (Figure 7A). Also banded on a denaturing gradient was DNA from cells labeled with 65 μM BrUdR for 48 hours. The relative amount of light:heavy strands was approximately 1:3, close to the anticipated ratio for plasmid which had replicated twice during the two cell generation incubation in density label (Figure 7B).

In short-term replication assays both the pNeo.Myc-2.4 plasmid, and its derivative pNeo.Myc-1.2 which deleted the extreme 5′ 1.2 kb of the c-myc insert (Materials and Methods), appeared exclusively in the heavy-light fractions of native CsCl gradients after labeling with 65 μM BrUdR for 24 hours [approximately one cell doubling time] (Figure 8; see also Table 2). That these heavy-light DNA molecules contained uniformly dense DNA single strands was confirmed by alkaline CsCl centrifugation (not shown). To determine whether pNeo.Myc-2.4 plasmid replication in short-term cultures obeyed similar temporal controls as did a chromosomal marker gene, cells transfected 48 hours earlier were pulsed with 65 μM BrUdR for 24 hours and total cellular DNA was isolated. After banding on neutral CsCl the DNA was hybridized first to the neo^r probe and then to a β-actin cDNA (45) (Figure 9). The density labeling patterns of the plasmid DNA and the β-actin genes were closely comparable, suggesting that the plasmid was rapidly incorporated into a replicating pool, and initiated replication approximately once per cell cycle. Additional experiments revealed that the BrUdR labeling of both the pNeo.Myc-2.4 plasmid and the β-actin genes was inhibited by 5 μM aphidicolin (C.M. and M.L., unpublished).

In control experiments BrUdR pulse labeling of HeLa cells transfected 48 hours earlier with the parent pdMMTneo(302-3) vector yielded only light-light plasmid. We also constructed the plasmid pNM.XB3 which contains the 3 kb c-myc DNA fragment extending 3′ from the XhoI site in exon I to the BglII site in intron II inserted into the SalI and BamHI sites of pdMMTneo(302-3). Two days post-transfection this plasmid incorporated 10-fold less density label in an overnight BrUdR pulse than did the chromosomal DNA in the same transfected cell culture, indicating that this control plasmid showed a significantly lower ability to initiate semiconservative replication (Table 2). Therefore, the presence of human c-myc DNA per se was not sufficient to confer efficient autonomous replication activity on the pdMMTneo(302-3) vector. As an additional control, a random clone was selected from a partial genomic library containing 2–4 kb HindIII restriction fragments of HeLa DNA cloned in pdMMTneo(302-3). This clone, pNM.HH1, also did not show semiconservative incorporation of BrUdR into extrachromosomal plasmid in the transient replication assay (Table 2).

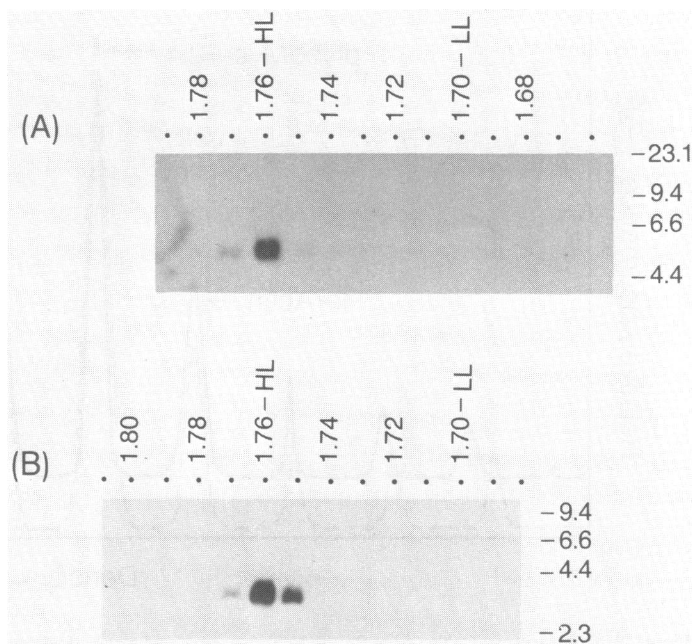

Figure 8. Transient replication analysis of c-myc plasmids. HeLa cells were labeled with 65 μM BrUdR for 24 hours (A) 9 days post-electroporation with pNeo.Myc-2.4 or (B) 3 days post-electroporation with pNeo.Myc-1.2. Hirt supernatant DNA was banded on neutral CsCl gradients and aliquots across the gradients were subjected to electrophoresis and blot hybridization to the neo^r probe. The central 14 fractions of each gradient (33 fractions) are shown. The top of each gradient is at the right. Approximate densities are indicated above each panel. HL and LL refer to the peak positions of heavy-light and light-light marker chromosomal DNAs. Size markers are indicated in kilobase pairs.

