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Clonal selection of autonomously replicating sequences from human HL60 cells *in vitro*

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CLONAL SELECTION OF AUTONOMOUSLY REPLICATING SEQUENCES FROM HUMAN HL60 CELLS IN VITRO

BY

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A dissertation submitted to the Graduate Faculty in Biomedical sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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ABSTRACT

CLONAL SELECTION OF AUTONOMOUSLY REPLICATING SEQUENCES FROM HUMAN HL60 CELL IN VITRO

by

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Each eukaryotic chromosome consists of linear double stranded DNA molecules that are bound to many nuclear proteins to form complex aggregates that then can be fit into distinct chromosomes. Although the lengths may vary in different eukaryotes, a controlled and efficient replication of the genome occurs once in every cell division and is essential to the survival of the animals. To replicate all of their genomes during the 10 hour synthesis phase of the cell cycle, the cells are thought to unwind 10^4 initiation sites and form bubbles where DNA synthesis starts. In spite of this number

of replication origins, the mechanisms and sequences involved in initiation of eukaryotic replication are not known, perhaps due to the large size of the genome, the complexity of DNA with nuclear proteins and the unavailability of appropriate assays.

This is a project to replicate in vitro the SV40 origin-containing plasmids using cellular extracts prepared from permissive (monkey COS7, CV1) and semi-permissive (human HL60) cell lines. I have analyzed the modes and intermediates of replication by two dimensional gel electrophoresis and by electron microscopy. Furthermore, I have used the replication efficient HL60 extracts to select and characterize cloned human (HL60) DNA fragments which can initiate replication as was tested by the above system. These replicating clones have then been used for in vivo transfection studies. Availability of these plasmids will enhance studies of human DNA replicons and the factors controlling over the initiation processes.

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INTRODUCTION

Each eukaryotic chromosome consists of a linear double stranded DNA molecule of variable lengths.These strands are wound around each other and further bound to many nuclear proteins to form complex aggregates that can then be packed into distinct metaphase chromosomes, in which the DNA is compacted 5000-10000 fold. This chromatin is a combination of basic proteins, the histones, and one long DNA molecule that is wrapped around histones H2A, H2B, H3 and H4. H1 is present between neighboring nucleosomes and its primary structure varies greatly between species in contrast to the other histones(119a, 99a).

Although overall DNA content may vary in different eukaryotes, a controlled and efficient replication of the genome occurs once in every S phase of cell division and is essential to the survival of all animals. To replicate all of its genome during the 10 hour synthesis phase of the cell cycle, a mammalian cell is thought to initiate replication at about 3×10^{4} replicons (83, 99, 119).

Most DNA molecules replicate bi-directionally and in a semi-conservative fashion. The mechanism involves the unwinding of the two parental strands by DNA helicases and duplication of these into two daughter hybrids by DNA polymerases.

Complex machinery including many proteins is responsible not only for replicating but also for segregating the daughter molecules and compacting them into chromatin , and, with the correct superhelicity, into chromatids (113a).

Most organisms' DNAs replicate as circular structures.Topoisomerases remove or produce tension in these molecules, allowing them to unwind for replication or to segregate the newly replicated molecules, respectively.Type I topoisomerases are thought to open up the double helix around the initiation site and allow a complex of few enzymes to get in , open up the two strands and start synthesizing short RNA primers which then will be elongated by DNA polymerases using free deoxynucleotides(151, 170).

All known DNA polymerases synthesize DNA in a 5'-3' direction. This unidirectional synthesis allows continuous DNA growth only on the leading strand and discontinuous segmented DNA synthesis on the lagging strand. A second function of the

DNA polymerase, a 3'-5' exonuclease activity ,removes the short RNA primers and fills in the gaps. The enzyme, DNA ligase, connects all fragments together(99, 119).

To duplicate all of its genome within few hours, mammalian cells are thought to contain many initiation sites. However,in spite of this number, the mechanisms and sequences involved in initiation of replication in higher eukaryotes are not known, perhaps due to the large size of the genome, the complexity of the proteins involved and unavailability of appropriate assays.

Prokaryotes and eukaryotes have DNAs with similar chemical properties, and even though eukaryotes have developed highly regulated and complex processes involving many proteins to replicate their large genomes, animal viruses present excellent models for studies of chromosomal replication since these viruses replicate mostly by the cell's replication machinery(86, 99).

Small animal viruses(such as SV40 and polyoma) present simple replication models. They form DNA-protein complexes in the nuclei with the hosts' histones and non histone proteins, and replicate using mostly the hosts' replication machinery. Since they contain one specific initiation origin, they

provide us with small chromatin molecules capable of autonomous replication in the permissive cells (86, 190, 191).

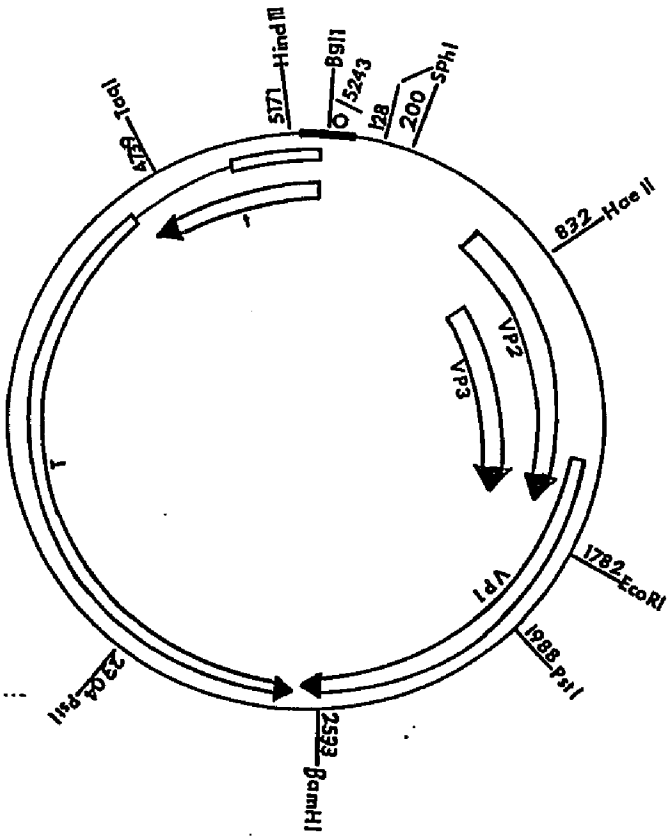
Furthermore, with the availability of in vitro replication systems using permissive cellular extracts, these viruses have revealed many functions of the viral and cellular proteins involved in the initiation, elongation and termination of viral DNA synthesis(10, 131).

Replication of SV40 DNA

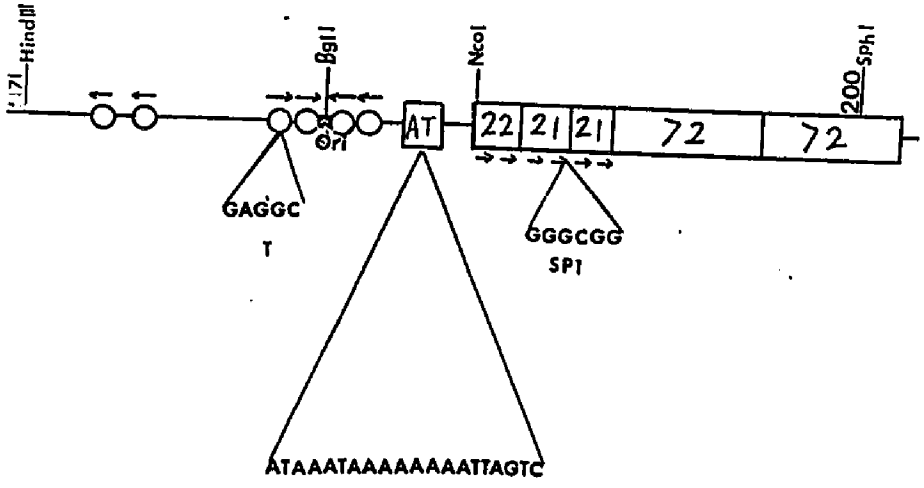
Papovaviruses represent simple replication models because of their small genomes and the use of one specific replication initiation site. They represent useful models for studying eukaryotic DNA synthesis since they interact with many of the cellular proteins(57, 86).

Simian virus 40(figure 1) is a 5.2 kb papovavirus that invades monkey cells (permissive), human cells (semi-permissive) and rodent cells (non- permissive). Its minichromosomes are supercoiled by 20-25 densely packed cellular nucleosomes. It replicates as double stranded circular chromatin molecule in the permissive host's nuclei. Its infection cycle (for permissive and semi-permissive cells) is divided into early and late phases. In the early phase two proteins, small and large T-antigens, are produced which stimulate host DNA synthesis and initiate viral DNA replication. In the late phase three structural proteins (coat)are produced which package the mature virions, which in turn kill the host. If, however, the early phase is disrupted (e.g. due to mutations at the origin), the viruses which have intact early genes may integrate into the host genome, constitutively express these genes, and keep the host in

Figure 1. A) The SV40 DNA map. B) The SV40 origin. Circles: T antigen binding sites.



SV40 – 5.3 kb



SV40 Origin

constant cell division and lead to transformation. Most cells are non permissive for SV40 infection which means, due to incompatibility of these cells' replication proteins with the viral sequences and proteins, no virus replication occurs, no late gene products are synthesized and no virions are produced. These cells either degrade the virus or dilute it out by cell division. Again in rare cases transformation occurs(57, 86).

The monkey kidney cell line COS family (figure 2) have SV40 origin- mutated molecules integrated into the genome. They express the early genes constitutively. These cells represent good hosts for studies of DNA replication of Templates containing the SV40 origin in vivo and in vitro (142, 176).

Large T antigen is a multi-functional phosphoprotein that regulates many of the viral replication processes by binding to four different regions at nucleotides GAGGC at the SV40 origin (166). Its helicase activity can unwind DNA substrates, and thus may initiate the viral replication (49). Furthermore, T antigen forms complexes with the cellular polymerase alpha-primase proteins and is thought to lead this aggregate to its binding sites at the origin and initiate the leading

Figure 2. A) Monkey kidney, COS 7, cells is a derivative of CV1 cells that has origin deleted SV40 sequences , transcribing T antigen, integrated into its genome.

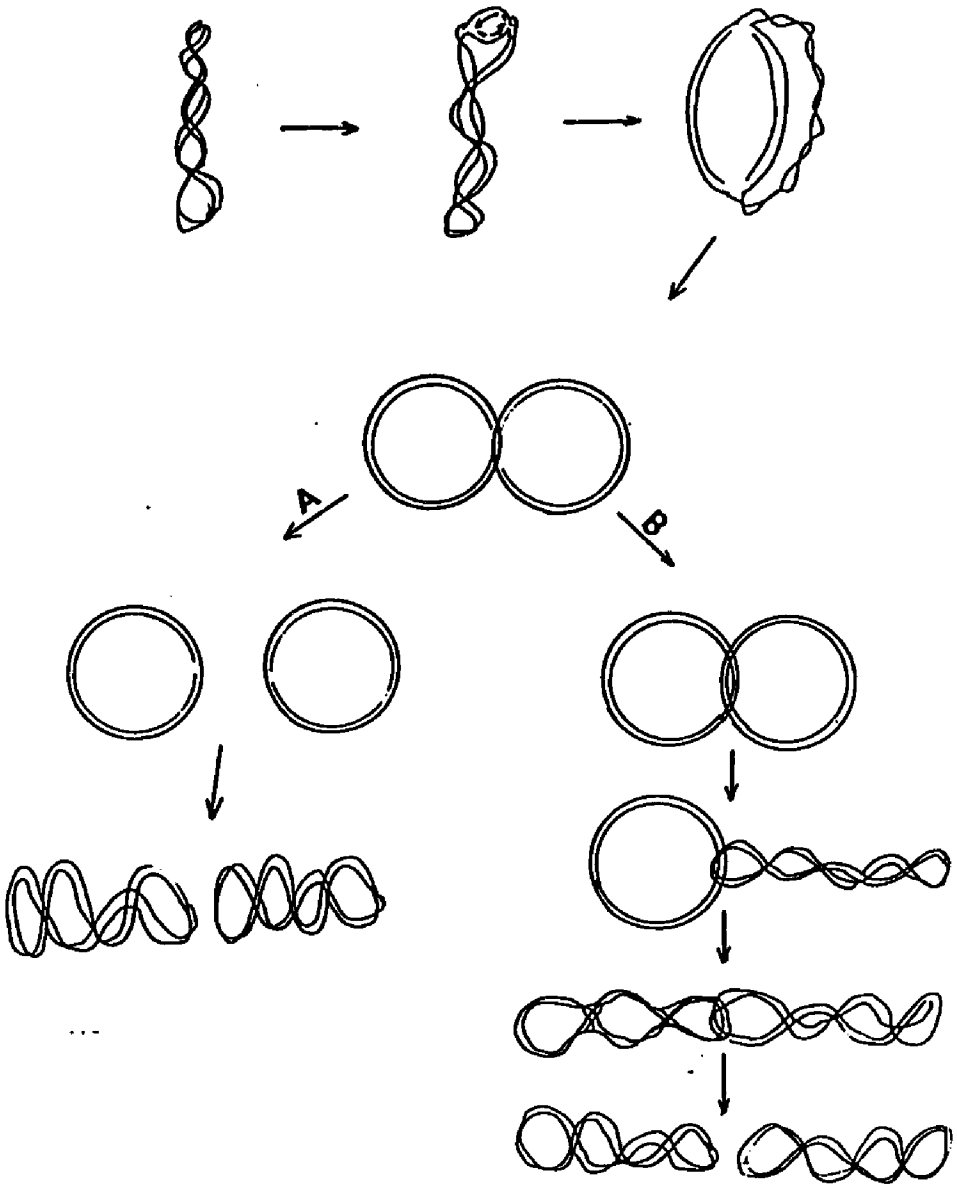
con 7

strand synthesis. Monoclonal antibodies to T antigen prevent this interaction with the polymerase alpha and inhibit the viral replication (172).

