ISAS - INTERNATIONAL SCHOOL FOR ADVANCED STUDIES

MAPPING DNA REPLICATION ORIGIN WITHIN HUMAN CHROMOSOMES

Thesis submitted for the Degree of

Magister Philosophie

Candidate: Silvia Diviacco

Supervisors: Prof. Arturo Falaschi

Dr. Mauro Giacca

Academic Year 1990/1991

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INTRODUCTION

DNA replication in eukaryotic cells is limited to a discrete part of the cell cycle known as the synthetic or S phase. During this time, the entire genome, along with its associated chromosomal proteins, must be efficiently and accurately duplicated to ensure that the structural and functional integrity of each chromosome is maintained.

Replication occurs within the context of an elaborate nuclear architecture consisting of a proteinaceous scaffold or matrix to which the DNA is attached at periodic intervals facilitating supercoiling and higher order chromatine structure.

A full understanding of the process of DNA replication in animal cells requires the identification and characterization of replicons and their cis-acting control elements. Although bacterial and viral replication origins have been isolated and their activity studied in vivo and in vitro, analysis of eukaryotic and genomic origins has been hampered both by the complexity of replication in these biological system and the lack of appropriate assay techniques for measuring origin function.

This thesis examines several aspects of the initiation of chromosomal DNA replication and focuses in particular on experimental approaches designed to localize and characterize origins of replication in eukaryotic cells.

1.1 REGULATION OF MULTIPLE ORIGINS OF DNA REPLICATION

Several years ago, Huberman and Riggs used an autoradiographic technique to demonstrate that replication initiates at multiple loci along mammalian chromosomal DNA fibers (1). Their studies showed that origins can be spaced 15-800 Kb apart and that most are bidirectional and established the existence of multiple replicons (chromosomal domains replicated from a single replication origin).

Prokaryotes regulate the action of a single replicon, while eukaryotes must coordinate the activity of tens of thousands of replicons.

The length of S phase in cells of different animals can vary over more than two orders of magnitude: this great variation is not due to differences in the rate of DNA synthesis by individual replication complexes and must be due to differences in origin usage: Callan's work (2) showed that fewer functioning origins result in an increase in inter-origin distance and, consequently, a longer time for adjacent replication forks to meet and a longer S phase.

Another important feature of eukaryotic chromosomal replication is that, on a local level replication origins tend to fire synchronously: chromosomes contain clusters of many replication origins that are activated at different times during S phase. It is possible that the nuclear structure has an organizational role: pulling together collinear arrays of replication origins into clusters that function synchronously (3).

1.2 ENTRY INTO THE S PHASE

Twenty years ago, Rao and Johnson (4) performed a classic series of cell fusion experiments that showed that when G1 and S phase cells are fused, the S phase cell exerts a dominant effect on the G1 nucleus, causing it to enter S phase much sooner than it otherwise would. Therefore there are some diffusible factors in the S phase cell that cause the onset of DNA replication in the G1 nucleus. This factor is also inactivated after S phase since G1 nuclei do not enter S phase precociously in G1-G2 fusions. It is also known that holding cells artificially at the G1-S boundary causes a large increase in the number of origins used after release, suggesting that the accumulation of some limiting initiation factor occurs during the block (5).

In the yeast Saccharomices cerevisiae and Schizosaccaromyces pombe, the initiation of DNA replication is controlled at a point called START: only if conditions are appropriate cells traverse START, thus becoming committed to initiate DNA replication and complete the remainder of the cell cycle.

While most biochemical experiments have looked at the G2-M transition, a function for the cdc2 kinase at the initiation of the S phase has been predicted from genetic studies done in S pombe, and it has clearly been demonstrated for CDC28, its homolog in S.cerevisiae.

D'Urso et al. (6) studied the regulation of DNA replication during the eukaryotic cell cycle in a system where cell free

replication of SV40 DNA was used as a model for chromosome replication.

A multiprotein complex, RF-S, was partially purified from human S phase cells based on its ability to activate DNA replication in extracts from G1 cells. RF-S contains a homologue of the $p34^{cdc2}$ protein kinase and this kinase is necessary for RF-S activity. It has been suggested that the limiting step in activation of the p34 kinase at the G1 to S transition may be its association with a cyclin since addition of cyclin A to a G1 extracts is sufficient to start DNA replication.

Nurse et al.(7) have recently shown that $p34^{cdc2}$ or a very related protein, is involved in the initiation of DNA replication in Xenopus egg extracts.

Reed et al. (8) demonstrated that in S.Cerevisiae CLN genes encode Cyclin homologs essential for progression from G1 to S phase. The CLN2 gene encodes a 62 Kd polypeptide that accumulates periodically peaking during G1 and decreasing rapidly thereafter; CLN2 polypeptide interacts with $p34^{CDC28}$ to form an active protein kinase complex.

1.3 EUKARYOTIC ORIGINS MAPPING TECHNIQUES

Experimental approaches designed to localize and characterize origins of replication are based on studies in bacterial systems, and fall into two general categories (Fig.1):

fig.1

APPROACHES TO THE IDENTIFICATION OF MAMMALIAN DNA REPLICATION ORIGINS

	Method	Region studied	Main conclusion	Reference
"REPLICATORS"	BrdUrd substitution *Dpn I* resistance *in vitro* replication	c-myc upstream region	autonomous replication of plasmids containing the region	McWhinney *et al.*, Nucl. Acids Res. 1990, 18, 1233
		random DNA sequences	selection of *ars* sequences	Frappier *et al.*, Proc. Natl. Acad. Sci. USA 1987, 84, 6668
		random DNA sequences in EBV-derived vectors	efficient replication for inserts >12.000 bp	Krysan *et al.*, Mol. Cell. Biol. 1989, 9, 1026
"MAPPERS"	2D-gels	DHFR	origin dispersed within 30 kb	Vaughn *et al.*, Cell 1990, 61, 1075
	protein synthesis inhibition	DHFR, β-globin, APRT	origin mapped within 15 kb	Cedar *et al.*, Cell 1989, 57, 909
	PCR of nascent DNA strands	DHFR, c-myc	origin mapped within 2.5 kb (DHFR) and 2 kb (c-myc)	Vassilev *et al.*, Mol. Cell. Biol. 1990, 10, 4685; Vassilev *et al.*, Mol. Cell. Biol. 1990, 10, 4899
	strand polarity switch	DHFR	origin mapped within 450 bp	Burhans *et al.*, Cell 1990, 62, 955

A) identification of fragments that are capable of autonomous replication and must contain the cis-acting elements necessary for initiation;
B) in vivo labelling protocols designed to detect the positions along the chromosome at which nascent chains are initiated.

A) ATTEMPTS TO ISOLATE ORIGINS BY PHENOTYPIC RESCUE: AUTONOMOUS REPLICATION IN ANIMAL CELLS

The most popular approach for detecting animal cell origins has been to search for sequences that confer the ability to replicate autonomously on small extrachromosomal plasmid elements. Genomic fragments are cloned into a vector (usually containing a selectable marker) and are transfected into a suitable host cell line. The genomic insert can either be an entire genomic library, or sequences suspected to contain an origin of replication based on independent in vivo studies. Although this method has been used successfully in prokaryotes and yeast, experiments in animal cells indicate that the requirement for episome replication may be more complex.

Several criteria are usually employed to demonstrate autonomous replication of transfected plasmids:
(i) demonstration of semiconservative replication by density shift (with previous labelling with BrUdR) by CsCl gradients
(ii) a short term assay in which low molecular weight DNA is sampled over the period of few days after transfection and the ability of the tranfected DNA to replicate autonomously is

determined by its resistance to the enzyme DpnI. This enzyme cannot cleave its recognition sequence (GATC) when the A residue is not methylated, as in DNA that has replicated in mammalian cells, but readly digests the transfected bacterial DNA, which is methylated

(iii) in vitro replication system: soluble cell free systems prepared from mammalian cells that are capable of replicating exogenous DNA template (9).

Initial studies on plasmid sequences injected into Xenopus oocytes, suggested , at least in these cells, that origins may have little sequence specificity (10). Such molecules were found to replicate semiconservatively and in a regulated fashion: each molecule replicated no more than once per cell cycle. All tested circular molecules replicated with approximately equal efficiency; no requirement for a specific origin sequence was detected.

A sequence near the 5' promoter of the Human c-myc gene that was previously implicated as a chromosomal origin of replication by in vivo labelling studies (11), has also been reported to persist as an autonomously replicating element for more than 300 cell generations and to increase in mass by 500-1000 fold over this time period (12).

A different laboratory (13) also reported the episomal maintenance of a sequence from a human c-myc promoter region (not the same sequence as above) both in tissue culture cells and by transmission through several generations of transgenic mice. This sequence is also reported to bind to the

c-myc protein which is suggested to regulate replication in this system (14).

These date therefore seem to suggest that the way is open for critical genetic studies that can mutate cloned origins in vitro and determine the key cis-acting elements required for origin function. But there continues to be some doubts about the identity of these fragments and the authenticity of the assay system because of the scarce reproducibility.

Recently Calos et al (5) suggested that any Human DNA fragment can replicate to a limited extent after transfection into human cells, provided that it is long enough. Sixty two random human genomic fragments ranging in size from 1 to 21Kb were cloned in a replication-defective EBV vector (obtained by removing a part of the oriP region) (15) and tested for their replication ability in the short term assay. A positive correlation has been found between replication strength and fragment length, indicating that large size is favoured for efficient autonomous replication in human cells.

This result implies that only a low degree of sequence specificity is required to initiate DNA replication in human cells or that cis regulatory element occur at very frequent intervals in human DNA. In the same work, they also used the same assay to test in human cells a random series of fragment derived from Escherichia coli chromosomal DNA: the bacterial fragments supported replication less efficiently than the human fragments. This result suggests that while the

sequences involved in replication in human cells are found frequently in human DNA, these sequences are rare in bacterial DNA.

