MOLECULAR AND CELLULAR BIOLOGY, Feb. 1989, p. 586–593
0270-7306/89/020586-08$02.00/0

Vol. 9, No. 2

Opposite Replication Polarity of the Germ Line c-*myc* Gene in HeLa Cells Compared with that of Two Burkitt Lymphoma Cell Lines

MICHAEL LEFFAK* AND C. DAVID JAMES†
Department of Biochemistry, Wright State University, Dayton, Ohio 45435

Received 21 July 1988/Accepted 17 November 1988

To study the cell type specificity of the direction of replication of the human c-*myc* genes and the relationship of replication polarity to transcriptional activity, we analyzed the directions of replication of the c-*myc* genes in two Burkitt lymphoma cell lines, CA46 and ST486, and in HeLa cells. On the basis of in vitro runoff replication of forks initiated in intact cells, we found that transcribed c-*myc* genes in the germ line configuration in HeLa cells were replicated in the direction of transcription from origins in the 5′-flanking DNA, while the repressed, unrearranged c-*myc* genes of CA46 and ST486 cells were replicated in the antitranscriptional direction. In contrast, the transcribed c-*myc* genes of CA46 cells were replicated in the transcriptional direction, while the translocated, amplified c-*myc* genes of ST486 cells showed no preferred polarity of replication. The data also provided evidence for the existence of an endogenous barrier to DNA polymerases in the flanking DNA immediately 5′ to the HeLa c-*myc* genes.

The replication of eucaryotic chromosomes is controlled through the selection of sites for the initiation of DNA synthesis (2, 6, 7, 10, 12, 14, 19, 21, 24, 32, 39) and through the temporal programming of the activity at these sites (10, 12, 19, 23). Combined with evidence for the existence of proteins which recognize the replication origins of viral minichromosomes (11, 17, 28, 44), these results suggest that chromosome replication can be regulated in *trans* by DNA sequence-specific protein binding.

That the activity of eucaryotic genes is related to the replication of chromatin is supported by several observations that transcribed genes are replicated early in the S phase of the cell cycle (8, 9, 16, 18) and by the demonstration that the efficient transcription of certain transfected genes requires the replication of their chromatin template (13, 42). Moreover, Miller and Nasmyth (31) have demonstrated that passage through the S phase is necessary for repression of the silent mating type loci in *Saccharomyces cerevisiae*. Smithies (37) has proposed that the processes of replication and transcription are linked such that transcriptionally active genes are replicated from upstream origins, whereas inactive genes are replicated from either upstream or downstream origins. Consistent with this proposal, we have shown that the avian histone H5 genes are replicated in the transcriptional direction in embryonic erythrocytes, where they are expressed, but in the antitranscriptional direction in lymphoblastoid cells and chicken embryo fibroblasts, where they are quiescent (43). In comparison, the avian alpha-pi and alpha-D globin genes are replicated in the transcriptional direction both in cells in which they are active (erythrocytes) and in cells in which they are inactive (lymphoblastoid cells and fibroblasts) (26; Y. I. Lindstrom and M. Leffak, manuscript in preparation).

To investigate further the relationship between replication polarity and transcriptional activity, we used the in vitro runoff replication (IVR) assay (26, 43) to characterize the replication of the human c-*myc* genes. In the Burkitt lymphoma cell line CA46, the coding exons of one c-*myc* allele have been translocated close to the immunoglobulin A (IgA) heavy-chain constant region (35). We found that the translocated, transcribed c-*myc* sequences in CA46 cells are replicated in the transcriptional direction, whereas the quiescent germ line sequences in these cells are replicated in the antitranscriptional direction. Similarly, in the Burkitt lymphoma line ST486 the quiescent, unrearranged c-*myc* gene is replicated in the antitranscriptional direction. In contrast, the germ line c-*myc* genes of HeLa cells are transcribed and are replicated in the transcriptional direction. The replication polarity of the human c-*myc* genes, therefore, shows cell type specificity, and the transcriptional activity of the c-*myc* genes is correlated with replication in the direction of transcription.

MATERIALS AND METHODS

Cell culture. HeLa, CA46, and ST486 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum and 50 μg of gentamicin per ml at 37°C in a 5% CO_2 atmosphere. CA46 and ST486 cells were generously provided by I. McGrath of the National Cancer Institute. Cells in logarithmic growth were labeled in vivo with 20 μg of bromodeoxyuridine (BrUdR) per ml or used for isolation of nuclei and preparation of a replication cocktail.

In vitro replication assay. In vitro replication mixtures were prepared essentially as described previously (26, 43), including a restriction enzyme at about 50 to 100 U/μg of DNA where indicated. DNA was purified by protease K digestion and organic solvent extraction and digested at 10 U of restriction enzyme per μg of DNA. High-specific-activity restriction enzyme preparations were purchased from Bethesda Research Laboratories, Inc., or New England BioLabs, Inc. DNA was centrifuged on neutral CsCl gradients, electrophoresed on agarose gels, and transferred to nitrocellulose or nylon membranes (Schleicher & Schuell, Inc.) (38).

Hybridization and densitometry. Hybridization and high-stringency washing of filters (0.015 M NaCl, 0.0015 M sodium citrate [pH 7.4], 0.1% sodium dodecyl sulfate) were by standard procedures (29, 39). The c-*myc* probes I and II are *Sst*I restriction fragments derived from a CH4A bacteriophage clone containing a 12.5-kilobase (kb) *Eco*RI restriction fragment of human genomic DNA (4, 36). The phage

* Corresponding author.
† Present address: Ludwig Institute for Cancer Research, Montreal, Quebec H4A3E6, Canada.

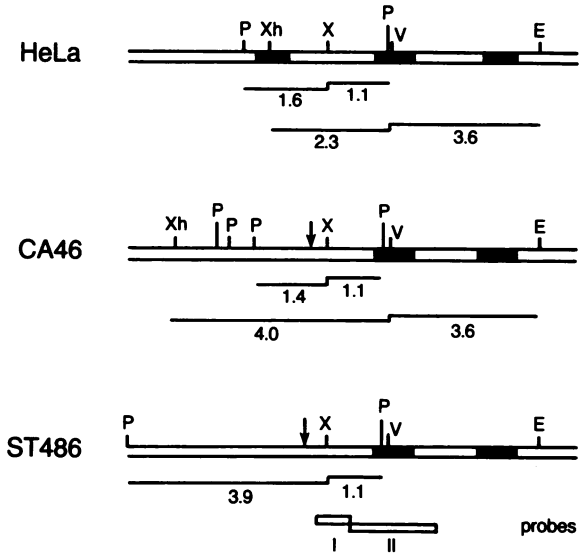

FIG. 1. Maps of unrearranged and translocated c-*myc* loci. The maps were redrawn from the data of Gelman et al. (15), Siebenlist et al. (36), and Showe et al. (35). Symbols: =, c-*myc* and flanking DNA; ▬, c-*myc* exons; ↓, translocation breakpoints. To the left of the arrows is the IgA constant region (in CA46 cells) or the IgM constant region (in ST486 cells). Only the structures of the translocated c-*myc* genes are shown for CA46 and ST486 cells. Jointed lines represent the restriction fragments discussed in the text. Abbreviations: P, *PstI*; Xh, *XhoI*; X, *XbaI*; V, *Eco*RV. The open rectangles at the bottom represent probe I (a 0.65-kb *SstI-SstI* fragment spanning the *XbaI* site in c-*myc* intron I) and probe II (a 1.7-kb *SstI-SstI* fragment spanning the *Eco*RV site in c-*myc* exon II).