Sundin and Varshavsky in 1980 revealed in vivo replication intermediates of SV40 virus (190). They showed series of catenated dimers intermediary to the final segregated hybrids (figure 3). These intermediates had been thought to be the result of bi-directional fork movement going through theta-like structures. Recent observations suggested another pathway that is preferred under normal conditions. They suggested that the SV40 DNA replication termination proceeds via late replicative intermediates to gapped daughter molecules; i.e. before the completion of replication, the remaining sections of the parental DNAs become unwound and release two monomeric circles with gaps of up to 50 bases that are filled rapidly by DNA synthesis (57, 204).

A third pathway indicates that the termination of SV40 DNA replication proceeds through hemicatenated structures containing two SV40 DNA molecules that are interlocked in the termination region via a single-stranded bridge. These may indicate a role for topoisomerase I in segregation of SV40 DNA replication products, since this enzyme makes single strand

Figure 3. Termination of SV40 replication.


A
B

breaks(175).

Analysis of in vitro SV40 DNA replication yielded consistency in replication initiation, elongation and termination mechanisms similar to in vivo SV40 DNA replication.

Li and Kelly in 1984 dissected the in vitro replication of SV40 origin containing plasmids in cellular extracts, confirming the results published by Ariga and co-workers(10, 131). They showed directly the initiation of replication by the help of viral T antigen at the SV40 origin.Before this , SV40 replication was performed in isolated nuclei or in SV40 infected cell lines. Li and Kelly in 1984 observed an authentic bi-directional replication that followed the in vivo replication products closely (131). The products of in vitro replication contained different superhelical density ranging from most supercoiled, form I to relaxed (nicked) circles, form II, as well as replication intermediates observed by Sundin and Varshavsky (191).However, the topoisomerases in the extracts seem to act on templates lacking the SV40 origin as well and producing similar topoisomeric DNA bands.

The sources of cellular replication proteins include monkey and human cell lines. These include multi-subunit DNA

polymerases, DNA primase, helicase, topoisomerases, DNA binding proteins and ligases.They can be extracted by low ionic buffers from crude homogenates,by high salt extraction of isolated nuclei, or by ultracentrifuge fractionation of the crude cellular extracts (8, 132, 209). These extracts are crucial for all steps of DNA synthesis, since non- permissive mouse cells have no replication activity (131,209). However,this specificity seems to arise from interactions between the viral T antigen and the cellular replicase during initiation or fork movement phases of replication. Other proteins involved in the replication process seem to be non-specific and interchangeable (172).

Large T antigen is the only viral gene product (though multi-functional) needed for in vitro initiation at SV40 origin sequences using cellular extracts from permissive cell lines(10,131). Large amounts of this protein became available from cells infected with SV40 or with modified adenovirus vectors containing the early genes (131, 184,196,197,209). Purification of T antigen by immunoaffinity chromatography contributed tremendously to this in vitro replication assay (169a).

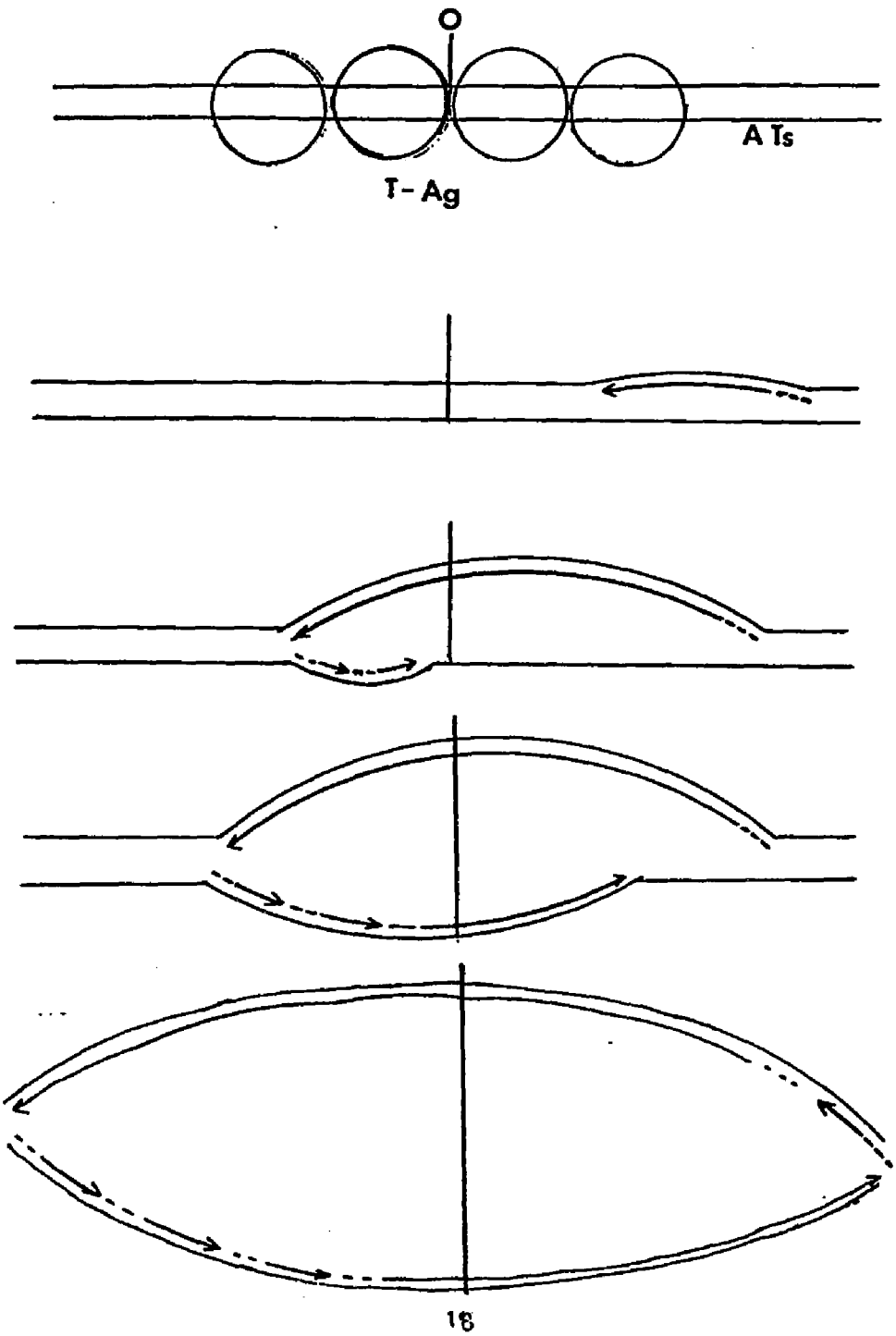
Wobbe and co-workers in 1986 dissected the initiation

process into two steps(210). First, a pre-elongation phase that is RNase A sensitive and initiates in the absence of dNTPs in the first 20 minutes of incubations.During this period, T antigen-replicase complexes are thought to interact with the T antigen binding sites near the SV40 origin sequences, unwind the double helix at the AT rich regions. The primase subunit of the replicase, then, copies the DNA template, making RNA primers of 6-9 nucleotides in the direction of early mRNA.

Second, in the elongation phase, DNA polymerases initiate DNA synthesis continuously on the leading strands and in segments on the lagging strands in the 5′ to 3′ direction. The primase is responsible for RNA synthesis of the Okazaki fragments as well as the origin. This is in agreement with Albert's "trombone model" and Kornberg's "replisome hypothesis" which, describe a replicase machinery at the moving forks where, two coupled polymerases elongate DNA replication. One polymerizing on the leading strands and the other, attached with a primase activity, elongating the lagging strands of the replication fork (86, 119, 170, 210).

Recent observations have suggested the involvement of PCNA(proliferating cell nuclear antigen)in SV40 replication

Figure 4. SV40 initiation of DNA replication.


O
A Ts
T - Ag

in vitro (154, 155). In the absence of PCNA, replication forks continue bi-directionally but only the Okazaki fragments are synthesized. The dependence of leading strand synthesis on PCNA (and therefore, polymerase delta), complements the above hypotheses that two different polymerases may be involved at the moving replication forks; one required for synthesis on the leading strands that does not contain a primase function but needs a proofreading, exonuclease 3' to 5', activity and a second polymerase synthesizing DNA on the lagging strands with a primase function needed for priming of Okazaki fragments. Polymerase delta and polymerase alpha support such functions, respectively (69, 82, 85, 119, 152, 154, 155, 170).

Aphidicolin, an inhibitor of polymerase alpha (and delta) which suppresses SV40 replication in vivo, has similar effects in the replication activities of the cellular extracts. It competes with dCTP incorporation into newly synthesized DNA strands and inhibits the elongation process by the polymerases. However , it has no effects on the initiation process by T antigen-replicase complex, leading to accumulation of early replication intermediates that contain a few hundred residues of unwound single stranded parental DNA (52).

SV40 DNA replicates as a circular structure containing host nucleosomes. Unwinding of the replication forks and segregation of the final products require single and double strand breaks in the SV40 genome. These are accomplished by cellular topoisomerases I and II. Camptothecin, an inhibitor of topoisomerase I, allows the enzyme to make a single strand break in the template DNA and inhibits the resealing process. Sogo and co-workers have used the inhibitor to locate topoisomerase I positions around the replication forks of the SV40 DNA,indicating topoisomerase- I processing at the growing forks (12). VM26 an inhibitor of topoisomerase II ,which produces double strand breaks , has no effects on chain elongation at the replication forks of SV40 DNA however, it inhibits maturation and segregation of daughter molecules indicating its function at the termination of SV40 replication (161). High salt concentrations also inhibit topoisomerase II, since in cells grown in hypertonic media, SV40 replication products consist of multiply interlocked catenated dimers (190, 191).This in vitro replication assay, using crude extracts, replicated template plasmids with a high fidelity when compared to assays that use purified polymerases alpha or beta. The error frequency of mammalian chromosomal DNA

replication-9 is about 10 and is controlled by the DNA polymerases during 5'-3' incorporation of complementary deoxynucleotides, by 3'-5' exonucleolytic editing of misinserted bases and, by post-replicative mismatch repair (69, 82, 85, 152).

Recent observations have shown that polymerase delta, once thought to function in DNA repair, may play a role in eukaryotic DNA replication as well as SV40 DNA replication in vitro(154). It contains a 3' to 5' exonuclease activity needed for proof-reading as well as an auxiliary protein that facilitates processive eukaryotic DNA replication. It lacks an associated primase activity that is needed for the lagging strand synthesis.It is more sensitive to DNA replication inhibitor aphidicolin than is polymerase alpha , while it is more active on poly dA/oligo dT templates. Its auxiliary component, PCNA (Proliferating Cell Nuclear Antigen or cyclin), is a 36 kd protein that does not bind to DNA strands but enhances and stabilizes the binding of polymerase delta to the templates. Interestingly this protein also enhances processive DNA synthesis by polymerase III in yeast cells, increasing the fidelity of replication (69, 82, 85, 152).

Replication of SV40 viral DNA in permissive cells

primarily involves bi-directional fork progression. However, rolling circles have been observed among isolated replicative intermediates(18,19). Rolling circle replication is thought to initiate from a nick in a DNA molecule perhaps at a specific site , as in the origin of phage OX174, and elongate unidirectional , displacing one of the parental strands. The leading strand is then elongated by DNA synthesis at one end of the active parental strand and is covalently linked to it; while the lagging strand is copied off the same strand. The other parental strand always stays as a circle and is used as a template for the leading strand. Without the need for re-initiation, these sigma structures can elongate their tails , generating long tandemly repeated polymers. If these are cut at specific sites, they release regular monomers of viral molecules.

When linear plasmids containing the SV40 origin are transfected into monkey cells that produce large T antigen, needed for the replication of SV40, DNA molecules forming long tandemly repeated segments are observed that are thought to be generated by a replication-related process (53,60).These intermediates form long smears on agarose gels and may indicate variable lengths of head to tail repeats of the

transfected plasmids. When these are digested by a linearizing restriction endonuclease, they migrate as , a monomer length band, indicating rolling circle replication. Similarly, when Herpes Simplex Virus 1 and 2-infected , non- SV40 replicating, rabbit skin cells are transfected with monomer SV40 molecules, head to tail concatemers of replicated SV40 molecules are observed (47).

When the DNA replication initiation inhibitor 2 deoxy-2' azidocytidine is added to cells transfected with polyoma origin containing plasmids, a high frequency of rolling circles is observed. Rolling circle replication does not need to re-initiate every time the replication complex passes through the origin and thus,their replication is not suppressed by the initiation inhibitor. Bi-directional replication however, needs re-initiation for every round of DNA synthesis, which results in the observed high frequency of rolling circles in the isolated replicative intermediates (18, 19).

Rolling circles have also been observed in the in vitro replication assays reported by Hurwitz and co-workers and Li and Kelly in long incubations (132,209). They maybe caused by inhibition of re-initiation or depletion of the initiation

protein (T antigen) at the time. They are generally thought to arise from breakage of one of the replication forks and continuation of replication by a unidirectional mechanism. Recent work using the topoisomerase I inhibitor Camptothecin, which breaks at one strand near the replication forks, showed enhanced production of sigma molecules (12).However,Sogo et al. never observed molecules with tails longer than monomer size DNA and ruled out any unidirectional DNA replication (12). The in vitro replication of SV40 DNA has allowed comprehensive studies of viral and cellular replication proteins.