On the other hand there is certainly no reason to assume that genomic origins are all capable of extrachromosomal replication. This perhaps highlights the fact that many chromosomal origins may have structural or topographical requirements that cannot be mimicked on episomal molecules.

B) MAPPING INITIATION OF DNA REPLICATION WITHIN CHROMOSOMES

Many different techniques have been used in order to map by physical methods the position along the chromosome at which initiation of DNA replication occurs.

In the following paragraphs the most promising current approaches are presented:

Asymmetrical nucleosome segregation

This technique has been developed by Cedar et al (16) and is based on the observation that under conditions of protein synthesis inhibition (presence of emetine) and therefore of limiting quantity of histone proteins, the segregation of nucleosomes on newly synthesized DNA is conservative, with almost all of the parental nucleosomes remaining on the leading portion of the replication fork(17). The DNA synthesized in the presence of emetine was labelled with BrUdR and isolated nuclei were treated with micrococcal

nuclease: the lagging part of the fork is completely digested since it is unprotected by nucleosome particles, while the leading double stranded DNA is isolated as mononucleosome-sized fragments. The newly synthesized BrUdR-label leading strand of the DNA is then purified by alkaline isopicnic gradient centrifugation, and hybridized with strand specific probes of the region. If there are fixed origins, then the nucleosome-protected fragments should hybridize selectively to the leading strand, and the position at which hybridization switches from one template strand to the other should define the origin (Fig.2).

This technique has been applied to DHFR (dihydrofolate reductase) domain and to an upstream locus of APRT (adenine phosphoribosyltransferase) gene. In Fig 2 there is a scheme that summarize the principle of this technique.

2-D gel electrophoresis

Both Huberman et al.(18) and Brewer and Fangman (19) reported the use of two dimentional (2-D) gel electrophoretic methods which allowed localization of the replication origins in the S. cerevisiae plasmids. Both methods have been subsequently applied also to eukaryotic chromosomal DNA.

In the neutral-neutral method of Fangman et al. (19) the behaviour of restriction fragments containing branched replication intermediates were analyzed in two-dimentional agarose gels where the first dimension separates molecules roughly according to their mass and the second dimension

METHODS FOR MAPPING MAMMALIAN DNA REPLICATION ORIGINS:

Asymmetrical nucleosome segregation

Handeli, S. *et al.* 1989. *Cell* 57,909-920

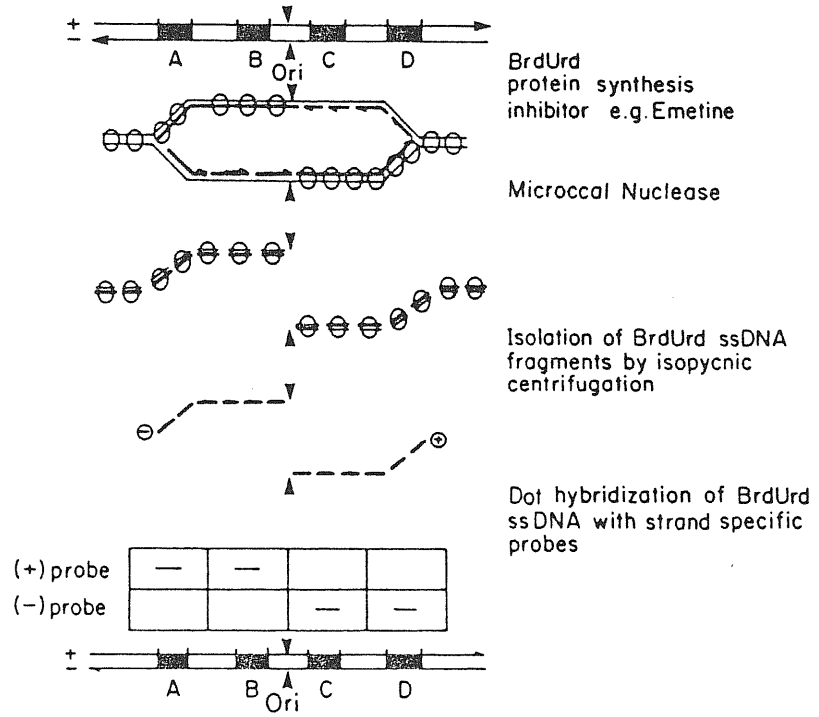


fig. 2

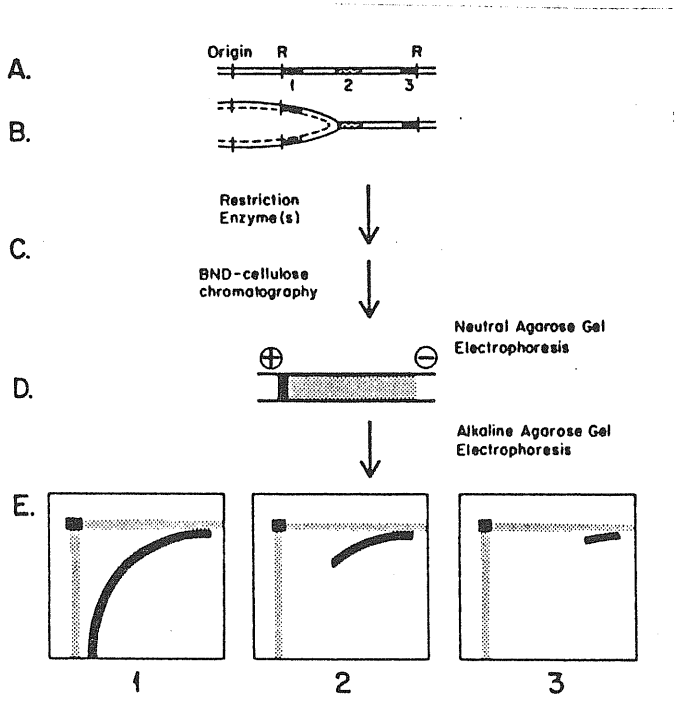
separates molecules according to their shape. In Fig 3 (on the right) the lower panels depict the expected migration in the two-dimentional agarose gel of the four types of the replication intermediates (RIs) shown above. In panel A a 1Kb restriction fragment that is replicated by a fork passing through the molecule from one end to the other (simple Y) is considered. The first dimension of the two-dimentional gel will separate the RIs according to their mass; in the second dimension the RIs will be retarded in their mobility by their branched shapes: a molecule that is exactly half-replicated will have three branches of equal length and will migrate most slowly. Therefore the RIs will produce a continuous arc that begins at the position of 1Kb linear molecule, has an inflection point at a mass equivalent to 1.5Kb linear molecules and ends at the position of 2Kb linear molecule. In panel B is described the RIs pattern obtained by bubbles (or eye forms) produced by initiation of replication in the center of the 1Kb restriction fragment. For bubbles there is a more complex relationship between the extent of replication and three dimentional shape of the molecule, however the pattern obtained in two dimentional gel is that shown in panel B. Another replicating form with double Ys produced by the approach of two forks that are initiated outside the fragment and meet in the exact center, is depicted in panel C: the linear pattern obtained derives from the fact that the four branches increase in length in direct proportion to the extent of replication. If bidirectional replication initiates at an asymmetrical position

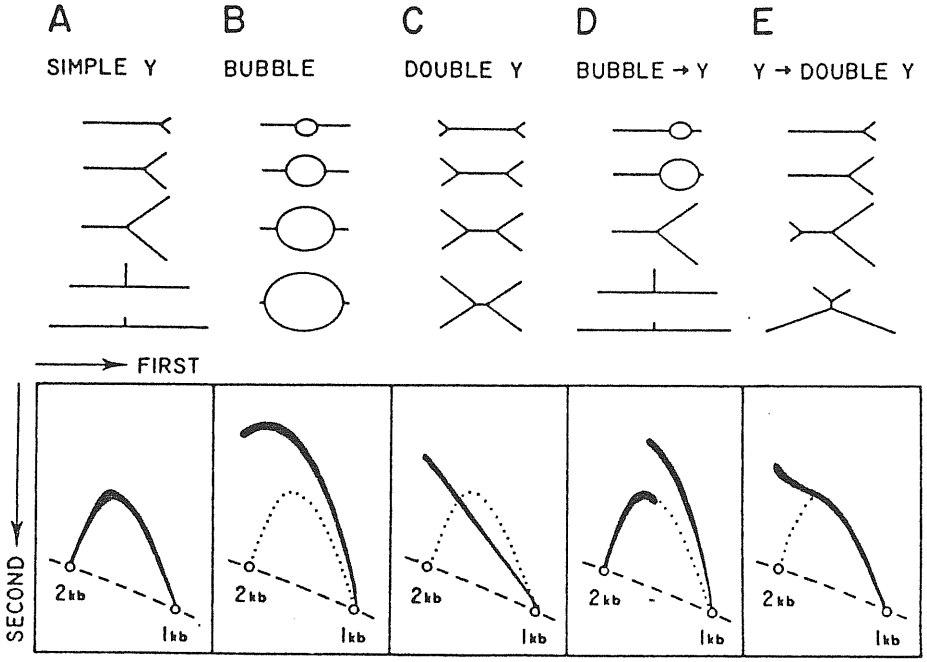
METHODS FOR MAPPING MAMMALIAN DNA REPLICATION ORIGINS:

2D-Gel Electrophoresis

fig.3

Nawotka, K.A. and Huberman, J.A. 1988. *Mol. Cell. Biol.* 8, 1408-1413
Brewer, B.J. and Fangman, W.L. 1987. *Cell* 51, 463-471





within the 1Kb fragment (panel D) then the RIs will begin as double structures but become simple Ys when a fork passes one of the restriction sites, yielding a gel pattern with a discontinuity.

