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Replication of proto-oncogenes early during the S phase in mammalian cell lines

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ABSTRACT

Members of several classes of proto-oncogenes replicate during the first third of S-phase in two human (K562 erythroleukemia and HeLa), one Chinese hamster (CHO) and eight mouse cell lines. These cell lines exhibit a variety of specialized functions characteristic of pre-B and B cells, T cells and erythroid cells. The proto-oncogenes studied include fos, myc, myb, abl, fes, fms, mos, raf, rel, sis, Ha-ras, Ki-ras, and N-ras. In K562 cells, amplified and rearranged c-abl genes show a pattern of temporal replication during S that is similar to the pattern observed for the 5' breakpoint cluster region (bcr) and the amplified Cλ light chain immunoglobulin genes. The c-Ki-ras related sequences in CHO cells provide one example of late replicating proto-oncogene sequences that are present in multiple copies. The cellular gene N-myc replicates late during S in some of these cell lines. In three pre-B cell lines in which N-myc specific transcripts have been detected, N-myc replicates earlier in the S phase than in the other cell lines studied here.

INTRODUCTION

The possible mechanisms by which potentially transforming DNA sequences known as proto-oncogenes become activated include: chromosomal rearrangements resulting in their association with promoters or enhancers, point mutation, and amplification, (for review, see e.g. ref. 1). All of these mechanisms involve steps in which the temporal order of replication of proto-oncogenes could play a significant role in their abnormal activation. While proto-oncogenes code for several different classes of proteins exhibiting diverse functions, it has been suggested that they are essential to cell growth and differentiation (1). Knowledge about the sensitivity of proto-oncogenes during specific stages of the S phase to events that ulitmately lead to their activation could be important to our understanding of the process of cellular transformation. In an attempt to explore the possible relationship between the temporal replication of a proto-oncogene and its genomic organization, our laboratory has initiated

studies on the temporal replication of a number of proto-oncogenes in several different mammalian cell lines.

We have previously studied the temporal replication of c-myc exons before and after chromosomal rearrangements (2). In the MPC11 cell line, the 5' portion of c-myc exon 1 has been translocated from chromosome 15 to chromosome 12 and replicates much later during S than the nonrearranged portion of this exon in these same cells. The remaining portion of myc exon 1 plus exons 2 and 3 are earlier replicating than the corresponding nonrearranged copies in MPC11. In this cell line, the rearranged earlier replicating c-myc sequences appear to be more actively transcribed than the later replicating nonrearranged c-myc sequences (3). Thus, chromosomal rearrangements involving c-myc exons are accompanied by changes in their temporal order of replication.

In the present study we show that before undergoing any genetic change in their structure or function, most of the proto-oncogenes replicate early during S phase in several different mammalian cell lines. We have used the technique of centrifugal elutriation, which we have previously shown can be used with a 5-bromodeoxyuridine (BUdR) labeling method to carry out these measurements (2,4). By chemically linking the bromouracil (BU) labeled DNA to diazobenyloxymethyl (DBM) paper, we are able to include several types of internal controls in each measurement. We have divided the S phase into four intervals so that we can not only determine whether a gene replicates early or late during S, but we can also compare the temporal replication of a particular proto-oncogene in different cell lines.

METHODS

Cell lines and culture conditions: The human erythroleukemia cell line K562 was grown unattached in tissue culture flasks (Falcon No. 3028) in minimal essential medium supplemented with non-essential amino acids, glutamine and 15% fetal bovine serum. The culture conditions for the murine Friend virus transformed erythroleukemia (MEL) cell line DS19, the mouse plasmacytoma cell lines MPC11 and S107, and a subclone of mouse L cell (L60T), were as previously described (2). The pre-B cell lines (5) kindly provided by Dr. Fred Alt were derived from the bone marrow of BALB/c (22D6 and 22D6-1017) or from fetal liver of NIH/Swiss mice (300-19). The radiation induced RL611 T-cell leukemia cell line (6) kindly provided by Dr. Allen Silverstone were grown in RPMI medium supplemented with 12% heat

inactivated (30 min. at 55°C) fetal bovine serum, antibiotics and 50 μM β-mercaptoethanol at 37°C in 5% CO_2 atmosphere. The Chinese hamster ovary (CHO) cell line, and the BALB/c 3T3 cell line were grown in monolayer culture in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum, and antibiotics, at 37°C in 5% CO_2 atmosphere. Trypsinization of monolayer cells was accomplished by first washing the cells in Hanks balanced salt solution and then treating with 0.25% trypsin (Difco 1:250) EDTA for 5-7 min. at 37°C. The cells were then detached by gentle agitation and fractionated by centrifugal elutriation.