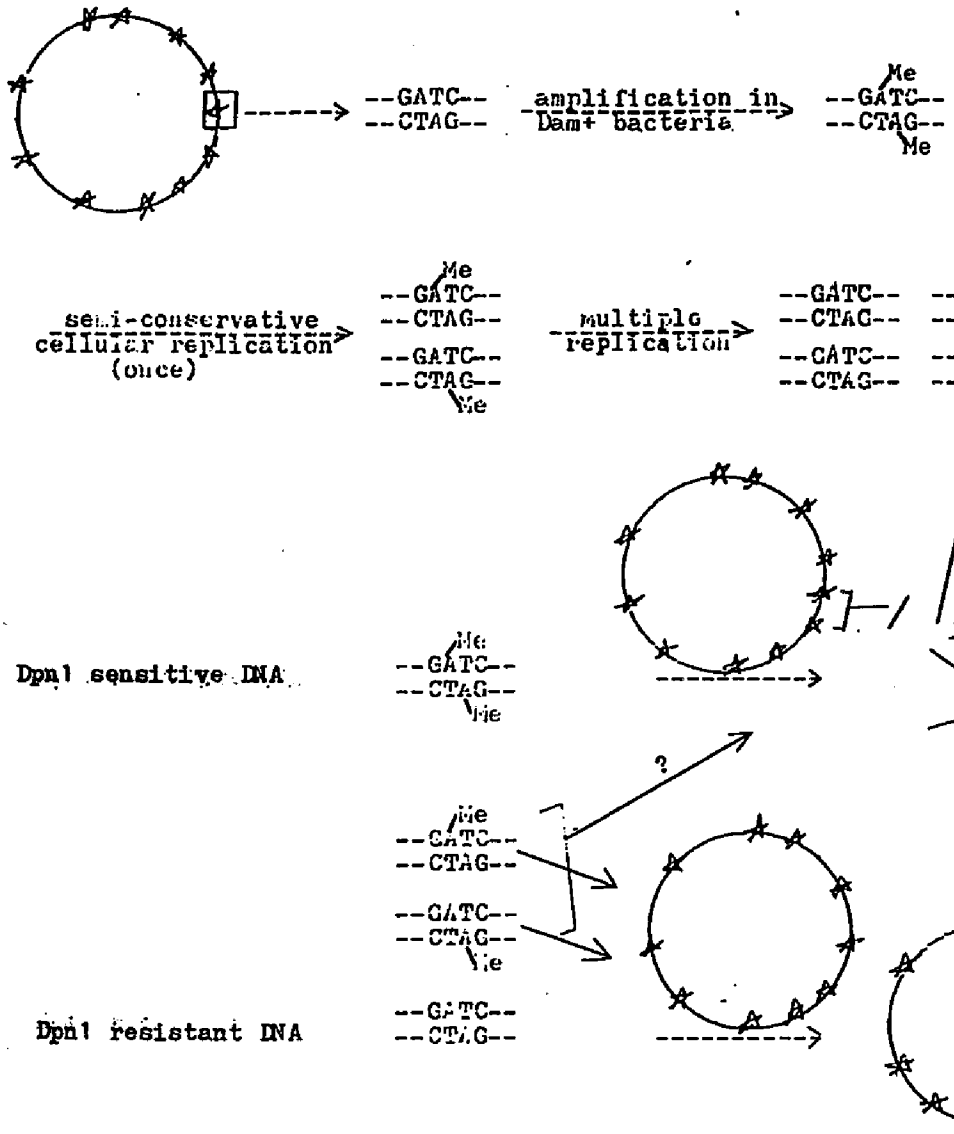
Another important approach towards identification of plasmid replication products is the use of bacterial methylation enzymes and endonucleases which cut either methylated or unmethylated sequences at specific locations. The restriction endonuclease DpnI cleaves the residues GATC only when the adenosines are methylated at both strands. This sequence is also the site for the E.Coli Dam methylase. When plasmids are propagated in a Dam+ strain of bacteria, both strands become methylated at that sequence and thus, these plasmids become sensitive to DpnI digestion(123). However, after one round of semi- conservative replication in

eukaryotic cells, the new daughter strands become unmethylated while the parental strands remain methylated, resulting in a hemi-methylated DNA molecule which is resistant to DpnI digestion. More than one round of semi-conservative replication results in unmethylated DNA molecules which are again resistant to enzymatic cleavage. The DpnI cleavage sites (GATC) are dispersed many times around the templates (pBR322 contains more than 20 DpnI sites in its genome). Thus unreplicated molecules are fragmented into small pieces by DpnI digestion and are easily distinguished from replicated templates that remain intact (figure 5).

DpnI resistance only applies to fully replicated molecules. However, newly initiated or partially replicated molecules are still digested at their unreplicated ends. To analyze these intermediates, two dimensional gel electrophoresis and electron microscopy are employed.

I have examined, in the first part of this thesis, products, intermediates and mechanisms of the in vitro replication of SV40. I have shown that plasmids containing the SV40 origin are capable of efficiently replicating through bi-directional fork movement as is observed by others as well as replication by unidirectional fork movement(10, 49, 131,

Figure 5. DpnI segregation between replicated vs. not replicated DNA templates. The adenosine residue in the sequence GATC is methylated upon amplification in Dam+ bacteria but is not methylated when the DNA replicates in eukaryotic cells. GATC is also the restriction site for DpnI.


--GATC--
--CTAG--
amplification in Dam+ bacteria
Me
--GATC--
--CTAG--
Me
semi-conservative cellular replication (once)
multiple replication
Dpn1 sensitive DNA
Dpn1 resistant DNA
?

132, 209). Furthermore, I show a novel class of molecules where two circular plasmids are connected by a linear double stranded bridge. These may be formed by a replication dependent strand transfer.

In the second part of this project, I have used the efficiently- replicating extracts from the above assays to select DNA fragments out of human genomic libraries that best initiate autonomous replication in vitro. This system will help identify sequences of eukaryotic origins of replication as well as cis-acting regulatory elements and will further identify the nuclear proteins involved in this consistently regulated complex network of eukaryotic DNA synthesis.

Eukaryotic DNA replication

A faithful and precise replication of the parental DNA is needed for the transfer of information to progeny. However, very little is known about this complicated process. The enzymatic process is carried out by a complex machinery of many proteins, which have mostly been studied through their interactions with viral sequences participating in viral DNA replication.

However, the sequences used during eukaryotic replication initiation are obscure and have been difficult to isolate. Several studies indicate that distinct DNA regions follow a sequential order of activation during the S phase and this order is maintained in successive generations (94).

Others have found sequence-directed bent DNA structures near the origins of replication of animal viruses, yeast ARS sequences and bacteriophages. These contain a series of adenine residues that play a role in binding of replication initiator proteins to the origin sequences (94, 98).

So far progress has been made successfully in DNA replication only in prokaryotes, animal viruses and lower eukaryotes. Autonomously replicating extrachromosomal DNAs have been discovered that initiate replication at specific

regions of the genome, such as rDNA in Physarum polycephalum (46a) and Tetrahymena thermophilia(36) and the 2 micron plasmids of yeasts (150, 151, 177). In vitro replication assays for small viruses have further shed light on eukaryotic replication machinery ; however, not on the eukaryotic sequences involved in the replication process.

In yeast cells considerable progress has been made in discovering autonomously replicating sequences (ARS) which can replicate extrachromosomal DNAs autonomously (37, 38, 64, 65, 145, 221). Their number (400) correlates with the estimated number of origins in yeast cells.

ARS sequences have also been isolated from higher eukaryotes that can replicate autonomously in yeast cells. However, their authenticity as origins of replication in their original organisms is not definite (11, 186, 188, 216, 220).

Others have searched for sequences that are similar to the origin of eukaryotic viruses which mostly use the replication machinery of their hosts. Simian virus 40 is a well studied candidate. Its origin shares homology with the widely dispersed Alu sequences. These are present about 2-5 x 10^5 times in the genome of many multicellular eukaryotes and are good candidates for chromosomal origins of DNA replication

(7, 106).

However, it has been difficult to confirm that Alu sequences can selectively initiate replication in a mammalian system, either in vitro or in vivo. Alu sequences are reportedly enriched five-fold over genomic levels in DNA segments isolated from zones of replication initiation in xeroderma pigmentosum cells (3). Alu sequences are also reportedly enriched among segments extruded from chromosomal DNA replication bubbles by branch migration (220). In neither of these studies has any functional relationship to initiation been established.

Upon transfection of plasmid BLUR8 (Bam Linked Ubiquitous Repeat) into COS7 cells , bi-directional replication initiating within the Alu insert was detected. In this case levels of completed circular DNA replication were low relative to those of plasmids bearing an SV40 origin of replication. However, constraints on initiation may be a regulatory feature of natural origins which affect studies of replication of plasmid clones. Deletion of a 15 bp segment of the BLUR8 Alu insert , including a potential T antigen binding site, prevented any replication, suggesting that T antigen or some cellular analog may influence initiation at Alu sequences

(50, 108).

Alu sequences have recently been reported to participate in recombination in human chromosomes. Sequences of both the high and low density lipoprotein receptor genes are rearranged in tissues with junction point occurring in Alu sequences. Mechanisms through which Alu sequences may recombine are not known but could involve DNA replication. A cellular Alu sequence has been observed to integrate into SV40 viral DNA in monkey kidney cells with junction points occurring in both segments near potential initiating sequences (58). The SV40 origin of replication has recently been observed to be particularly susceptible to recombination in vitro.

Alu sequences are transcribed in cells by RNA polymerase III. The putative promoter for transcription lies within the 15 bp sequence required for plasmid replication(108).The function of Alu sequence transcripts is not known but has been suggested to be used as RNA primers for DNA synthesis.

Binding of T antigen to the Alu insert of BLUR8 has been reported(7). Human cells however, do not normally make T antigen; thus, cellular sequences binding the protein may respond to one or more cellular proteins fulfilling analogous functions.

Studies on the initiation of DNA replication within Alu sequences in vitro have been controversial. Ariga reported T antigen dependent initiation of plasmid BLUR8 and semi-conservative DNA replication(7).No DpnI resistance studies were performed. In contrast Li and Kelly (132) reported virtually insignificant generation of DpnI resistant replication products using constructs with the BLUR8 insert in a different plasmid. Here we address this controversy by examining replication of BLUR8 in a system based on monkey COS 7 cell extracts supplemented with purified T antigen (132).

Ariga and co-workers recently isolated and cloned ,in segments , a human lung cancer gene(C-K-ras) into pBR322. Two clones hybridized extensively to SV40 origin sequences and further,these two clones were shown to replicate autonomously in vivo and in vitro (9).

In order to identify cellular analogs of SV40 large T antigen , the multifunctional protein (including initiation of SV40 DNA replication), the same researchers, have been able to replicate transfected SV40 origin- containing plasmids in HL60 and Raji cell lines (101). These cells express the oncogene, c-myc, at high levels which is indicated to enhance the replication of the SV40 sequences in vivo. The replication

was 1/100 of that in monkey COS1 cells (in COS1 cells, copy number= $5x\ 10^4$ - $1x\ 10^5$) and was inhibited by co-transfected C-myc antibody (102).

Abnormal expression of C-myc has been observed in various tumors. It has amino acid sequence homology to adenovirus E1A protein and can substitute for the SV40 T antigen in SV40 DNA replication. It binds to pARS65, an autonomously replicating sequence from mouse liver cells, and promotes plasmid DNA replication in vivo and in vitro. Its levels are high in proliferating cells and low in quiescent cells. Thus it may be closely involved in unregulated cellular DNA replication and amplification leading to immortalization (11, 101).

Ariga and co-workers isolated sequences from the human genome that bind to the C-myc protein, and interestingly, 90% of their clones replicated autonomously in human and mouse cell lines (101, 102).

SV40 origin sequences, therefore, seem to have homology to many sequences that are normally or abnormally correlated with eukaryotic DNA synthesis. Thus, the SV40 DNA may be a small eukaryotic replicon that suitably is studied as a eukaryotic chromosomal origin by many researchers.

Recently several investigations have led to selection of

mammalian replication elements. Origin-enriched sequences (ORS) have been isolated from monkey CV1 cells by collection and purification of nascent DNAs that have replicated early in S phase after synchronization by serum starvation(112, 220). Sixteen branch migration extruded DNA fragments from early replication bubbles have been studied extensively and some resemble the Alu sequences.

They can replicate autonomously in monkey and human cell lines in vivo and in vitro, as determined by bromodeoxyuridine incorporation and resistance to DpnI digestion. However, the small sizes of the inserts may lack upstream or downstream regulatory elements (220).

Similarly, replication initiation sequences (RIS) have been studied from mouse DNA cross linked with Trioxsalen. Short DNA fragments synthesized between the crosslinks are isolated and purified by density centrifugation (3).

Others have studied gene amplification, where large amounts of a particular gene are accumulated in a short period. This is thought to be caused mostly by multiple rounds of DNA replication of sequences surrounding the gene.

Molecular and cytogenetic analysis of many amplified regions in mammalian tumor cells have shown that the sizes of

amplified regions vary and are generally larger than the target genes ; with each amplicon containing the corresponding gene plus a DNA replication origin (28, 35, 88).

In higher organisms gene amplification may be controlled by multiple rounds of DNA replication per cell cycle from essential origin sequences. In D. Melanogaster, the Chorion gene is amplified by DNA replication initiated at sequences upstream of the gene and involves 100 kbps (35, 51, 178).

A rolling circle mode of replication is also observed in genomic amplifications (of insects, fish and amphibians). Particularly, the genes for rRNA, needed for production of ribosomes which synthesize proteins necessary for the rapid development, are amplified 4,000 fold in the oocytes of Xenopus Laevis. Rolling circles and circular DNA molecules have been observed during and after the rRNA amplifications (70a).

The isolation of amplified dihydrofolate reductase gene (DHFR) in methotrexate resistant chinese hamster ovary cells (CHO) let Heintz and co-workers to localize a region of 4-6 kbp that is needed for replication and is located 14 kbp from 3' end of the gene in each amplified unit (28, 35,88).

Similarly, Carrol and co-workers, working with CAD gene

(multifunctional protein containing carbamylphosphate synthetase, aspartate transcarbamylase and dihydrooratase), inserted the gene into random genomic locations and selected cells with highly amplified CAD genes. A 250 kbp extrachromosomal element was found containing tandem array of donated CAD genes plus a functional origin of DNA replication (35, 202).

However , the resolution of these studies have not led to any specific nucleotide sequences, nor has there been a reliable system to test their authenticity as origins of replication used in other locations or with other genes.

In this project, I have taken advantage of a reliable in vitro replication system plus a sensitive assay to segregate between replicated and parental molecules, to isolate and study replicating clones from human genomic libraries. This is the first general method of isolating autonomously - replicating sequences that can be adapted to any organism, with a feasible assay to study effects of specific proteins or elements necessary for DNA replication.

I have inserted fragments of human HL60 DNA into bacterial plasmids and have selected the clones that initiate replication in HL60 soluble extracts. These clones have been

digested with excess DpnI and the undigested, replicated products have been used to transform a Dam+ strain of E.Coli. Appropriate colonies have been then selected and recycled through this system several times to choose the clones which replicate most efficiently in vitro. Electron microscopy and gel electrophoresis have been used to confirm specific initiation at human fractions of the plasmids. In other experiments, I have tested for homology between these clones and repetitious mammalian sequences. Finally , I have used the replicating plasmids to transfect human and monkey cell lines to test in vivo replication and stabilization.

