MOLECULAR AND CELLULAR BIOLOGY, July 1989, p. 2881–2889
0270-7306/89/072881-09$02.00/0

Vol. 9, No. 7

Temporal Order of Gene Replication in Chinese Hamster Ovary Cells

JANOS TALJANIDISZ,[1,2] JANUSZ POPOWSKI,[1] AND NILIMA SARKAR[1,2]*

Department of Metabolic Regulation, Boston Biomedical Research Institute, 20 Staniford Street, Boston, Massachusetts 02114,[1] and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115[2]

Received 28 December 1988/Accepted 30 March 1989

To investigate the molecular basis of the regulatory mechanisms responsible for the orderly replication of the mammalian genome, we have developed an experimental system by which the replication order of various genes can be defined with relative ease and precision. Exponentially growing CHO-K1 cells were separated into populations representing various stages of the cell cycle by centrifugal elutriation and analyzed for cell cycle status by flow cytometry. The replication of specific genes in each elutriated fraction was measured by labeling with 5-mercuri-dCTP and [^{3}H]dTTP under conditions of optimal DNA synthesis after cell permeabilization with lysolecithin. Newly synthesized mercurated DNA from each elutriated fraction was purified by affinity chromatography on thiol-agarose and replicated with the large fragment of *Escherichia coli* DNA polymerase I by using [α-^{32}P]dATP and random primers. The ^{32}P-labeled DNA representative of various stages of the cell cycle was then hybridized with dot blots of plasmid DNA containing specific cloned genes. From these results, it was possible to deduce the nuclear DNA content at the time each specific gene replicated during S phase (C value). The C values of 29 genes, which included single-copy genes, multifamily genes, oncogenes, and repetitive sequences, were determined and found to be distributed over the entire S phase. Of the 28 genes studied, 19 had been examined by others using in vivo labeling techniques, with results which agreed with the replication pattern observed in this study. The replication times of nine other genes are described here for the first time. Our method of analysis is sensitive enough to determine the replication time of single-copy genes. The replication times of various genes and their levels of expression in exponentially growing CHO cells were compared. Although there was a general correlation between transcriptional activity and replication in the first half of S phase, examination of specific genes revealed a number of exceptions. Approximately 25% of total poly(A) RNA was transcribed from the late-replicating DNA.

It has long been recognized that eucaryotic chromosomes do not replicate as a single element; rather, different sections of chromosomes synthesize their DNA at characteristic times during S phase of the cell cycle, which indicates that each chromosome consists of multiple units of replication (65, 66). The existence of subchromosomal replication units in mammalian cells was demonstrated directly, using autoradiography, by Cairns (17) and Huberman and Riggs (42). These experiments also demonstrated that the chronological order of replication of specific DNA segments is invariant from one cell generation to the next (42). Further studies showed that the replication units (replicons) into which chromosomal DNA is organized range in size from 50 to 330 kilobases and appear to initiate in synchronous clusters (25 to 100 replicons per cluster), spaced irregularly along the chromosome and activated in a sequential fashion throughout S phase (35, 41). Examination of specific genomic DNA sequences showed that replication follows a distinct temporal order that is maintained from one cell generation to the next. For example, early replication of ribosomal DNA cistrons in CHO cells was independently observed by three groups, using a variety of cell synchronization procedures (2, 4, 61). A different temporal order of ribosomal gene replication was reported for other eucaryotic cell types, with ribosomal DNA replicating late in S phase in kangaroo rat cells (31) and throughout S phase in HeLa (4), yeast (32), and Friend (26) cells. Other examples are the globin DNA sequences, which are found to replicate early in mouse erythroleukemia cells synchronized by centrifugal elutriation (26, 29). Using a different approach based on selective photodegradation, D'Andrea et al. found that both the ribosomal DNA and the dihydrofolate reductase (DHFR) sequences replicated almost entirely during the first quarter of S phase in CHO cells (23). The careful studies of Schildkraut and co-workers showed that the temporal order of replication of various segments of the immunoglobin gene corresponds to the linear order of these segments on the chromosome (14, 16) and that chromosomal rearrangement of a gene can change dramatically the replication time of the gene (18). Similarly, studies on the replication of simian virus 40 integrated into the host genome indicated that the time of replication of this single-copy DNA sequence depends on its location in the genome (51). Holmquist and co-workers studied the replication times of several protein-coding genes and middle-repetitive sequences and postulated that transcriptionally active genes are replicated early in S phase but that inactive genes may be either early or late replicating (33, 40).

The studies summarized above suggest that the replication of genes occurs in a specific temporal order that may have a functional significance. However, existence of order implies regulation, yet nothing is known about the molecular mechanisms that might be involved in the temporal order of gene replication. To facilitate the study of the replicative process and its temporal control, we have developed a relatively simple and versatile method for determining gene replication order. This method involves separation of cultured cells at various replication stages by centrifugal elutriation, followed

* Corresponding author.

TABLE 1. Hybridization probes for specific sequences[a]

Sequence	Organism	Plasmid	Source	Reference
v-*mos* oncogene	Virus	pHT10	ATCC	13
neu proto-oncogene	Virus	pSV2 neu NT	R. A. Weinberg	9
SINE	Hamster	pB4	G. A. Holmquist	40
HPRT	Hamster	pHPT20	ATCC	15
c-*abl* proto-oncogene	Mouse	p102	N. Sonenberg	
c-*fos* proto-oncogene	Mouse	pc-fos	R. A. Weinberg	52
β-Actin	Human	p221	N. Oliver	
HMG-CoA reductase	Hamster	pRed227	ATCC	20
v-*erbA* oncogene	Virus	perbA	R. A. Weinberg	28
CAD	Hamster	pCAD41	G. W. Wahl	67
DHFR	Hamster	pB67H1	L. A. Chasin	19
v-*sis* oncogene	Virus	pv-sis	ATCC	58
v-Ha-*ras* oncogene	Virus	pHB11	ATCC	25
N-*ras* proto-oncogene	Human	pNR-sac	R. A. Weinberg	55
Encephalin	Human	pHPE-9	M. Comb	22
α-Globin	Mouse	pα-globin	N. Sonenberg	56
c-*myc* proto-oncogene	Human	pJ1 pHc-myc	R. A. Weinberg	49
v-*myc* oncogene	Virus	pmyc 3-Pst	ATCC	1
Thymidine kinase	Hamster	pHa-TK-1	J. A. Lewis	50
Cytochrome P1-450	Mouse	pHAV-cat5	D. K. Biswas	47
APRT	Hamster	pH-2	L. A. Chasin	
β-Globin	Human	pVAL	F. Bunn	48
Fibronectin	Human	pNO200	N. Oliver	57
Line (A)	Hamster	pC-1	G. P. Holmquist	40
N-*myc* proto-oncogene	Human	pNb-6	R. A. Weinberg	60
LINE (B)	Hamster	pB-7	G. P. Holmquist	40
v-Ki-*ras* oncogene	Virus	pHiHi-3	R. A. Weinberg	25
Apoferritin	Human	pB1	F. Bunn	37
p21 proto-oncogene	Human	pCDC K-76	R. A. Weinberg	