Centrifugal elutriation and isolation of BU-DNA. Exponentially growing cells were pulsed with BUdR (20 μg/ml) either for 2 hrs. (mouse and Chinese hamster cell lines) or 2.25 hrs. (K562) before centrifugal elutriation. The use of centrifugal elutriation and flow microfluormetric analyses to obtain four distinct but overlapping cell classes (I-IV) of S-phase cells on the basis of DNA content and size is described elsewhere (2,4,7). Total cellular DNA was then isolated from each cell class as described previously (2). After complete digestion with EcoRI endonuclease the BU-substituted DNA was separated from unsubstituted DNA by preparative ultracentrifugation.

DNA transfer and hybridization: The EcoRI digested BU-DNA (4 μg) from each cell class (I-IV) was electrophoresed in an 0.8% agarose gel for 16-18 hours and transferred to DBM paper by a modification of the procedure of ref. 8. The various nick-translated ^{32}P-labelled proto-oncogene probes were individually hybridized to DBM transfers in the presence of 40-50% formamide, 10% dextran sulphate, 1 X Denhardt's and 5 X SSPE (SSPE is 0.18 M NaCl; 0.01 M NaH_2PO_4, 1 mM EDTA) at 37°C for 16-18 hours. The filters were then washed at either high (0.1 x SSPE, 0.1% SDS, 52°C) or low stringency (2 x SSPE, 0.1% SDS, room temperature) depending on the homology of a given probe with the DNA on the filter. The DBM transfers were then exposed to X-ray film with intensifying screens for several days at -70°C. In most instances, the same DBM transfer was used to hybridize different oncogene probes after removing the previous probe by treating the filters with 0.4M NaOH at 37°C for 15 minutes. Quantitation of the signal was accomplished by measuring the areas of densitometric scans. The interval during the S phase in which a particular oncogene replicated was determined as described (2,4). The DBM transfers of pre-B cell synchrony experiments were kindly provided by Dr. Susan Stuart (9).

Isolation of total cellular RNA: Total cellular RNA from each of the cell lines was isolated by a modification of a previously described procedure (10). Briefly, 2.5 x 10^8 cells from each cell line was washed in 0.15 M NaCl, 10 mM tris (pH 7.5) and 1.5 mM $MgCl_2$. The cells were lysed in 0.1 M sodium acetate, 1 mM EDTA pH 5.2 and 0.5% SDS. The lysate was then extracted with phenol saturated with acetate: EDTA buffer at 60°C. The interphase of the lysate and the organic phase were extracted again with one half the original volume of acetate: EDTA buffer and the two aqueous phases were combined. This was extracted twice with a mixture of phenol, chloroform and isoamyl alcohol (25:24:1) and finally ethanol precipitated at -20°C. Approximately 20 µg of total RNA was fractionated in formaldehyde-agarose gels (1.2%), transferred to nitrocellulose, prehybridized and then hybridized overnight with ^{32}P-labelled probes at 65°C. The filters were washed at low stringency at 65°C in 2 X SSC containing 0.1% SDS (0.1 xSSC is 0.15M NaCl and 0.015M Na citrate)and high stringency at 55°C in 0.1 X SSC containing 0.1% SDS. The filters were then exposed to X-ray films as described earlier.

