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SHORT NOTE

Replication of the Dihydrofolate Reductase Genes on Double Minute Chromosomes in a Murine Cell Line

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The purpose of this study is to determine the kinetics of the replication of intrachromosomal versus extrachromosomal amplified dihydrofolate reductase (DHFR) genes. Previous studies reported that the DHFR gene, when carried intrachromosomally on a homogeneously staining region, replicates (as a unit) within the first 2 h of the S phase of the cell cycle. We wished to determine if the extrachromosomal location of the amplified genes carried on double minute chromosomes effects the timing of their replication. Equilibrium cesium chloride ultracentrifugation was used to separate newly replicated (BUdR-labeled) DNA from bulk DNA in a synchronized cell population. Hybridization with the cDNA for the DHFR gene allowed us to determine the period of time within the cell cycle in which the DHFR DNA sequences were replicated. We found that, in contrast to intrachromosomal dihydrofolate reductase genes that uniformly replicate as a unit at the beginning of the S phase of the cell cycle, dihydrofolate reductase genes carried on double minute chromosomes (DMs) replicate throughout the S phase of the cell cycle. These results suggest that control of replication of extrachromosomal DNA sequences may differ from intrachromosomal sequences. © 1990 Academic Press, Inc.

INTRODUCTION

Several studies have documented the temporal order of replication of DNA sequences within the genome [1, 2]. Evidence has been accumulating which suggests that actively transcribed genes are generally replicated early within the S phase of the cell cycle while nontranscribed or heterochromatic areas of DNA replicate late in S. The switching of a gene from an inactive state to an active one may be accompanied by a corresponding switch in its timing of replication [3]. "Housekeeping" genes such as dihydrofolate reductase have been reported to replicate early in the S phase of the cell cycle, as well as late [3–5].

Murine cells in culture have been found to generate resistance to the chemotherapeutic drug methotrexate (MTX), by a variety of molecular mechanisms. One widely studied mechanism is amplification of the dihydrofolate reductase (DHFR) gene, which results in an increased cellular content of the target enzyme of the drug [6, 7]. Early studies on the karyotypic abnormalities which accompanied gene amplification revealed that the amplified genes could be carried intrachromosomally, on homogeneously staining regions (HSRs), or extrachromosomally, in the form of double minute chromosomes (DMs) [8, 9]. Other studies demonstrated that the enzyme produced by the amplified DHFR genes was physically, biochemically, and immunochemically identical to the enzyme produced by the single copy gene whether they were carried on HSRs [10, 11] or DMs [12]. Several replication studies on the DHFR genes in mouse, hamster, and human cell lines have shown that when carried intrachromosomally, as single copies or multiple copies in an HSR, the DHFR genes are replicated early in S [4, 5, 13–15]. However, a recent study has reported replication of a single copy DHFR gene in the second half of the S phase in a murine cell line [3]. The present study focuses on the replication of the DHFR genes carried on double minute chromosomes. Does the extrachromosomal location of the DHFR genes alter the temporal replication of the gene? In this study we find that DMs, carrying the DHFR genes, are replicated once per cell cycle and that, in contrast to the synchronized early replication of the amplified DHFR genes carried on the HSR, replication of the DHFR genes on DMs is asynchronous and occurs throughout the S phase of the cell cycle.

MATERIALS AND METHODS

Cell and culture conditions. The cell line 3T6 R50-MS6-clone A was derived as described in Farnham and Schimke [16]. The cell line

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is resistant to 50 μM MTX and contains approximately 40 to 50 times as many dihydrofolate reductase genes as do sensitive cells [17]. These genes are contained on extrachromosomal elements (approximately 30 double minute chromosomes per cell) [16]. The cells are maintained in Dulbecco's modified Eagle's medium in 5% CO_2. The medium is supplemented with 10% dialyzed fetal calf serum, 50 μM MTX, 100 units of penicillin and 100 μg of streptomycin/ml. These cells are maintained in Ham's F-12 medium without glycine, hypoxanthine, or thymidine (GIBCO) at 10% CO_2. The medium is supplemented with dialyzed serum, MTX, penicillin, and streptomycin as described above.