Table 2. BrUdR Labeling of Plasmids in Short-Term Replication Assays[a]

Plasmid	Insert[b]	BrUdR incorporation[c]
pNeo.Myc-2.4	2.4 kb c-myc 5′ DNA	+
pNeo.Myc-1.2	1.2 kb c-myc 5′ DNA	+
pdMMT.neo(302-3)	none	–
pNM.XB3	3.0 kb c-myc gene DNA	–
pNM.HH1	2.5 kb genomic DNA	–

a; HeLa cell cultures were incubated overnight with 50–100 μM BrUdR 48–72 hours post-transfection. Density labeling of plasmid and chromosomal DNAs were compared by densitometry (Methods). b; plasmid inserts were in the pdMMTneo(302-3) vector: pNeo.Myc-2.4, 2.4 kb HindIII/XhoI DNA fragment of c-myc 5′ flanking DNA; pNeo.Myc-1.2, 1.2 kb SstII/XhoI DNA fragment of c-myc 5′ flanking DNA; pNM.XB3, 3 kb XhoI/BglII DNA fragment extending from c-myc exon I to intron II; pNM.HH1, 2.5 kb random HeLa genomic DNA HindIII fragment. c; +, equivalent percentages of chromosomal and plasmid DNAs were density labeled; –, plasmid labeling was 0–10% of chromosomal DNA density labeling.

Sensitivity of pNeo.Myc-2.4 to methylation-sensitive restriction enzymes

As an additional test of the replication of pNeo.Myc-2.4 the plasmid recovered from long-term HeLa cultures was subjected

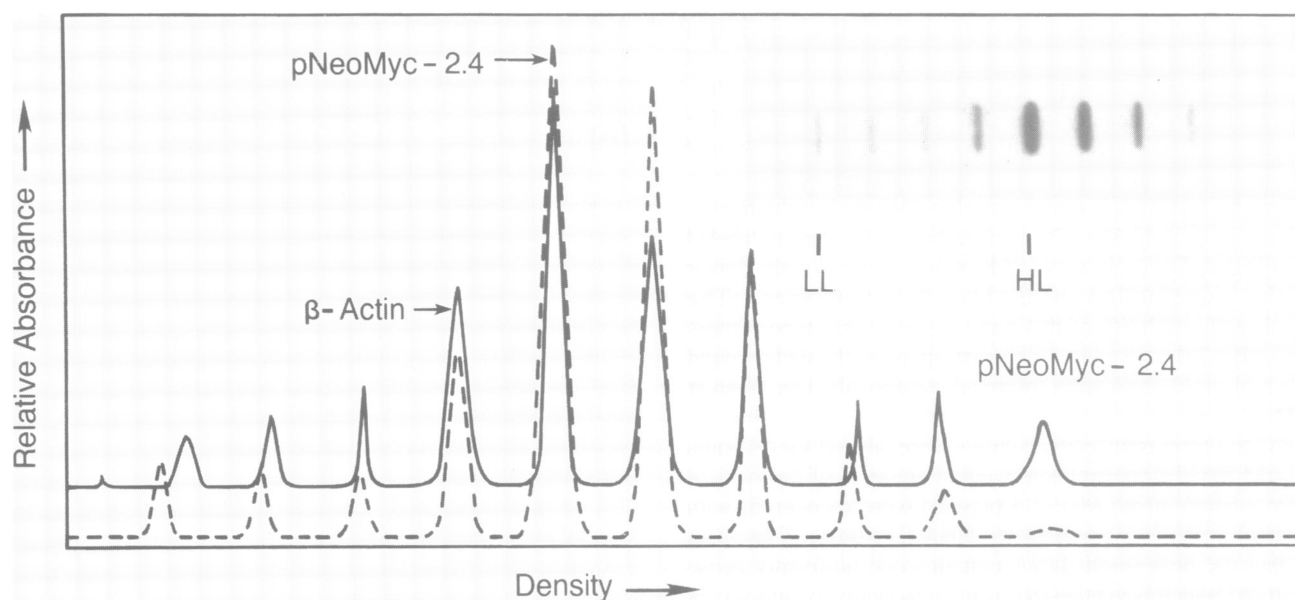

Figure 9. Comparison of pNeo.Myc-2.4 and chromosome replication. HeLa cells 48 hours post-transfection were labeled with 65 μM BrUdR for 24 hours and total cellular DNA was banded on neutral CsCl. Aliquots across the gradient were slot-blotted to the neo^r probe. The blot was stripped of probe and rehybridized to a β-actin probe (34). The inset shows the autoradiogram after hybridization to the neo^r probe (central 12 of 33 fractions shown). The signals from the two hybridizations were compared by densitometry. The top of the gradient is at the left.