MATERIALS AND METHODS

DNA templates. Plasmid pSVOD (3.3 kb), a gift of Dr. T. Maniatis, is a pML2 derivative containing a 232 bp SV40 insert spanning the origin of replication and from which one 72 bp enhancer sequence has been partially deleted (30). Plasmid pML2 (2.9 kb) is a pBR322 derivative with a 1.37 kb deletion including poison sequences reportedly inhibitory to replication in animal cells (28). Plasmid pSV2-neo (5.6 kb) includes, in addition to pBR322 sequences, an SV40 insert of 323 bp spanning the origin of replication and both enhancer sequences. It also possesses the E. coli neomycin resistance gene and additional SV40 sequences 3' to it. This plasmid does not have poison sequences deleted (33). Plasmids and SV40 DNA are purified by standard procedures employing CsCl gradient centrifugation and contained >80% form I DNA.

Preparation of genomic libraries. Genomic DNAs from, human HL60 cells were isolated and purified before digestion with restriction endonucleases BamHl or Sau3A. They were run

on a 1% agarose gel and the DNA fragments of 0.5- 4.0 kb were excised from the gel and the DNA was purified by elutip columns or Geneclean according to manufacturers suggestions.

The plasmid pML2 was linearized with BamH1 and its ends were dephosphorylated to suppress self-ligation. The genomic DNAs were ligated to pML2 according to Maniatis et al.(136).

Preparation of cell extracts. Monkey COS 1, COS 7 and CV 1 cells and human HeLa cells were grown in 75 cm2 flasks in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and antibiotics. Human HL60 cells were grown in suspension cultures in RPMI 1640 plus 10% fetal calf serum and antibiotics. Cells were harvested at 80% confluence and extracts prepared by an adaptation of the method of Li and Kelly (131). Monolayer cells were washed once with 0.14M NaCl containing 10 mM sodium phosphate, pH 7.0 (PBS) and released from the flasks by washing with PBS containing 2.5 mM EDTA. The loose cells were washed rapidly two times with 5 ml of cold hypotonic buffer (20 mM HEPES, pH 7.5, 5.0 mM KCl, 1.5 mM MgCl2 and 1.0 mM dithiothreitol), followed by centrifugation at 2000 x g for 5 min, and resuspended in two volumes of the same buffer. After swelling on ice for 10 min,

cells were disrupted by 20 strokes of a B-pestle Dounce homogenizer. The lysate was kept on ice 45 min and then clarified by centrifugation at 12,000 x g for 20 min. Aliquots were stored at -70 c.

DNA replication conditions. Reaction mixtures (50 ul) contained 300 ng of DNA in 30 mM HEPES, pH 7.5, 7.0 mM MgCl2, 0.5 mM dithiothreitol, 100 uM each dATP, dGTP and dTTP, 10 uM a-32P-dCTP (15-30 Ci/mmole), 200 uM each GTP, UTP and CTP, 4.0 mM ATP, 40 mM phosphocreatine, 10 ug creatine phosphokinase (Sigma), and 30 uL COS7 cell extract (added last). Reactions were supplemented with purified T- antigen as indicated in the text at approximately levels used to study T-antigen binding to sequences at the SV40 origin (131). Incubation was for times indicated at 37 C. Reactions were stopped by addition of EDTA to 10 mM. RNase A was then added to 5.0 ug/ml and incubation continued for 15 min. Proteinase K was then added to 200 ug/ml and incubation continued for 30 min. DNA was purified at 4 C. by two phenol-chloroform extractions, one chloroform extraction and one ether extraction followed by ethanol- acetate precipitation. The purified DNA products were used for gel electrophoresis or electron microscopy.

For recombination and clonal selection experiments similar conditions were used. pre-incubations contained the same contents and the plasmid products were purified as above before addition to new in vitro replication mixtures.

All restriction endonucleases were used according to manufacturers suggestions except for DpnI incubations, where NaCl concentrations was raised to 200 mM.

Agarose gel electrophoresis. In vitro replication gel electrophoresis usually was done in 20 cm plates containing 1.5% agarose gel in tris-acetate buffer or tris-phosphate buffer and was run at 80 v for 16 hours.

2-dimensional gel electrophoresis. Labeled DNA from the replication reaction was dissolved in 10 ul of 0.5% SDS, 10 mM EDTA, pH 8.0, and 8% sucrose. First dimensional native gel electrophoresis was carried out in 80 mM Tris- HCl, pH 7.5, 5.0 mM sodium acetate, 1.0 mM EDTA on 20 cm 1.5% agarose gels at 60 v for 20 hrs. For the alkaline dimension 1.0 cm strips were cut out of the first gel and soaked in buffer containing 30 mM NaOH, 2.0 mM EDTA and 1.0 mM EGTA for 1 hr and then cast into 1.8% agarose gels containing the same buffer. Electrophoresis was again at 60 v for 20 hrs.

Autoradiography. The gels were dehydrated and placed on two pieces of Kodak films without the intensifier screen. The films were usually exposed within 24 hours.

Electron microscopy. DNA samples from replication reactions were purified as described and dissolved to a concentration of 1.0 ug/ml in 50% (vol/vol) formamide, 0.1 M Tris-HCl, pH 8.5, 10 mM EDTA. Cytochrome c was added to a concentration of 50 ug/ml, and samples were spread onto a hypophase of 20% (vol/vol) formamide, 0.01 M Tris-HCl, pH 8.5, 1.0 mM EDTA. The spread DNA was picked up on collodion grids, stained with uranyl acetate and rotary shadowed with Pt/Pd as described (16). Double-stranded Øx174 DNA was included in selected samples to serve as a length standard. Measurements were made on enlarged prints with a Numonics digital integrator.

DNA transfection by CaPO4. For each 75cm2 flasks 10 ug of plasmid DNA was used. It was first precipitated with ethanol acetate and rinsed with 70% ethanol. The pellet was dissolved in 305 ul of 1mM Tris-HCl, 0.1 mM EDTA pH 7.6. 305 ul of 0.5 M CaPO42 was added and gently mixed 610 ul of 2x HB (280 mM NaCl, 50 mM HEPES, 1.5 mM Na2HPO4.H2O, pH 7.1). It was mixed gently and let stand for 15-30 minutes. The precipitate was mixed by pipeting once up and down and it was added to the flasks (containing 5ml medium).

The cells were incubated for 4 hours at 37 C. The cells were then washed 2x with PBS and new medium was added. The cells were harvested at 0, 24, 48, 72 hours after transfection. The low molecular plasmid DNA was collected by Hirt procedure as follows: The cells were rinsed 2x with ice cold 10mM Tris-HCl, 10 mM EDTA pH 7.5 and 2ml of hot (60 C) 1% SDS, 10 mM EDTA and 10 mM Tris-HCl was added to each flask. The cells were scraped down and transferred to 15 ml corex tubes. Then 0.5 ml 5M NaCl was added. Then the Corex tube was covered with parafilm and inverted gently 10 times and let stand at 4 C overnight. The DNA solution was spun at 10,000 RPM for 35 minutes at 4 C. The supernate was transferred to

new tubes and extracted few times with phenol-chloroform and the DNA was precipitated with ethanol acetate. For replication assays DNA products were linearized with a restriction enzyme and then digested with DpnI, run on an agarose gel and hybridized with appropriate probe.

Southern transfer.The gel was incubated in 0.25 M HCl for 15 minutes to hydrolyze the DNA. Then it was incubated in 0.4N NaOH-0.6M NaCl for 30 minutes at room T to denature the DNA. Then 1.5M NaCl-0.5M Tris-HCl, pH 7.5, was added for 30 minutes at room T to neutralize the gel. GeneScreenPlus was cut to exact size of the gel and was rinsed in water and laid into 10x SSC.A piece of Whatman paper was placed in a gel box,both sides hanging over.The gel was put on top and added the GeneScreenPlus with side B in contact with the gel. Few whatman papers and a stack of paper towels were placed on top. Then 10x SSC was added up to the gel and transfer was continued overnight. The GeneScreenPlus was removed and placed in 0.4M NaOH for 1 minute.Then 0.2M Tris-2X SSC was added and the screen was blotted dry between folds of Whatman paper 20x SSC: 3M NaCl, 0.3M Nacitrate, pH 7.

Prehybridization of blots. GeneScreenPlus was added to a heat sealable bag and 30 ml prehybridization solution and 250 ug/ml denatured salmon sperm DNA was added. The bag was placed on a rotor at 68 C for at least 6 hours.

Hybridization of blots.The prehybridization solution was replaced with 30 ml hybridization solution plus denatured salmon sperm DNA and the denatured probe. The bag was sealed and placed in the 68 C oven for 16 hours. The GeneScreenPlus was washed with 2x SSC for 10 minutes at room T twice. Then it was washed with 2x SSC-1% SDS for 30 minutes at 65 C twice. Then it was wash with 0.1x SSC for 30 minutes at 37 C twice. The filter was wraped with saran wrap and was autoradiographed.

Prehybridization solution: Ficoll 0.1%;polyvinylpiyrrilodine 0.1%; NaCl -Nacitrate 5x SSC; NaH2PO4, Na2HPO4 50 mM; EDTA pH 8, 5 mM; Dextran Sulfate 5%; adjust to pH 7, filter and add pure BSA 0.1%; SDS 1%. Hybridization solution: Ficoll 0.02%; polyvinylpiyrrilodine 0.02%; NaCl- Nacitrate 2x SSC; NaH2PO4, Na2HPO4 20 mM; EDTA pH 8, 2 mM; Dextran Sulfate 10%; adjusted to pH 7 and filtered then pure BSA was added to 0.02%; SDS 1%.

Random primer labeling reaction. The DNA was fragmented and run on 1% low melting agarose gel. The desired band was cut out and was boiled with 3 ml H2O/g of gel for 7 minutes to denature the DNA. The labeling reaction was carried at room T for 3-5 hours as follows: H2O to total of 50 ul; 10 ul oligo-labeling buffer; 2 ul of BSA (10 mg/ml); DNA in agarose (up to 35 ul); 5 ul [32p]dCTP (3000 Ci/mmol); 2 units of large fragment of E. coli DNA polymerase I.

The reaction was stopped by addition of 200 ul of a solution containing 20 mM NaCl, 20 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.25% SDS and 1uM dCTP.

OLB: Solution O: 1.25M Tris-HCl, 0.125M MgCl2, pH 8.0. Stored at 4 C. Solution A: 1 ml solution O + 18 ul 2-mercaptoethanol + 5 ul dATP, dTTP, dGTP of 0.1M solution. Stored at -20 C. Solution B: 2M HEPES, pH 6.6(NaOH). Stored at 4 C.Solution C: Hexadeoxyribonucleotides in TE at 90 OD units/ml. Stored at -20 C. Solutions A,B,C were mixed in 100:250:150 ratio to make OLB.

Preparation of competent bacterial cells. The colonies were from newly streaked SOB agar plate and dispersed in 10 ml of culture medium. The cells streaked from a frozen stock or

a fresh stab 16-20 hours prior to initiating liquid growth. We used a culture volume of 1:10. The cultures were shaken at 200 rpm at 37 C for 4-6 hours (4-7 x 7 10 cells/ml). The cells were collected in 50 ml polypropylene centrifuge tubes and chilled on ice for 10 minutes.

The cells were centrifuged at 1000 xg for 15 minutes at 4 C and resuspended in 1/3 of original volume in RF1 and chilled on ice for 15 minutes (DH1 and JM101) to 2 hours (HB101). The cells were centrifuged at 1000 xg and the pellet was resuspended in 1/12.5 of original volume in RF2, chilled on ice for 15 minutes. They were frozen in chilled screw cap tubes in liquid nitrogen and stored at -70 C.

Transformation of competent bacterial cells. The tubes were removed from the freezer and thawed at room temperature. The DNA solution in <20 ul was added of to 200 ul of competent cells. The tubes were mixed gently and chilled on ice for 60 minutes. We heat shocked the cells at 42 C water bath for 90 seconds and chilled on ice. 800 ul of medium was added and it was shaken at 37 C for 30 minutes (tetracycline resistance) or 60 minutes(ampicilin resistance). Then, for plasmid amplification 200 ml of NZY plus 40 ug/ml ampicilin was added

and it was shaken at 200 rpm for 10-12 hours at 37 C.

For agar plates, NZY plus 1.5% agar was autoclaved and cooled to 55 C before adding 40 ug/ml of a 25mg/ml Ampicilin solution. 10-15 ml of NZY was poured as bottom agar into each plate and let cool to room temperature. For top agar, we autoclaved NZY plus 1% agar and cooled to 55 C to add Ampicilin and the transformed bacteria. The tube was mixed well and poured onto the bottom agar. The plates were incubated at 37 C overnight.

RF1: RbCl 100 mM; MnCl2.4H2O 50 mM; potassium acetate 30 mM; CaCl2.2H2O 10 mM; Glycerol 15%; pH 5.8 with acetic acid and filter. RF11: MOPS 10 mM; RbCl2 10 mM; CaCl2.2H2O 75 mM; Glycerol 15%; pH 6.8 with NaOH and filter.

Rapid plasmid purification. The cells were grown in 200 ml of NZY plus Ampicilin overnight without chloramphenicol amplification.The cells were then collected into screw cap bottles by spinning at 4000 xg for 10 minutes at 4 C.

I resuspended the cells in 45 ml of STET buffer (50 mM Tris-HCl pH8.1; 50 mM EDTA; 8% Sucrose; 5% Triton X-100). This tube was transferred to Nalgene polypropylene tube and added 1 ml of a 10 mg/ml freshly made lysozyme solution in TNE

buffer (0.1 M NaCl; 0.01 M Tris-HCl pH 8.0; 1 mM EDTA) and left at room temperature for 10 minutes.

The tubes were held in a boiling water for 90 seconds and then spun at 36600 xg at room temperature for 30 minutes. The plasmid DNA in supernatant was then precipitated with an equal volume of isopropanol for 1 hour at -20 C.