Cell synchronization. Cells were synchronized according to the method of Mariani *et al.* [18, 19]. Briefly, cells were grown in a 150-cm flask (Costar Plastics, Cambridge, MA). The medium was drained and 4 ml of prewarmed media was added. The flask was tapped several times to dislodge the mitotic cells and they were transferred to a 25-cm flask. After 1 h, the medium was replaced with fresh prewarmed medium to remove dead cells. This is designated as $t = 0$.

Radioisotope labeling of DNA. DNA was prelabeled by exposing exponentially growing cells to 0.02 μCi/ml [^{14}C]thymidine ([*methyl*-^{14}C]thymidine, 40–60 mCi/mmol, New England Nuclear) for three to four generations. The ^{14}C was removed 24 h before mitotic selection so that precursor pools could be cleared. To determine the rates of [^{3}H]thymidine incorporation during the S phase of the cell cycle, synchronous populations selected by mitotic shake-off were labeled for 20 min at 37°C at 1-h intervals in the presence of 2.0 μCi/ml of [^{3}H]thymidine ([*methyl*-^{3}H]thymidine, 6.7 Ci/mmol, New England Nuclear). The pulse was terminated by the addition of ice-cold Hanks' balanced salt solution with unlabeled thymidine (10 μg/ml). After removal of the above solution, lysis buffer was added to the plates and incubated at room temperature for 10 min. The plates were scraped into tubes and incubated at 37°C for 3 h or room temperature overnight. An equal volume of trichloroacetic acid (10% wt/vol; 4°C) was added, and the extracts were kept on ice for 30 min. Trichloroacetic acid-precipitable material was collected on Whatman glass fiber filters (GF/C) and washed with 5% trichloroacetic acid. Filters were dried and the amount of radioisotope was determined by immersion in Liquiscent (National Diagnostics, Somerville, NJ). Rates of [^{3}H]thymidine incorporation were normalized to the quantity of [^{14}C]thymidine present.

5-Bromodeoxyuridine density labeling. For DNA density labeling, cells were pulsed in 10 μM BrdU for 30 min. This concentration of BrdU provided adequate substitution to achieve maximal shift of DNA on a cesium chloride gradient without significantly affecting the growth rate of the cells (data not shown).

DNA preparation and CsCl density equilibrium centrifugation. DNA is prepared for CsCl centrifugation as described in Mariani and Schimke [5] except that DNA was sheared by only five passes through a 25-gauge needle. After separation on CsCl gradients, the substituted and unsubstituted peaks were pooled and the DNA was dialyzed and used for slot blot analysis.

Synthesis and labeling of oligonucleotides. The intronic 32mer and exonic 30mer were chemically synthesized on a polymer support using the phosphoramidite nucleoside chemistry [20]. An Applied Biosystems 380A DNA synthesizer was used. Appropriate sequences were selected from the 3′ region of exon 1 and the 5′ region of intron 1. After the oligomers were converted to diesters, they were precipitated, purified on a 10% polyacrylamide gel, and quantitated. Typically, 1–2 nmol of each of the synthetic oligomers was labeled with T4 polynucleotide kinase and γ-labeled dATP, separated from unincorporated label by chromatography on a 10-ml column of Sephadex G-50, and used in hybridization to detect homologous sequences on nitrocellulose filters.

Preparation of DNA and hybridization of slot blots. Gene copy number was quantiated using the slot hybridization assay as previously described [21]. Briefly, trypsinized cells (2×10^5) are rinsed in Hanks' balanced salt solution containing 1% serum and pelleted. Cells are lysed by the addition of solution A (0.01 M Tris–HCl, 0.01 M EDTA, 0.01 M NaCl, 0.2% SDS) and treated with RNase A and Proteinase K. The sample is extracted with phenol:chloroform (1:1) and then chloroform alone. The DNA is denatured by the addition of NaOH, neutralized by the addition of ammonium acetate, and immediately applied to nitrocellulose. After application the filter is briefly rinsed in 5× SSPE and baked *in vacuo* for 2 h at 80°C. The filter is then hybridized with cloned ^{32}P-labeled DHFR [5] or HMG CoA reductase [22] cDNAs under aqueous conditions [23]. The filters are washed, dried, exposed to preflashed X-ray film, and developed.