Characteristics of probes: The c-myc probe (pc-myc Bam/Bam) is a 5.5 kb Bam HI fragment which contains exons 1, 2 and 3 of c-myc gene as well as flanking sequences cloned from S107 DNA (11). Plasmid p-Mc-myc 54 (kindly provided by Dr. Ken Marcu) is a 2.2 kb cDNA clone of mouse c-myc mRNA in pBR322 (3). The v-abl probe (pAB3Sub3) consists of 2.3 kb of v-abl sequences inserted at the HindIII-Bam HI site of pBR322 (12). The v-Ki-ras probe is the 1.0 kb EcoRI fragment of clone HiHi3 (13) and v-Ha-ras probe is clone BS-9 (14). The c-mos probe consists of a 900 bp AvaI and HindIII fragment cloned in pBR322 (15). v-myb is the 1.0 kb Bam HI fragment of clone HAX4 (16); the v-sis probe consists of the 1.0 kb PstI-XbaI fragment of v-sis sequences without helper (17). The v-fes probe is a 0.5 kb PstI fragment from Pst-4 which contains the SR fragment of v-fesst (18). The p-fos-1 probe is a 1.1 kb Pst I fragment containing oncogene sequences of FBJ murine osteosarcoma virus (19). The Cλ probe consists of 0.7 kb Bgl II-EcoRI genomic segment of Cλ Mcg gene (λ1) (20). The N-mycl probe (kindly provided by Drs. R. DePinho and F. Alt) is a 1.6 kb DNA Cla-EcoRI fragment of the pN7.7 murine N-myc genomic clone which contains exon 3 (21). The praf-1 DNA probe is derived from 3611-murine sarcoma virus proviral DNA and cloned into the SalI site of pBR322 (22). The N-ras-1 probe is derived from human promyelocytic cell line HL-60 and cloned into the EcoRI site of puc-12 (23). The v-rel probe (EcoRI-rel) is a subclone of clone 2-20-4

containing rel specific sequences cloned in pBR322 (24). The breakpoint cluster region (5' bcr) probe is a PvuII-PstI c-DNA fragment cloned in reversed orientation into pSP64 (25) kindly provided by Dr. John Groffen. The authenticity of each probe included in this study was confirmed by excising the inserted segment with the appropriate restriction enzyme and comparing the size with that which was originally reported.

RESULTS

Most proto-oncogenes studied replicate during the first half of the S phase: The temporal order of replication of each proto-oncogene was determined in mammalian cells fractionated according to their position in the cell cycle by centrifugal elutriation. The cells are fractionated into four classes (I-IV) according to their position in the cell cycle and EcoRI segments of BU-DNA replicated during four intervals of the S phase is isolated as previously described (4). This EcoRI digested DNA is size fractionated and chemically linked to DBM paper for hybridization with nick-translated oncogene probes. Since the paper is used many times, the replication of the different proto-oncogenes is compared using the same BU-DNA samples. In addition, we have used the replication times of immunoglobulin genes that we have previously measured as internal controls (2).

Using these techniques we have measured the replication time of the different classes of proto-oncogenes in ten murine cell lines including the erythroid MEL cell line, two B cell plasmacytoma cell lines (S107 and MPC11), three Abelson transformed pre-B cell lines (22D6, 22D6-1017 and 300-19P), a radiation induced T cell leukemia cell line (RLδ11), a subclone of mouse L cell (L60T), and the BALB/c 3T3 cell line. In addition, we have studied a human erythroleukemia cell line (K562), the HeLa cell line and a Chinese hamster ovary cell line (CHO).

A common feature observed is that most of these proto-oncogenes replicated during the first third of the S phase in most of the cell lines examined (Table 1). This was determined by analyzing the FMF profiles from the fractions obtained by centrifugal elutriation by the method described by Brown et al. (9). A representative DBM transfer showing an early replicating proto-oncogene (c-mos) is shown in Fig. 1.

N-myc replicate earliest in the S phase in the pre-B cell lines: N-myc did not replicate during the first half of the S phase in every cell

line examined. In HeLa, L-60T, CHO, 3T3 and RL611, N-myc sequences replicated during the second half of the the S phase (Table 2). In MEL, S107, and MPC11, replication of N-myc was during the second interval or the