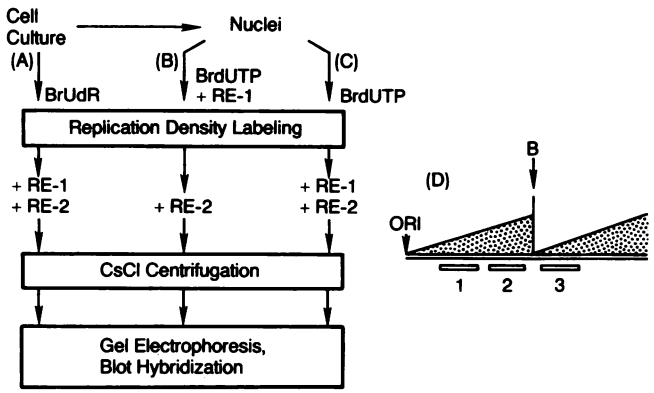

FIG. 2. DNA density labeling. (A) In vivo labeling. An asynchronous culture of cells was labeled with BrUdR for about one-half of a cell generation; DNA was purified, digested with restriction enzymes 1 (RE-1) and 2 (RE-2), and banded on neutral CsCl; and the dense DNA (density, 1.75 to 1.78 g/ml) was analyzed by electrophoresis and blot hybridization. (B) In vitro labeling in the presence of a restriction enzyme. Isolated nuclei were incubated in a replication cocktail containing BrdUTP and restriction enzyme 1. DNA was purified, digested with restriction enzyme 2, and banded on neutral CsCl, and the dense DNA was electrophoresed and blot hybridized. (C) In vitro labeling in the absence of a restriction enzyme. Isolated nuclei were incubated in a replication cocktail containing BrdUTP. DNA was purified, digested with restriction enzymes 1 and 2, and banded on neutral CsCl, and the dense DNA was electrophoresed and blot hybridized. (D) Model for graded-density labeling during in vitro replication. Symbols: =, replicating DNA; ORI, replication origin; ▭, probed fragments; B, barrier to replication fork movement. The amplitude of the wedge at each point reflects the relative degree of density labeling of the DNA beneath it. See the text for a discussion.

clone was generously provided by P. Leder. X-ray film bands were scanned and quantitated with an LKB Ultroscan 2200 soft-laser densitometer and the Gelscan 2190 software package. Reconstruction experiments presented previously have demonstrated the linearity of the instrument response over the range of 0.05 to 3.0 absorbance units (26). In the present work, each hybridized filter was subjected to multiple film exposures to ensure that the film response was linear with respect to the autoradiographic signal. Overexposures of some of the autoradiograms actually scanned are shown to emphasize faint bands (see Fig. 5 and A1).

RESULTS

IVR assay. In the Burkitt lymphoma cell lines CA46 and ST486, the coding exons of one c-*myc* allele have been translocated from chromosome 8 to chromosome 14, into the switch regions of the IgA and IgM heavy-chain genes, respectively. The breakpoints on chromosome 8 in these cell lines fall within 20 base pairs of one another in intron I of the c-*myc* gene, and the translocations result in head-to-head arrangement of the c-*myc* and immunoglobulin constant-region genes (15, 35) (Fig. 1). The translocated c-*myc* exons are transcribed as efficiently in these cells as in human lymphoblastoid cell controls, whereas expression of the unrearranged genes is depressed more than 20-fold (1, 3, 5, 33, 40).

The polarity of c-*myc* replication was determined with an IVR assay which has been described in detail previously (26). Briefly, nuclei were isolated from an asynchronous population of cells and incubated in a nucleotide cocktail containing the density label bromo-dUTP (BrdUTP). Under these conditions, replication is limited to the extension of DNA chains initiated in the intact cells (C. D. James, Ph.D. thesis, Wright State University, Dayton, Ohio, 1986). In the presence of a barrier to the progression of fork movement (for instance, a restriction enzyme cleavage) origin-distal DNA fragments are enriched in density label relative to origin-proximal fragments located on the same side of the barrier (Fig. 2D; compare fragments 1 and 2); origin-proximal DNA fragments are enriched in density label compared with origin-distal fragments located immediately across the barrier (Fig. 2D; compare fragments 2 and 3). Incorporation of BrdUTP across the c-*myc* loci was analyzed by purification of the in vitro-replicated DNA, secondary restriction enzyme cleavage, isopycnic centrifugation, and blot hybridization (Fig. 2B). The relative enrichment for BrdUTP during in vitro replication was normalized by comparing the signal ratio of c-*myc* fragments in dense in vitro-replicated DNA with that obtained by labeling c-*myc* with BrUdR in vivo (Fig. 2A) (26, 43).

In the absence of a nearby barrier to replication fork movement, little difference should be detected in the patterns of density labeling of DNAs replicated in vitro (Fig. 2C) and in vivo. To test this, CA46 nuclei were subjected to in vitro replication and the IVR DNA was purified, digested with *XbaI* and *PstI*, banded on CsCl, electrophoresed, and blot hybridized to probe I (an *SstI-SstI* fragment spanning the *XbaI* site in c-*myc* intron I). Similar stoichiometries of dense DNA fragments were obtained after in vivo density labeling and after in vitro replication of DNA in the absence of externally imposed barriers (Fig. 3).

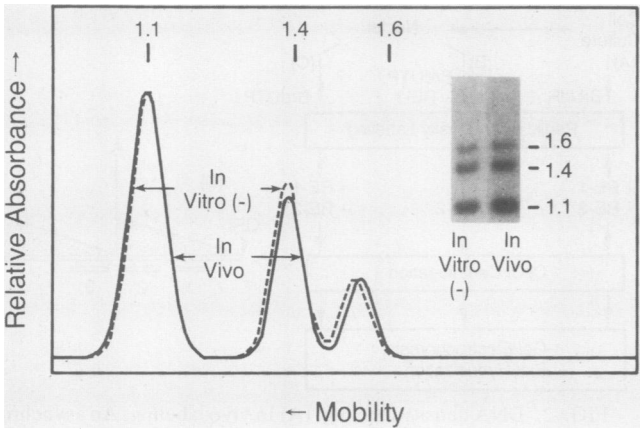

FIG. 3. IVR of CA46 nuclei without in situ restriction enzyme digestion. CA46 nuclei were isolated from an asynchronous cell culture and submitted to the IVR density-labeling reaction as outlined in Fig. 2C. DNA was purified from these nuclei [in vitro (−)-labeled DNA] and from CA46 cells labeled for 6 to 10 h in culture with BrUdR (in vivo-labeled DNA). Each DNA sample was cleaved to completion with *Xba*I and *Pst*I and banded on neutral CsCl gradients run in parallel. Dense DNAs (1.75 to 1.78 g/ml) were isolated from each gradient, electrophoresed on the same gel, and blot hybridized together to probe I. Inset, Autoradiogram of dense DNA from the in vitro (−) replication reaction (left lane) and after in vivo labeling (right lane). DNA sizes are in kilobases. The scans were scaled to normalize the signals of the 1.1-kb bands.