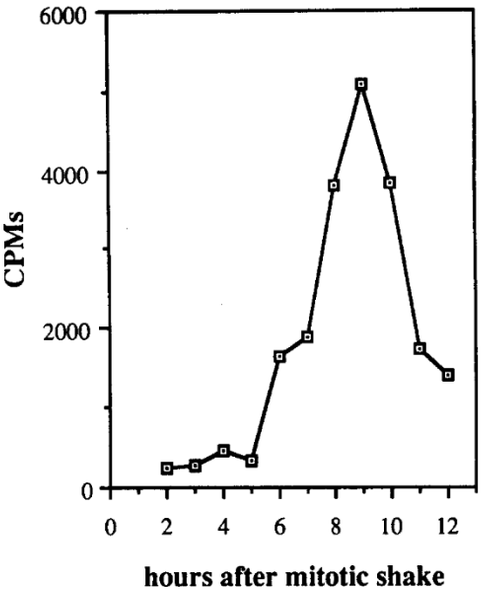

FIG. 1. Cells were synchronized by mitotic shake and plated into 25-cm flasks. At hourly intervals the cells were labeled with [^{3}H]thymidine for 20 min. [^{3}H]Thymidine incorporation is expressed relative to 5×10^5 cells. Each point is the average for duplicate plates per time interval.

RESULTS

3T6 R50-MS6-clone A is a mouse cell line which carries approximately 50 copies of the dihydrofolate reductase gene extrachromosomally, as double minute chromosomes. These cells were synchronized by mitotic shake as described in detail by Mariani and Schimke [5]. At hourly intervals a small aliquot of cells was labeled with [^{3}H]thymidine to monitor DNA replication. Figure 1 shows the incorporation of [^{3}H]thymidine during a complete cell cycle as a measure of the synchrony of the population. The mouse cells entered the S phase of the cell cycle during the 5th h after mitotic shake. The cells' progress into the G2 phase and mitosis is complete by the 12th h. The cell cycle kinetics of these cells are similar in duration and synchrony to those measured in Chinese hamster ovary cells [5] where the amplified copies of the DHFR gene are carried intrachromosomally on a homogeneously staining region.

At each 30-min interval during the S phase of the cell cycle a sample of cells was incubated in the presence of bromodeoxyuridine. Incorporation of this density label

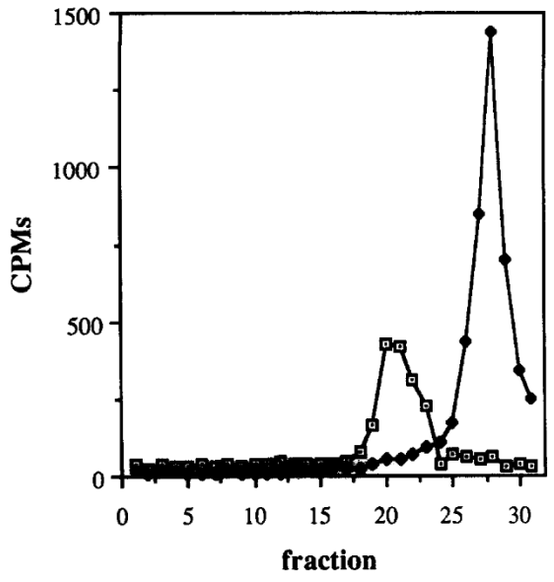

FIG. 2. Cells were labeled for 30 min with BUdR from 8.5 to 9.0 h after the mitotic shake in this representative sample. DNA was harvested and isolated in CsCl gradients as described in Mariani and Schimke [5]. Heavy–light (newly replicated) DNA was obtained by pooling fractions 18–23 (open squares). Nonsubstituted (nonreplicated) DNA was obtained by pooling fractions 27–31 (solid diamonds).

into DNA as it is being synthesized allows for the separation of newly replicated DNA from bulk unreplicated DNA during CsCl gradient centrifugation. A typical gradient is shown in Fig. 2. DNA was isolated from the substituted (heavy–light) as well as the unsubstituted (light–light) peaks and applied to nitrocellulose filters for slot blot analysis. Hybridization of probes with the heavy–light fraction of each gradient indicates when the gene is replicated. Figure 3 (left panel) shows hybridization of these samples with a cDNA probe for the HMG-CoA reductase gene. Replication of this single copy housekeeping gene occurs within a discrete interval of the S phase of the cell cycle, 8.0 h after mitotic shake. Previously published studies by Mariani and Schimke [5] demonstrated that replication of the DHFR genes in a homogeneously staining region occurs in a synchronous fashion, as a unit, early in the S phase of the cell cycle. Figure 3 (right panel) shows hybridization of the same DNA samples with a cDNA probe for the DHFR gene. In contrast to the discretely timed replication of HMGCoA reductase gene, DHFR gene sequences on double minute chromosomes are replicated throughout the S phase of the cell cycle.