Table 1

Temporal Order of Replication of Proto-oncogenes in Different Cell Lines

Cell Line	Proto-oncogene	Size (kb) of EcoRI Segment (a)	Relative Concentration of Segments in BU-DNA Isolated from Cell Class (b) I	II	III	IV
MEL	c-abl	28.0	68.6	25.5	5.4	0.4[A]
	c-fes	11.0	67.6	25.3	5.5	1.6[D]
	c-fms	8.0	45.9	45.9	7.5	0.1[B]
	c-fos	5.5	40.8	41.2	14.5	3.4[C]
	c-Ha-ras	22.0	75.6	21.3	2.3	0.8[B]
	c-Ki-ras	14.0	55.9	33.3	10.0	0.8[C]
	c-mos	15.0	62.8	33.9	2.0	1.2[A]
	c-myb	5.3	49.5	21.8	20.6	8.0[D]
	c-myc	21.0	49.2	28.0	16.1	6.7[A]
	c-raf	2.9	59.4	38.4	1.5	0.6[B]
	c-rel	5.5 4.8	32.0 43.6	44.3 48.1	17.8 4.7	5.8[D] 3.6D
S107	c-abl	28.0	29.4	33.6	29.5	7.6[A]
	c-fms	7.4	62.0	23.2	13.7	1.0[B]
	c-fos	5.5	52.1	32.3	14.7	1.0[B]
	c-Ha-ras	22.0	49.0	34.4	13.6	5.8[C]
	c-Ki-ras	14.0	55.9	33.0	9.7	1.4[D]
	c-mos	15.0	56.9	32.5	8.6	1.8[B]
MPC11	c-abl	28.0	46.2	27.6	23.7	2.5[B]
	c-fos	5.4	66.1	24.0	5.5	4.4[D]
	c-Ha-ras	22.0	69.8	23.2	4.4	2.8[C]
	c-Ki-ras	14.0	50.9	36.7	7.0	5.5[D]
	c-mos	15.0	52.2	33.8	12.1	1.9[A]

Cell Line	Proto-oncogene	Size (kb) of EcoRI Segment (a)	Relative Concentration of Segments in BU-DNA Isolated from Cell Class (b) I	II	III	IV
L-Cell	c-fos	5.1	61.5	20.9	17.5	0.1[C]
	c-Ha-ras	20.0	60.3	31.9	7.8	0.0[C]
	c-myc	21.0	39.7	34.8	19.7	5.7[C]
3T3	c-fos	5.7	54.1	38.2	5.6	2.1[B]
	c-myc	21.0	16.4	33.7	42.4	7.5[B]
22D6	c-fos	5.6	17.0	60.4	22.0	0.1[B]
	c-Ha-ras	20.0	66.0	28.6	5.3	0.0[C]
	c-myc	31.0	66.0	19.2	8.5	6.3[D]
300-19P	c-Ha-ras	27.0	63.8	28.0	6.9	1.3[C]
	c-myc	23.0	53.9	34.4	7.5	4.1[C]
RL♂11	c-fms	9.4	55.3	34.0	6.8	3.8[B]
	c-fos	5.5	46.1	37.0	14.1	2.8[B]
	c-Ha-ras	19.0	61.4	31.4	3.7	3.3[B]
	c-myc	19.0	38.1	42.2	2.8	16.8
	c-raf	3.4	50.4	34.6	11.8	3.2[A]
K562	c-Ha-ras	25.0	59.0	37.6	2.5	0.9[D]
	c-Ki-ras	2.8	33.3	30.8	20.9	15.0[D]
	c-mos	2.5	52.3	29.0	12.0	6.5[C]
	c-myc	13.5	60.0	30.4	9.5	0.4[C]
	c-raf	9.5	65.2	28.2	5.7	0.9[B]
		6.4	34.6	34.3	20.1	11.0[B]
	N-ras	6.5	54.9	35.6	10.0	0.0[B]
	c-sis	23.0	58.4	30.4	8.5	2.6[D]
HeLa	N-ras	6.5	60.4	34.1	3.3	2.1[C]
CHO	c-abl	25.0	47.3	31.8	15.6	5.2[C]
	c-fes	4.3	86.1	11.0	2.2	0.6[D]
	c-fos	6.0	55.5	20.8	12.4	11.4[C]

Cell Line	Proto-oncogene	Size (kb) of EcoRI Segment (a)	Relative Concentration of Segments in BU-DNA Isolated from Cell Class (b) I	II	III	IV
	c-Ha-ras	12.0	54.0	32.6	8.4	4.8[C]
	c-mos	9.0	46.6	29.2	15.4	8.8[D]
	c-raf	9.5	67.2	18.2	7.7	6.7[C]

a The segment sizes are similar to those reported previously.

b Cell Classes (I-IV) were selected on the basis of DNA content and size (see Materials and Methods). Each cell class had synthesized BU DNA at a specific interval of S. The relative concentrations of EcoRI segments in each of the four intervals (expressed as % of the total concentration) determines the temporal order of replication (2,4).