Polarity of c-*myc* replication in CA46 cells. The strategy for determining the replication polarities of the c-*myc* genes in CA46 cells involved introducing a barrier to fork movement in the form of an *Xba*I cut during the nuclear IVR reaction. Subsequent cleavage of the DNA by *Pst*I would then allow comparison of the signal from the density-labeled DNA fragments 5′ to the barrier (the 1.6-kb *Pst*I-*Xba*I fragment of the unrearranged c-*myc* sequences and the 1.4-kb *Pst*I-*Xba*I fragment from the translocated sequences) to the signal from the density-labeled 1.1-kb *Xba*I-*Pst*I fragments 3′ to the barrier.

Restriction enzyme digestion in isolated nuclei is normally incomplete (26, 27), and the unrearranged and translocated c-*myc* genes may show differential sensitivity to digestion in situ, which results in unequal contributions to the signal from the common 3′ DNA fragment. Therefore, a normalized 5′:3′ fragment ratio in dense DNA, N, was calculated for each experiment on the basis of the signals of the DNA fragments not cleaved in situ, to account quantitatively for the effect of differential restriction enzyme digestion (see Appendix). The values of N are indicated in the text and are shown in Table 1. Empirically, consideration of differential cleavage of the unrearranged and translocated c-*myc* genes was found in all of the experiments only to enhance the magnitude of the enrichment or depletion of DNA fragments evident from direct qualitative examination of the data.

When in vitro replication of CA46 nuclei was performed in the presence of *Xba*I, a decrease was observed in the density labeling of the germ line 5′ 1.6-kb *Pst*I-*Xba*I fragment compared with its neighboring 3′ 1.1-kb *Xba*I-*Pst*I fragment. Conversely, there was an increase in the density labeling of the 5′ 1.4-kb *Pst*I-*Xba*I fragment at the translocation compared with its neighboring 3′ 1.1-kb fragment (Fig. 4A). N was 0.39 for the unrearranged gene, and N was 2.95 for the translocated gene (Table 1). Here, a value of N of less than unity indicates that the fragments 5′ to the *Xba*I barrier are farther from the replication origin than fragments 3′ to the *Xba*I site. Thus, the depletion of the germ line 5′ fragment relative to the 3′ fragment suggests that the unrearranged gene is replicated from a downstream origin. Conversely, the enrichment of the 5′ fragment of the translocated gene indicates that the translocated gene is replicated from an upstream origin.

TABLE 1. Calculation of normalized ratios of 5′-3′ DNA fragment signals for IVR reactions

Cells	Restriction enzyme(s)[a]		*R1*[b]	*R2*[c]	N^G[d]	N^T[e]
	1	2				
CA46		*Xba*I-*Pst*I[f]	0.65	0.72	1.07	1.10
CA46		*Xba*I-*Pst*I[g]	0.88	0.25	0.94	1.07
CA46	*Xba*I	*Pst*I	0.82	0.24	0.39	2.95
CA46	*Eco*RV	*Xho*I-*Eco*RI	0.47	0.66	0.43	2.15
ST486	*Xba*I	*Pst*I	0.59	0.42	0.18	0.97

[a] 1, Restriction enzyme used for in situ digestion of nuclei; 2, restriction enzyme used for secondary digestion of purified DNA.
[b] *R1* is the ratio of the signals from the unrearranged and translocated c-*myc* sequences remaining undigested by restriction enzyme 1.
[c] R2 is the ratio of the percentages of the signals from the 3′ c-*myc* fragments in the in vitro- and in vivo-replicated samples.
[d] N^G, Value for the germ line gene.
[e] N^T, Value for the translocated gene. In multiple repeats of each experiment, the calculated values of N^G and N^T did not vary by more than 0.20.
[f] Partial (25%) digestion with *Xba*I.
[g] Partial (80%) digestion with *Xba*I.

These conclusions were confirmed by subjecting CA46 nuclei to the IVR reaction in the presence of *Eco*RV. After purification, the DNA was digested to completion with *Eco*RI and *Xho*I. The data (Fig. 4B) show that, compared with the 3′ 3.6-kb *Eco*RV-*Eco*RI fragments, the germ line 5′ 2.3-kb *Xho*I-*Eco*RV fragment was depleted in dense DNA (N = 0.43), while the 5′ 4.0-kb fragment of the translocated gene was enriched (N = 2.15).

Polarity of c-*myc* replication in ST486 cells. In ST486 cells, the c-*myc* coding exons have been translocated into the IgM heavy-chain constant region (15). Blot hybridization to total genomic DNA with c-*myc* and immunoglobulin gene sequence probes showed that the translocated sequences were duplicated subsequent to the translocation (C. D. James and M. Leffak, submitted for publication). When replication was performed in ST486 nuclei in the absence of restriction enzyme cutting, followed by digestion of the purified DNA with both *Xba*I and *Pst*I, the density gradient distribution of the in vitro-replicated DNA fragments was the same as that of the in vivo-labeled fragments (Fig. 5A). To determine whether the transcriptionally repressed c-*myc* gene is replicated from a 3′ origin in these cells as in CA46, ST486 nuclei were allowed to replicate in vitro in the presence of *Xba*I. The DNA from these nuclei was subsequently digested with *Pst*I and analyzed as described above. The germ line 5′ 1.6-kb *Pst*I-*Xba*I fragment was strongly depleted in dense DNA (N = 0.18) relative to the 3′ 1.1-kb *Xba*I-*Pst*I fragments, however, there was no appreciable enrichment or depletion of the 5′ 3.9-kb *Pst*I-*Xba*I fragments derived from the translocation (N = 0.97) (Fig. 5B; Table 1). These data indicate that the unrearranged c-*myc* gene is preferentially replicated from a 3′ replication origin in ST486 cells. In contrast, the translocated c-*myc* genes are either replicated in opposite directions in different cells in the population or the c-*myc* genes within each duplicated pair are replicated with opposite polarities.