[a] Abbreviations: SINE and LINE, short and long, respectively, interspersed repetitive sequence; HPRT, hypoxanthine phosphoribosyltransferase; HMG-CoA, hydroxymethylglutaryl coenzyme A; CAD, carbamyl-phosphate synthetase-aspartate transcarbamylase-dihydro-orotase; DHFR, dihydrofolate reductase; APRT, adenine phosphoribosyltransferase; ATCC, American Type Culture Collection, Rockville, Md.

by labeling of nascent DNA in permeable cells with 5-mercuri-dCTP (HgdCTP) to allow the rapid and efficient selective isolation of newly synthesized DNA on a thiol affinity column (5–8, 10, 11, 63). Using this procedure, we have determined the replication timing of 29 genes which span almost the entire S phase of CHO cells. The ability to study the replication time of single-copy genes also allowed us to reexamine the hypothesis of Holmquist et al. (33, 39) regarding the relationship between the transcriptional activity and replication time of a gene.

MATERIALS AND METHODS

Cell culture. Chinese hamster ovary cells (CHO K1; ATCC CCL61) were grown in suspension at 37°C in spinner flasks, using minimal Eagle medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco Laboratories) and 33 μg of proline per ml.

Specific gene probes. Genomic and cDNA clones of various specific genes were obtained from the sources described in Table 1.

Centrifugal elutriation and cell fractionation. Cells were harvested at a density of 4×10^5 cells per ml by centrifugation at $650 \times g$ for 10 min and suspended in minimal Eagle medium containing 1% fetal bovine serum. The cell suspension (2.5×10^7 cells per ml) was introduced into the chamber of an elutriator rotor (model JE6B; Beckman Instruments, Inc.) maintained at 2,250 rpm in a J21 centrifuge (Beckman), and cells were elutriated by increasing flow rates in a stepwise manner (16 to 50 ml of minimal Eagle medium plus 1% fetal bovine serum per min) at constant rotor speed and temperature (22°C). Fractions elutriated at different pump speeds were collected by centrifugation and used for the in vitro DNA replication assay. The first one or two fractions containing primarily G1 cells and the last few fractions containing cell aggregates were discarded. A total of 5×10^8 cells were separated into nine elutriated fractions for DNA synthesis, and recovery of cells after elutriation was 85 to 90%. A sample (10^6 cells) from each fraction was stained with propidium iodide in 0.1% sodium citrate (50 μg/ml) for 15 min at 0°C, fixed in 35% ethanol, and monitored for DNA content per cell by flow cytometry, using a fluorescence-activated cell sorter (Becton-Dickinson and Co.) (29).

Calculation of C values. As a cell progresses through S phase, the nuclear DNA content, expressed as C value (a C value of 1 corresponds to a haploid DNA content per cell) increases from 2 to 4. Thus, the C value provides a quantitative measure of the progress of a cell through S phase. Since the flow cytometry profile gives the distribution of DNA content in a cell population, it can be used to calculate the average DNA content (C value) of a sample. For convenience, the cell population of each elutriated fraction was divided into 12 classes on the basis of DNA content, and the fraction of the cell population corresponding to each class was determined by the appropriate area under the flow cytometry profile. These values were then averaged to yield the mean DNA content (or C value) of the elutriated fraction.

In vitro DNA replication and isolation of HgDNA. A total of 10^7 cells from each elutriated fraction were washed twice with buffer A (150 mM sucrose, 80 mM KCl, 35 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4], 5 mM KPO_4 [pH 7.4], 5 mM $MgCl_2$, 0.5

mM $CaCl_2$), resuspended in 100 μl of the same buffer, and permeabilized by treatment with 200 μg of lysolecithin per ml for 2 min at 0°C (53). Permeabilization was monitored by trypan blue uptake. The cells were then diluted in DNA synthesis mixture to give final concentrations of 10 mM phosphoenolpyruvate, 0.25 mM ATP, 0.1 mM each CTP, GTP, and UTP, 0.25 mM each dATP and dGTP, 0.05 mM HgdCTP, 0.02 mM [^{3}H]dTTP (5 mCi/mmol), 1 mM dithiothreitol, and 5 μg of pyruvate kinase per ml. After 1 h of incubation at 37°C, the cells were lysed in a mixture of 0.6% sodium dodecyl sulfate (SDS), 100 mM NaCl, 20 mM EDTA, and 50 mM Tris (pH 7.9). Proteinase K (0.1 mg/ml, final concentration) was added to the mixture, followed by incubation at 37°C for 1 h. The lysate was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and three times with ether. After concentration to 50 to 100 μl by butanol extraction, the DNA solution was passed through a Bio-Gel P-4 column (0.8 by 25 cm; Bio-Rad Laboratories) preequilibrated with 50 mM Tris (pH 7.5)–50 mM NaCl–1 mM EDTA. The material eluting at the void volume was collected and sonicated to shear the DNA to about 400- to 800-base-pair fragments. After denaturation by heating for 5 min at 100°C, HgDNA was purified by chromatography on thiol-agarose (Affigel 401) as previously described, with a recovery of 85 to 95% of input HgDNA (5, 6). HgDNA from each fraction was labeled to high specific activity (10^8 to 10^9 cpm/μg) with [α-^{32}P]dATP, using the hexanucleotide primer extension method as described previously (27) except that 10 times less primer (14 A_{260} units per ml) was used.

Hybridization. Purified plasmid DNA was denatured by heating at 65°C for 1 h in 0.3 N NaOH and bound to a nylon membrane (MSI 10836; 0.45 μm pore size) for hybridization in the presence of 2 M ammonium acetate. The dot blots were dried at 80°C for 2 h and then washed at 55°C for 1 h in a large volume of 2× SSC (SSC is 0.15 M NaCl plus 0.015 sodium citrate)–1% SDS to remove any loosely bound DNA. The dots were then prehybridized for at least 1 h and hybridized for at least 24 h at 37°C in the following hybridization mixture: 0.75 M NaCl–0.05 M NaH_2PO_4–0.005 M EDTA–0.1% Ficoll (Pharmacia Fine Chemicals)–0.1% polyvinylpyrrolidine–0.1% bovine serum albumin–1% SDS–0.2 mg of denatured herring sperm DNA per ml–50% deionized formamide. DNA probes had specific activities of at least 5 × 10^8 cpm/μg, and 2 × 10^6 cpm/ml was used for hybridization reactions. After hybridization, the dot blots were washed twice for 15 min each time in 100-ml volumes of 2× SSC at room temperature, twice in 2× SSC–1% SDS at 55°C for 30 min and three times at 37°C for 20 min, and finally twice in 0.2× SSC at 37°C for 15 min (64). Dot blots were dried and subjected to autoradiography. Individual dots were then excised and counted, using a toluene-based scintillation cocktail. Efficiency of hybridization determined under this experimental condition was 17%.