The second method (19) uses two-dimentional neutral-alkaline gel electrophoresis followed by hybridization with short probe sequences and is briefly described in Fig 3 (on the left). A shows a DNA fragment containing a replication origin and two sites for restriction endonuclease (R) and short segments 1,2,3 are cloned sequences uses as hybridization probes. The DNA fragment is assumed to be replicated by fork travelling from left to right. DNA is isolated from cells, cut with restriction enzyme R, enriched for fork-containing molecules by chromatography through BND cellulose (which interact with the single stranded DNA) and electrophoresed through a neutral agarose gel: nonreplicating molecules form a sharp band (vertical bar in D) whereas the replicating molecules from a trailing smear: molecules beginning replication migrate just behind the nonreplicating molecules, while almost fully replicated molecules are the most retarded. The gel lane containing replicating DNA is rotated 90° and run in a second dimension gel soaked in alkaline buffer in order to denature DNA. During second-dimension electrophoresis, the four strands of each replicating molecule separate to produce two lines : a horizontal line composed of parental strands (which are the same site in all replicating molecule) and an arc containing nascent strands of increasing size. The DNA is then

tranferred onto a nylon membrane and hybridized with probes 1,2 and 3. The probe closest to the origin detects all the nascent strands (long and short ones) and the probes farthest away detects only the longest nascent strands.

Okazaky fragment polarity

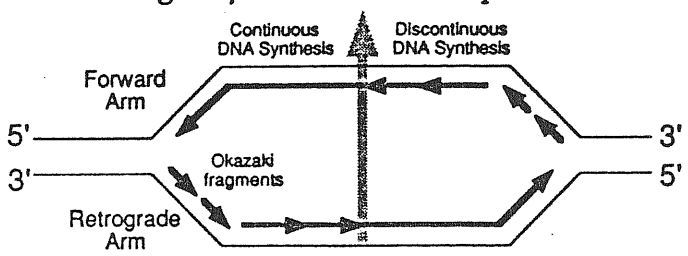
This method developed by Burhans et al. (20) is based on the observation that when DNA replication proceeds bidirectionally from specific sites, the resulting transition from discontinuous to continuous DNA synthesis that occurs on each strand of DNA, defines an origin of bidirectional replication (OBR) (Fig4). Therefore hybridization of Okazaky fragments to cloned strand-specific DNA templates that span the initiation site will reveal the transition between discontinuous and continuous DNA synthesis. Cells are synchronized at the G1/S boundary in order to obtain a large population of replication forks within the initiation zone and after release from the aphidicolin block the replicating DNA is labelled with [32P] dATP and BrUdR. The DNA extracted is then fractionated by gel electrophoresis, and Okazaky-sized fragments (25-300Kb) are recovered by electroelution and selectively precipitated with antiBrUdR antibodies. The Okazaky fragments are then used to probe the two complementary strands that have been cloned into single stranded M13 DNA and immobilized on nitrocellulose membranes (dot-blot), representing selected sequences in and outside of the initiation zone.

METHODS FOR MAPPING MAMMALIAN DNA REPLICATION ORIGINS:

Okazaki fragment polarity

Burhans, W.C. *et al.* 1990. *Cell* 62,955-965

Origin of Bidirectional Replication



Experimental Protocol

1. Synchronize cells at G1/S border
2. Permeabilize cells
3. Label nascent DNA for 1.5 min with [α-^{32}P]dATP & BrdUTP
4. Fractionate DNA by denaturing gel electrophoresis
5. Electroelute Okazaki fragments and high MW DNA
6. Immunoprecipitate nascent DNA with anti-BrdU antibodies
7. Hybridize nascent DNA to cloned ssDNA templates
8. Quantify autoradiographs by densitometry

fig.4

PCR of nascent DNA strands

This PCR-based method for origin mapping has been developed by Vassilev et al. (21) and is schematically presented in Fig5.

For any origin of replication situated near a genomic DNA segment of known sequence, three pairs of oligonucleotides, complementary to both DNA strands encompassing a putative origin, can be synthesized. These will serve as primers for specific amplification of the region between each pair by the polymerase chain reaction. An additional set of oligonucleotides are synthesized as probes for each amplified segment. The neosynthesized DNA labelled with BrUdR and H3-deoxycytidine, obtained from unsynchronized cells, can be denatured and size fractionated by alkaline sucrose gradient centrifugation and then isolated by immunoprecipitation with antiBrUdR antibodies. If initiation takes place at a specific origin then lengthening strands will progressively include the three chosen segments. PCR amplification can then be performed on each separated strand fraction with all the three primer sets. Slot blotting and hybridization then reveals the fraction containing the shortest length of nascent DNA that includes each segment and the patten of hybridization of strand size fractions to the various probe will show the direction of fork progression.

The advantages of this method are that it avoids the use of metabolic inhibitors, does not require synchronized cells and can detect replication through single copy sequences.

METHODS FOR MAPPING MAMMALIAN DNA REPLICATION ORIGINS:

PCR of nascent DNA strands

Vassilev, L. and Johnson, E.M. 1990. *Mol. Cell. Biol.* 10, 4899-4904
Vassilev, L. *et al.* 1990. *Mol. Cell. Biol.* 10, 4685-4689

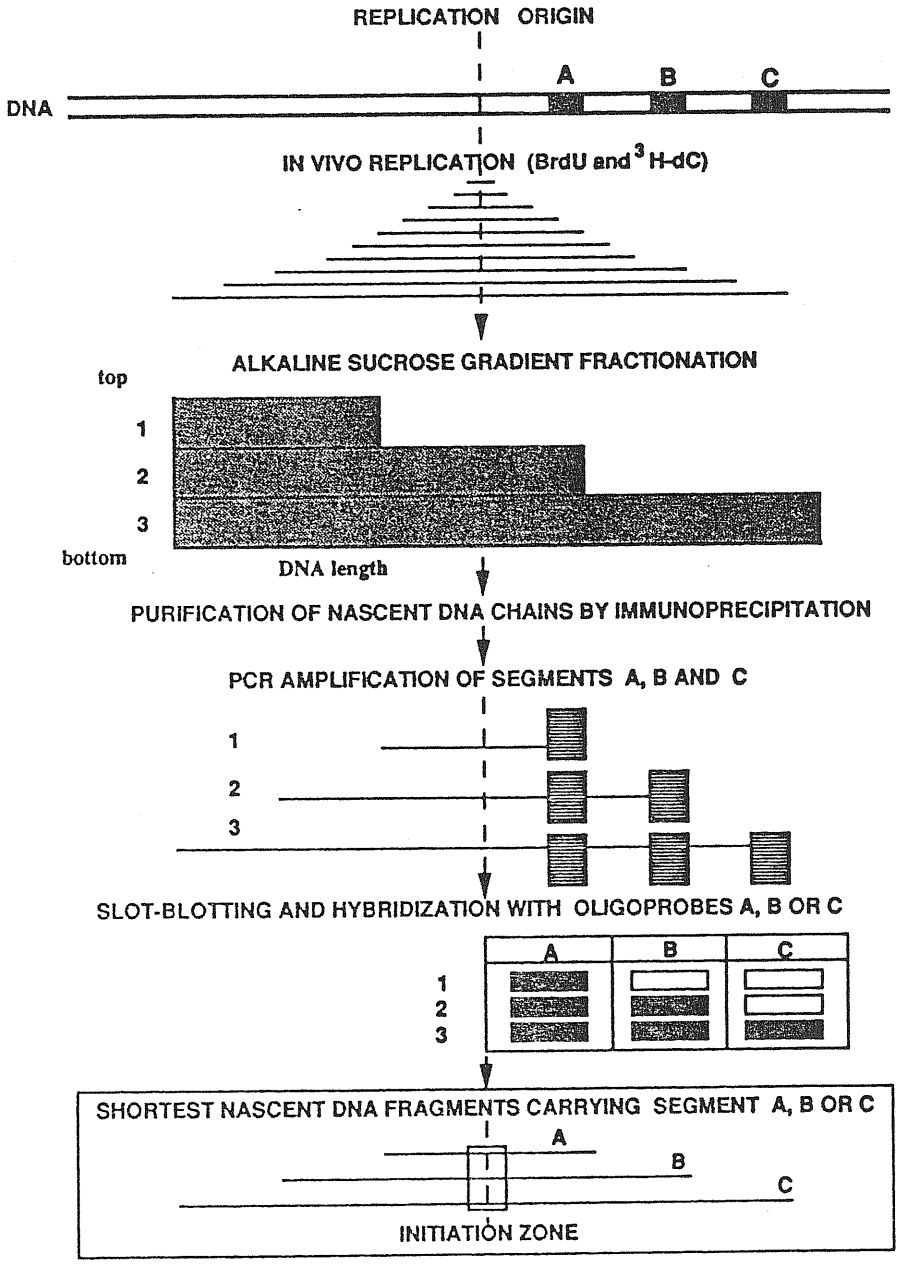


fig. 5

The PCR amplification method was also used to study nascent chain distribution in the 5' flanking region of the c-myc gene in human cells (22). The results of this study suggest that an origin resides roughly 1.5 kb upstream from exon 1, in a zone encompassing fragments reported to replicate autonomously in human cells (12).

1.4 THE DIHYDROFOLATE REDUCTASE LOCUS

Most of these methods have been applied to the DHFR (dihydrofolatoreductase) gene, that at present represents the most studied putative origin of replication, even if it still unclear if it contains one or more sites of initiation or if replication initiates in a broad zone encompassing the entire DHFR domain. Attempts to analyze replication intermediates at a suspected origin using specific hybridization probes on Southern blots are relatively difficult without either amplifying the signal emanating from an origin or employing synchronization methods that substantially increase the percentage of replication intermediates.