Polarity of c-*myc* replication in HeLa cells. Because the repressed c-*myc* genes in CA46 and ST486 cells appeared to

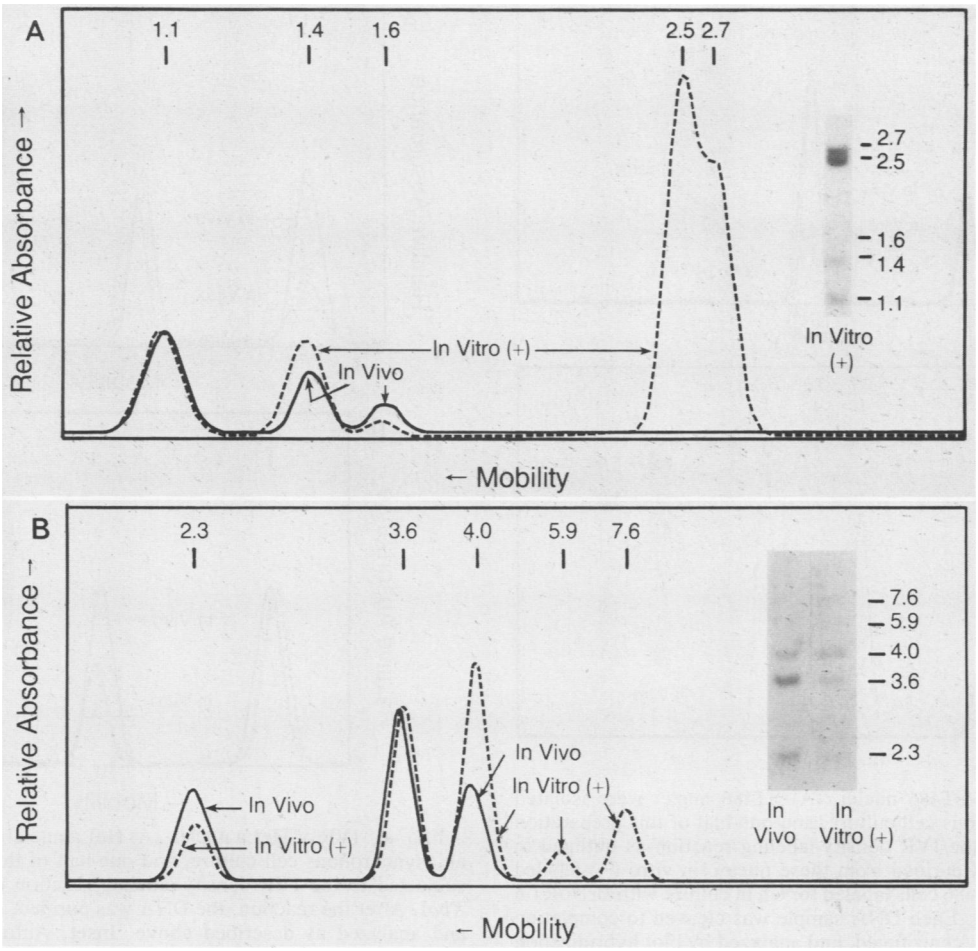

FIG. 4. IVR of CA46 nuclei with digestion in situ. (A) CA46 nuclei were incubated in the IVR reaction mixture in the presence of *Xba*I as outlined in Fig. 2B. DNA was purified, digested to completion with *Pst*I, and analyzed as outlined in Fig. 2B, by hybridization to probe I. Inset, Autoradiogram of the dense DNA from the in vitro (+) reaction. The scan compares the blot hybridization pattern of the dense in vitro (+) DNA with that of DNA density labeled in vivo after normalization of the 1.1-kb band signals. (B) CA46 nuclei were submitted to the IVR reaction in the presence of *Eco*RV. DNA was purified from these nuclei, digested to completion with *Eco*RI and *Xho*I, and analyzed by hybridization to probe II. Inset, Autoradiogram of the dense DNAs from the in vitro reaction and from in vivo labeling. The in vivo-labeled DNA was digested to completion with *Eco*RV, *Eco*RI, and *Xho*I. The scans compare the hybridization patterns of the in vitro (+)- and in vivo-labeled DNAs after normalization of the 3.6-kb band signals. DNA sizes are in kilobases.

be replicated in an antitranscriptional direction, it was of interest to determine the replication polarities of the c-*myc* genes in HeLa cells, in which these sequences are in the germ line configuration and are actively transcribed (20, 34). The data (Fig. 6A) show that in vitro replication of HeLa nuclei in the presence of *Xba*I, followed by DNA digestion with *Pst*I, led to about 2.5-fold enrichment of the 5′ 1.6-kb *Pst*I-*Xba*I fragment in dense DNA over the 3′ 1.1-kb *Xba*I-*Pst*I fragment, demonstrating that the c-*myc* genes in HeLa cells are replicated in the direction of transcription. (These experiments compared fragments on opposite sides of a single restriction enzyme cut site at equivalent homologous chromosomal loci and were not influenced by the occurrence of incomplete digestion products [26].)

Surprisingly, when HeLa nuclei were replicated in the absence of a restriction enzyme and the purified DNA was digested with *Xba*I and *Pst*I, blot hybridization of the dense DNA revealed about twofold depletion of the 5′ 1.6-kb *Pst*I-*Xba*I fragment (Fig. 6B). This confirmed that the germ line c-*myc* genes are replicated from 5′ replication origins but implied also that an endogenous replication fork barrier exists in the 5′-flanking DNA close to the c-*myc* gene. On the basis of the sensitivity of the IVR assay (26), the distance to this barrier was estimated to be no more than 4 to 5 kb.

DISCUSSION

The unrearranged c-*myc* genes are preferentially replicated in the transcriptional direction in HeLa cells and in the antitranscriptional direction in CA46 and ST486 cells. Translocation of the c-*myc* coding exons in these Burkitt lymphoma cell lines results in a shift in their direction of replication. In conjunction with our previous results, these data indicate that the replication polarity of a gene can vary with both cell type and chromosomal location. Moreover, for the histone H5, alpha globin, and c-*myc* genes examined, transcriptional activity is correlated with replication in the transcriptional direction.

It should be noted that the present results reveal only the preferred direction of replication of the c-*myc* genes. For instance, if all restriction enzyme target sites were cut in situ before passage of the replication fork, the IVR model (Fig.