The precipitate was centrifuged at 12000 xg for10 minutes and washed in 66% ethanol/ 0.9% NaCl. Then we desiccated the pellet to dryness and resuspended the pellet in 9.6 ml TE buffer containing 0.1% SDS. 200 ul of a 1 mg/ml stock solution of RNase A was added and the mixture was incubated at 37 C. 200 ul of a 10 mg/ml proteinase K solution was added and incubated at 65 C for 10 minutes.

This solution was extracted with phenol;chloroform, chloroform and ether. The DNA was precipitated with ethanol acetate and spun at 12000 xg for 10 minutes. The pellet was desiccated to dryness and resuspended in 0.4 ml of TE . 50 ul of RNase A solution was again added and incubated at 37 C. 50 ul Proteinase K solution was added and incubated at 65 C for 10 minutes. The DNA was extracted as above and precipitated with ethanol acetate.

DNA purification by CsCl gradient. For 27 ml total, DNA solutionis added in 16 ml plus 1 g CsCl/ml and 2 ml Ethidium Bromide (10 mg/ml).The rest of the ultracentrifuge tube was filled with TE and 1 g/ml CsCl.It was spun at 40000 rpm in VTI 50 rotor for 24 hours. The bottom DNA band was taken out by a needle and was extracted with isoamyl alcohol or isobutanol few times to take out the Ethidium Bromide.

The DNA was then dialyzed against several changes of TE buffer and extracted with phenol-chloroform, chloroform and ether and was precipitated with ethanol acetate and checked in an agarose gel.

RESULTS

In vitro replication of SV40

Simian Virus 40 appears to be an excellent model for studying eukaryotic chromosomal replication machinery, because it replicates as a circular minichromosome with one replication origin and requires only one viral protein, the T antigen. Although this is a multifunctional protein, the initiation, the elongation and the termination events use similar mechanisms to the ones of the hosts chromosomes. Therefore, a detailed understanding of its replication would accumulate information for analysis of eukaryotic DNA synthesis. However, only in vitro assays can reveal specific roles of different proteins and their binding sites involved in initiation, elongation or termination of this complex event.

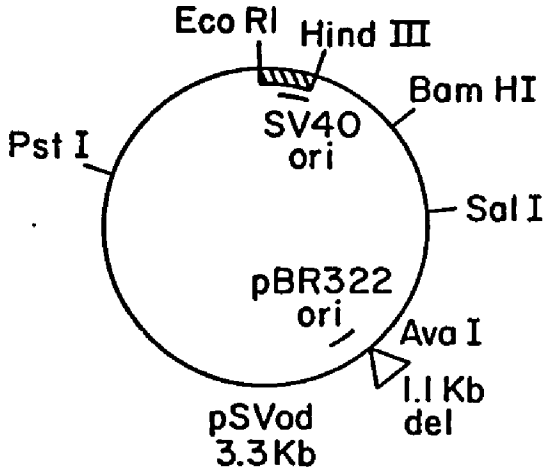
Several laboratories have been able to initiate SV40 replication in vitro, mostly due to availability of purified T antigen and a sensitive replication assay (10, 131, 132, 209).

We studied the replication of plasmids containing the SV40 origin ,pSVOD and pSV2neo (figure 6), in a crude hypotonic monkey CCS7 cellular extract. Plasmids pBR322 and pML2 (figure 7) were used as repair and background synthesis controls. The templates were incubated in the replication mixture at 37 C and the products were analyzed by gel electrophoresis and electron microscopy as described in the methods.

Initially, we used extracts from COS7 cells which produce T antigen constitutively and have been reported to initiate SV40 replication in vitro (10, 131). However, only after addition of large amount of purified T antigen (0.5 ug/50 ul reaction) our extracts could carry all of the SV40 replication stages.

In order to determine the types of replication intermediates that participate in replication in vitro, we performed detailed electrophoretic analyses of replication intermediates of two SV40-based plasmids used for these

Figure 6. Maps of SV40 origin containing plasmids pSVOD and pSV2neo. Diagonally hatched regions represent SV40 sequences.


Eco RI
Hind III
Bam HI
SV40
ori
Pst I
Sal I
pBR322
ori
Ava I
1.1 Kb
del
pSVod
3.3 Kb

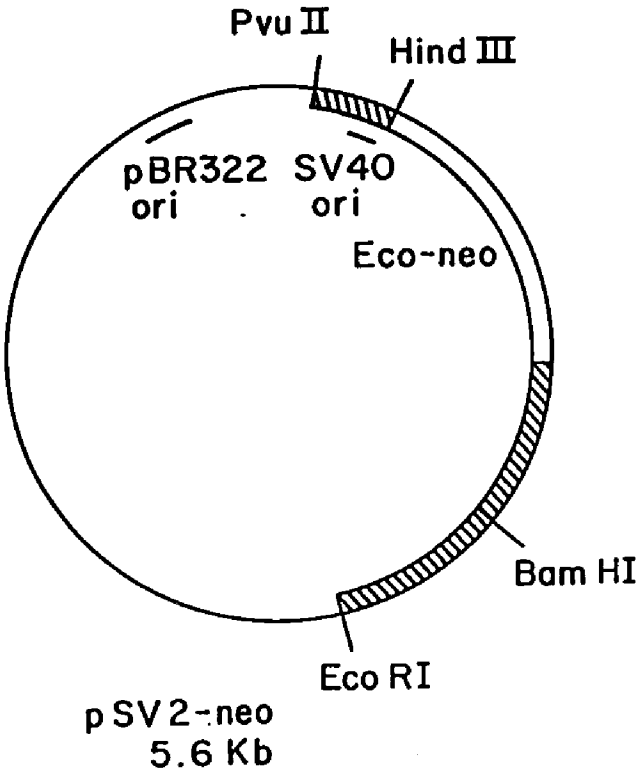
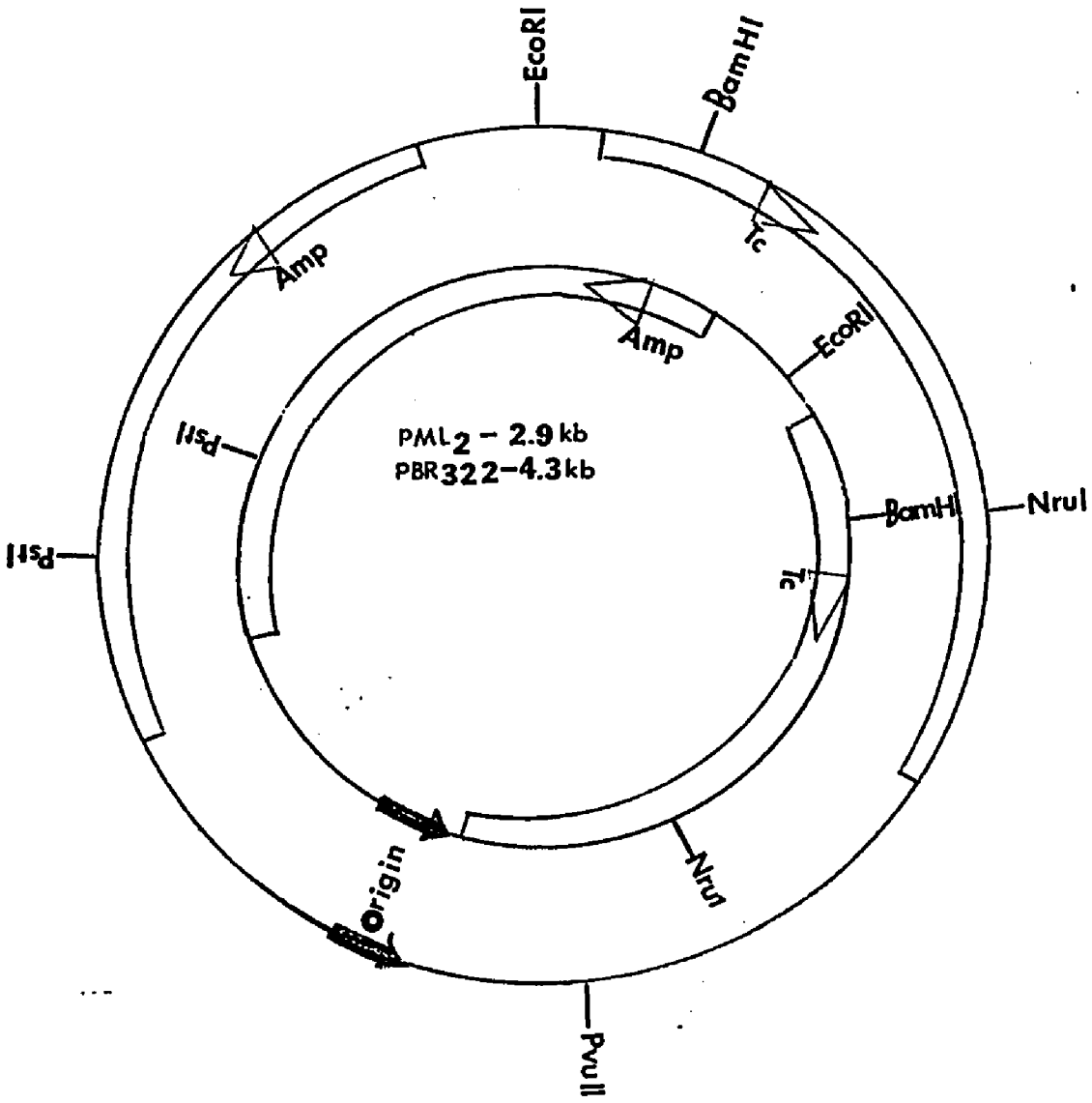

Pvu II
Hind III
pBR322
ori
SV40
ori
Eco-neo
Bam HI
Eco RI
pSV2-neo
5.6 Kb

Figure 7. Maps of plasmids pML2 and pBR322.

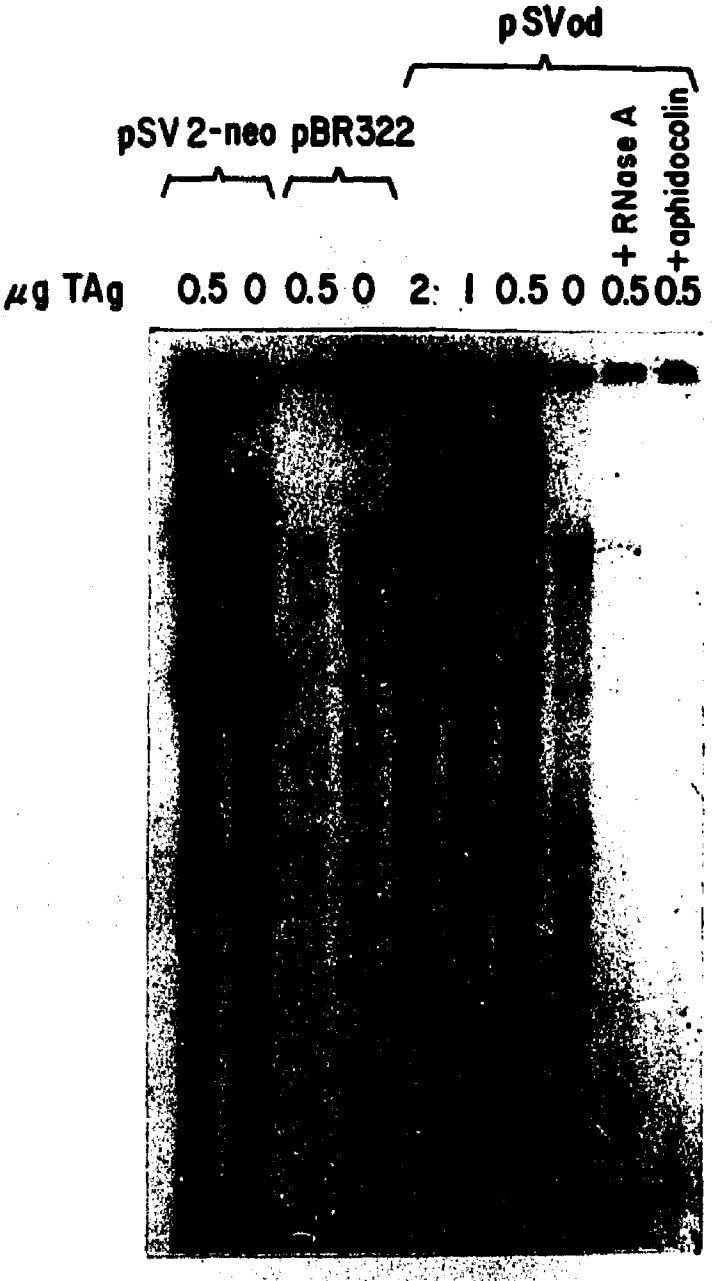

EcoRI
BamHI
Tc
EcoRI
Amp
Amp
PstI
PML2 – 2.9 kb
PBR322–4.3 kb
BamHI
NruI
Tc
PstI
Origin
NruI
PvuII

studies. Synthesis of pSVOD and pSV2-neo plasmid DNA is shown in Fig 8A, which demonstrates the T-antigen requirement for generation of labeled intermediates. The plasmids were incubated in the replication mixture and the purified DNA products were run on a 1.5% agarose gel as described in the methods. We find that at high final concentrations of cellular extract (>6 mg protein/ml) the enhancing effect of added T-antigen peaks at concentrations of 0.5-1.0 ug/ 50 ul reaction.

Analysis of P^{32} incorporation showed that approximately 20 pmoles of dCTP were incorporated per reaction with plasmid pSVOD without added T-antigen and 95 pmoles per reaction with T-antigen (0.5 ug). Approximately 40 pmoles of dCTP were incorporated per reaction with pSV2-neo without added T-antigen and 135 pmoles with T-antigen (0.5 ug). Less than 5 pmoles were incorporated with pBR322 either with or without added T- antigen. Differences in labeling of pSVOD and pSV2-neo in the absence of added T-antigen are consistent and may reflect different efficiencies of replication of the two plasmids using the endogenous T-antigen.

Figure 8A. In vitro replication of plasmids pSVOD and pSV2neo, T-antigen dependence replication of SV40 origin containing plasmids: pSVOD, pSV2neo and control pBR322 DNA were subjected to in vitro replication conditions with p^{32}dCTP as described in methods. Shown are gel autoradiographs of labeled DNA. Each 50 ul reaction was supplemented with purified T antigen at the levels indicated. Indicated reactions included RNase A (10 ug/ml) or Aphidicolin (0.1 mM).