ABCD All exposures were within the linear response range of the X-ray film. Each film has been classified according to the degree of nonspecific background hybridization observed after autoradiography. We estimated the maximum error by calculating the normalized relative concentrations in two different ways. The band intensity used to calculate the normalized concentrations was always corrected for the background by subtracting the average value of the background film optical density adjacent to the band. To determine the maximum error in this calculation, either the maximum or the minimum amount of unevenly distributed background optical density adjacent to the band was subtracted from the band intensity and the relative concentrations were calculated as described in Methods. The difference between the two results obtained by this calculation is the maximum percent error listed for each result: A. less than 10%, B. 10-15%, C.15-20%, D.20-30%.
In order to determine the accuracy of this approach we compared different measurements of the temporal replication of three IgCH genes in the MEL cell line. We used three independent elutriation experiments for these studies. For one of the elutriation experiments, we prepared several different DBM transfers containing identical amounts of BU-DNA from each of the four intervals of S. The average standard deviation was about 10 % (4). This error estimate also includes the error due to unevenly distributed background on the X-ray films described above.

middle of S. Replication of N-myc was earliest in the pre-B cell lines 22D6, 22D6-1017 and 300-19P, in which N-myc specific transcripts have been first demonstrated by Zimmerman et. al, 1986 (26) and also confirmed in the present report (Fig. 2). We did not detect steady state levels of N-myc in total RNA from other cell lines in which we measured the temporal replication. By comparison, c-myc transcripts were detected in all of the cell lines studied (Fig. 2). In all of these cell lines, with the exception of 3T3 (Table 1), c-myc replicated during the first third of S. In the 3T3 cell line, analysis of FMF profiles (9) indicated that c-myc replicated during the middle of S.

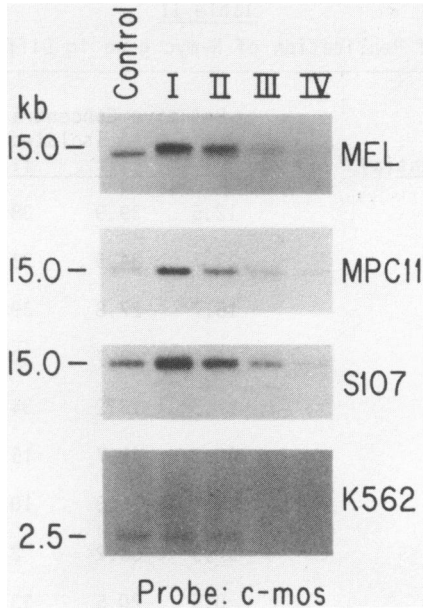

Figure 1. Replication of c-mos sequences during the first third of S in different cell lines. The control lane contains 4 μg of EcoRI digested DNA isolated from exponentially growing cells before elutriation. The probe used was a 900 bp AvaI and HindIII fragment containing the c-mos sequences (15). The segment sizes in each cell line are the same as previously reported. The DBM transfers of DNA from MEL, MPC11 and S107 cells were washed at high stringency, whereas, the K562 DNA transfer was washed at low stringency.

Replication of amplified c-abl, bcr and Cλ light chain genes in K562 cells: In K562 cells, c-abl, 5' bcr and Cλ light chain constant region immunoglobulin genes are amplified 4-8 fold (25,27-28), and are located on a single marker chromosome (29). Our observations (Fig. 3, Table III) show that EcoRI segments containing c-abl, 5' bcr and Cλ light chain genes replicate at similar times during S. The unrearranged Cλ gene in the MEL cell line exhibited a temporal replication pattern similar to that of Cλ in K562 cells (data not shown).

Multiple copy c-Ki-ras related sequences in CHO replicate late in S: Previously, it has been shown that multiple copies of c-Ki-ras related sequences are present in the CHO cell line (30). We have determined, from the areas of densitometric scans of autoradiograms such as those shown in Fig. 4, that there are 30-35 fold more c-Ki-ras sequences present in Chinese hamster cells than in mouse cell lines. Both Chinese hamster skin

Table II

Temporal Order of Replication of N-myc gene in Different cell lines.