Poly(A) RNA isolation and hybridization. Total RNA was prepared from 600 ml of an exponentially growing culture by a modification of the guanidinium isothiocyanate method (21). In brief, cells were lysed in 4 M guanidinium isothiocyanate and 0.1 M 2-mercaptoethanol, and total RNA was isolated after addition of CsCl to 0.5 g/ml; the sample was layered on 1.2 ml of 5.7 M CsCl and centrifuged at 35,000 rpm for 24 h at 20°C in an SW50.1 rotor. The RNA pellet was dissolved in sterile water and precipitated twice with 2 volumes of ethanol. Poly(A) RNA was isolated by oligo(dT)-cellulose chromatography as described previously (64). Poly(A) RNA was denatured in 6× SSC containing 7.5% formaldehyde at 65°C for 15 min, and samples were spotted onto a prewetted nylon membrane (Micron Separations Inc.), using a minifold filtration system [2 μg of poly(A) RNA per dot]. *Escherichia coli* tRNA was also spotted as a control. RNA blots were hybridized with ^{32}P-labeled nick-translated plasmids (2 × 10^6 cpm for each blot in 400 μl of hybridization buffer) containing specific genes or sequences, using the procedure described for DNA hybridization except that poly(A) (200 μg/ml) instead of salmon sperm DNA was included in the hybridization mixture. The filters were washed, and individual dots were cut and directly counted as described above. Poly(A) RNA dots were also hybridized with ^{32}P-labeled HgDNA isolated from the permeabilized cells from various stages of S phase by the same procedure.

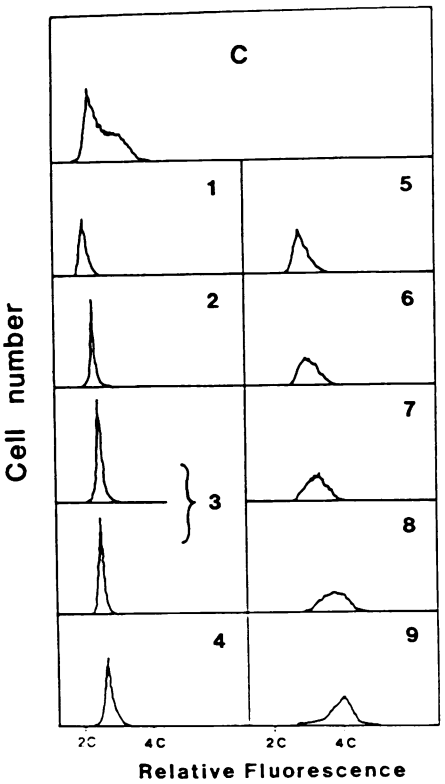

FIG. 1. Flow cytometric analysis showing cellular DNA content of CHO cells fractionated by centrifugal elutriation. The profiles are histograms showing cell number versus DNA content, measured by fluorescence and expressed as C values. A C value of 1 is the haploid DNA content of the CHO cells. The top profile was obtained from exponentially growing cells before elutriation; the others were obtained from samples of successive fractions collected during centrifugal elutriation (see Materials and Methods). (Fraction 3 was obtained by pooling two fractions with very similar profiles.)

RESULTS

Separation of CHO cells at different stages of replication by centrifugal elutriation. Exponentially growing CHO cells in suspension culture were separated into nine fractions by centrifugal elutriation, which separates cells on the basis of size into populations at different stages of the cell cycle (29). Each fraction was monitored for the distribution of cell cycle phases by flow cytometry after staining with propidium iodide. This analysis showed a progressive increase in DNA content with increased elution time (Fig. 1). The average C values of the elutriated fractions, determined as described in Materials and Methods, progressed from 2.03 in fraction 1 to

TABLE 2. S-phase replication times of various genes in CHO cells

Gene or gene product[a]	Relative extent of replication in cells from elutriated fraction at given C value									Avg replication time of gene (C value)
	2.03	2.29	2.41	2.70	2.98	3.17	3.52	3.80	3.95	
v-*mos* oncogene	29.5	34.1	16.7	3.9	4.9	0.0	0.0	7.1	3.8	2.21
neu proto-oncogene	29.5	37.5	23.4	2.5	0.0	2.6	0.1	2.3	2.1	2.23
SINE	29.0	33.6	23.8	6.8	1.5	0.6	0.0	2.4	2.2	2.23
HPRT	22.5	29.2	19.6	3.8	7.1	7.2	7.1	2.7	0.8	2.23
c-*abl* proto-oncogene	36.4	20.2	17.8	10.4	4.4	4.0	5.6	0.9	0.4	2.24
c-*fos* proto-oncogene	23.8	20.9	28.0	4.6	4.9	6.3	10.3	0.8	0.5	2.24
β-Actin	21.1	23.0	29.1	3.7	4.6	5.7	11.8	0.5	0.5	2.25
HMG-CoA reductase	21.0	25.6	28.9	4.6	5.3	4.4	9.3	0.7	0.2	2.25
v-*erbA* oncogene	30.0	42.0	15.2	10.3	2.1	0.4	0.0	0.0	0.0	2.26
CAD	19.5	27.5	31.3	8.0	6.8	0.5	4.9	0.0	1.6	2.26
DHFR	23.3	29.7	32.1	7.5	2.7	2.1	2.6	0.0	0.0	2.26
v-*sis* oncogene	14.5	21.9	18.1	12.4	4.7	8.2	6.4	6.6	7.3	2.33
v-Ha-*ras* oncogene	15.7	18.1	21.1	12.3	5.1	8.3	6.0	7.4	5.8	2.33
N-*ras* proto-oncogene	17.8	19.6	18.6	14.7	9.8	6.1	6.3	3.2	3.9	2.33
Encephalin	28.3	19.7	15.5	15.6	12.7	3.6	2.3	1.6	0.6	2.38
α-Globin	8.4	41.6	22.1	11.2	6.8	4.7	2.9	1.1	1.2	2.39
c-*myc* proto-oncogene	18.6	17.7	14.0	16.7	10.1	6.5	6.2	5.3	5.0	2.42
v-*myc* oncogene	8.7	18.5	25.6	10.9	4.8	6.4	9.2	5.5	7.2	2.42
Thymidine kinase	2.2	4.4	25.7	20.1	12.8	15.7	10.9	6.4	1.8	2.84
Cytochrome P1-450	4.6	0.0	14.6	23.6	25.8	8.9	10.5	8.1	3.9	2.89
APRT	1.2	0.9	11.6	19.4	26.8	15.6	11.0	6.5	7.1	2.95
β-Globin	2.8	5.6	6.6	6.3	11.6	13.9	14.0	15.7	23.5	3.58
Fibronectin	7.2	3.3	8.2	5.8	7.0	14.9	16.3	16.5	20.6	3.66
LINE (A)	0.1	4.3	1.3	4.1	10.3	12.2	14.8	15.8	37.1	3.66
N-*myc* proto-oncogene	5.4	4.6	6.5	7.1	9.3	15.1	13.7	15.3	22.9	3.67
LINE (B)	5.5	4.9	8.0	8.8	5.7	11.4	16.9	16.6	22.3	3.69
v-Ki-*ras* oncogene	2.0	4.2	5.4	5.9	6.3	13.2	18.6	19.7	24.7	3.69
Apoferritin	1.6	4.0	6.9	5.1	5.5	12.4	18.3	21.4	24.9	3.70
p21 proto-oncogene	0.7	0.2	4.7	2.8	1.6	8.2	16.4	24.3	41.1	3.84

[a] Abbreviations are as given in the footnote to Table 1.