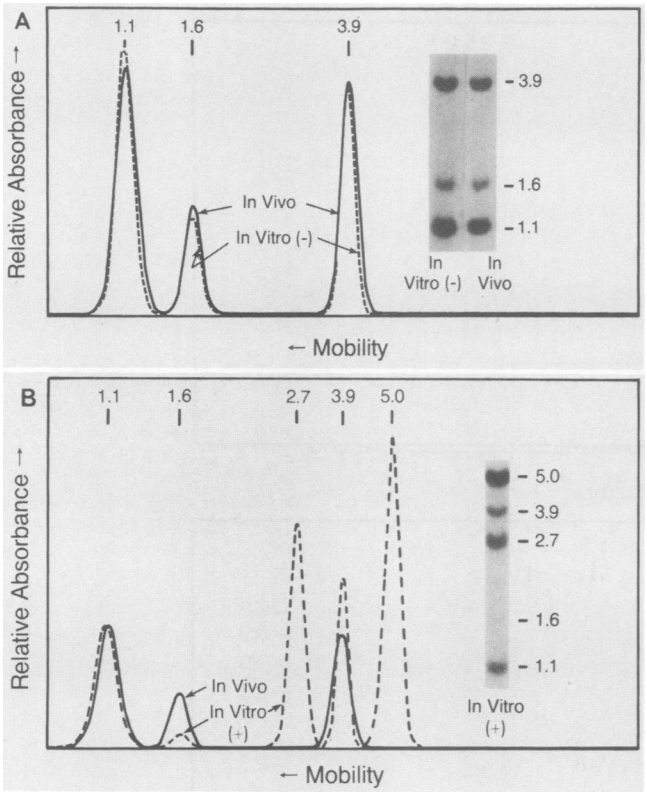

FIG. 5. IVR of ST486 nuclei. (A) ST486 nuclei were isolated from an asynchronous cell culture, and one-half of this preparation was submitted to the IVR density-labeling reaction as outlined in Fig. 2C. DNA was purified from these nuclei [in vitro (−)-labeled DNA] and from ST486 cells labeled for 6 h in culture with BrUdR (in vivo-labeled DNA). Each DNA sample was cleaved to completion with *Xba*I and *Pst*I, centrifuged, and analyzed by blot hybridization to probe I. Inset, Autoradiogram of dense DNAs from the in vitro (−) replication reaction (left lane), and after in vivo labeling (right lane). (B) The sister sample of the ST486 nuclear preparation used in the experiment of panel A was submitted to the IVR reaction in the presence of *Xba*I. DNA was purified and digested to completion with *Pst*I. The DNA was banded on CsCl in parallel with the DNAs shown in panel A, electrophoresed on the same gel, and hybridized on the same filter as these DNAs. Inset, Autoradiogram of dense DNA from the in vitro reaction. The scans compare the hybridization patterns of the dense in vitro (+)- and in vivo-labeled DNAs after normalization of the 1.1-kb band signals. DNA sizes are in kilobases.

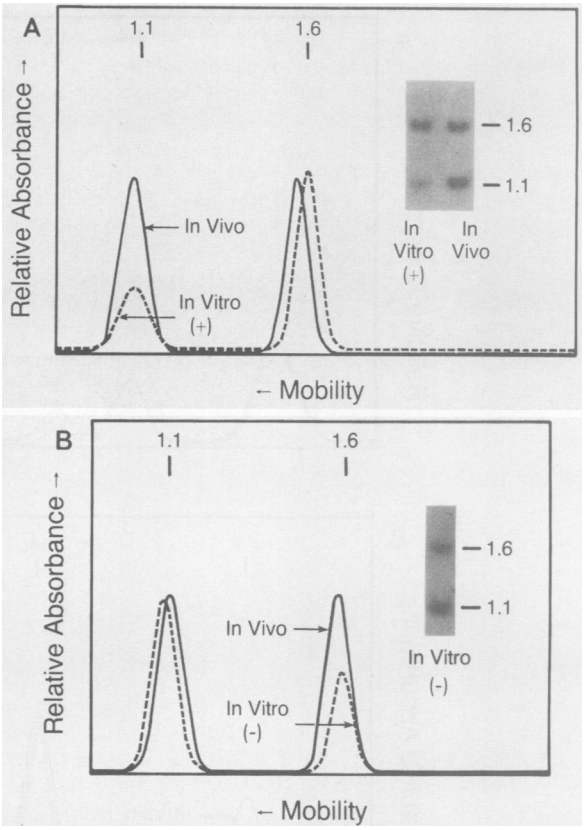

FIG. 6. IVR of HeLa nuclei. (A) HeLa nuclei were isolated from an asynchronous cell culture, and one-half of this preparation was subjected to the IVR density-labeling reaction in the presence of *Xba*I. After the reaction, the DNA was purified, digested with *Pst*I, and analyzed as described above. Inset, Autoradiograms of the dense DNAs from the in vitro (+) reaction and from in vivo labeling of HeLa cells in culture. The scans of the IVR- and in vivo-labeled DNAs were normalized to the signals from the 1.6-kb bands. (B) The sister sample of the HeLa nuclear preparation used in the experiment of panel A was submitted to the IVR reaction in the absence of a restriction enzyme. The DNA was digested with *Pst*I and *Xba*I, banded on CsCl in parallel with the DNAs shown in panel A, electrophoresed on the same gel, and hybridized to probe I. The scans compare the hybridization patterns of the dense in vitro (−)- and in vivo-labeled DNAs after normalization of the 1.1-kb band signals. DNA sizes are in kilobases.

2D) predicts that virtually no signal should be observed in dense DNA from fragments which occur immediately adjacent to a replication barrier on the downstream side. Nevertheless, such downstream fragments (e.g., the 1.6-kb *Pst*I-*Xba*I fragment) are evident in the blots shown in Fig. 4 and 5. This could be due to (i) incomplete resolution of heavy and light DNAs, (i i) a population of cells replicating the c-*myc* genes in the direction opposite to the preferred polarity, or (iii) in situ restriction enzyme cutting after the 5′ and 3′ fragments have been entirely replicated. The present results, therefore, are likely to represent a minimum estimate of the replication polarity preference observable were it possible to fractionate heavy versus light DNAs completely and eliminate the contribution of uniformly labeled c-*myc* loci to the data.

Although a preferred direction of replication of the germ line c-*myc* genes in HeLa cells can be diagnosed and shown to be opposite to that in CA46 and ST486 cells, these results do not prove that all c-*myc* genes which replicate in the same direction initiate replication at the same origin. This view is nonetheless appealing in light of the cell type specificity of alpha globin, histone H5, and c-*myc* gene replication polarity and the demonstration of unique origin sequences for the replication of DNA viral minichromosomes (17, 28, 40, 41) and the yeast 2μm plasmid (6, 24).

If the proximity of a gene to an active replication origin determines its replication polarity, the present results imply that the active origin closest to the c-*myc* gene in HeLa cells is in the 5′-flanking DNA. In CA46 and ST486 cells, either this origin is inactivated and the next closest active origin is in the 3′-flanking DNA or the 5′ origin remains functional but a closer 3′ origin is activated. We have shown that a DNA sequence within 2.5 kb 5′ to the HeLa c-*myc* genes imparts autonomous replication activity to a selectable plasmid transfected into HeLa cells (30) and that bidirectional repli-

cation initiates within 3.5 kb 5′ to the c-*myc* gene in HeLa chromosomes (C. McWhinney and M. Leffak, submitted for publication). This suggests that the endogenous barrier to replication fork movement 5′ to the c-*myc* genes in HeLa cells may be the germ line c-*myc* chromosomal replication origin. In contrast, in vitro replication of CA46 or ST486 nuclei offers no indication of an origin close to the 3′ side of the unrearranged c-*myc* genes. Thus, the antitranscriptional direction of germ line c-*myc* gene replication in Burkitt lymphoma cells suggests that the origin active in HeLa cells is suppressed in Burkitt lymphoma cells by factors which act in *trans*.