To determine if the late replicating DHFR sequences may be due to the replication of intronless pseudogenes we performed the following analysis. We synthesized a 32-base oligomer homologous to a portion of the first intron in the DHFR murine sequence (5′ catgaccctactcagtgaaccgcctcagtttc 3′) and a 30-base oligomer homologous to exon 1 of the same gene (5′ gcggaggccagggtaggtctccgttcttgc 3′). Pseudogenes are usually processed and occur in the genome without the original intronic segments. If the late-replicating DHFR sequences are due solely to replication of pseudogenes of DHFR, we would expect that the oligomer complimentary to intron 1 of DHFR would only hybridize with newly replicated DNA during the early part of the S phase of the cell cycle. If the nonprocessed genes on DMs are replicating both early and late we would expect to see hybridization of both oligomers to be distributed in a pattern similar to that seen for the hybridization with the cDNA for the DHFR gene in Fig. 3 (right panel). Hybridization of the intronic sequence or the exonic sequence with the DNA samples in Fig. 3 generated a pattern identical to that seen with the cDNA probe for the DHFR gene (data not shown). Similar hybridization patterns with intronic, exonic, and cDNA DHFR sequences demonstrate that the detected DHFR genes which are replicating during the latter part of S are not processed pseudogenes.

DISCUSSION

We have studied the replication of the DHFR DNA sequences which are carried on DM chromosomes in physiologically synchronized cell populations in order to compare the replication of extrachromosomally located amplified DNA sequences with that of intrachromosomally located amplified copies.

Gross replication of DNA sequences carried on DM chromosomes has been studied by Barker *et al.* [24] using autoradiography. Their results indicated that the

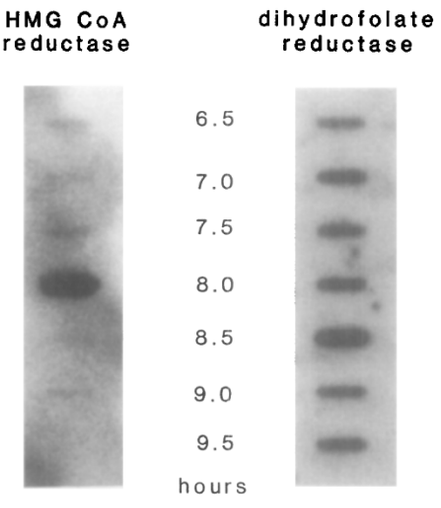

FIG. 3. Equal amounts of CsCl purified heavy–light DNA (1 μg) from each window of the S phase of the cell cycle was applied to nitrocellulose. [^{14}C]Thymidine, which indicated total DNA, was present in equal intensity in each slot (data not shown). The filter was hybridized with the cDNA of HMG CoA reductase (specific activity 1.8×10^8 dpm/μg DNA) and autoradiographed. The filter was stripped with NaOH, rechecked for the presence of the DNA by monitoring the ^{14}C intensity, and rehybridized with the cDNA for the DHFR gene (specific activity 1.6×10^8 dpm/μg DNA).

DNA within the double minute chromosomes replicated during the S phase, once per cell cycle [24]. Similarly, studies on a CAD episome yielded the same results using BUdR labeling and CsCl centrifugation [25]. In our studies, replication of the DHFR sequences in the presence of BUdR results in a shift of newly replicated DNA to a higher density in CsCl gradients (production of heavy–light DNA). In no instances were DHFR sequences located in doubly substituted (heavy–heavy) DNA, a result which would have indicated multiple rounds of replication within a single cell cycle. Our results, therefore, are consistent with those previously reported by Barker *et al.* [24], which demonstrated that DM chromosomes replicate once during the S phase of the cell cycle. Also consistent with previous reports [24] was our finding that replication of DHFR sequences (i.e., DM-containing DNA) did not occur outside of the S phase of the cell cycle (data not shown).