One way to amplify the signal from a given initiation site is to study replication intermediates in the amplified domain (amplicons) of drug-resistent cells. When Chinese Hamster Ovary cells are selected for their ability to grow in the presence of a drug methotrexate, lines emerge that have amplified the DHFR gene many times. A line called CHOC 400 contains about 1000 copies of the DHFR gene arranged as tandem repeats within the chromosome(23).

The replication pattern of the amplified locus has been studied in several laboratories by a variety of methodologies. Hamlin et al (24) cloned the entire 240Kb amplicon in a series of overlapping cosmids: in pulse labelling studies on synchronized cultures, they previously showed that initiation in the DHFR amplicon occurs somewhere within a 28Kb region that maps downstream of the DHFR gene. Subsequent studies (25) in which the intrinsic labelling patterns of amplified restriction fragments are analyzed by in gel renaturation suggested that there are actually two preferred zone of initiation within the 28 Kb initiation region, the centers of which are spaced about 22Kb apart (ori Beta and ori Gamma). Supporting evidence for this proposal came from experiments in which synchronized cells were cross-linked with trioxalen just prior to entry into the S period. The DNA synthesized between cross-link on either side of origins was labelled with bromodeoxyuridine (BrUdR), separated on sucrose gradients and precipitated with antibodies anti BrUdR. When this DNA was used as probe on cosmids and plasmids from the DHFR domain, the same two rough zones of early labelling were detected (26).

Hamlin et al (20) later described a third early firing initiation locus in the larger DHFR amplicon of DC3F/A3 cells. The new initiation locus (ori alpha) maps approximately 240 Kb upstream from ori beta.

The mapping technique of "asymmetrical nucleosome segregation" by Cedar has been used also to study the origin of

replication in DHFR locus: the results of this analysis confirmed the locations of the initiation zones (Alpha and Beta) identified previously.

A further confirmation that initiation of DNA replication in DHFR locus occur within a very narrow zone in the region of ori Beta came from two independent studies: Burhans et al. using the method of "Okazaky fragment polarity" (27) and identified an origin at a specific site within a 0.45Kb sequence approximately 17Kb downstream from the 3' end of the DHFR gene; Vassilel at al. (21) using the technique of "PCR of nascent DNA strands", located an origin of bidirectional DNA replication in a region of about 2.5Kb centered approximately in the same region.

In order to examine replication intermediates in the DHFR locus more directly, Hamlin et al.(28) applied the two different two-dimentional electrophoretic mapping methods to an analysis of the DHFR domain in CHO 400 cells. This analysis surprisingly revealed that initiation occurs in vivo at many random sites scattered throughout the entire 28-30Kb initiation locus encompassing ori beta and ori gamma and not just at two single sites. This unexpected result was obtained with both log and synchronized cells(28). The same result was also obtained when the single copy DHFR locus in drug-sensitive CHO cells was examined by the two different two dimentional gel mapping methods(29).

Because it is difficult to quantitate the relative number of initiation occurring per unit length of DNA in these

experiments, it is not yet known whether there are more initiation events occurring over the two peaks of early labelling that were identified in earlier studies and thus it is difficult to know if these data are compatible with the previous ones.

From all these results it is clear that at the moment it is not known whether initiation of nascent DNA chains occurs at fixed or at random sites in vivo. It is possible that any of the methods described above could contain potential artifacts since they are all relatively new and since there is no other known, fixed chromosomal origin from a mammalian source to serve as a positive control.

However it is also possible that both sets of results are valid in the context of the experiments in which they were obtained. For example it may be that in vivo, chromatin domain structure forces a large region of the DNA around an origin to melt upon interaction of the origin with a trans-acting initiation protein. Once the large region is melted, the replication machinery per se may be able to initiate nascent chains anywhere within the large melted region. In the in vitro situation wherein chromatin structure is undoubtedly distinguished, interaction with an initiation protein may result in melting only within a narrow zone around the origin itself, resulting in initiation of nascent strands in a narrow zone.

1.5 THE GENOMIC CLONE pB48 AND A NOVEL APPROACH TO MAP HUMAN DNA REPLICATION ORIGIN

Some years ago in our laboratory some sequences that replicated immediately after the onset of S phase were isolated using the synchronization of human promielocytic HL60 cells(30). Cells were synchronized at the G1/S border by two blocks with aphidicolin. About 200 sequences synthesized immediately after the entry into the S phase were cloned. The newly synthesized DNA showed a significant increase in snap-back DNA and no enrichment in repeated sequences. The two largest clones (pB48 , 1560bp and pLC46, 716bp) were chosen for further analysis. they were sequenced and studied in detail. The longest fragment (pB48) contains many characteristic features showing its possible regulatory role in the cell (Fig.6):

- an element of the human Alu family, present in the last 189 nucleotides
- a 600bp long CpG-rich region with the properties of an HTF island (high frequency of Hpa II sites and CpG dinucleotide)
- three possible thermodynamically stable stem-loop structures with stability greater than -27 Kcal/mol
-three sequences homologous (70-100%) to the central palindrome in the origin of human Papova virus JCV, two regions homologous (83-100%) to the binding site II of the human Papova virus BK T antigen

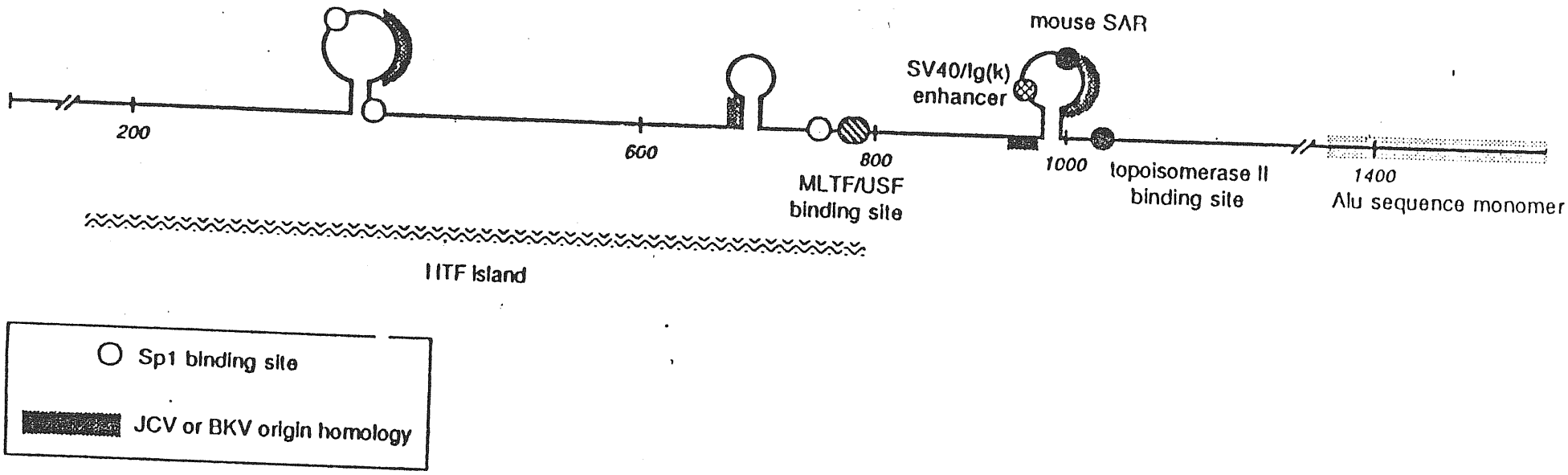

mouse SAR
SV40/Ig(k)
enhancer
200
600
800
1000
1400
MLTF/USF
binding site
topoisomerase II
binding site
Alu sequence monomer
IITF island
Sp1 binding site
JCV or BKV origin homology

fig.6

- some transcriptional signals in the CpG-rich region: three Sp1 binding sites located within a short range, in the stem and loop area; a 9bp sequence homologous to the SV40 enhancer.
-pB48 contains a 17bp sequence that specifically binds to nuclear proteins. This sequence (TTCGTCACGTGATGCGA), which is palindromic (with one mismatch) in the twelve central nucleotides, turned out to be very similar to the upstream element of the Major Late Promoter of Adenovirus 2, which is bound by a human factor (USF-MLTF). We have recently demonstrated (31) that at least three human nuclear proteins specifically recognize this sequence, and that binding motif is a general cis-regulatory element conserved throughout evolution as the target of a number of nuclear factors that share the same DNA binding specificity albeit in the context of different functions. We also showed that this sequence is able to promote transcription when cloned in the correct orientation upstream of a reporter gene.

In order to investigate a larger chromosomal region containing the pB48 fragment, we isolated from the human placenta DNA library a 13.7Kb clone, whose location was subsequently mapped on the short arm of chromosome 19 band 13.3 by in situ hybridization. Hybridization experiments with DNA replicated at different S phase intervals using this region as probe proved that it is indeed completely replicated within the first two minutes of the S phase. A restriction map was defined and several subclones were obtained; eventually most of the sequence was determined. Sequence data analysis

revealed a homology with the cDNA of mouse lamin B2. The transcriptional pattern of the region was studied in detail: at least five different mRNAs were evidenced by Northern blotting experiments.

In order to demonstrate that pB48 genomic region really represents an origin of DNA replication in HL60 cells we performed an experiment in which, by quantification of the DNA neosynthesized in specific regions of the chromosome, it is possible to follow the movement of the replication fork and the distance from the origin. In order to obtain this result we combined two advanced techniques:

-purification of nascent newly-synthesized DNA strands from parental strands, after BrdU incorporation, by immunoaffinity chromatography

-amplification of specific DNA sequence with a new method of quantitative PCR

In the first part of the experiment the single strand DNA neosynthesized after the entry in S phase has been purified from HL60 cells synchronized with aphidicolin. The neoreplicated DNA has been analyzed by two different approaches; the first was a dot-blot assay in which an array of fragments of the genomic region of pB48 was used as probe for hybridization to the human DNA obtained from the previous purification.