Cell Line	Size of EcoRI Segment(a)	Relative Concentration of Segments in BU-DNA Isolated from Cell Class(b) I	II	III	IV
MEL	6.3	12.5	39.9	38.8	8.9^{A}
S107	8.8	23.7	34.9	33.3	8.1^{B}
MPC11	7.4	16.7	27.8	39.3	16.1^{B}
L Cell	6.1	4.1	9.7	33.9	52.3^{C}
3T3	8.7	17.8	13.2	34.0	35.0^{B}
22D6	8.8	40.2	41.7	15.5	2.4^{B}
22D6-1017	9.0	65.1	16.3	10.0	8.6^{A}
300-19P	8.4	57.9	38.2	2.2	1.6^{B}
RLd11	9.6	0.5	20.5	53.8	25.1^{C}
HeLa	1.9	2.3	4.4	22.5	70.7^{B}
CHO	7.7	14.9	27.4	27.6	30.0^{B}

a, b and A,B,C and D as described in Table I.
The S phase for 22D6-1017 was divided into 6 intervals rather than 4 intervals. The sum of the percents for the first two and last two intervals are included in the first and last columns respectively since these S phase intervals are equivalent to the first and last intervals for the other cell lines.

and spleen showed a similar level of amplification. The proto-oncogene c-Ha-ras which is closely related to c-Ki-ras in both structure and function did not exhibit amplification in Chinese hamster cells. The pattern of replication in CHO cells of c-Ha-ras sequences are shown in Fig. 4 and the results for c-Ki-ras EcoRI sequences are shown in Fig. 4 and Table 3. It is clear that, c-Ha-ras genes replicate during the first half of S, while the multiple copies of c-Ki-ras sequences replicate during the second half of S.

DISCUSSION

In the present study, the temporal order of replication has been determined for 14 different oncogenes representing several different

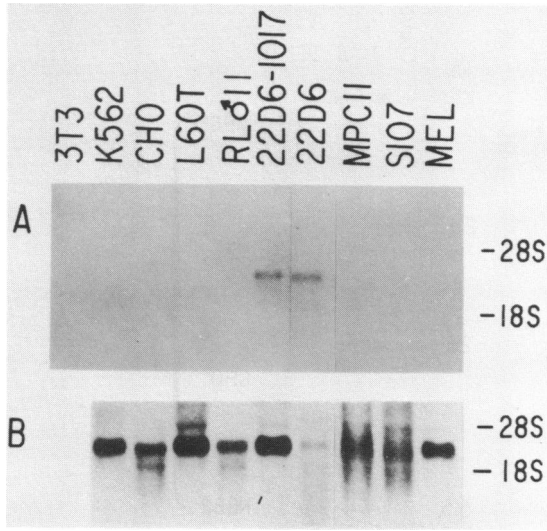

Figure 2. c-myc transcripts were detected in almost all of the cell lines studied while N-myc transcripts were detected only in the pre-B cell lines. Approximately 20 µg of total cellular RNA from each of the cell line listed above was fractionated through formaldehyde-agarose gel (1.2%) and transferred to nitrocellulose (see methods). The transfer was hybridized to nick-translated pN-myc-1 probe (A) or the pMc-myc54 probe (B). N-myc specific transcripts were observed only in the pre-B cell lines, while c-myc transcripts were detected in all of the cell lines (the presence of c-myc transcripts in 3T3 has been reported after serum stimulation (47-48), as well as in exponentially growing 3T3 cells (personal communication, Dr. Fillipo Cavalieri, Columbia University, College of Physicians and Surgeons, New York).

classes of proteins such as GTP binding proteins, growth factors, protein kinases and nuclear associated proteins. We have demonstrated that most of these proto-oncogenes replicate predominantly during the first third of the S phase in 11 cell lines derived from differentiated tissues such as T-and B-lymphoid cells and erythroid cells. This is true in all three mammalian species studied, irrespective of the chromosomal locations of the proto-oncogenes.