Specific replication of the DHFR genes has been studied previously by investigators using amplified cell lines. In these studies, the DHFR genes were carried on a single HSR [5, 13, 14] or two HSRs [15]. In all cases studied, the DHFR gene replicated in the first half of the S phase of the cell cycle even when the gene sequences were clustered on two different chromosomes. The studies of Mariani and Schimke refined the window of replication even further and demonstrated that the amplified genes replicated during the first 2 h of the S phase [5]. The replication of amplified DHFR sequences as they are carried on DM chromosomes is an area which has been less extensively studied. Our studies have used bromodeoxyuridine incorporation of DNA (during replication) to separate replicated DNA from nonreplicated bulk DNA. We have then probed each pool of replicated DNA to determine the presence of DHFR sequences in each window of the cell cycle. We find that DHFR sequences are present in each window. Poor synchronization of the cell population would also give rise to this result. Even though the synchronization profile of the R50 cells looked good (see Fig. 1) we have hybridized our aliquots of replicated DNA with an independent probe, HMG CoA reductase, to analyze the replication of a single gene sequence in our samples. The hybridization profile in Fig. 3 demonstrates the excellent degree of synchrony attained in this set of experiments and rules out this explanation as a possible basis for the pattern of hybridization observed.

An alternative explanation for the extensive hybridization of DHFR sequences throughout the cell cycle would be the detection of pseudogenes in the genomic DNA. Recent studies of the eucaryotic genome have indicated the existence of pseudogenes, copies of specific genes which are not expressed. Such intronless pseudogenes have been reported for the DHFR gene in human cell lines [26, 27]. In this study we examined whether the hybridization of DHFR sequences in the late part of the S phase of the cell cycle is due to the presence of intronless pseudogenes. We have synthesized an oligomer homologous to 32 base pairs in the first intron of the mouse DHFR gene. Hybridization of this sequence throughout the S phase of the cell cycle (paralleling the hybridization pattern of the entire gene) suggests that the late-replicating sequences which are homologous to DHFR are not attributable to the late replication of intronless pseudogenes.

DHFR genes carried on double minute chromosomes replicated throughout the S phase of the cell cycle while DHFR genes carried within the chromosome replicate only within a discrete window of the S phase. One interpretation of this result is that the DHFR sequences carried on DM chromosomes are not subject to the same regulation of replication as those genes carried on the chromosome. This regulation could either be at the level of initiation or elongation of replication. It could be that the initiation of replication of the DHFR genes occurs early in the S phase (in both intrachromosomal and extrachromosomal locations) but that elongation of DNA sequences on DM chromosomes proceeds slowly. Alternatively, these genes may be initiated for replication throughout the S phase of the cell cycle. Other possible explanations include differences in the flanking regions of DNA, higher order chromatin structure or differential access to replication complexes. The first possibility would seem to be less likely because of the large amount of flanking DNA which is amplified along with the DHFR gene; estimated sizes range from 135 to 10,000 kb [28, 29]. Reports indicate that an origin of replication is located relatively near the 3′ end of the DHFR gene in CHO cells [30] suggesting inclusion of replication regulatory sequences within the amplified unit. Preliminary information has been published which may address the second possibility. Ultrastructural studies have shown that DM chromosomes contain nucleosomal chromatin which is organized into typical higher order fibers [28]. Electron microscopy of Miller spread preparations of DM chromosomes demonstrated thick chromatin fibers that are organized into loops and radiate from a central structure. These structures are reminiscent of the scaffold structures observed in genomic DNA and have been postulated to regulate transcription as well as replication of active genes [31]. Interestingly, the electron microscopy also revealed that DM chromosomes are often linked through chromatin strands and may be topologically linked circular molecules which have not been resolved. It is possible that these concatenated structures could alter the progression of DNA elongation and yield the results reported in this paper.

The data presented in this paper suggest that the DHFR sequences present on DMs are not subject to the same regulation of replication as those genes found intrachromosomally. Further studies are needed to determine the molecular basis for this observation.