In vitro Replication of Plasmids pSVod and pSV2-neo: T-antigen Dependence



Results with negative control aphidicolin are as observed by others using in vitro SV40 DNA replication and indicate that most elongation is carried out by DNA polymerase alpha (or delta) (52, 131). Inhibition of plasmid replication in vitro by RNase A has also previously been reported which may be due to inhibition of RNA primer synthesis either at the origin or for priming of DNA synthesis at the lagging strands (209). The insignificant labeling of control plasmid pBR322 affirms that initiation is selective for the SV40 origin. Labeling of topoisomers of pML2 is due to unspecific topoisomerase- replicase activities as seen elsewhere (137).

Several aspects of pSVod labeling shown in Fig 8A are notable. Fully supercoiled form I of the plasmid is not labeled. The most highly labeled products correspond to the position of open-circular form II and of high molecular weight replication intermediates, seen as a diffuse series of bands above form II. These bands are consistent with Sundin and Varshavsky 's results including catenated dimers of monomeric SV40 DNA in forms of A, B and C corresponding to two nicked or gapped circles, one open circle and one supercoiled circle, and two supercoiled circles, respectively. The segregation of

daughter strands occurs from A to B to C and then to single monomeric supercoiled circles(190). These intermediates appear at early incubations and disappear into topoisomers at later time points (>2 hours), indicative of their resolvance into monomeric forms. Catenated dimers form when DNA replication machinery passes the termination sites of the genome before the two molecules separate and as a consequence, the daughter molecules become catenated. Topoisomerase II segregates these dimers to monomer circles by introducing double strand breaks and is thought to be involved in the termination event. In SV40 DNA replication reactions where Topoisomerase II inhibitor, VM26, has been used or otherwise in cells grown in hypertonic media, the products of the DNA replication contain multiply interlocked catenated dimers and linear molecules, respectively (161).

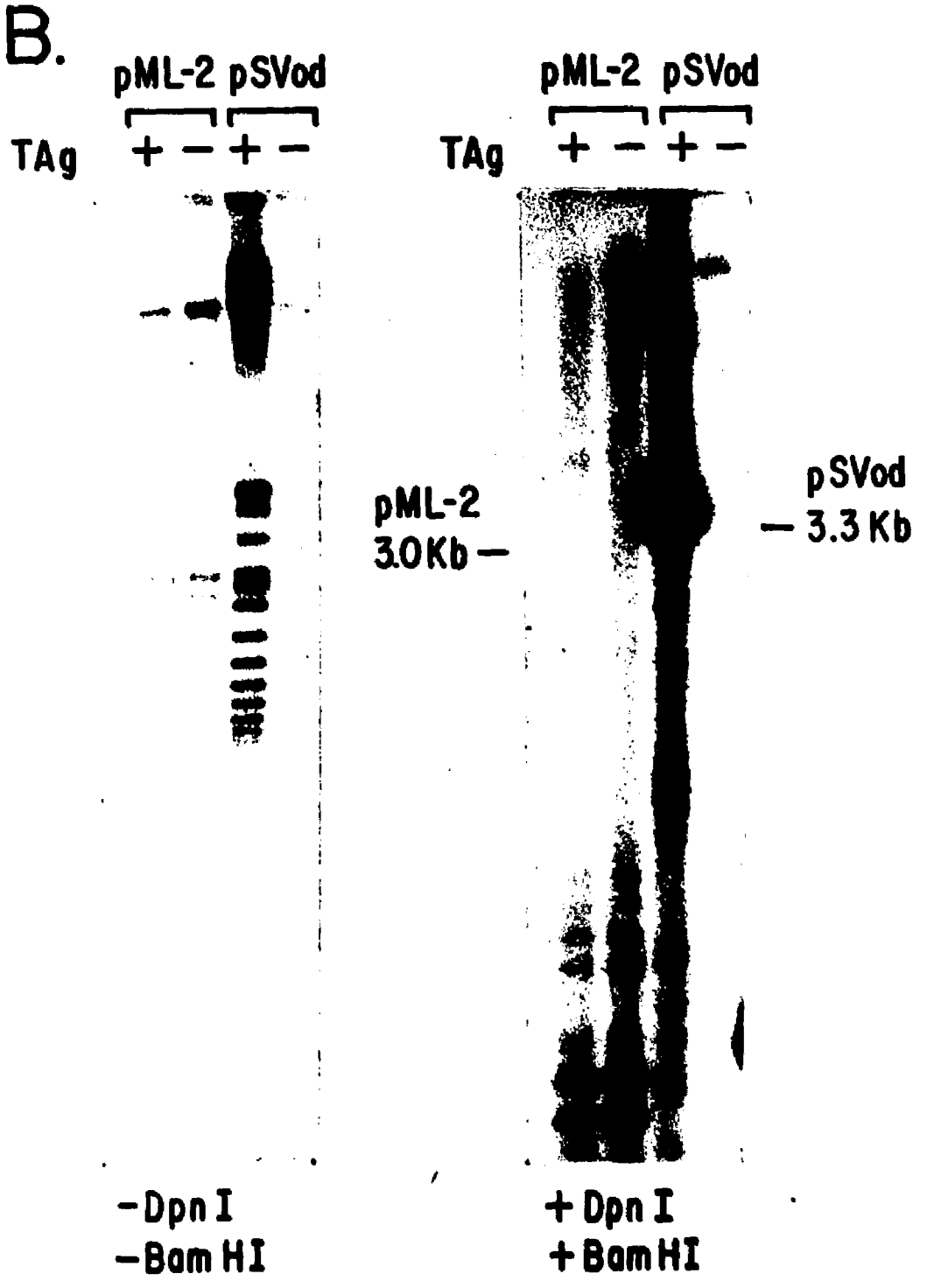
Recent observations have indicated another pathway for termination of SV40 DNA replication where, the daughter molecules separate before the replication machinery reaches the termination sites (90-95% replication completed) and as a result, two gapped (<50 bps) DNA molecules are produced that are subsequently filled by the replicase (204). Both

mechanisms may be active in our system ,since we observe catenated dimers as well as monomeric topoisomers.

A series of labeled bands below form II correspond to circular pSVOD at various levels of supercoiling. These labeled circular molecules are consistent with their being resolved end products of plasmid replication via bi-directional fork progression. Repair labeling could also contribute to generating these series of bands. Full supercoiling of completed circles to form I DNA reportedly depends on inclusion of a nuclear extract in the reaction mixture or in the presence of ethidium bromide in the gel electrophoresis and is selective for replicated molecules (49, 184). Densitometry of labeled gel bands shows that these circular replication products account for 35% of overall labeling at 2 hrs. The remainder of labeling is accounted for by more slowly-migrating replication intermediates.

These results were confirmed by DpnI assays; that DpnI only digests plasmids that are propagated in a Dam+ strain E. coli (thus are methylated). However, semi-conservative replication of these methylated templates, using cellular extracts would yield hemi-methylated (one round of

Figure 8B. In vitro replication of plasmids pSVOD and control pML2: Plasmids pSVOD and pBR322 were subjected to in vitro replication conditions as described in methods in the presence or absence of T antigen. Both plasmids were digested with BamHI prior to digestion with DpnI.


B.
pML-2
pSVod
TAg
+ − + −
pML-2
3.0Kb
pSVod
3.3 Kb
-Dpn I
-Bam HI
+ Dpn I
+ Bam HI

replication) or unmethylated molecules that are resistant to DpnI digestion (123). The restriction site GmeATC is present several times in our plasmids which when digested with DpnI yield DNA fragments smaller than 500 bps.

FIG 8B depicts the DpnI digestion of replication products of plasmids pSVOD and pML2. The templates were incubated in the replication mixture as figure 8A. The purified products were linearized by BamHI and digested with DpnI. Depicted are linear forms of pSVod (3.3 kb) that remain resistant to DpnI digestion and a series of smaller bands corresponding to DpnI fragmented, repaired molecules or otherwise,Cairns structures. A high molecular weight DNA that is resistant to DpnI digestion migrates about 20 kbp. These molecules may correspond to long cellular DNAs present in the extracts and/or tangled catenated dimer end products of replication(173a, 204). Control pML2 does not replicate in this system and thus there are no linear band (2.9 kb) apparent. However, repair labeling is present as smaller DpnI fragments.

Figure 9 shows a neutral- neutral 2D gel of PSVOD. The plasmid pSVOD was incubated in the replication mixture and

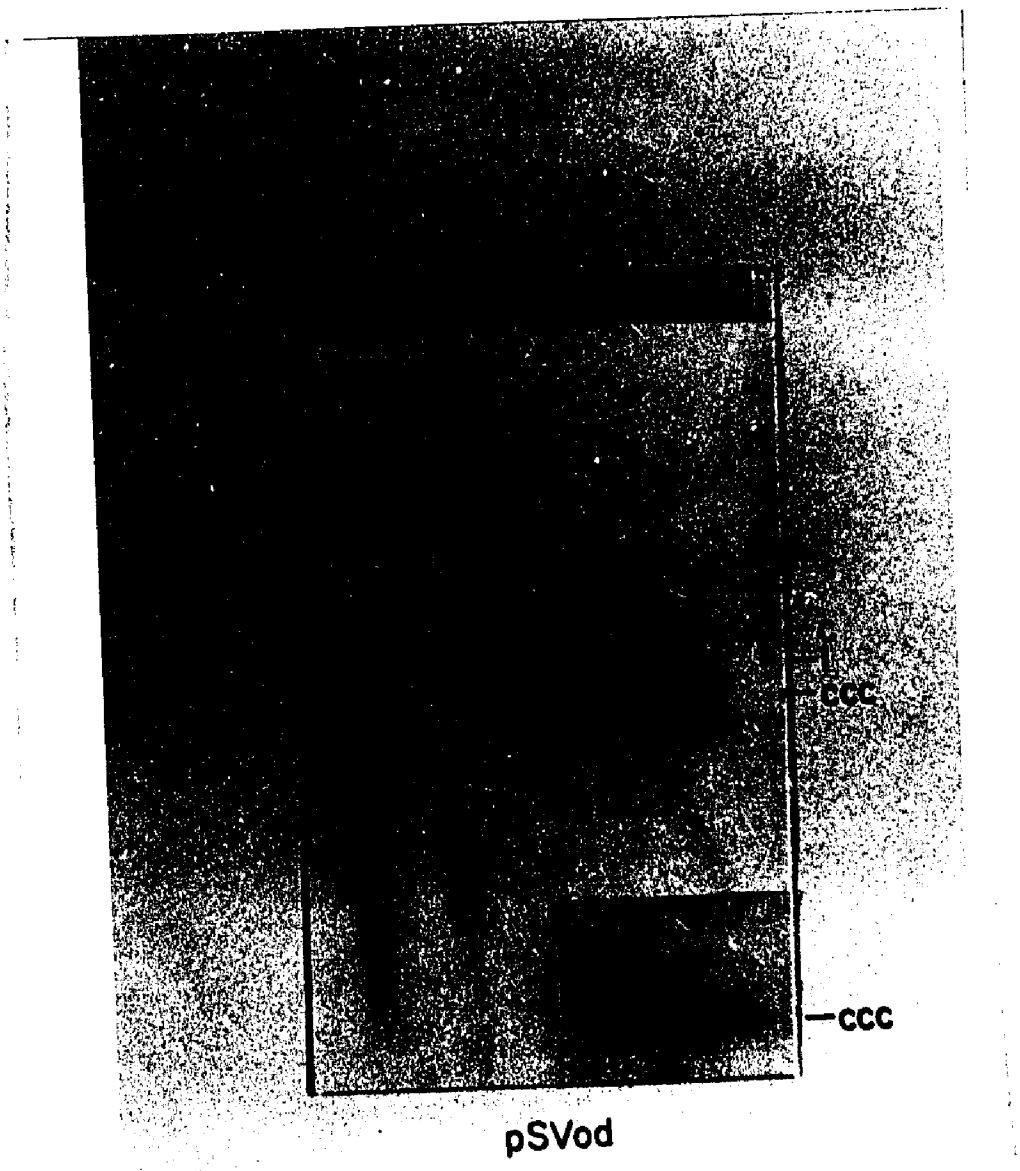
Figure 9. 2-dimensional neutral-neutral gel electrophoresis: plasmid pSVOD was subjected to in vitro replication conditions with p^{32}dCTP and 0.5 Ug of T antigen. Gel lane was cut from the first dimension and digested partially with BamHI. This was then run in the second dimension as described in the methods. Lambda DNA, cut with Hind III , was run as the marker. L= linear DNA; C= circular DNA; D= catenated dimers.


D
C
L
23 Kb
9.4
6.5
4.3
2.3
2.0
D
C
L

run in a first neutral dimension. The lane corresponding to pSVOD was cut out and digested with excess BamHI. This was then run in a second neutral dimension. The enzyme did not get through the gel efficiently and thus a partial digestion of pSVOD replication products are depicted. The digested products consist of topoisomers that in the second dimension run at L-linear; uncut materials include topoisomers with different supercoiling that in the first dimension range from form I DNA to form II; also seen are nicked or gapped molecules at C; and at last high molecular weight molecules at D, extending from C to top of the gel, corresponding to sigma structures, catenated dimers, and late Cairns structures.

Neutral-alkaline 2-dimensional gel electrophoresis of pSVOD (figure 10). The plasmid pSVOD was incubated in the replication mixture and the purified products were run on a first neutral dimension. The lane corresponding to pSVOD was cut out and placed in a second alkaline gel and run in alkaline conditions to separate the two strands of DNA molecules. As shown in Figure 10, most of the labeled intermediates migrate as covalently-closed circles (ccc) which do not separate under the denaturing conditions. The major

Figure 10. 2-dimensional neutral-alkaline gel electrophoresis of pSVOD: plasmid pSVOD was treated in the replication conditions plus 0.5 Ug of T antigen . Gel lane corresponding to pSVOD from the first neutral electrophoresis was cut and ran in a second alkaline gel as described in the methods. ccc- denatured, covalently closed , double stranded , circular DNA; l- single stranded linear DNA; c- single stranded, circular DNA; Io- covalently closed, relaxed circular DNA; hsi I,II- denatured, high molecular weight synthetic intermediates.


ccc
ccc
pSVod

form of these migrates at the position of form II circles and thus consists of non-supercoiled, closed double stranded molecules (form Io), as seen in the inset. Form II circular DNA, which migrates at the position of form Io in the first dimension, appears in the alkaline dimension as a closed-circular single strand (c) and a full-length linear strand (l).