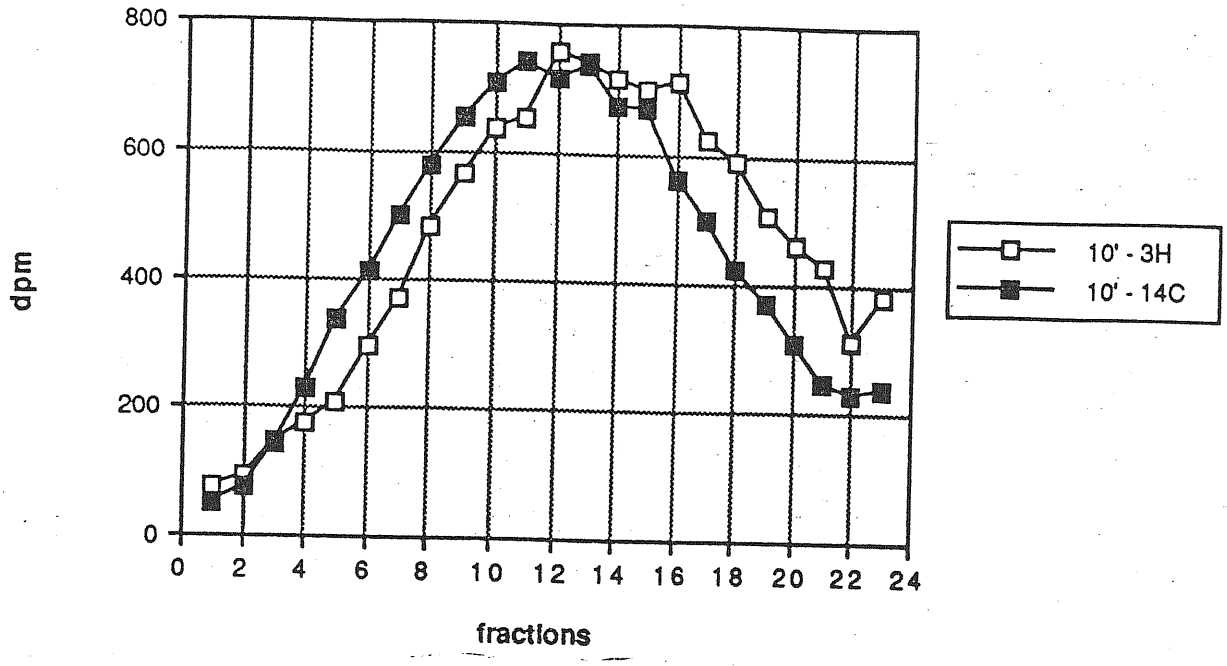
Since the sensitivity of the dot-blot experiment is very low we decided to use the purified DNA also as a template for PCR amplification (using set of primers spaced at discrete intervals) whose sensitivity is several order of magnitude higher than traditional hybridization. We rendered our PCR analysis quantitative by coamplification within the same tube of the template DNA and of a standard DNA (competitor) of known concentration that shares with the template sequence the same primer sites. The preliminary results obtained are presented in "Results".

RESULTS

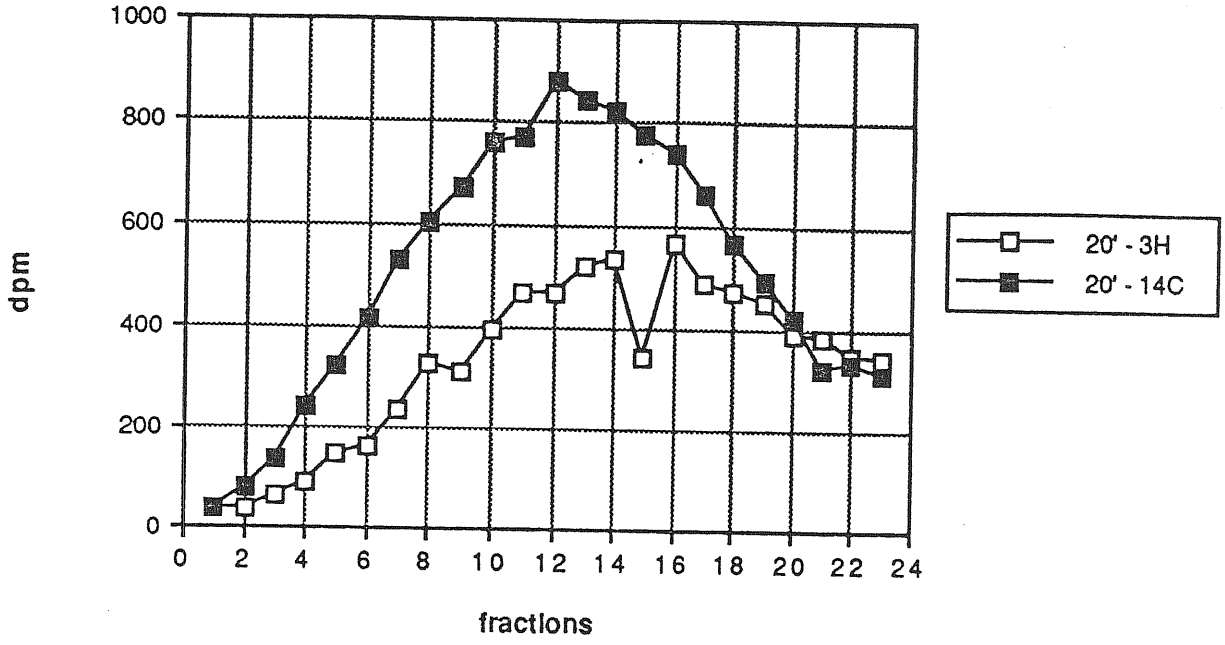
2.1 PURIFICATION OF BrdUrd SUBSTITUTED DNA BY IMMUNOAFFINITY CHROMATOGRAPHY

The DNA neosynthesized at the onset of S phase has been purified from HL60 cells synchronized with aphidicolin (see Materials and Methods). The genomic DNA was extracted, denatured and size fractionated on 5 to 30% neutral sucrose gradients. In Fig.(7) are presented three gradients obtained respectively at 10,20,30min. after release from aphidicolin. The high quantity of ^{14}C can be explained with the very high percentage of dead cells after treatment with aphidicolin. These fractions where then purified from the parental DNA by a immunoaffinity column obtained with mouse anti BrUdR monoclonal antibody.

The purification of BrdUrd-substituted DNA is a step common to many experimental approaches to the study of nascent DNA chains. Commercially available anti-BrdUrd monoclonal antibodies have been recently used for purification of newly synthesized DNA strands by immunoprecipitation . It has been reported that this method allows the recovery of about 85% of the BrdUrd-substituted DNA with a 200-fold enrichment with respect to total parental DNA . Other authors, however, lost up to 80% of the BrdUrd labelled DNA by immunoprecipitation and achieved only a 20 to 40-fold purification .



Gradient 20 min.



Gradient 30 min.

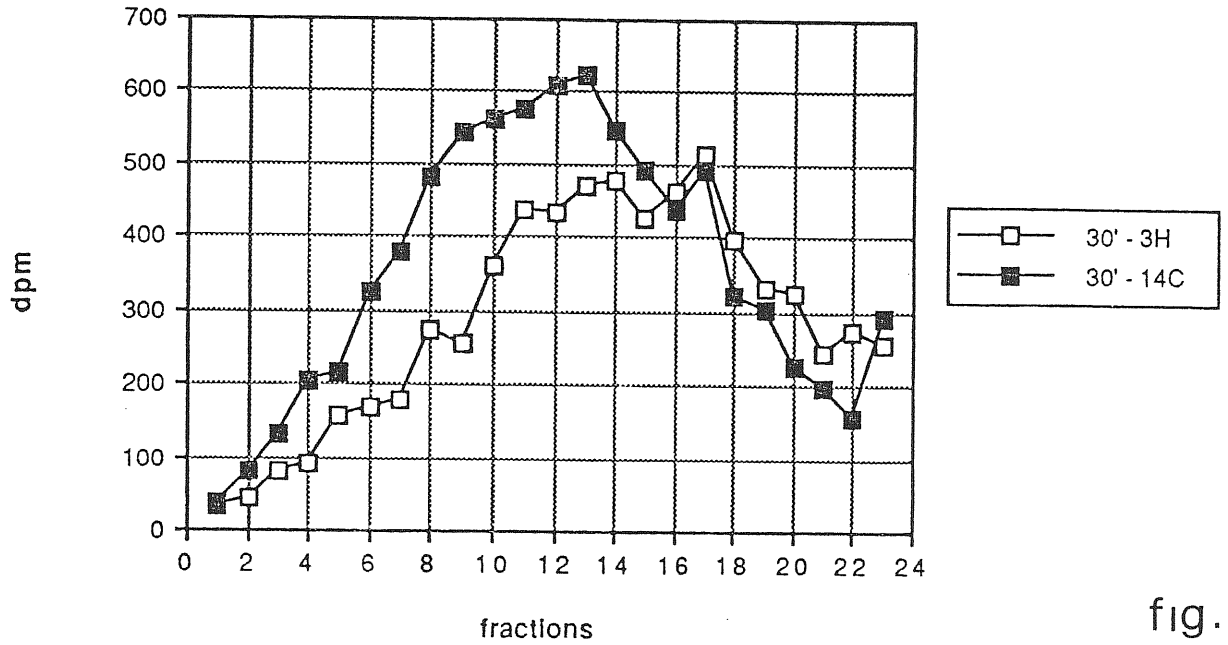


fig.7

Results from different experiments, as shown in table 1, demonstrates that purification of BrdUrd DNA by immunoaffinity chromatography is quantitative and specific since the recovery of BrdUrd DNA in the eluate is greater than 90% of the total BrdUrd DNA loaded and virtually no parental DNA is present in the purified sample.