3.95 in fraction 9 (Table 2). Pulse-labeling with [^{3}H]thymidine showed that the rates of DNA synthesis were greatest for the fractions with the intermediate C values (Fig. 2).

Labeling of DNA at various replication stages with HgdCTP. To label DNA at various replicative phases, cells from each elutriated fraction were permeabilized to small molecules by treatment with lysolecithin (53) and incubated in the presence of HgdCTP and [^{3}H]dTTP under conditions of optimal DNA synthesis. The rate and extent of DNA synthesis in permeable cells was not affected by replacement of HgdCTP by dCTP (Fig. 3). The rate of DNA synthesis in permeable cells from an exponential culture was 30 to 50 pmol/10^6 cells per h. Incorporation of radioactive precursors

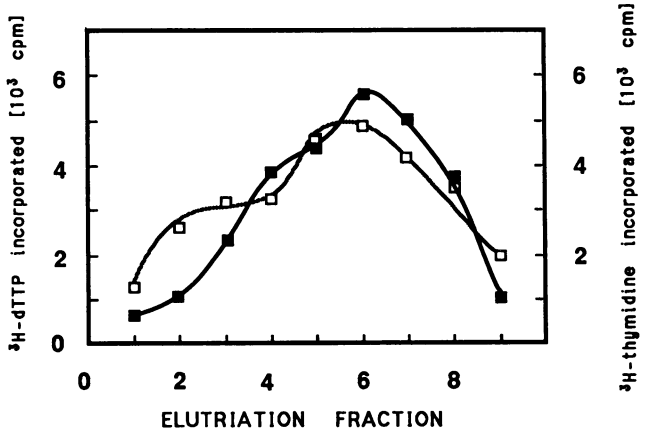

FIG. 2. DNA synthesis by CHO cells fractionated by centrifugal elutriation. Cell samples (10^5 cells) from the elutriated fractions described in the legend Fig. 1 were pulse-labeled with [^{3}H]thymidine (1 μCi/ml) for 60 min at 37°C or permeabilized and incubated for DNA synthesis with [^{3}H]dTTP for 60 min at 37°C as described in Materials and Methods. The labeled samples were analyzed for acid-insoluble radioactivity: [^{3}H]thymidine (□) or [^{3}H]dTTP (■).

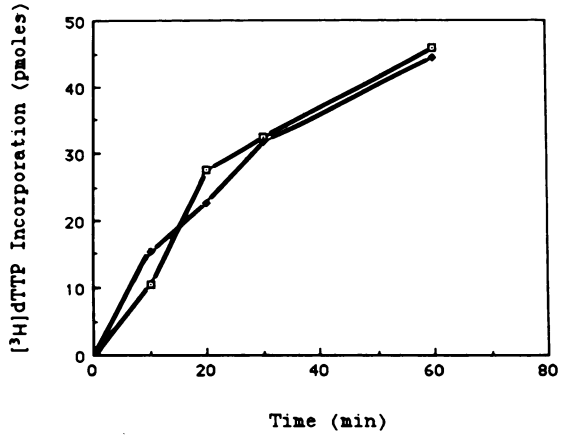

FIG. 3. Kinetics of DNA synthesis in permeable CHO cells. DNA synthesis by lysolecithin-treated permeable CHO cells (2 × 10^6) was measured as described in Materials and Methods, using either dCTP (⊡) or HgdCTP (■), with [^{3}H]dTTP as the labeled substrate.

into DNA by the permeabilized cells in the elutriated fractions paralleled the rate of thymidine incorporation in the corresponding intact cells (Fig. 2). This finding suggested that incorporation of deoxyribonucleotides by the permeable cells reflected DNA replication, a conclusion supported by the sensitivity of this process to inhibition by low levels of aphidicolin (50% inhibition by 0.3 μg/ml) and arabinosyl CTP (50% inhibition by 2 μg/ml) (59). The average size of the HgDNA was determined by agarose gel electrophoresis after mild alkali digestion (0.3 N NaOH for 15 min at 25°C) and found to range from 3 to 10 kilobases (data not shown).

Analysis of newly synthesized DNA for specific DNA sequences. Newly synthesized HgDNA from the various elutriated fractions representing different stages of the replication cycle was isolated by affinity chromatography on thiol-agarose, with a recovery of 85 to 90%. DNA of high specific activity corresponding to the HgDNA from each elutriated fraction was prepared by synthesis of the complementary strands with Klenow fragment, using [α-^{32}P]dATP and random hexanucleotide primers (27). The labeled DNA was analyzed for specific gene sequences by hybridization with dot blots containing an excess of plasmid DNA carrying specific cloned genes (64). Under the conditions used, radioactivity hybridized with the immobilized plasmid probe DNA increased linearly with increasing input of ^{32}P-labeled DNA (data not shown). Accordingly, dot hybridization could be used to provide a direct measure of the concentration of the probed sequence in the ^{32}P-labeled DNA. The extent of hybridization of a ^{32}P-labeled copy of HgDNA was determined for the nine elutriated fractions, yielding an estimate of the extent of replication of that specific sequence in each of the fractions. From this information and the measured average C values of the nine elutriated fractions, the average C value for the replication of each of the probed sequences was calculated as a weighted average. The C values deduced for the DNA sequences used for screening the newly synthesized DNA fractions covered almost the entire S phase, ranging from very early replicating (C value of 2.3) to late-replicating sequences (C value of 3.84) (Table 2). A wide variety of genes, including single-copy genes, multifamily genes, oncogenes, and repetitive sequences, were used in this experiment.

Transcription of genes and replication order. A relationship between replication order and gene expression has been suggested by observations from many laboratories; in higher eucaryotes, transcriptionally active genes replicate early in tissues in which they are expressed but late in other tissues (24, 33, 34, 36). To estimate the transcriptional potential of the chromosome segments replicating at various times, we hybridized the various DNA fractions to total poly(A) RNA isolated from exponentially growing CHO cells. Purified poly(A) RNA was immobilized on nylon membranes and hybridized separately with DNA replicating at various stages of S phase. ^{32}P-labeled replicas of HgDNA isolated from the elutriated cell fractions were prepared as described above. As shown in Fig. 4, 74% of total poly(A) RNA hybridized to DNA replicating in the first half of S phase, with C values of between 2 and 3.