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REFERENCES

1. Calza, R. E., Eckhardt, L. A., DelGiudice, T., and Schildkraut, C. L. (1984) *Cell* **36,** 689–696.
2. Iqbal, M. A., Chinsky, J., Didamo, V., and Schildkraut, C. L. (1987) *Nucleic Acids Res.* **15,** 87–103.
3. Hatton, K. S., Dhar, V., Brown, E. H., Iqbal, M. A., Stuart, S., Didamo, V. T., and Schildkraut, C. L. (1988) *Mol. Cell. Biol.* **8,** 2149–2158.
4. Doggett, N. A., Cordeiro-Stone, M., Chae, C-B., and Kaufman, D. G. (1988) *Mol. Carcinog.* **1,** 41–49.
5. Mariani, B. D., and Schimke, R. T. (1984) *J. Biol. Chem.* **259,** 1901–1910.
6. Alt, F. W., Kellems, R. E., Bertino, J. R., and Schimke, R. T. (1978) *J. Biol. Chem.* **253,** 1357–1370.
7. Schimke, R. T. (1984) *Cell* **37,** 705–713.

7a. Stark, G. R., and Wahl, G. M. (1984) *Annu. Rev. Biochem.* **53,** 447–503.

8. Biedler, J. L., and Spengler, B. A. (1976) *Science* **191,** 185–187.
9. Kaufman, R. J., Brown, P. C., and Schimke, R. T. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5669–5673.
10. Alt, F. W., Kellems, R. E., and Schimke, R. T. (1976) *J. Biol. Chem.* **251,** 3063–3074.
11. Bostock, C. J., Clark, E. M., Harding, N. G. L., Mounts, P. M., Tyler-Smith, C., Van Heyningen, V., and Walker, P. M. P. (1979) *Chromosoma* **74,** 153–177.
12. Morandi, C., and Attardi, G. (1985) *J. Biol. Chem.* **256,** 10169–10175.
13. D'Andrea, A. D., Tantravahi, U., Lalande, M., Perle, M. A., and Latt, S. A. (1983) *Nucleic Acids Res.* **11,** 4753–4774.
14. Hamlin, J. L., and Biedler, J. L. (1981) *J. Cell. Physiol.* **107,** 101–114.
15. Kellems, R. E., Leys, E. J., Harper, M. E., and Smith, L. M. (1982) *in* Gene Amplification (Schimke, R. T., Ed.), pp. 81–88, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
16. Farnham, P. J., and Schimke, R. T. (1985) *J. Biol. Chem.* **260,** 7675–7680.
17. Brown, P. C., Beverley, S. M., and Schimke, R. T. (1981) *Mol. Cell. Biol.* **1,** 1077–1083.
18. Mariani, B. D., Slate, D. L., and Schimke, R. T. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 5985–5989.
19. Kaufman, R. J., and Schimke, R. T. (1981) *Mol. Cell. Biol.* **1,** 1069–1076.
20. Matteucci, M. D., and Caruthers, M. H. (1981) *J. Amer. Chem. Soc.* **103,** 3185–3191.
21. Brown, P. C., Tlsty, T. D., and Schimke, R. T. (1983) *Mol. Cell. Biol.* **3,** 1097–1107.
22. Luskey, K. L., Faust, J. R., Chin, D. J., Brown, M. S., and Goldstein, J. L. (1983) *J. Biol. Chem.* **258,** 8462–8469.
23. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
24. Barker, P. E., Drwinga, H. L., Hittleman, W. N., and Maddox, A-M. (1980) *Exp. Cell Res.* **130,** 353–360.
25. Carroll, S. M., Gaudray, P., De Rose, M. L., Emery, J. F., Meinkoth, J. L., Nakkim, E., Subler, M., Von Hoff, D. D., and Wahl, G. M. (1987) *Mol. Cell. Biol.* **7,** 1740–1750.
26. Masters, J. N., Yang, J. K., Cellini, A., and Attardi, G. (1983) *J. Mol. Biol.* **167,** 23–36.
27. Chen, M., Shimada, T., Moulton, A. D., Harrison, M., and Nienhuis, A. W. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7435–7439.
28. Hamkalo, B. A., Farnham, P. J., Johnston, R., and Schimke, R. T. (1985) *Proc. Natl. Acad. Sci. USA.* **82,** 1126–1130.
29. Stark, G. R., Debatisse, M., Giulotto, E., and Wahl, G. M. (1989) *Cell* **57,** 901–908.
30. Heintz, N. H., Milbrant, J. D., Greisen, K. S., and Hamlin, J. L. (1983) *Nature (London)* **302,** 439–442.
31. Vogelstein, B., Pardoll, D. M., and Coffey, D. S. (1980) *Cell* **22,** 79–85.

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