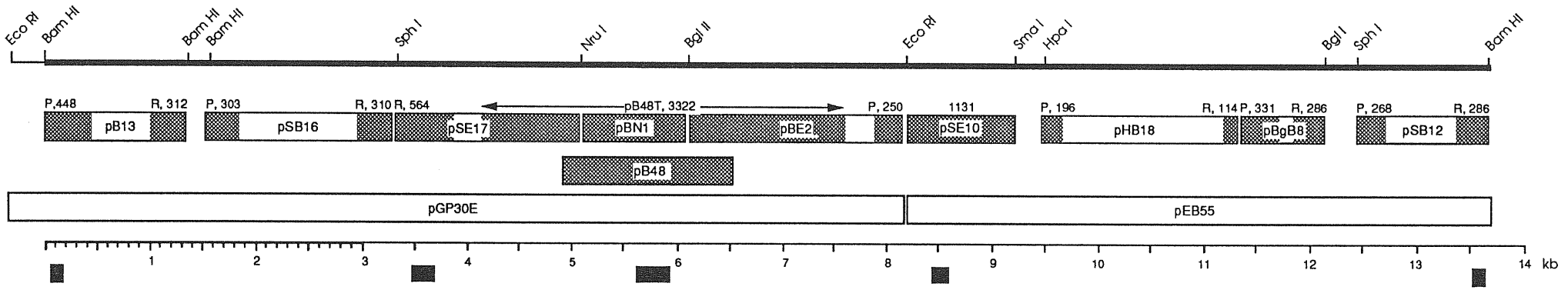
2.2 DOT-BLOT HYBRIDIZATION

In an experiment of purification of BrUdR substituted DNA from synchronized HL60 cells, the fractions obtained by sucrose gradients and chromatography have been pooled in 5 different aliquots indicated in Fig 9 as P2,P3,P4,P5 and P6 with respectively increasing average size. These aliquots have been hybridized in a Dot-Blot assay with four probes (SB16, BN1, SE10 and BgB8) that span the genomic clone pB48. (see Fig. 8) The aim of this experiment is to find an enrichment of nascent DNA in proximity of initiation of replication.

In the Dot-Blot assay we have hybridized as a control (for standardization of efficiency of hybridization) different amounts of HL60 genomic DNA, lambda DNA and each plasmids used as probe for hybridization The experiment is shown in the picture in Fig.9 and the details of the experiment are presented in the table below.

The only sure information that can be obtained by this experiment is that, if the intensity of hybridization of the HL60 genomic DNA dilutions and BrUdR purified DNA are compared, the five pools are all enriched in pB48 with respect

fig. 8



primer	location	sequence
B13SX	54-73	5'-CCTCAGAACCCAGCTGTGGA-3'
B13DX	219-209	5'-GCCAGCTGGGTGGTGATAGA-3'

PCR product: 166 bp

PCR profile: 94°C 1' — 55°C 1' — 72°C 1'

primer	location	sequence
B48SX	883-902	5'-TAGCTACACTAGCCAGTGAC-3'
B48DX	1108-988	5'-AGATGCATGCCTAGCGTGTT-3'

PCR product: 226 bp

PCR profile: 94°C 1' — 52°C 1' — 72°C 1'

primer	location	sequence
SE10SX	238-257	5'-GTTCTGGCAGTTCGCTTAGA-3'
SE10DX	377-358	5'-AGGCTGCTACGTGATGTGAA-3'

PCR product: 140 bp

PCR profile: 94°C 1' — 52°C 1' — 72°C 1'

primer	location	sequence
SB12SX	2156-177	5'-CACACCTTGGCCTGGTAGGTCT-3
SB12DX	265-246	5'-GAGTACGACTTCAAGATGGC-3'

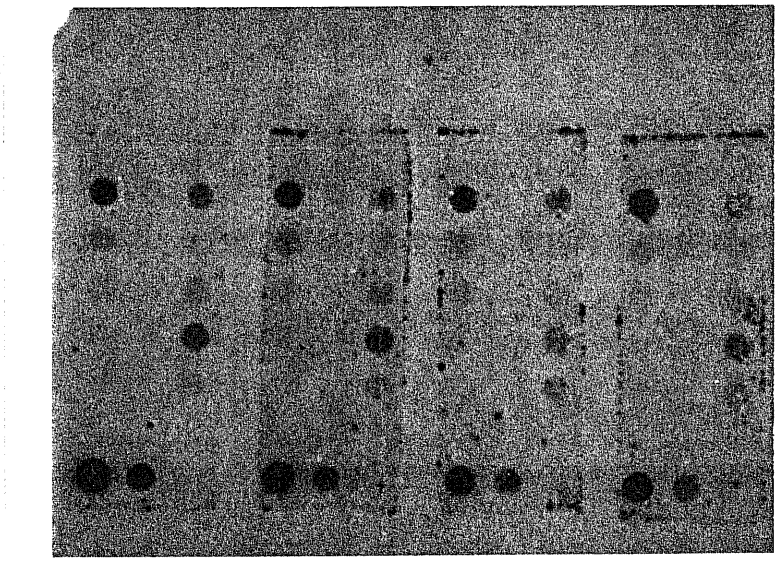
PCR product: 110 bp

PCR profile: 94°C 1' — 60°C 1' — 72°C 1'

primer	location	sequence
SE17SX	188-207	5'-AATTTCCACTCCACAGCCGT-3'
SE17DX	395-376	5'TAGGAGTGACCCTAGTGACT-3'

PCR product: 208 bp

PCR profile: 94°C 1' — 52°C 1' — 72°C 1'



SB16			BN1			SE10			BgB8		
1000	1000	P2	1000	1000	P2	1000	1000	P2	1000	1000	P2
100	100	P3	100	100	P3	100	100	P3	100	100	P3
50	50	P4	50	50	P4	50	50	P4	50	50	P4
25	25	P5	25	25	P5	25	25	P5	25	25	P5
12.5	12.5	P6	12.5	12.5	P6	12.5	12.5	P6	12.5	12.5	P6
6	6		6	6		6	6		6	6	
2	0.5		2	0.5		2	0.5		2	0.5	

HL60 DNA [ng]

Lambda DNA [ng]

^{3}HdCyt - BrUdR DNA

Probes [pg]

fig.9

to total DNA. Furthermore there is an enrichment of specific DNA in pool5 for SB16 and BN1 with respect to SE10 and BgB8. The result is not clear and sufficient probably because the quantity of DNA used was too low and therefore is not still possible to draw a conclusion.

Since for a good Dot-Blot assay a higher quantity of DNA must be tranferred on the membrane and since the BrUdR substituted DNA isolated after the gradients and the immunoaffinity column is necessarily very low, we decided to perform also an experiment of quantitatve PCR in order to detect very little amount of template DNA.

2.3 QUANTITATIVE PCR

Since its introduction the Polymerase Chain Reaction has rapidly become the method of choice for the detection of nucleic acids present in very little amounts in biological samples. The sensitivity of the technique is several orders of magnitude higher than traditional techniques (such as nucleic acids hybridization), and, in the best conditions, it practically corresponds to the limit of the presence of a single molecule. A major problem with PCR, however, is its problematic reproducibility, which is commonly experienced by most of the groups utilizing the technique for very different porpoises. Some of the reasons for this variability can be abolished by careful standardization: carefully controlled reagents and Taq polymerase (including batch), standardization of the procedures for obtaining the sample to be amplified, use of

reliable thermal cyclers (including, for example, the careful control of the internal temperature for each single well). Even in the most controlled experimental conditions, however, it is common to experience some variability, especially in product yield, from experiment to experiment and even from tube to tube within the same experiment. Since the final product derives from exponential amplification of the starting template, minor differences in amplification efficiencies will result in large differences in the overall product yield, especially if the amount of initial template is low. However, since PCR is just the method of choice when the amount of the template is low , a reliable method for quantitation is absolutely required.

Several authors have observed a linear relationship between input template and amplification product within the exponential range of amplification This range, however, is strictly dependent on the abundance of the starting material and is heavily influenced by the presence of spurious proteins and nonionic detergents (in samples prepared by rapid cell lysis), differences in sample preparation, machine performance, reaction conditions, and inhibitors of PCR.

For all these reasons, although semiquantitative data can be obtained readily with dilution curves, quantitative analysis is cumbersome and difficult.

An approach to overcome these tube-to-tube variations has been the coamplification within the same tube of a reference template.

The principle of the technique is that any variable influencing amplification should affect both the reference and the template under study similarly, if the reaction is maintained into its exponential phase. For this reason, in order to obtain a linear response to template concentrations, the primers, nucleotides, and polymerase must remain in large excess. Nevertheless, it is a commonly experienced problem that the nature of the amplified sequences and of the primers have a largely unpredictable influence on the efficiency of amplification. All these reasons indicate that it is impossible directly to compare the relative abundance of different targets without previously empirically determine all the amplification parameters for each primer set.

The most reliable approach to quantitative PCR so far described relies on the coamplification of reference sequences which share with the target sequence the same primer sites, so that the two sequences compete for the same primer set (competitive PCR). The two amplified products can be recognized because of their different lengths, or for the presence of a mutation in the competitor which create a novel restriction site or can be resolved by TGGE.

Provided that the sizes of template and competitor are not very different (since the efficiency of amplification is inversely proportional to the size of the amplified fragment) this is the technique of choice for quantitation by PCR because both the targets share the same priming sites and so are equally influenced by any variable affecting amplification.

Furthermore, the ratio between template and standard remains constant during extensive PCR cycling, and amplification can be performed over many cycles so that PCR products can be visualized and quantitated by ethidium bromide staining.
Usually the target DNA is coamplified with a dilution series of competitor DNA of known concentration. The relative amounts of target DNA versus competitor can be measured and since the starting concentration of the competitive template is known, the initial concentration of the target DNA can be determined.