In K562 cells, c-abl, 5' bcr and λ light chain constant region immunoglobulin genes are amplified 4-8 fold (27-28), and are located on a single marker chromosome (29), however, the distance between the two genes is still unknown. Because both c-abl and λ genes are amplified in K562 cells, these authors suggested that both these genes might be located in the same amplification unit on the marker chromosome. Previous studies from our laboratory indicate that genes that are in the same chromosomal

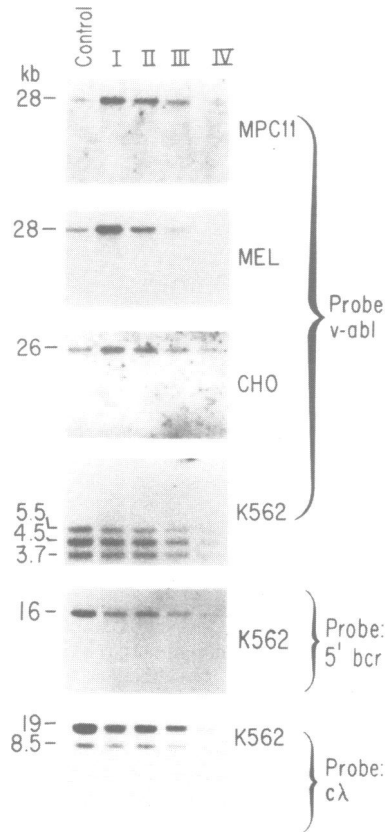

Figure. 3. Replication of c-abl, 5'-bcr and Cλ light chain sequences in K562 cells. The K562 DBM transfer was first hybridized to a c-abl probe consisting of 2.3 kb of v-abl sequences inserted at the HindIII-BamHI site of pBR322, erased and rehybridized to a Cλ light chain gene probe (0.8 kb BglII-EcoRI fragment of Cλ1 gene) and erased again and rehybridized to a 5' bcr c-DNA probe. The segment sizes of c-abl, 5' bcr and Cλ EcoRI sequences are similar to those reported previously.

location usually replicate at similar times during S (2). While genome walking experiments indicate that the distance between 3' c-abl and 5' bcr sequences present on the Ph^1 marker chromosome in K562 cells is greater than 99 kb (25) the similarity in the replication times of c-abl, 5' bcr and λ light chain genes observed in the present study is consistent with their being less than 200 kb apart (Hatton et al., manuscript in preparation).

The replication of c-Ki-ras in CHO cells provides one example of late

Table III

Replication of Rearranged and Multiple Copy Proto-oncogene Sequences

Cell Line	Size (kb) of EcoRI Segment (a)	Relative Concentration of Segments in BU-DNA Isolated from Cell Class (b) I	II	III	
K562					
c-abl	5.5	44.4	35.9	13.5	6.3[A]
	4.5	36.5	34.9	20.6	8.1[A]
	3.7	35.5	31.4	23.2	9.9[A]
5' bcr	16.0	37.5	36.6	23.2	9.9[A]
Cλ	19.0	29.8	34.6	24.4	11.3[A]
	8.5	30.5	34.6	24.9	10.2[A]
CHO					
c-Ki-ras	7.0	6.0	14.1	17.5	62.5[A]
	5.4	8.7	18.7	36.2	37.2[A]
	2.3 + 3.4	3.6	8.0	45.9	42.5[A]

a, b and A,B,C, and D as described in Table I.

replicating cellular oncogenic related sequences. However, we cannot eliminate the possibility that one or a few c-Ki-ras sequences are also early replicating (see Fig. 4). Previously it has been shown that multiple

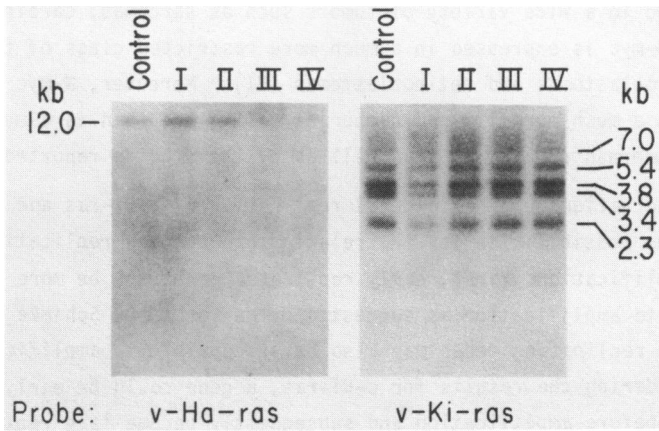

Figure 4. In CHO cells, c-Ha-ras sequences replicate early and c-Ki-ras sequences replicate late in S. The transfer was hybridized to the v-Ha-ras probe, erased and rehybridized to the v-Ki-ras probe.