to digestion with DpnI or MboI. Under the appropriate conditions DpnI will cleave its target sequence, GATC, only when the central adenine residues are methylated, whereas MboI will digest this sequence only when the adenines on each strand are unmethylated. Dilution of the adenine methylation pattern established in dam^+ *E. coli* as judged by resistance of supercoiled plasmid DNA to DpnI digestion and sensitivity to MboI digestion is taken as evidence of DNA replication in the eucaryotic host (16, 19, 43, 46). A HeLa cell subculture which showed low levels of miniplasmid approximately 450 cell doublings after transfection was used for the purification of pNeo.Myc-2.4 monomer by Hirt extraction and CsCl/EtBr centrifugation (Materials and Methods). Plasmid DNA was mixed with an excess of linearized pUC18 which had been cloned in dam^+ *E. coli*. As shown in Figure 10 (lane 1), the recovered supercoiled pNeo.Myc-2.4 was resistant to an amount of DpnI which completely digested the internal pUC18 reference plasmid. In contrast, the internal pUC18 reference was stable to MboI digestion under conditions which resulted in the complete loss of the pNeo.Myc-2.4 plasmid (Figure 10, lane 2) and the contaminating chromosomal DNA (not shown). These results suggest that each of the >25 potential DpnI target sites has lost its bacterial methylation through replication in HeLa, and that the plasmid is capable of rereplication, leading to MboI sensitivity.

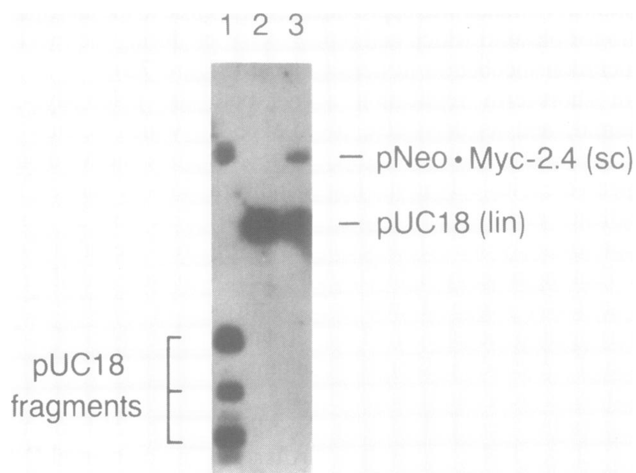

Figure 10. Digestion of recovered pNeo.Myc-2.4 with methylation-sensitive restriction enzymes. Low molecular weight supercoiled DNA was extracted from HeLa cultures approximately 18 months (ca. 450 cell generations) post-transfection, mixed with an excess of pUC18 (Materials and Methods) and digested with DpnI (lane 1) or MboI (lane 2). Lane 3, the DNA mixture before digestion with DpnI or MboI. The migration positions of linear pUC18, supercoiled pNeo.Myc-2.4, and the DpnI digestion fragments of pUC18 are indicated.

DISCUSSION

The bidirectional movement of replication forks initiating within 3.5 kb upstream of the c-myc gene of HeLa cells is consistent with the suggestion that the previously detected barrier to DNA polymerases in the 5′ c-myc flanking DNA (32) is a chromosomal origin of replication. That the plasmid pNeo.Myc-2.4 containing the 5′ flanking region of the human c-myc locus persists as an extrachromosomal element in HeLa cells supports the contention that the presence of the c-myc flanking DNA imparts to the plasmid the ability to be recognized by the replication machinery of HeLa cells, although by itself the recovery of a fraction of pNeo.Myc-2.4 DNA as monomer-sized plasmid does not directly preclude retention due to repeated excision of chromosomally integrated DNA. However, in the absence of detectable chromosomally integrated copies of the plasmid, the long-term stability and amplification of pNeo.Myc-2.4 imply that it is able to replicate autonomously. Hybridization intensity comparisons with known amounts of c-myc DNA indicate that the extrachromosomal c-myc sequences are present on average at