Since natural competitor sequences are not often available, however, the major problem suffered by competitive PCR, is the construction of competitors, which can be often a tedious and long work of mutagenesis and cloning. We used a new method for the construction of such competitive templates, that allow their quantitation and use without any need for cloning.

2.4 CONSTRUCTION AND QUANTITATION OF COMPETITOR

We constructed a competitor sequence that differs from the target sequence for 20 bp located inside the amplified fragment. In this way the two fragments can be easily resolved in a 8% polyacrilamide gel. We obtained the competitor by a "recombinant PCR" procedure.Two PCR products that overlap in sequence are produced by two separate reactions.These products contain the 20 bp addition (the overlapping region) as part of one of the two primer used for amplification. These overlapping primary products are then recovered from

polyacrilamide gel by touching with a tip of a needle the corresponding band stained in ethidium bromide, then denatured and annealed together, producing two possible heteroduplex products. The heteroduplex that has recessed 3'ends is then filled by Taq DNA polymerase to produce a fragment that is the sum of the two overlapping products. A subsequent reamplification of this fragment with only the right and left primers ("out-side" primers) results in the enrichment of the full length secondary products (fig.10). In this way fragments containing the insertions can be obtained only by using PCR.

The competitor has been subsequently quantified directly by evaluating the amount of incorporated [32P] dCTP in a PCR cycle.(see Matherials and Methods).

In order to assess if the quantitation of the competitors was reliable and to test the applicability of the system, we performed an experiment of coamplification of genomic DNA (obtained from HL60 cells) with five different competitors encompassing the region of genomic pB48 (about 10Kb) . Fig. 8 shows a schematic restriction map of pB48 and the plasmids in which it has been subcloned. The five regions which have been amplified are those limited by the five pairs of primers indicated and the position in pB48 is indicated by black boxes. Three different dilutions of each competitor containing respectively 60000 (dilution "E") 6000 (dilution"F") and 600 (dilution"G") molecules in 5 µl were coamplified with 10ng of genomic DNA (corresponding to 3000 copies of a single copy

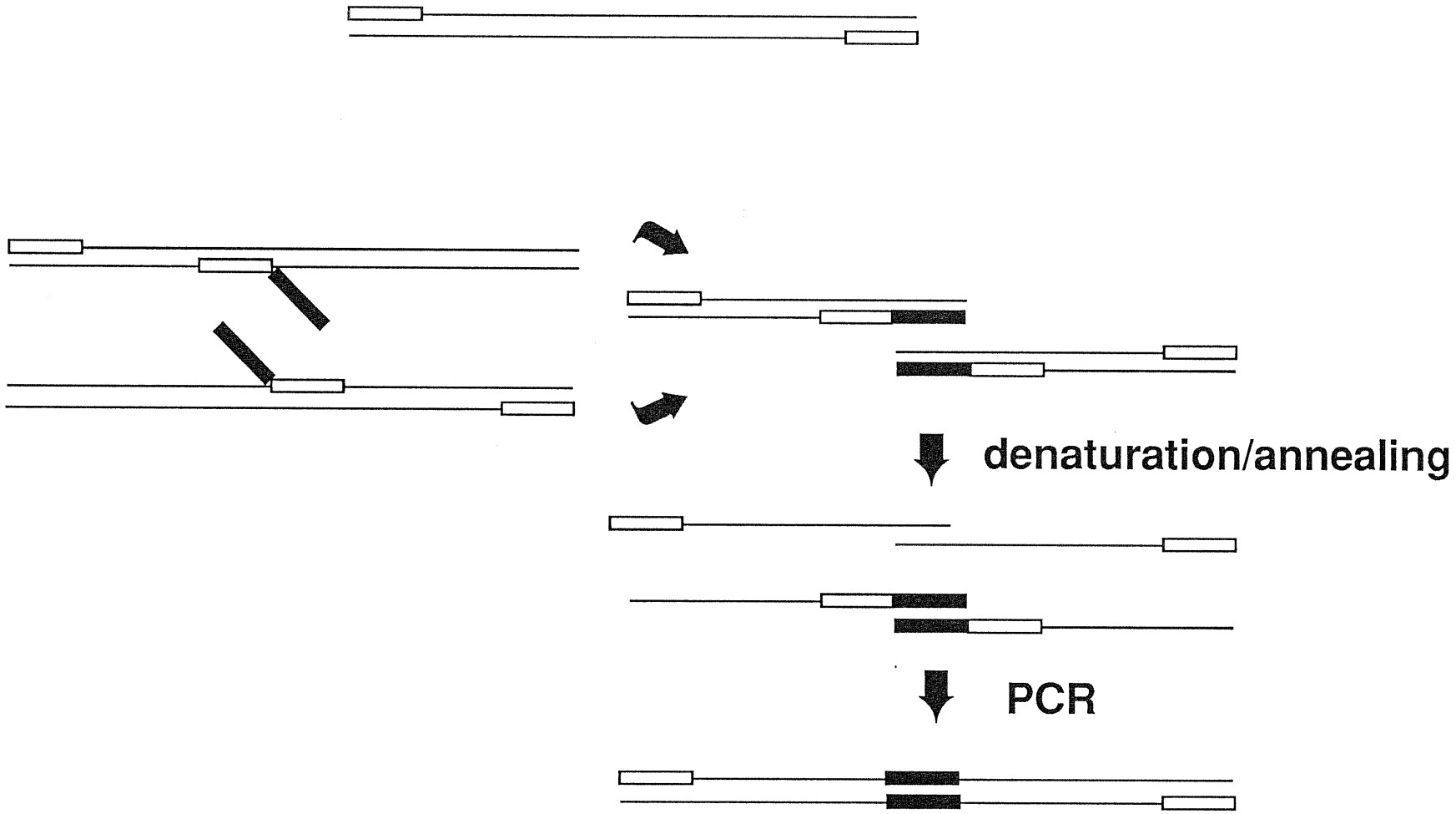


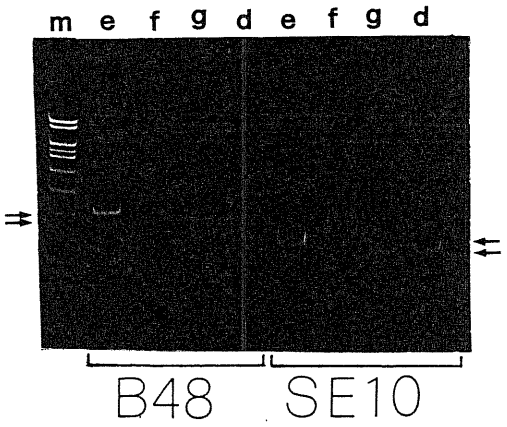
fig.10

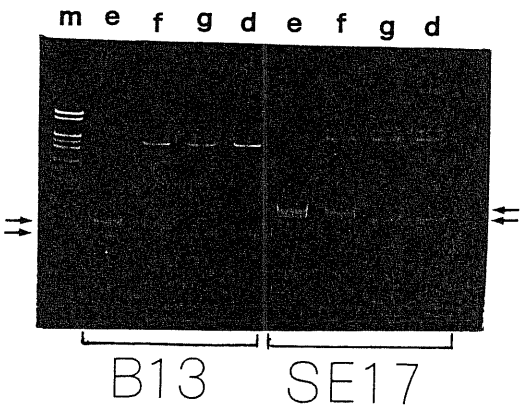
gene) with a PCR profile as shown in Fig 8. As a control the genomic DNA has been amplified also without any competitor.

The results are shown in the pictures in Fig 11 : for each competitor can be noticed that there is a nearly equivalence in lanes F (6000 molecules of competitor and 3000 of genomic DNA) while in lane E there is an excess of competitor (60000 molecules of competitor and 3000 of genomic DNA) and in lane G there is an excess of genomic DNA (600 molecules of competitor and 3000 of genomic DNA). Lanes D represent the amplification of genomic DNA alone and lanes M are molecular weight markers.

This experiment shows that the quantification of competitors calculated directly by evaluating the amount of incorporated [32P]dCTP in a PCR amplification was exact (at least of 1 order of magnitude) and that this technique can can be easily used to quantify a template DNA of unknown concentration.

We used this method to amplify the BrUdR substituted DNA together with the five competitors, to quantify the nascent DNA in different regions on pB48 in order to follow the movement of the replication fork. At the moment we are analyzing some fractions of BrUdR-DNA obtained at different time after release from the aphidicolin block . The experiments are still not complete, but the preliminary results seem to confirm those obtained from the Dot-Blot described in 2.2. A conclusive result will come only after analysis of





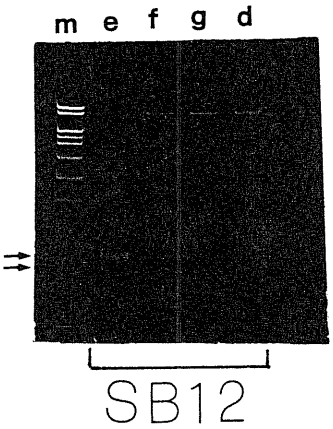


fig.11

several gradients obtained at different times from synchronized and non-synchronized cells.

MATERIALS AND METHODS

3.1 CELLS, LABELING AND ISOLATION OF DNA

HL60 human promielocitic cells were cultured in RPMI 1640 medium (Gibco) containing 50μmoli/ml gentamicin, 10% fetal calf serum and 5mM glutamine.