The transcribed c-*myc* genes are preferentially replicated from upstream origins in HeLa and CA46 cells, as are the active histone H5 and alpha globin genes in chicken erythrocytes. However, the generalization that the leading strand for replication is the sense strand for transcription cannot hold for genes in which both strands of the DNA duplex are transcribed (e.g., see reference 22). This apparent discrepancy can be resolved by speculating that the polarities of replication and transcription are not linked in an obligatory fashion but that regulatory sequences which are frequently but not exclusively found 5′ to genes can facilitate both replication and transcription.

APPENDIX

Calculation of *N*, the normalized 5′:3′ fragment ratio in dense DNA. The contributions of the germ line and translocated c-*myc* genes to the total 3′ fragment signals of in vitro-replicated c-*myc* DNA were calculated for each experimental repeat on the basis of the extent of in situ restriction digestion. As demonstrated below, apart from the influence of replication polarity, these values yielded the same 5′:3′ fragment signal ratios as DNA labeled in vivo.

In the absence of in situ restriction enzyme digestion, the pattern of in vitro replication labeling of c-*myc* DNA in ST486 and CA46 nuclei is equivalent to its labeling pattern in vivo. Thus, the fraction of the replicated c-*myc* DNA which is derived from the germ line locus is equivalent to the mole fraction of the germ line gene relative to all c-*myc* genes in the cell population, X_g (X_g equals one-half in CA46 cells and one-third in ST486 cells [James and Leffak, submitted]). Similarly, the fraction of the dense c-*myc* DNA which is derived from the translocation is equivalent to the c-*myc* mole fraction of the translocated sequences, X_t. In the replicated DNA, as

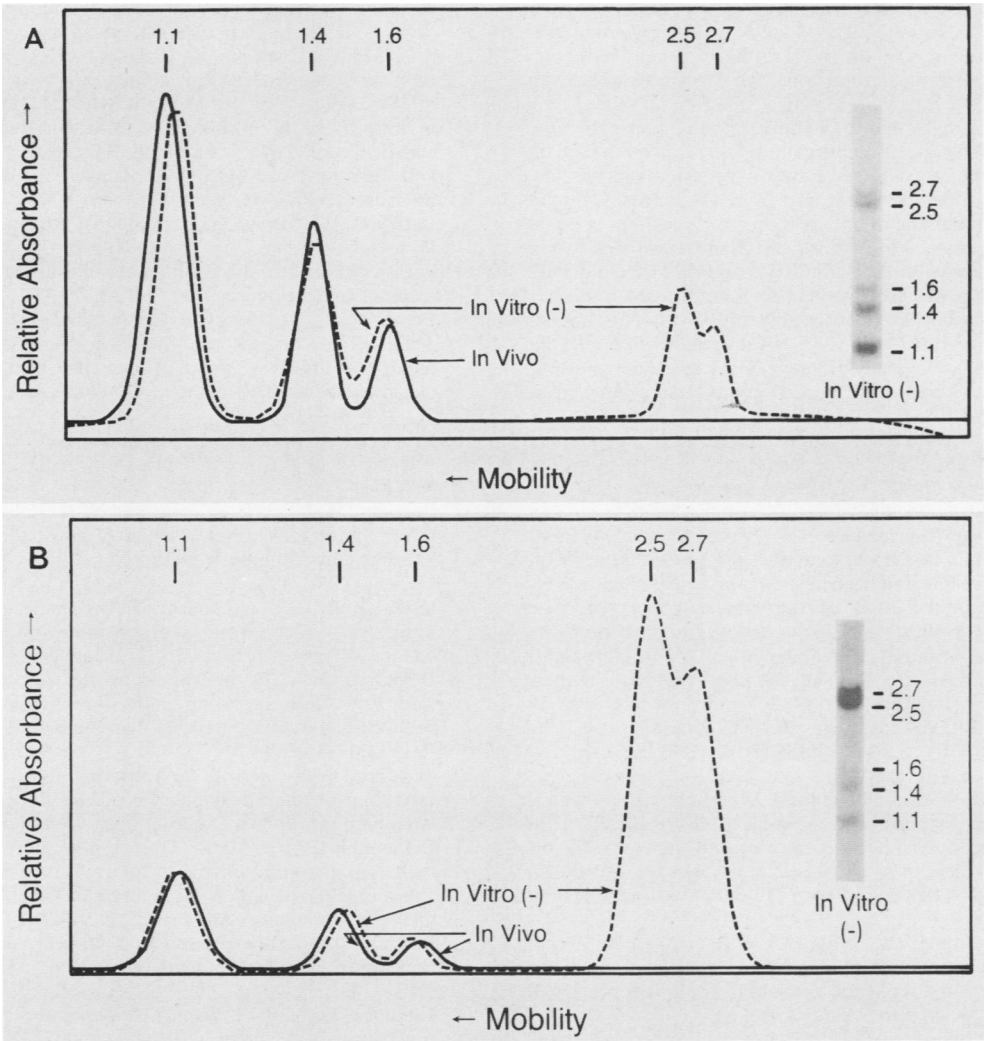

FIG. A1. Reconstruction of an IVR reaction in CA46 nuclei with partial in situ restriction enzyme digestion. CA46 nuclei were submitted to IVR incubation as for Fig. 3, and the purified DNA was cleaved to about 80% (A) or 25% (B) completion with limiting amounts of *Xba*I. The DNA was digested completely with *Pst*I and banded on CsCl. The scans compare the patterns of blot hybridization to probe I of dense DNAs from the in vitro reaction and from in vivo labeling. The scans of the IVR- and in vivo-labeled DNAs were normalized to the signals from the 1.1-kb bands. Insets, Autoradiograms of the dense DNA from the in vitro reaction. DNA sizes are in kilobases.

in total DNA, $X_g + X_t = 1$. When replication occurs in the presence of a restriction enzyme, the fraction X_g can be divided into a fraction of c-*myc* genes which has been cut in situ, B_g, and a fraction which has not, AB_g: $X_g = AB_g + B_g$. Similarly, B_t or AB_t represents, respectively, the fraction of c-*myc* genes which has or has not undergone digestion in situ and derives from the locus of the translocation. Let *R1* equal the ratio of the fractions of the c-*myc* genes which have not been digested in situ (but all of which are digested by restriction enzyme 2 (RE-2): $R1 = AB_g/AB_t = (X_g - B_g)/(X_t - B_t)$. The value of *R1* is obtained from the ratio of the blot hybridization signals of the partial-digest fragments (digested only by the RE-2). Let *R2* equal the sum of the fractions of the c-*myc* genes which have been digested in situ: $R2 = B_g + B_t = (3'_{ivr})/(3'_{iv})$. The percentage of the total hybridization signal of an in vivo-labeled DNA sample (digested to completion) which is due to the 3′ c-*myc* DNA fragments, $3'_{iv}$, can be used as a standard to determine the completeness of the in situ restriction digestion. Thus, *R2* equals the ratio of the signals of the 3′ DNA fragments (expressed as percentages) in the in vitro and in vivo samples. Substituting the values for B_t and X_t into the equation describing *R1* and solving for B_g yields $B_g = [(X_g)(1 + R1) - R1(1 - R2)]/[1 + R1]$. The fraction B_g can be converted to an autoradiographic signal value (expressed as a percentage of the total signal), $G^{3'ivr}$, by $G^{3'ivr} = B_g \times 3'_{iv}$. The 5′:3′ fragment ratio for the germ line locus in the in vitro sample is calculated by dividing the signal from the 5′ fragments of the germ line c-*myc* gene, $G^{5'ivr}$, by $G^{3'ivr}$. The value *N* for the germ line gene equals the 5′:3′ fragment ratio for the germ line locus in the in vitro sample normalized to the same ratio for the in vivo sample: $N = [G^{5'ivr}/G^{3'ivr}]/[G^{5'iv}/G^{3'iv}]$.