To assess the level of transcription of specific DNA sequences with known replication times (C values), blots of total poly(A) RNA were separately hybridized with various nick-translated plasmid probes containing the specific genes or sequences. The relative levels of transcription of specific genes or sequences in exponentially growing CHO cells are given in Table 3. Only 4 of 26 genes or sequences examined were not significantly transcribed under our experimental

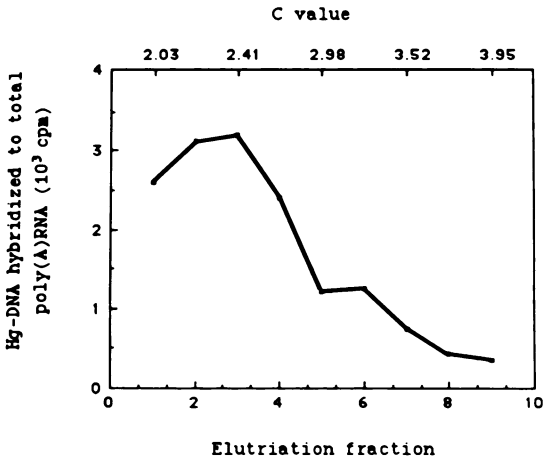

FIG. 4. Hybridization of [^{32}P]HgDNA synthesized by CHO cells at various stages of S phase with total poly(A) RNA. Exponentially growing cells were separated into populations representing various stages of S phase, using centrifugal elutriation, and HgDNA synthesis was done after permeabilization as described in Materials and Methods. [^{32}P]HgDNA from each elutriated fraction was hybridized with total poly(A) RNA immobilized on a nylon membrane.

conditions of cell growth and mRNA analysis. Levels of expression of the remaining genes varied considerably (10- to 15-fold), as expected since we analyzed a wide variety of cellular functions, such as intermediary metabolism, protooncogenes, structural proteins, carrier proteins, hormones, and some intermediate repetitive sequences.

DISCUSSION

The nature of the regulatory mechanisms responsible for the orderly replication of the mammalian genome is largely unknown. As a first step toward developing an understanding of these mechanisms, we have developed an experimental system by which the replication order of various genes can be defined with relative ease and precision. Earlier studies on the replication order of specific genes involved prior labeling of the cells with bromodeoxyuridine (BUdR), followed by synchronization using centrifugal elutriation (18, 26, 29) or fluorescence-activated cell sorting (23). One modified version is to synchronize the cells first by metaphase arrest and then label the cells with BUdR (33). The analysis of DNA replication in BUdR-labeled cells is then effected by isopycnic centrifugation and restriction analysis, a procedure that not only is time consuming but limits the potential temporal resolution because of the need for relatively extensive labeling with BUdR (16). An alternative procedure developed by D'Andrea et al. (23) uses selective photodegradation of BUdR-labeled DNA and thus does not require physical separation based on density, but it shares with all subtractive methods of analysis the difficulty of detecting weak signals. Our method involves labeling of replicating DNA after the isolation of synchronous cell populations. The labeling is done in permeabilized cells with mercurated nucleotides, and the newly synthesized DNA can be isolated by a simple chromatographic procedure. Since there is no lower limit on the size of the DNA to be fractionated, very short labeling periods can be used.

Analysis of the temporal order of gene replication by the method described here is predicated on the assumption that our labeling procedure is specific for normally replicating

TABLE 3. Steady-state level of transcription of various genes in exponentially growing CHO cells

Category	Gene or gene product[a]	Replication time (C value)	^{32}P hybridized with specific gene probe[b] (cpm)
Intermediary metabolism	HPRT	2.23	1,090
	HMG-CoA reductase	2.25	490
	CAD	2.26	1,930
	DHFR	2.26	500
	Thymidine kinase	2.84	3,590
	Cytochrome P1-450	2.89	9,470
	APRT	2.95	3,650
Oncogenes and proto-oncogenes	v-*mos*	2.21	<100
	neu	2.23	3,600
	c-*abl*	2.24	<100
	erbA	2.26	520
	N-*ras*	2.33	1,750
	v-Ha-*ras*	2.33	850
	v-*sis*	2.33	<100
	c-*fos*	2.34	5,100
	c-*myc*	2.42	4,350
	N-*myc*	3.67	780
	v-Ki-*ras*	3.69	3,020
	p21	3.84	2,330
Structural proteins	β-Actin	2.25	8,000
	Fibronectin	3.66	4,670
Carrier proteins	β-Globin	3.58	<100
	Apoferritin	3.70	1,410
Hormones	Encephalin	2.38	840
Repetitive sequences	SINE	2.23	3,660
	LINE (B)	3.69	4,410

[a] Abbreviations are as given in the footnote to Table 1.

[b] Total poly(A) RNA (1.4 μg) from approximately 2×10^7 CHO cells was immobilized as dot on a nylon membrane and hybridized with ^{32}P-labeled nick-translated gene probe. Hybridized counts on the dot were counted and corrected for the blank, subtracting the value for the *E. coli* tRNA dot (1.4 μg) in the same blot for each probe.

DNA. This assumption is supported by earlier experiments showing that the cell permeabilization procedure used allows DNA synthesis in a cell cycle-dependent manner (53) as well as continuous initiation of replication of the simian virus 40 genome in permeabilized infected monkey kidney cells (59). Moreover, our results with the inhibitors arabinosyl CTP and aphidicolin are consistent with the idea that the synthesis of HgDNA in this system reflects normal DNA replication. A potential advantage of the permeabilized cell system is the possibility of studying the effect of exogenous compounds on the replication order of chromosomes and specific genes. The recent demonstration that monoclonal antibodies to DNA polymerase α are capable of entering lysolecithin-permeabilized human cells and inhibiting nuclear DNA replication makes possible new approaches to the study of chromosome replication order in the permeable cells (54).

A critical evaluation of our approach is the comparison of the replication times of specific genes obtained by our procedure with those obtained by other methods that use in vivo labeling of cells. Such a comparison must necessarily be mostly qualitative, since the results of others are often expressed in terms of early or late replication times rather than in terms of C values. Our results with the *DHFR*, CAD, and *HPRT* genes are in good agreement with the in vivo labeling results of Goldman et al. (33). The observed early replication of the α-globin gene agreed with three independent determinations (26, 29, 33); early replication of the hamster short interspersed repetitive sequence and late replication of the long interspersed repetitive sequences were also observed by Holmquist and co-workers (40); and early replication of most (9 of 12) oncogenes was consistent with the observations of Iqbal et al. (44). The only exception is that the *APRT* gene, which was observed early by Goldman et al. (33), was found rather later in our study (C value of 2.95). With this exception, these comparisons, although largely qualitative, serve to confirm the validity of our experimental approach.