Figure 10 affirms that repair synthesis contributes little to our overall plasmid labeling. No repair intermediates are visible as a vertical smear below any of the major replication products. The predominant replication intermediates are seen as two curved smears that extend to high molecular weight, one of which emanates from form II circular DNA (hsi I in Fig 10). These cannot be theta forms of replicating molecules since theta forms would yield no denaturation product longer than full-length linear plasmid.

Nor is it likely that these smears represent catenated dimeric molecules. In a gel system similar to ours catenated dimers have been observed as a series of discrete spots (190). Upon longer exposure or at early stages of replication, we see such a series of discrete spots extending

approximately horizontally below hsi II. These do not extend toward form II circular DNA as does hsi I. The most cogent explanation for a long, continuously-labeled DNA single strand is that it arises from plasmid molecules through rolling circle elongation. Beneath the hsi smear the second, lighter smear extends to very low molecular weight, curving asymptotically to the first-dimension position of form II or form Io circular DNA (1si in Fig 10). The upper hsi smear could thus represent the continuous "tail" of rolling circle synthesis and the lower smear smaller discontinuous synthetic products, down to Okazaki fragments, denatured from the longer labeled products directly above.

Another, darker smear, from material migrating more slowly in the first dimension, most likely represents the unligated newly-made strands from molecules having nearly completed bi-directional replication, i.e., late theta-forms. Above the dimeric form of pSVOD at 6.6 kb there is a discontinuity in the hsi smear consistent with the possibility of rolling-circle intermediates involving dimeric forms of plasmid (hsi II). Complexities in the labeling of high-molecular-weight intermediates are illuminated by

electron microscopy.

Figure 11 shows bi-directional replication of plasmids pSVOD, pSV2neo and plasmid BLUR8. The templates were incubated in the reaction mixture without labeled nucleotides. The purified products were spread for electron microscopy as described in the methods. DNA molecules from different stages of replication are shown: A, B, C: newly initiated replication bubbles; D, E, F : late Cairn structures; G, H, I : catenated dimers; J : segregating daughter molecules.

Rolling circle replication generates circles with long tails when We spread aliquots of replication reaction mixtures for electron microscopy as described in the experimental procedures. Fig 12 shows several representative sigma forms that are derived from unidirectional replication. Rolling circle structures that may be derived from broken theta structures , as suggested by others ,are shown in figure 13 (132, 209).

The percentages of various plasmid molecules observed as different replication intermediates are presented in Table IA. Of the plasmids tested, pSVOD molecules are visualized replicating most frequently, about 17% observed as either

Figure 11. Electron microscopic visualization of plasmid replication intermediates. Purified DNAs of plasmids pSVOD, pSV2neo and BLUR8 were subjected to in vitro replication conditions and spread for electron microscopy as described in the methods. A, B, C : replication bubbles. D, E, F : late Cairns structures. G, H, I : catenated dimers. J: segregating plasmids. The bars are 100 nm.

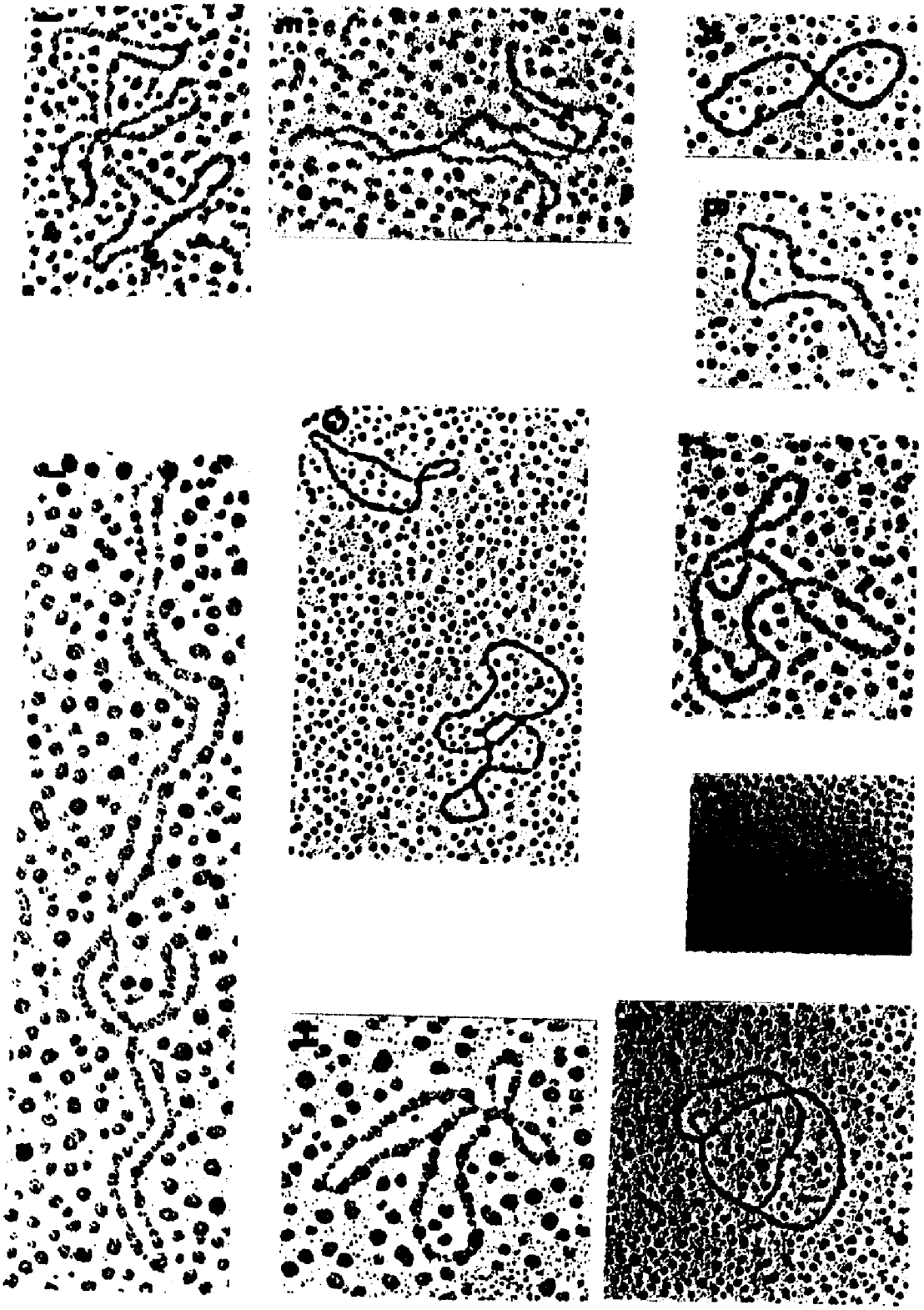


Figure 12. Electron microscopic visualization of plasmid rolling circle replicative intermediates. Purified DNAs of plasmids pSVOD, pSV2neo and BLUR8 were subjected to in vitro replication conditions for 2 hours and spread for electron microscopy as in methods. The bars are 100 nm.

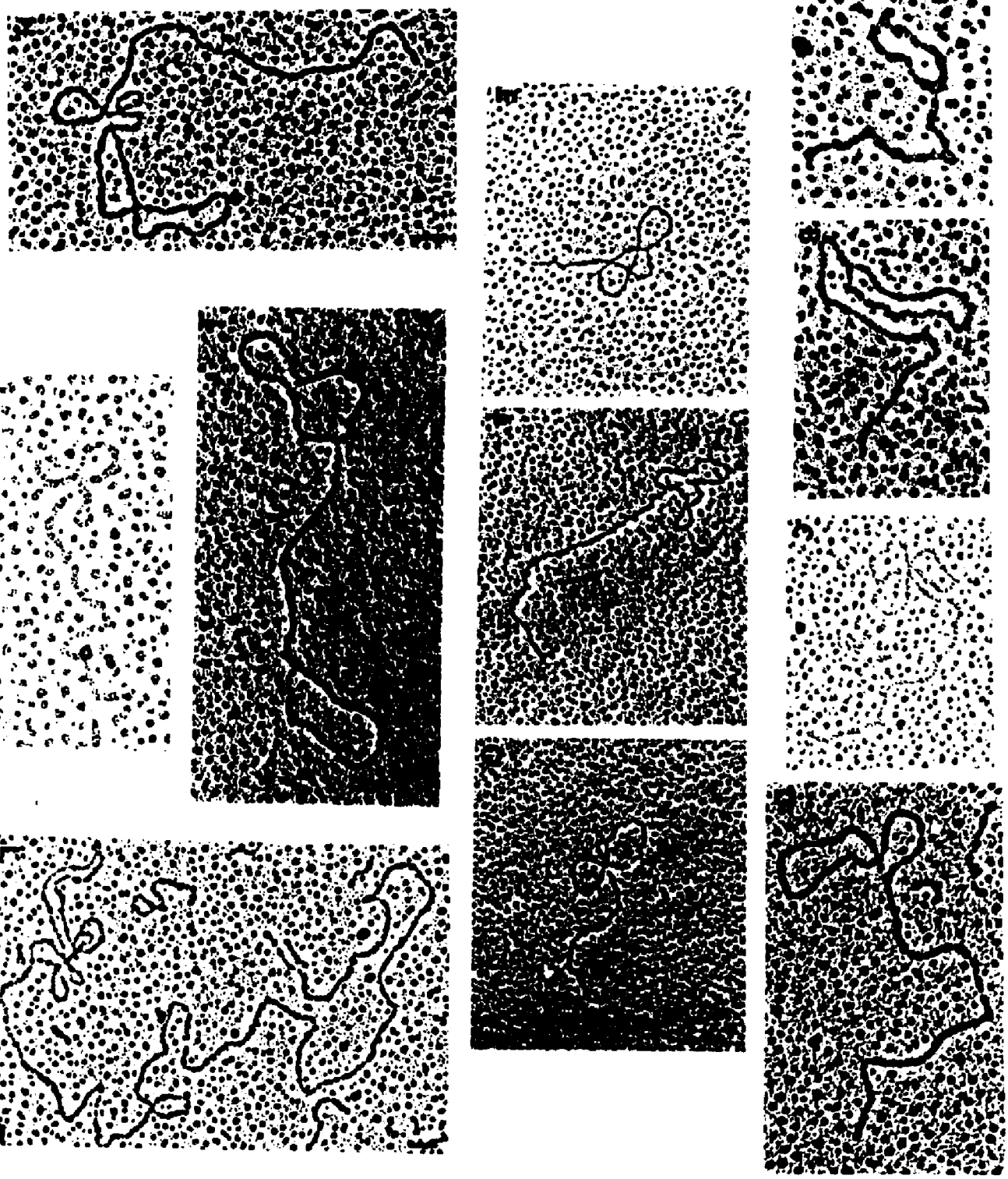


Table 1. A. Plasmid molecular forms after 2 hours incubation in the in vitro replication system.

a. Aliquots of plasmids pSVOD, pSV2neo, BLUR8 or pML2 were subjected to in vitro replication conditions in the presence of T antigen for 2 hours after which DNA was purified and spread for electron microscopy as described in methods. All molecules containing clearly identifiable circles were scored.

b. 1000 molecules of each plasmid type were scored.

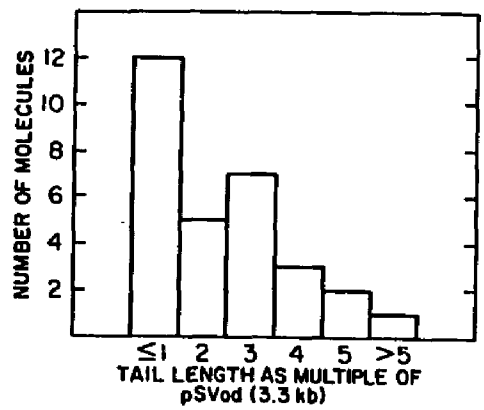
c. Two such molecules were scored.

B. length of free DNA ends in sigma form pSVOD molecules. DNA of plasmid pSVod was subjected to in vitro replication conditions and spread for electron microscopy as in A. lengths of free DNA ends on 30 sigma form molecules were measured and plotted as multiples of plasmid length.

A. PLASMID MOLECULAR FORMS AFTER 2 HOURS INCUBATION

PLASMID MOLECULAR FORM[a]	PERCENT OF MOLECULES[b]			
	pSVod	pSV2-neo	BLUR8	pML2
Circles, no replication evident	76.9	85.0	94.4	93.8
Theta forms	7.7	5.3	3.4	0
Sigma forms	9.0	5.3	1.1	0
Catenated dimers	0	1.1	1.1	3.1
Circular dimers	5.1	2.2	0	3.1
Dual sigma forms (2 equal circles)	1.3	1.1	<1[c]	0

B. LENGTHS OF FREE DNA ENDS IN SIGMA FORM pSVod MOLECULES



theta or sigma forms, nearly 1.5-fold higher than pSV2-neo. Nine per cent of pSVod molecules and 5% of pSV2-neo molecules are seen at 2 hrs as sigma structures in which a single double- stranded linear tail protrudes from a plasmid circle (Table IA). No sigma structures are seen with control pML2.