HL60 cells were labelled for 3 days with 0.2 μM ^{14}C-thymidine (52 mCi/mmol, Amersham, UK), synchronized with aphidicolin (Sigma Chemical Co., USA) as previously described (32) and then labelled for 10-30 minutes, in presence of 1μg/ml of aphidicolin, either with 100 μM ^{3}H-BrdUrd (2.5Ci/mmol, Amersham) or with 1 μM ^{3}H-deoxycytidine (21.5Ci/mmol, Amersham) in presence of 100 μM cold BrdUrd (Boehringer Mannheim GmbH, FRG). All the subsequent steps were performed in the dark or with an orange safety light to prevent photodamage to BrdUrd-substituted DNA. Genomic DNA was extracted, alkali-denatured in 0.5 M NaOH for 10 min in ice, dialyzed against TE buffer ph 8 for 3 hours at 4°C for neutralization, and size fractionated on 5 to 30% neutral sucrose gradients as previously described (32). The size of the gradients varied according to the amount of DNA to be loaded (7-8 μg DNA per ml of gradient). Aliquots of each fraction were used for counting radioactivity. Pools of 4-6 fractions were dialysed against 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 mM EDTA (TBSE) and concentrated down to an appropriate

volume (1.5 to 4 ml) with polyethylenglycol 6000 (Merck, FRG), if necessary.

The immunoaffinity column was prepared as follows. Goat anti-mouse IgG (Zymed Laboratories Inc., USA) was coupled to CNBr activated Sepharose 4B (Pharmacia LKB Biotechnology, Sweden) according to the manufacturer's protocol (2.5 mg protein/ml gel). 0.5 ml of the coupled Sepharose was poured into a Poly-Prep 2 ml disposable chromatography column (Bio-Rad, USA) and washed with >30 ml of TBSE. The column was then closed with the bottom stopper leaving 0.5 ml of buffer above the gel surface. Mouse anti-BrdUrd monoclonal antibody (mAb, Becton Dickinson, USA) was added at a final concentration of 3 µg/ml, the column was capped with the top stopper and incubated for 2 hours at room temperature with slow agitation. The immunoaffinity column was then washed with 10 ml of TBSE and the excess buffer was allowed to drain out. Different amounts of DNA (0.1-10 µg in 1.5-4 ml TBSE), containing ^{14}C-thymidine labelled strands (3.47 x 10^3 d.p.m./µg) and ^{3}H-BrdUrd labelled strands (3.3 x 10^6 d.p.m./µg) were denatured for 2 minutes in boiling water, chilled on ice and added to the column. After 2 hours incubation at room temperature with slow agitation, unbound DNA was allowed to drain out and one aliquot was assayed for radioactivity (^{3}H and ^{14}C). The Sepharose was washed with 4 ml of TBSE and bound ^{3}H-BrdUrd DNA-anti-BrdUrd mAb complexes were eluted with 2 ml of 150 mM NaCl adjusted to pH 11.5 with NH_4OH. The eluate was

neutralized and treated with proteinase K (200 µg/ml) at 37° C overnight. One aliquot of eluate was assayed for radioactivity (^{3}H and ^{14}C). DNA was extracted once with phenol:chloroform:isoamylic alcohol (25:24:1) and once with chloroform:isoamylic alcohol (24:1) and precipitated with ethanol. To completely wash out the residual DNA, the coupled Sepharose was washed with 5 gel volumes of 150 mM NaCl adjusted to pH 11.5 with NH_4OH and 5 gel volumes of 100 mM glycine/HCl pH 2.5, followed by re-equilibration with 30-40ml of TBSE. The column was stored at 4°C after addition of 100 µl of 1% thimerosal (0.05% final, Sigma), leaving 1.5 ml of buffer above the gel surface. The same goat anti-mouse IgG Sepharose could be used for purification at least 25-30 times without any loss of activity.

3.2 OLIGONUCLEOTIDES SYNTHESIS AND PCR AMPLIFICATION

Oligonucleotides primers used for amplification of BrUdR-substituted DNA were chemically synthesized with an Applied Biosystem 380B DNA synthesizer apparatus with reagents from the same company. The exact position and sequence of oligonucleotides and the PCR cycle profile is indicated in Fig 8. Polymerase chain reactions were performed in a final volume of 100µl containing : DNA of interest as template (picograms amounts), 10mM Tris HCl pH8.3, 50 mM KCl; 1.5mM MgCl2; 0.2mM each of dATP,dCTP, cGTP and TTP; 1µµ of each oligonucleotides primer and 2.5 units of Taq DNA Polymerase (Perkin Elmer Cetus). Amplification was carried

out in a Perkin Elmer Cetus DNA Thermal Cycler. The DNA is then extracted once with chloroform (in order to eliminate the mineral oil) and one-fifth of the amplified DNA is resolved in a 8% polyacrilamide gel and stained with Ethidium Bromide.

3.3 CONSTRUCTION OF COMPETITOR FOR QUANTITATIVE PCR

The competitor for the quantitative PCR has been obtained by two subsequent amplification. For the first amplification two primers complementary to two sequences internal to the target template amplified by "outside" primers used for the second amplification (which are the same used for amplification of BrUdR substituted DNA) are synthesized. They anneal to contiguous sequences and each contain in 5' end 20 nucleotides complementary to the 5' end of the other.

In the first amplification two partial fragments are obtained by amplification with standard PCR conditions with one outside and one internal primer: the two partial products obtained will contain an overlapping region of 20bp. These primary products are eluted from a polyacrilamide gel by touching with the tip of a needle the corresponding band stained with Ethidium Bromide. The tip was soaked in 50 µl of distilled water and 5µl of each elution was used for subsequent amplification in standard PCR conditions with only the outside primers. In order to allow the formation of the heteroduplex products the reaction is first denatured at 94° for 1 min., then taken at 50° in 10 min., then kept at 50° for 2 min., and then at 72° for 5 min. Then the reaction is amplified

using a PCR cycle profile as follows: the first 5' cycles: 94° for 1 min., 37° for 30 sec. and 72° for 30 sec.; cycle 6 to 10: 94° for 1 min., 42° for 30 sec., 72° for 30 sec.; cycle 11 to 30: 94° for 1 min., 55° for 30 sec and 72° for 30 sec. A subsequent reamplification of the secondary products will allow enrichment of the full length products and quantification of it.

3.4 Quantitation of competitor

Quantitation of competitors was directly obtained by evaluating the amount of incorporated 32PdCTP in a PCR cycle as follows. A small amount of competitor DNA was obtained from an acrylamide gel by touching with the tip of a needle the corresponding band stained by ethiudium bromide. The tip was immersed in 100 µl of distilled water and 1 µl was used as template for amplification. The amplification mixture (50 µl) contained the standard amount of cold dCTP (10 nmoles, as described above) plus 0.2 µl (0.66 pmoles) of P32-labeled dCTP (Amersham, U.K., 3000 Ci/mmol, 10 µCi/ml), corresponding to 6.2x106 cpm, as experimentally evaluated by Cerenkov counting in a beta-counter.

The amplification products were resolved on a polyacrilamide gel and the radioactive band eluted in 100 µl of water. 5 µl were counted and the concentration of competitor was evaluated in accordance to the final specific activity of dCTP (cold plus radioactive) and the number of nucleotides incorporated.

3.5 BLOTTING AND HYBRIDIZATION

DNA was blotted onto Schlicher & Schuell Nytran Membranes using a Dot-Blot apparatus. The four plasmids used as probes were labelled with [32P] dCTP (3000Ci/mmol), Amersham) by the Random Primed DNA Labelling kit (Boeringher 1004760) at 37° for 45 min. Labelled DNA was purified from unincorporated label by a passage through Sephadex G50 columns (Quick spin columns, Boeringher). For hybridization with labelled DNA probes membranes were preincubated overnight in prehybridization solution (6X SSC, 5X Denhart's reagent, 0.5% SDS, 250 µg/ml yeast t-RNA) in the 68° oven with gentle agitation.

They were hybridized overnight in the same solution at 68° with labeled probe (10^6 cpm/ml) and washed twice for 1hr at 65° with 2X SSC, 1% SDS.

DISCUSSION

Studies on DNA replication and its regulation in eukaryotic cells have been limited in part by the lack of useful genetic approaches , but also by the feeling that the mechanism of DNA replication in eukaryotic cells will merely reflect that of bacteria and phages. In fact mapping of replication origins in E.coli and prokaryotic and eukaryotic viruses to a few hundreds of nucleotides or less suggested that eukaryotic chromosomal origins might correspond to short stretches of defined nucleotide sequence. Indeed, chromosomal replication origins in the yeast S.cerevisiae correspond to specific DNA segments of a few hundreds nucleotides or less. However the identification of replication origins in higher eukaryotes has proven to be more difficult than in yeast, mostly because of the complexity of the higher eukaryotic genome.

At the moment, the most studied putative origin of replication is the DHFR locus, which has been analyzed by every technique described up to now. These different approaches lead to different results and did not clarify if there are only two (or three) sites of initiation or if replication initiates in a broad zone. These contradictory conclusions can perhaps be accommodated by a replication model proposed by Hubermann (34) in which melting of large stretches of the helix occurs prior to the actual initiation of nascent strands.

Clearly new approaches will have to be devised to look at mammalian origins in different ways, with a broad-minded attitude that recognizes that mammalian origins may be more complex than those of viruses, yeast or bacteria. However many improvements have been made in helping to understand the initiation of replication and many techniques seem to be promising for future progress.

We have cloned a 13Kb DNA fragment that is synthesized at the onset of S-phase in HL60 Synchronized cells. This genomic region is actively transcribed, contains several potential transcription regulatory elements and therefore it is a good candidate for containing an origin of replication. In order to follow the movement of the replication fork along this region, we have used an improved PCR mapping technique (quantitative PCR) on newly synthesized BrUdR-DNA.

Although all the techniques available at the moment can be exhaustive in explaining initiation of DNA replication, we think that the method we developed has a great sensitivity, can give reproducible results and allows a very precise mapping of origins along chromosomal DNA.

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