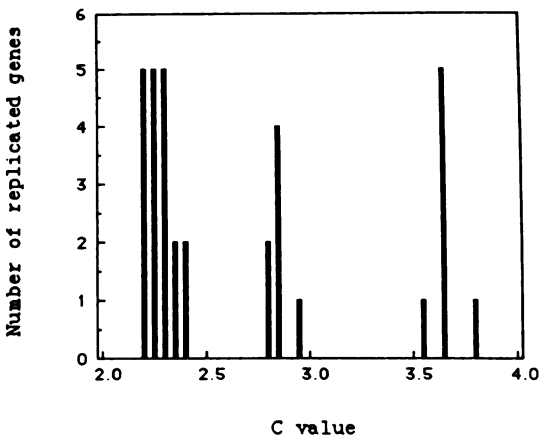

FIG. 5. Clusters of genes replicated at various C values during S phase of the cell cycle. The number of genes replicated at a particular C value is computed from Table 2 and plotted against the C value.

We selected a wide variety of DNA sequences for analysis: single-copy genes (e.g., CAD and *HPRT*); multifamily genes (e.g., α-globin); oncogenes, because of their close relationship to cell growth (12) and involvement in DNA synthesis (30, 43, 62); and repetitive sequences, some of which may act as mammalian replicons (3, 46). This is the first time that the replication order of various genes or sequences spanning the whole S phase of a single cell line has been quantitatively documented in terms of C values. The 28 gene probes studied here included 12 oncogenes (v-*myc* and c-*myc* recognized the same sequence). The oncogenes were replicated in two sharply defined groups; nine were replicated between C values of 2.2 and 2.42 and three were replicated between C values of 3.67 and 3.84, with none in between (Table 3). At this stage, it is not possible to speculate on the significance of the bimodal replication of this class of genes. Another interesting observation is the absence from our diverse collection of gene probes of any that replicate during the middle of S phase, between C values of 3.0 and 3.5. The replication times of the genes studied were not randomly distributed throughout S phase but formed distinct clusters (Fig. 5). Admittedly, we have studied only 28 genes and the gaps in replication times may be coincidental. On the other hand, they may somehow be related to a 3C pause in the middle of S phase of the type described by Holmquist et al. as an almost complete cessation of DNA synthesis demarcating the transition from early replication (R bands) to late replication (G bands) (38, 39).

Our analysis of transcription from most of these genes and its relationship to replication order supports the views of Holmquist et al. (33, 38, 39). When DNA replicating at different times in S phase was analyzed for transcribed sequences complementary to poly(A) RNA from exponential

cells, most (75%) of the active genes were found to reside in the early-replicating DNA (Fig. 4). Nevertheless, about 25% of poly(A) RNA was transcribed from late-replicating DNA. Our results are not entirely consistent with earlier experiments involving hybridization of nick-translated G-band (late) and R-band (early) DNAs with total poly(A) RNA, which showed no difference in transcriptional activities of early and late DNAs (38). The discrepancy may be due to different methods used for the isolation of early- and late-replicating DNA, our procedure accomplishing more complete separation. Our results were confirmed by the individual analysis of gene probes with known replication times for transcriptional activity (Table 3). Most (73%) of the transcriptionally active genes replicated early in S phase. Nevertheless, certain expressed genes, e.g., those for fibronectin and apoferritin, replicated late, an observation that cannot be adequately explained by the model of Holmquist et al. (38). Similarly, Hatton et al. (36) observed that the *DHFR* gene and one of the genes encoding a histone (H_2A) replicate late in the mouse L60T cell line despite being transcriptionally active.

A survey of the C values of the genes examined in this study shows that all genes whose products have housekeeping functions (i.e., are involved in intermediary metabolism) replicate during the first half of S phase. Oncogenes and proto-oncogenes fall into two classes: six expressed and three unexpressed genes replicate during the first quarter of S phase, and three replicate in the fourth quarter. As mentioned above, two genes, those encoding fibronectin and apoferritin, are of special interest because they are actively expressed yet replicate in the last quarter of S phase. Poly(A) RNA transcripts complementary to repetitive sequences were also found, in agreement with earlier studies on CHO cells (45). Transcriptional activity was associated with both early- and late-replicating repetitive sequences. As far as the time of transcription of a gene is concerned, it is completely independent of the time of replication, for most of the genes studied here are transcribed throughout the cell cycle and not just in S phase. In general, the replication order of 5S RNA genes in *Xenopus laevis* (34) and the elegant work of Dhar et al. on β-globin gene domains (24) support the idea, much elaborated by Holmquist (39), that genes which are actively expressed replicate early and that the switching of genes from early to late replication reflects commitment of the genes to quiescence. Nevertheless, our observation that about 25% of the expressed genes are late replicating suggests that the model of Holmquist (39) may be somewhat oversimplified.

Very little is known about the mechanism by which early- and late-replicating domains of eucaryotic chromosomes are regulated. The importance of chromosomal location for the temporal order of replication of a gene (18) and the association of nuclease-sensitive open chromatin configuration with early-replicating segments (24) have been demonstrated. The ease and convenience of our procedure provides the opportunity to extend these studies to additional classes of genes in CHO cells in order to determine whether DNA replication is indeed bimodal, examine the effect of chromosomal translocations (for example, of the *DHFR* gene in the mutant CHO cell lines DG24 and DG46), and study in more detail the interesting phenomenon of actively transcribed genes that replicate late. But most important, our experimental approach, which uses the study of DNA replication in permeabilized cells, offers the opportunity to perturb the replication process by the use of agents that cannot enter intact cells in order to obtain information on possible regulatory mechanisms.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM25022 from the National Institute of General Medical Sciences and by Public Health Service Biomedical Research Support Group grant RR 05711 from the National Institutes of Health Biomedical Research Support Program.

We thank all of the scientists listed in Table 1 for their gifts of recombinant plasmid probes. We are grateful to H. Paulus for critical reading of the manuscript and to A. Basnakyan for computer assistance in organization of the results.