roughly 10−50 copies per cell in stable cultures. Additionally, the pNeo.Myc-2.4 plasmid is lost from HeLa cells at a rate of approximately 5% per cell generation in the absence of drug selection. This rate is approximately four orders of magnitude greater than the rate of spontaneous loss of yeast chromosomes (47, 48), but is several fold slower than the anticipated rate of loss of an unstable nonreplicating plasmid through simple dilution at mitosis, as if the presence of the plasmid is stabilized by a mechanism such as nonrandom partitioning or autonomous replication. The increase in the amount of the transfected plasmid, taken together with the inability of the parental vector pdMMTneo(302-3) to be retained in nonintegrated form or to incorporate BrUdR in transient replication assays in vivo argue that the c-myc sequences contain one or more sites for the initiation of DNA replication or are able to induce such sites in the vector.

Within 48 hours after transfection a substantial proportion of the pNeo.Myc-2.4 plasmid is recoverable in supercoiled form, suggesting the acquisition of nucleosomal packaging, as reported for other transfected DNAs (49). The time-dependent appearance of pNeo.Myc-2.4 oligomer and, in some transfected cultures, deletion derivatives demonstrates that the plasmid is a substrate for cellular recombination enzymes in this form.

Density labeling experiments detected heavy-light and heavy-heavy pNeo.Myc-2.4 DNA on native CsCl gradients, and uniformly heavy pNeo.Myc-2.4 single strands on alkaline CsCl. Thus the majority of bromodeoxyuridine incorporation into the plasmid is not due to dispersive labeling or DNA repair; instead individual plasmid molecules undergo repeated rounds of semiconservative replication. This BrUdR incorporation follows a schedule similar to that of genomic replication and is sensitive to aphidicolin. In experiments not shown, we have also found that the c-myc 5′ flanking DNA which confers ARS activity on pdMMTneo(302-3) also allows the plasmid pRSV.Neo (42) to persist as an extrachromosomal element in HeLa cells under G418 selection for more than 85 cell generations, and allows semiconservative density labeling of pNeo.Myc-2.4 within 48 hours after transfection into human 293S cells (S. Berberich, personal communication). Therefore, the ARS activity of the c-myc origin is not specific to the pdMMTneo(302-3) vector or to HeLa cells.

Although our experiments have focused on the ability of the c-myc origin to impart ARS activity to the pdMMTneo(302-3) vector, we initially identified this origin based on its activity in the HeLa chromosome; the presence of a replication origin within ca. 2 kb 5′ to the HeLa c-myc gene has recently been confirmed by L. Vassilev and E. Johnson (personal communication). In contrast, several laboratories have shown that the the identification of mammalian replication origins based first on the ability of these sequences to confer ARS activity to selectable plasmids in long-term cell cultures is problematic, possibly because replication initiation and nuclear retention functions are not normally co-isolated in random genomic DNA fragments (52−54).

In summary, our view that the pNeo.Myc-2.4 plasmid can replicate autonomously in HeLa cells rests on the sum of the following evidence: (1) the plasmid containing c-myc sequences has been maintained continuously in extrachromosomal form for greater than 300 cell generations, (2) more than 500−1000 times the mass of plasmid taken up during the initial transfection has been recovered from stable cultures, (3) chromosomally integrated copies of the plasmid were not detected in cells displaying extrachromosomal pNeo.Myc-2.4, (4) the plasmid showed semiconservative incorporation of BrUdR in both long-term and transient transfected cultures, (5) the recovered plasmid was resistant to DpnI digestion and sensitive to MboI digestion. In contrast, the parent vector pdMMTneo(302-3) did not display any of these properties. Moreover, the absence of mycoplasmal contamination of these cultures, the synchrony of pNeo.Myc-2.4 plasmid and actin gene replication, and the sensitivity of pNeo.Myc-2.4 replication to aphidicolin together suggest that the plasmid replicates within the transfected HeLa cells through the activity of the major cellular replicative DNA polymerases. In conjunction with the demonstration that replication forks proceed divergently from this region in intact chromosomes these data argue that the chromosomal origin of bidirectional replication of the c-myc gene is located within 2.4 kb 5′ to the gene itself. The possibility that the activity of this origin of replication can be modulated by factors which act in *trans* has been raised by experiments showing that the transcriptionally repressed germ-line c-myc genes in CA46 and ST486 Burkitt lymphoma cell lines are replicated from downstream origins whereas the transcribed c-myc genes of HeLa cells are replicated in the transcriptional direction from a nearby 5′ origin (32; and vide infra).

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