copies of c-Ki-ras related sequences are present in the CHO cell line (30), and in the Chinese hamster lung fibroblast cell line V79 (31). Similar multiple copies of c-Ki-ras related sequences were found in different Chinese hamster cell types in our study. However, the degree of sequence homology between the multiple copies of the c-Ki-ras in Chinese hamster DNA has not been determined. It is not known whether any of the c-Ki-ras related sequences are pseudogenes or whether the additional copies of c-Ki-ras in Chinese hamster cells are located at several chromosomal sites or are tandemly arranged in a single site. The levels of c-Ki-ras related transcripts in total cellular RNA of CHO cells was approximately twenty-fold less than that observed in K562 cells as calculated by densitometric analysis (data not shown). The c-Ki-ras sequences may be pseudogenes dispersed throughout the genome and not represent amplified oncogene sequences that can be expressed.

In many of the cell lines studied here, N-myc, replicated later during S than most of the proto-oncogenes examined. In these cell lines, N-myc did not exhibit any apparent amplification or rearrangement. We did not detect steady state levels of N-myc transcripts in any of the cell lines in which N-myc replicated during the middle of S or during the second half of S. In the three pre B cell lines (22D6, 22D6-10-17 and 300-19P) however, in which N-myc replicated very early during S, we determined that it was transcribed. It has recently been shown (26,32-34) that N-myc exhibits a restricted pattern of expression. While many proto-oncogenes such as c-myc are expressed in a wide variety of tumors such as sarcomas, carcinomas and leukemias, N-myc is expressed in a much more restricted class of tumors such as neuroblastomas and retinoblastomas (21). Moreover, N-myc is expressed in a much more limited number of cell lines than is c-myc as shown by Zimmerman et al., (26) as well as by the studies reported here.

Our observations on the temporal replication of c-Ki-ras and N-myc suggest three possibilities for the relationship between replication time and gene amplification. First, early replicated genes may be more susceptible to amplification as suggested by Marianni and Schimke (35), Second, late replicating genes may also be susceptible to amplification. Third, considering the results for c-Ki-ras, a gene could be early replicating before amplification and subsequently become late replicating after amplification. Studies are in progress in our laboratory to further explore the possible relationship between temporal replication and gene amplification.

It has been suggested that the early portion of the S phase is sensitive to genetic alterations by mutagens that act during DNA replication. Point mutations in the c-Ki-ras gene have been implicated in the process by which this oncogene can transform normal cells (36). The chemical carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), has been shown to mutagenize specific gene loci preferentially at the time of DNA replication in E. coli and in yeast (37). Treatment of normal guinea pig cells with MNNG results in transformation and is accompanied by point mutations in the ras gene (38). Cell cycle studies in synchronized mammalian cells have demonstrated that the early part of the S phase was the period of maximal transformation from exposure to MNNG (39-40). These studies suggest that proto-oncogenes are more susceptible to mutations in cells in which they are early replicating.

It has also been suggested that early replicating genes may be more susceptible to amplification. Previous studies have shown that dihydrofolate reductase (DHFR) genes could be amplified many fold in mouse and Chinese hamster cells grown in the presence of methotrexate (for review see 41). These amplified DHFR sequences (42-43) and the corresponding single copy DHFR genes in the same CHO cells, before amplification (35,44) were shown to replicate early in S. Moreover, it has been shown that when DNA synthesis resumes after hydroxyurea (HU) treatment of CHO cells, the previously synthesized DNA preceding the HU block is rereplicated during the same S period resulting in the amplification of DHFR genes (35). Furthermore, treatment of cells with carcinogens also has been shown to result in amplification of DNA (45-46). Based on these results, it has been proposed (35) that if amplification by carcinogens proceeds by a mechanism similar to that shown for HU, then early replicating genes would by more susceptible to amplification. This mechanism would make proto-oncogenes more susceptible to amplification in cells when they undergo replication early during the S phase.

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