This derivation assumes that the hybridization response of the 3′ fragment of the unrearranged c-*myc* gene is the same as that of the identical 3′ fragment of the translocated gene. This analysis also requires that the probes, which come from sequences 3′ to the translocation breakpoint and hybridize to the same sequences on the partial-digest fragments of the translocated and germ line genes, hybridize equivalently within the limits of detection to these partial-digest fragments. This has been verified by hybridization of probe I to CA46 and ST486 cell DNAs digested only with *Pst*I and by hybridization of probe II to CA46 cell DNA digested with *Eco*RI and *Xho*I. It is stressed that the analysis does not assume that the probes hybridize equivalently to the partial and complete digestion products.

As an example, consider a reaction containing 10^6 replicating nuclei, involving 10^6 germ line c-*myc* genes and 2×10^6 translocated c-*myc* genes ($X_g = 1/3$ and $X_t = 2/3$). Assume arbitrarily that, as a result of in situ restriction digestion, 10% of the germ line c-*myc* genes and 25% of the translocated c-*myc* genes are cut by RE-1; subsequent digestion with RE-2 is complete. *R1* is the ratio of the signals from the germ line and translocated c-*myc* sequences not cut by RE-1 and equals the ratio of the copy numbers of these sequences: $R1 = (9 \times 10^5)/(1.5 \times 10^6) = 0.6$. *R2* is the ratio of the hybridization signals of the 3′ DNA fragments in the in vitro and in vivo samples and equals the ratio of the copy numbers of these sequences: $R2 = (6 \times 10^5)/(3 \times 10^6) = 0.2$. Solving for B_g and B_t, $B_g = 0.03333$, $G^{3'ivr} = 0.03333 \times 3'_{iv}$, $B_t = 0.16667$, and $T^{3'ivr} = 0.16667 \times 3'_{iv}$. As anticipated, fractions B_g and B_t are equal to the respective c-*myc* mole fractions of the germ line and translocated genes multiplied by their digestion efficiencies. Thus, the extent of RE-1 cutting can be used to predict the values of $G^{3'ivr}$ and $T^{3'ivr}$, the contributions of the germ line and translocated genes to the observed 3′ fragment signal in in vitro-replicated DNA, except for the influence of the graded-density labeling of restriction fragments due to runoff replication.

In the present example, the ratios $G^{5'ivr}/G^{5'iv}$ and $G^{3'ivr}/G^{3'iv}$ each equal 0.1, the efficiency of cutting of the germ line genes; thus, $N = 1.0$. In an actual in vitro replication reaction, the disproportionate labeling of the DNAs on either side of the RE-1 cut will make *N* greater or less than unity.

To test the applicability of these calculations further, a series of reconstruction experiments was performed to mimic the effects of incomplete *Xba*I digestion in situ. Nuclei were submitted to the IVR density-labeling reaction in the absence of a restriction enzyme, and DNA was purified. The DNA was digested partially with *Xba*I and to completion with *Pst*I, banded on CsCl, and analyzed by blot hybridization. Figure A1 shows the results of two such reactions in which the DNA was digested to about 80% (Fig. A1A) and 25% (Fig. A1B) completion with *Xba*I. The normalized ratios of the 5′ and 3′ fragment signals (*N* values) for these samples are close to unity (Table 1), as expected for reactions run in the absence of exogenously imposed barriers.

ACKNOWLEDGMENTS

C.D.J. was supported by a predoctoral research fellowship from the Wright State University Biomedical Sciences doctoral program. This work was supported by a Public Health Service grant (GM35877) from the National Institute of General Medical Sciences and a grant from the American Cancer Society.

We thank P. Leder for generously providing the human c-*myc* and immunoglobulin gene clones used in these experiments and I. McGrath for the CA46 and ST486 cell lines. We also thank J. Huberman for constructive criticism of the manuscript.

LITERATURE CITED

1. **Adams, J., S. Gerondakis, E. Webb, L. M. Corcoran, and S. Cory.** 1983. Cellular myc oncogene is altered by chromosome translocation to an immunoglobulin locus in murine plasmacytomas and is rearranged similarly in human Burkitt lymphomas. Proc. Natl. Acad. Sci. USA **80**:1982–1986.
2. **Ariga, H., T. Itani, and S. M. Iguchi-Ariga.** 1987. Autonomous replicating sequences from mouse cells which can replicate in mouse cells in vivo and in vitro. Mol. Cell. Biol. **7**:1–6.
3. **ar-Rushdi, A., K. Nishikura, J. Erikson, R. Watt, G. Rovera, and C. Croce.** 1983. Differential expression of the translocated and the untranslocated c-myc oncogene in Burkitt lymphoma. Science **222**:390–393.
4. **Battey, J., C. Moulding, R. Taub, W. Murphy, T. Stewart, H. Potter, G. Lenoir, and P. Leder.** 1983. The human c-myc oncogene; structural consequences of translocation into the IgH locus in Burkitt lymphoma. Cell **34**:779–787.
5. **Bernard, O., S. Cory, S. Gerondakis, E. Webb, and J. M. Adams.** 1983. Sequence of the murine and human cellular myc oncogenes and two modes of myc transcription resulting from chromosome translocation in B lymphoid tumors. EMBO J. **2**:2375–2383.
6. **Brewer, B. J., and W. L. Fangman.** 1987. The localization of replication origin on ARS plasmids in S. cerevisiae. Cell **51**: 463–471.
7. **Burhans, W. C., J. E. Selegue, and N. H. Heintz.** 1986. Isolation the origin of replication associated with the amplified Chinese hamster dihydrofolate reductase domain. Proc. Natl. Acad. Sci. USA **83**:7790–7794.
8. **Calza, R. E., L. A. Eckhardt, T. DelGiudice, and C. L. Schildkraut.** 1984. Changes in gene position are accompanied by a change in time of replication. Cell **36**:689–696.
9. **D'Andrea, A. A., U. Tantrahavi, M. Lalande, A. Perle, and S. A. Latt.** 1983. High resolution timing of replication of specific DNA sequences during S phase of mammalian cells. Nucleic Acids Res. **11**:4753–4774.
10. **de Cicco, D. V., and A. C. Spradling.** 1984. Localization of a cis-acting element responsible for the developmentally regulated amplification of Drosophila chorion genes. Cell **38**:45–54.
11. **DePamphilis, M. L.** 1988. Transcriptional elements as components of eucaryotic origins of replication. Cell **52**:635–638.
12. **Edenberg, H. J., and J. A. Huberman.** 1975. Eukaryotic chromosome replication. Annu. Rev. Genet. **9**:245–284.
13. **Enver, T., A. C. Brewer, and R. K. Patient.** 1988. Role for DNA replication in β-globin gene activation. Mol. Cell. Biol. **8**: 1301–1308.
14. **Frappier, L., and M. Zannis-Hadjopoulos.** 1985. Autonomous replication of plasmids bearing monkey DNA origin-enriched sequences. Proc. Natl. Acad. Sci. USA **84**:6668–6672.
15. **Gelman, E. P., M. C. Psallidopoulos, T. S. Papas, and R. Dalla-Favera.** 1983. Identification of reciprocal translocation sites within the c-myc oncogene and immunoglobulin mu locus in a Burkitt lymphoma. Nature (London) **306**:799–803.