LITERATURE CITED

1. **Alitalo, K., J. M. Bishop, D. H. Smith, E. Y. Chen, W. W. Colby, and A. D. Levinson.** 1983. Nucleotide sequence of the v-myc oncogene of avian retrovirus MC-29. Proc. Natl. Acad. Sci. USA **80**:100–104.
2. **Amaldi, F., D. Giacomoni, and R. Zito-Bignami.** 1969. On the duplication of ribosomal RNA cistrons in Chinese hamster cells. Eur. J. Biochem. **11**:419–423.
3. **Ariga, H., T. Itani, and S. M. 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.
4. **Balaz, J., and C. L. Schildkraut.** 1971. DNA replication in synchronized cultured mammalian cells. II. Replication of ribosomal cistrons in thymidine-synchronized HeLa cells. J. Mol. Biol. **57**:153–158.
5. **Banfalvi, G., S. Bhattacharya, and N. Sarkar.** 1985. Selective isolation of mercurated DNA by affinity chromatography on thiol matrices. Anal. Biochem. **146**:64–70.
6. **Banfalvi, G., and N. Sarkar.** 1983. Analysis of the 5'-termini of nascent DNA chains synthesized in permeable cells of *Bacillus subtilis*. J. Mol. Biol. **163**:147–169.
7. **Banfalvi, G., and N. Sarkar.** 1985. Origin and degradation of the RNA primers at the 5' termini of nascent DNA chains in *Bacillus subtilis*. J. Mol. Biol. **186**:275–282.
8. **Banfalvi, G., A. Sooki-Toth, N. Sarkar, S. Csuzi, and F. Antoni.** 1984. Nascent DNA chains synthesized in reversibly permeable cells of mouse thymocytes. Eur. J. Biochem. **139**:553–559.
9. **Bargmann, C. J., M.-C. Hung, and R. A. Weinberg.** 1986. Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185. Cell **45**:649–657.
10. **Bhattacharya, S., and N. Sarkar.** 1981. Study of deoxyribonucleic acid replication in permeable cells of *Bacillus subtilis* using mercurated nucleotide substrates. Biochemistry **20**:3029–3034.
11. **Bhattacharya, S., and N. Sarkar.** 1982. Transforming activity of mercury-substituted DNA synthesized *in vitro* by permeable cells of *Bacillus subtilis*. J. Biol. Chem. **257**:1610–1612.
12. **Bishop, J. H.** 1985. Viral oncogenes. Cell **42**:23–28.
13. **Blair, D. G., W. L. McClements, M. K. Oskarsson, P. J. Fishinger, and G. F. V. Woude.** 1980. Biological activity of cloned moloney sarcoma virus DNA: terminally redundant sequences may enhance transformation efficiency. Proc. Natl. Acad. Sci. USA **77**:3504–3508.
14. **Braunstein, J. D., D. Schulze, T. DelGiudice, A. Furst, and C. L. Schildkraut.** 1982. The temporal order of replication of murine immunoglobulin heavy chain constant region sequences corresponds to their linear order in the genome. Nucleic Acids Res. **10**:6887–6902.
15. **Brennand, J., D. S. Konecki, and C. T. Caskey.** 1983. Expression of human and Chinese hamster hypoxanthine-quanine phosphorylsyltransferase cDNA recombinant in cultured Lesch-Nyhan and Chinese hamster fibroblast. J. Biol. Chem. **258**: 9593–9596.
16. **Brown, E. H., M. A. Jqbal, S. Stuart, K. S. Hatton, J. Valinsky, and C. L. Schildkraut.** 1987. Rate of replication of the murine immunoglobulin heavy-chain locus: evidence that the region is part of a single replicon. Mol. Cell. Biol. **7**:450–457.
17. **Cairns, J.** 1966. Autradiography of HeLa cell DNA. J. Mol.