16. **Goldman, M. A., G. P. Holmquist, M. C. Gray, L. Caston, and A. Nag.** 1984. Replication timing of genes and middle repetitive sequences. Science **224**:686–692.
17. **Griffin, B., M. Fried, and A. Cowie.** 1974. Polyoma DNA: a physical map. Proc. Natl. Acad. Sci. USA **71**:2077–2081.
18. **Guinta, D. R., J. Y. Tso, S. Narayanswami, B. Hamkalo, and L. J. Korn.** 1986. Early replication and expression of oocyte-type 5S RNA genes in a Xenopus somatic cell line carrying a translocation. Proc. Natl. Acad. Sci. USA **83**:5150–5154.
19. **Hand, R.** 1978. Eucaryotic DNA: organization of the genome for replication. Cell **15**:317–325.
20. **Hann, S. R., and R. N. Eisenmann.** 1984. Proteins encoded by the human c-myc oncogene: differential expression in neoplastic cells. Mol. Cell. Biol. **4**:2486–2497.
21. **Heintz, N. H., J. D. Milbrandt, K. S. Greisen, and J. L. Hamlin.** 1983. Cloning of the initiation region of a mammalian chromosomal replicon. Nature (London) **302**:439–441.
22. **Henikoff, S., M. Keene, K. Fechtel, and J. Fristrom.** 1986. Gene within a gene: nested Drosophila genes encode unrelated proteins on opposite DNA strands. Cell **44**:33–42.
23. **Huberman, J. A.** 1987. Eucaryotic DNA replication: a complex picture partially clarified. Cell **48**:7–8.
24. **Huberman, J. A., L. D. Spotila, K. A. Nawotka, S. M. El-Assouli, and L. R. Davis.** 1987. The in vivo replication origin of the yeast 2 micron plasmid. Cell **51**:473–481.
25. **James, C. D., and M. Leffak.** 1984. Replacement synthesis labeling of recombinant DNA molecules using the E. coli exonuclease III/DNA polymerase enzyme pair. Anal. Biochem. **141**:33–37.
26. **James, C. D., and M. Leffak.** 1986. Polarity of DNA replication through the avian alpha-globin locus. Mol. Cell. Biol. **6**:976–984.
27. **Lipchitz, L., and R. Axel.** 1976. Restriction endonuclease cleavage of satellite DNA in intact bovine nuclei. Cell **9**:355–364.
28. **Lusky, M., and M. R. Botchan.** 1986. Transient replication of bovine papillomavirus type 1 plasmids: cis and trans requirements. Proc. Natl. Acad. Sci. USA **83**:3609–3613.
29. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **McWhinney, C., and M. Leffak.** 1988. Episomal persistence of a selectable plasmid containing human c-myc DNA. Cancer Cells **6**:467–471.
31. **Miller, A. M., and K. Nasmyth.** 1984. Role of DNA replication in the repression of the silent mating type loci in yeast. Nature (London) **312**:247–251.
32. **Montoya-Zavala, M., and J. L. Hamlin.** 1985. Similar 150 kilobase DNA sequences are amplified in independently derived methotrexate-resistant Chinese hamster cells. Mol. Cell. Biol. **5**:619–627.
33. **Nishikura, K., A. ar-Rushdi, J. Erikson, R. Watt, G. Rovera, and C. Croce.** 1983. Differential expression of the normal and of the translocated human c-myc oncogenes in B cells. Proc. Natl. Acad. Sci. USA **80**:4822–4826.
34. **Saito, H., A. C. Hayday, K. Wiman, W. S. Hayward, and S. Tonegawa.** 1983. Activation of the c-myc gene by translocation: a model for translational control. Proc. Natl. Acad. Sci. USA **80**:7476–7480.
35. **Showe, L. C., M. Ballantine, K. Nishikura, J. Erikson, H. Kaji, and C. M. Croce.** 1985. Cloning and sequencing of a c-*myc* oncogene in a Burkitt's lymphoma cell line that is translocated to a germ line alpha switch region. Mol. Cell. Biol. **5**:501–509.
36. **Siebenlist, U., L. Hennighausen, J. Battey, and P. Leder.** 1984. Chromatin structure and protein binding in the putative regulatory region of the c-myc gene in Burkitt lymphoma. Cell **37**:381–391.
37. **Smithies, O.** 1982. The control of globin and other eukaryotic genes. J. Cell. Physiol. **1**:137–143.
38. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
39. **Spradling, A. C., and A. P. Mahowald.** 1981. A chromosome inversion alters the pattern of specific DNA replication in Drosophila follicle cells. Cell **27**:203–209.
40. **Taub, R., I. Kirsch, C. Morton, G. Lenoir, D. Swan, S. Tronick, S. Aaronson, and P. Leder.** 1984. Activation of somatic mutation of the translocated c-myc gene in Burkitt lymphoma cells. Cell **36**:339–348.
41. **Tooze, J.** 1980. DNA Tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. **Treisman, R., M. R. Green, and T. Maniatis.** 1983. Cis and trans activation of globin gene transcription in transient assays. Proc. Natl. Acad. Sci. USA **80**:7428–7432.
43. **Trempe, J. P., Y. I. Lindstrom, and M. Leffak.** 1988. Opposite replication polarities of transcribed and nontranscribed histone H5 genes. Mol. Cell. Biol. **8**:1657–1663.
44. **Wirack, D. O., L. E. Chalifour, P. M. Wassarman, W. J. Muller, J. A. Hassell, and M. L. DePamphilis.** 1985. Sequence-dependent DNA replication in preimplantation mouse embryos. Mol. Cell. Biol. **5**:2924–2935.