Biol. 15:372–373.
18. **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.
19. **Carothers, A. M., G. Urlaub, N. Ellis, and L. A. Chasin.** 1983. Structure of dihydrofolate reductase gene in hamster ovary cells. Nucleic Acids Res. 11:1997–2012.
20. **Chin, D. J., G. Gil, D. H. Russel, L. Liscum, K. L. Luskey, S. K. Basu, H. Okayama, P. Berg, J. L. Goldstein, and M. S. Brown.** 1984. Nucleotide sequence of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase. Nature (London) 308:613–617.
21. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. Biochemistry 18:5294–5299.
22. **Comb, M., P. H. Seeburg, J. Adelman, L. Eiden, and E. Herbert.** 1982. Primary structure of the human Met- and Leu-enkephalin precursor and its mRNA. Nature (London) 295:663–666.
23. **D'Andrea, A. D., U. Tantravahi, M. Lalande, M. A. Perle, and S. A. Latt.** 1983. High resolution analysis of the timing of replication of specific DNA sequences during S phase of mammalian cells. Nucleic Acids Res. 11:4753–4774.
24. **Dhar, V., D. Mager, A. Iqbal, and C. L. Schildkraut.** 1988. The coordinate replication of the human β-globin gene domain reflects its transcriptional activity and nuclease hypersensitivity. Mol. Cell. Biol. 8:4958–4965.
25. **Ellis, R. W., D. DeFeo, T. Y. Shih, M. A. Gondo, H. A. Young, N. Tsuchida, D. R. Lowy, and E. M. Scolnick.** 1981. The p-21 src genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. Nature (London) 292:506–511.
26. **Epner, E., R. A. Rifkind, and P. A. Marks.** 1981. Replication of α and β globin DNA sequences occurs during early S phase in murine erythroleukemia cells. Proc. Natl. Acad. Sci. USA 78:3058–3062.
27. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6–13.
28. **Frykberg, L., S. Palmieri, H. Beug, T. Graf, M. J. Hayman, and B. Vennstrom.** 1983. Transforming capacities of avian erythroblastosis virus mutants deleted in the erbA or erbB oncogenes. Cell 32:227–238.
29. **Furst, A., E. H. Brown, J. D. Braunstein, and C. L. Schildkraut.** 1981. α-Globin sequences are located in a region of early replicating DNA in murine erythroleukemia cells. Proc. Natl. Acad. Sci. USA 78:1023–1027.
30. **Ganon, F. V., and D. P. Lane.** 1987. p53 and DNA polymerase α compete for binding to SV40 T antigen. Nature (London) 329:456–458.
31. **Giacomoni, D., and D. Finkel.** 1972. Time of duplication of ribosomal RNA cistrons in a cell line of Potorus tridactylis (rat kangaroo). J. Mol. Biol. 70:725–728.
32. **Gimmler, G. M., and E. Schweizer.** 1972. rDNA replication in synchronized culture of *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. 46:143–149.
33. **Goldman, M. A., G. P. Holmquist, M. C. Gray, L. A. Caston, and A. Nag.** 1984. Replication timing of genes and middle repetitive sequences. Science 224:686–692.
34. **Gunita, D. R., J. Y. Tso, S. Narayanswami, B. A. Hamkalo, and L. T. Korn.** 1986. Early replication and expression of oocyte-type 5S RNA genes in a Xenopous somatic cell line carrying a translocation. Proc. Natl. Acad. Sci. USA 83:5150–5154.
35. **Hand, R.** 1978. Eukaryotic DNA: organization of the genome for replication. Cell 15:317–325.
36. **Hatton, K. S., V. Dhar, E. H. Brown, M. A. Iqbal, S. Stuart, V. T. Didamo, and C. L. Schildkraut.** 1988. Replication program of active and inactive multigene families in mammalian cells. Mol. Cell. Biol. 8:2149–2158.
37. **Hentze, M. W., S. Keim, P. Papadopoulos, S. O'Brien, W. Modi, J. Drysdale, W. J. Leonard, J. B. Harford, and R. D. Klausner.** 1986. Cloning, characterization, expression and chromosomal localization of a human ferritin heavy-chain gene. Proc. Natl. Acad. Sci. USA 83:7226–7230.
38. **Holmquist, G., M. Gray, T. Porter, and J. Jordan.** 1982. Characterization of Giemsa dark- and light-band DNA. Cell 31:121–129.
39. **Holmquist, G. P.** 1987. Role of replication time in the control of tissue-specific gene expression. Am. J. Hum. Genet. 40:151–173.
40. **Holmquist, G. P., and L. A. Caston.** 1986. Replication time of interspersed repetitive DNA sequences in hamsters. Biochim. Biophys. Acta 868:164–177.
41. **Housman, D., and J. A. Huberman.** 1975. Changes in the rate of DNA replication fork movement during S phase in mammalian cells. J. Mol. Biol. 94:173–181.
42. **Huberman, J. A., and A. D. Riggs.** 1968. On the mechanism of DNA replication in mammalian chromosomes. J. Mol. Biol. 32:327–341.
43. **Iguchi-Ariga, S. M. M., T. Itani, M. Yamaguchi, and H. Ariga.** 1987. c-myc protein can be substituted for SV40 T antigen in SV40 DNA replication. Nucleic Acids Res. 15:4889–4899.
44. **Iqbal, M. A., J. Chinsky, V. Didamo, and C. L. Schildkraut.** 1987. Replication of proto-oncogenes early during the S phase in mammalian cell lines. Nucleic Acids Res. 15:87–103.
45. **Jelinek, W. R., and C. W. Schmid.** 1982. Repetitive sequences in eukaryotic DNA and their expression. Annu. Rev. Biochem. 51:813–844.
46. **Johnson, E. M., and W. R. Jelinek.** 1986. Replication of a plasmid bearing a human Alu-family repeat in monkey COS-7 cells. Proc. Natl. Acad. Sci. USA 83:4660–4664.
47. **Jones, P. B. C., D. R. Galeazzi, J. M. Fisher, and J. P. Whitlock.** 1985. Control of cytochrome P_1-450 gene expression by dioxine. Science 227:1499–1502.
48. **Kraimer, A. R., T. Maniatis, B. Rushim, and M. R. Green.** 1984. Normal and mutant human β-globin pre-mRNAs are faithfully and efficiently spliced *in vitro*. Cell 36:993–1005.
49. **Land, H., A. C. Chen, J. P. Morgenstern, L. L. Parada, and R. A. Weinberg.** 1986. Behavior of *myc* and *ras* oncogenes in transformation of rat embryo fibroblasts. Mol. Cell. Biol. 6: 1917–1925.
50. **Lewis, J. A.** 1986. Structure and expression of the Chinese hamster thymidine kinase gene. Mol. Cell. Biol. 6:1998–2010.
51. **Marchionni, M. A., and D. J. Raufa.** 1981. Replication of viral DNA sequences integrated within the chromatin of SV40-transformed Chinese hamster lung cells. Cell 26:245–258.
52. **Miller, A. D., T. Curran, and I. M. Verma.** 1984. c-fos protein can induced cellular transformation: a novel mechanism of activation of a cellular oncogene. Cell 36:51–60.
53. **Miller, M. R., J. J. Castellot, Jr., and A. B. Pardee.** 1978. A permeable animal cell preparation for studying macromolecular synthesis. DNA synthesis and the role of deoxyribonucleotides in S phase initiation. Biochemistry 17:1073–1080.
54. **Miller, M. R., R. G. Ulrich, T. S. Wang, and D. Korn.** 1985. Monoclonal antibodies against human DNA polymerase-α inhibit DNA replication in permeabilized human cells. J. Biol. Chem. 260:134–138.
55. **Murray, M. J., J. M. Cunningham, L. F. Parada, F. Dautry, P. Lebonitz, and R. A. Weinberg.** 1983. The HL-60 transforming sequence. A ras oncogene coexisting with altered myc genes in hematopoietic tumors. Cell 33:749–757.
56. **Nishioka, Y., and P. Leder.** 1979. The complete sequence of a chromosomal mouse α-globin gene reveals elements conserved throughout vertebrate evolution. Cell 18:875–882.
57. **Oliver, N., R. F. Newby, L. T. Furcht, and S. Bourgeois.** 1983. Regulation of fibronectin biosynthesis by glucocorticoids in human fibrosarcoma cells and normal fibroblasts. Cell 33:287–296.
58. **Robbins, K. C., S. G. Devare, and S. A. Aaronson.** 1981. Molecular cloning of integrated Simian sarcoma virus: genome organization of infectious DNA clones. Proc. Natl. Acad. Sci. USA 78:2918–2922.
59. **Sarkar, N., J. F. List, and G. Banfalvi.** 1987. Replication of the origin region of simian virus 40 DNA in permeabilized monkey cells. Eur. J. Biochem. 168:263–268.
60. **Schwab, M., J. Ellison, M. Busch, W. Rosenau, H. E. Varmus, and J. M. Bishop.** 1984. Enhanced expression of the human gene

N-myc consequent to amplification of DNA may contribute to malignant progression of neuroblastoma. Proc. Natl. Acad. Sci. USA **81**:4940–4944.

61. **Stambrook, P. J.** 1974. The temporal replication of ribosomal genes in synchronized Chinese hamster cells. J. Mol. Biol. **82**:303–313.
62. **Studzinski, G. P., Z. S. Brewi, S. C. Feldman, and R. A. Watt.** 1986. Participation of c-myc protein in DNA synthesis of human cells. Science **234**:467–469.
63. **Taljanidisz, J., R. S. Decker, Z.-S. Guo, M. L. DePamphilis, and N. Sarkar.** 1987. Initiation of simian virus 40 DNA replication *in vitro*: identification of RNA-primed nascent DNA chains. Nucleic Acids Res. **15**:7877–7888.
64. **Taljanidisz, J., P. Karnik, and N. Sarkar.** 1987. Messenger ribonucleic acid for the lipoprotein of the *E. coli* outer membrane is polyadenylated. J. Mol. Biol. **193**:507–515.
65. **Taylor, J. H.** 1960. Asynchronous duplication of chromosomes in cultured cells of Chinese hamster. J. Biophys. Biochem. Cytol. **7**:455–464.
66. **Taylor, J. H.** 1963. DNA synthesis in relation to chromosome reproduction and the reunion of breaks. J. Cell. Comp. Physiol. **62**:73–86.
67. **Wahl, G. M., R. A. Padgett, and G. R. Stark.** 1979. Gene amplification causes overproduction of the first three enzymes of UMP synthesis in N-(phosphonacetyl)-L-aspartate-resistant hamster cells. J. Biol. Chem. **254**:8679–8689.