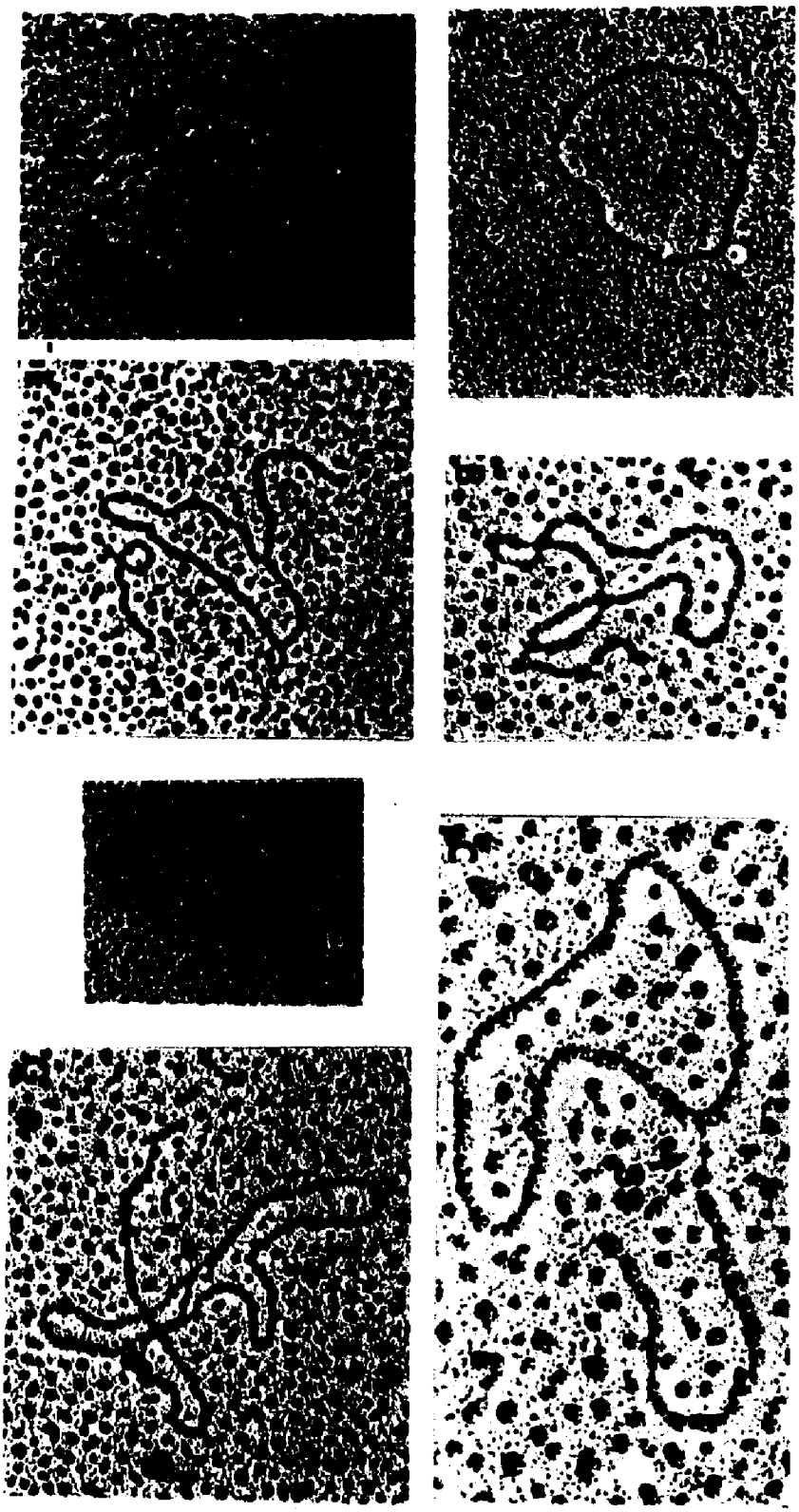
The percentage of overall replication forms seen as sigma structures by E.M. correlates well with the percentage of plasmid labeling detected as slowly-migrating continuous DNA strands, as seen for pSVOD in Fig 10. This supports the notion that the hsi labeling in Fig 10 represents the formation of rolling circles seen as sigma forms. Sixty per cent of scored sigma structures possess tails which are longer than plasmid monomer length, further indicating that they are rolling circles (table 1B). Table 1B may underestimate the number of sigma form molecules with tails less than one monomer length since many such molecules may not be easily visualized or may branch migrate prior to spreading. If so, the preponderance of sigma form could be at one monomer length or less, which agrees with figure 10.

The structure of the sigma-form molecules strongly indicates that they are replication intermediates. Approximately 50% of such molecules possess a short single-strand segment linking the double- stranded tail to the circle. This would be expected if those tails represent lagging-strand DNA synthesis. The average length of such single-stranded segments viewed in our experiments was 0.8 kb. An example of a single- stranded segment on a sigma form of pSVod is shown in Fig 13.

Our observations are in general agreement with other reports that sigma forms are the predominant branched species after 2 hrs in the replication reaction(132, 209). Here we show definitively that they are rolling circles. As also observed by those workers, we find a higher percentage of rolling circle forms at longer incubation times.

Sigma structures have been observed in experiments using the topoisomerase I inhibitor, Camptothecin, which allows the enzyme to make single stranded breaks, but inhibits the resealing process thereby, producing nicks around the SV40 replication forks. However, in those experiments, no sigma structures were observed with tails longer than one monomer

Figure 13. Electron microscopic visualization of plasmid rolling circle intermediates that may have been derived from broken theta structures. Legend is the same as in figure 12.



plasmid, suggesting the derivation of the sigma structures from broken theta forms.

In our system, we observe a high percentage of sigma structures with short tails (that may correspond to broken theta forms) as well as molecules containing tails many times the size of monomer plasmid (figure 12 vs. figure 13), indicating an unidirectional DNA elongation process.

When compared at one incubation time, 2 hrs, a variety of conditions did not alter the percentage of replicating molecules observed as rolling circles. Examining pSVOD synthesis at 2 hrs, we varied dCTP concentration, temperature, presence or absence of an ATP- regenerating system and concentration of extract protein. We also examined synthesis on template plasmid that was primarily form II rather than form I. None of these conditions altered the ratio of sigma structures to theta structures observed by electron microscopy Thus increased rolling circle formation is not simply a function of degeneration in the replication system.

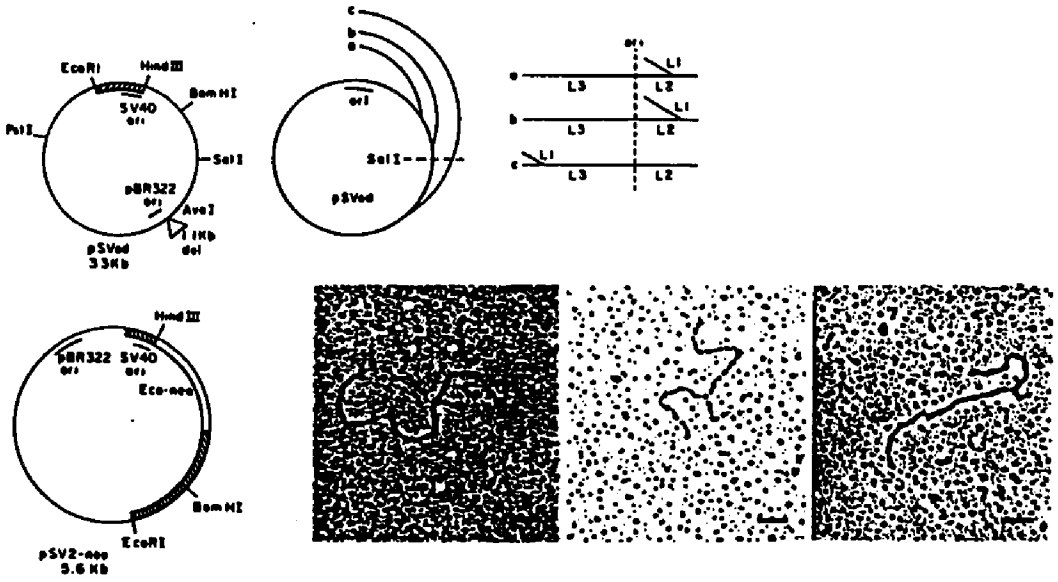
Rolling circles may be more readily visualized in vitro than are theta structures since theta structures are resolved in vitro after extending one length while rolling

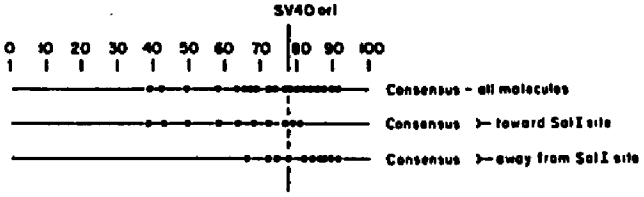
circles continue. A rapid resolution of theta structures is consistent with the relatively high labeling of closed circular forms of DNA (c and ccc) seen in Fig 10.

Bjursell has developed methods for mapping initiation of unidirectional DNA synthesis on plasmid circles (18, 19). He reported that free ends of polyoma rolling circles in mouse cells mapped to the viral origin of replication. Using the same electron microscopic mapping methodology we find that free ends generated on plasmid molecules in vitro map to the SV40 origin. Results can be described as follows (figure 14). When replicating molecules are cleaved with a restriction enzyme, and if the replication fork of a given newly-initiated molecule has not passed the restriction site once, fragments will be generated that possess three unequal arms, two of which (L1+L2) add up to the plasmid circumference and the third of which (L3) is the tail in its early stage.

We cleaved pSVOD, replicated in vitro for 2 hrs, with either Sal I or Pst I and selected molecules for analysis which had one parental arm >80% of plasmid length. This value was used because Sal I and Pst I each cleave approximately 20% of the circumference on either side of the SV40 origin.

Figure 14. Mapping of rolling circle initiation sites by restriction cleavage of replication intermediates and electron microscopy. Aliquots of plasmid DNAs were subjected to in vitro conditions as described in methods, purified and treated with restriction endonuclease SalI or PstI and spread for electron microscopy. L1= the longer arm of the parental strand. L2= the shorter arm of the parental strand. L3= the tail. Cleavage produces Y-shaped molecules. If the replication fork has not passed the cleavage site, Y-shaped molecules with three unequal arms are produced.


EcoRI
HindIII
BamHI
SV40 ori
PstI
SalI
pBR322 ori
AvaI
1 Kb del
pSVod 3.3Kb
c
b
a
ori
SalI
pSVod
ori
L1
a
L3
L2
L1
b
L3
L2
L1
c
L3
L2
HindIII
pBR322 ori
SV40 ori
Eco-neo
BamHI
EcoRI
pSV2-neo 5.6 Kb


SV40 ori
0 10 20 30 40 50 60 70 80 90 100
Consensus - all molecules
Consensus ⟩— toward SalI site
Consensus ⟩— away from SalI site

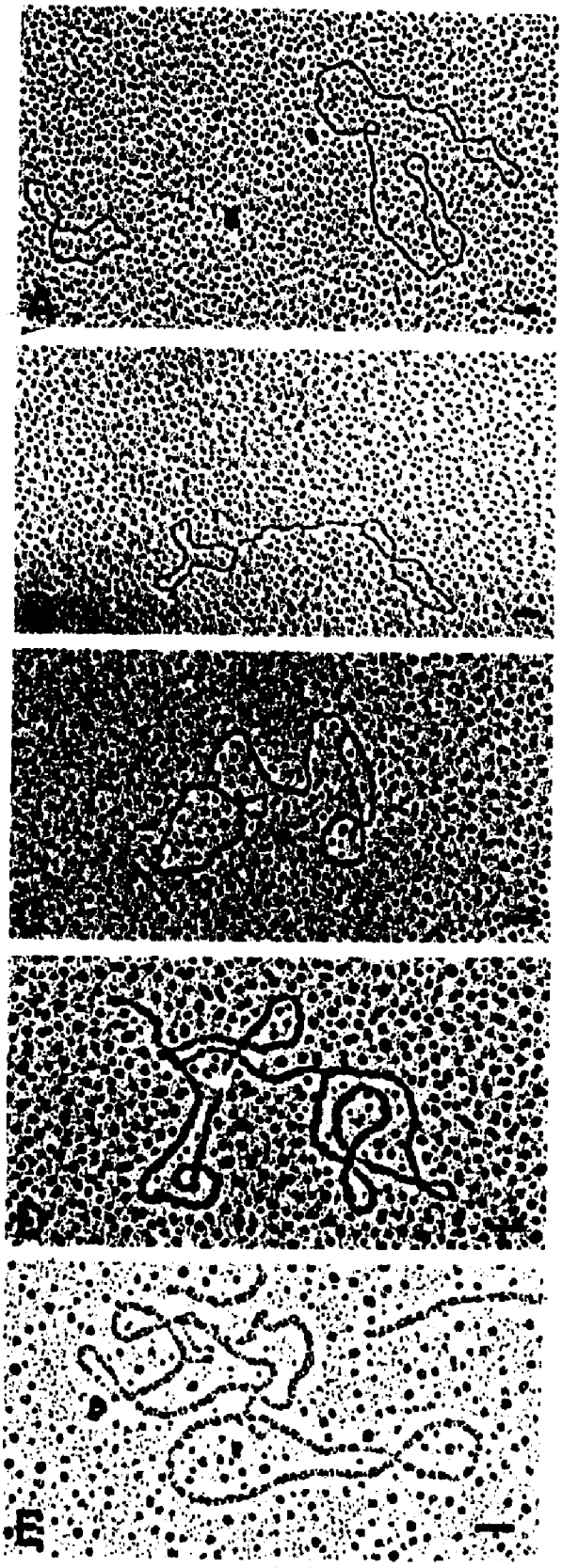
Note that this selection procedure does not exclude molecules from analysis that may have initiated anywhere on the plasmid circle. The selection does insure that if any replication forks have initiated at the SV40 origin, they are progressing along the short arc toward the given restriction site. We plotted correlations of L3 length with L1 length for 16 such molecules cleaved with Sal I. The regression line for these points had a slope of 1.11 and extrapolated to an initiation point corresponding to the SV40 origin (L1 = 80.2%, L2 = 19.8%). The correlation coefficient for the points was 0.88. Very similar results were obtained when replicating pSV0D was cleaved with restriction enzyme Pst I.

This indicates that forks initiating at the SV40 origin may progress either to the Sal I site or to the Pst I site. We wanted to be sure that the selection procedure described by Bjursell did not in our case prefer some aberrant class of molecules, i.e., intermediates with stalled forks. (Note that there is no evidence in the hsi I labeling of Fig 10 for such aberrant molecules.) Therefore, we additionally analyzed 10 molecules whose branch points were >20% of the plasmid length from an end.

Plotting of potential origins showed clustering about the SV40 origin, assuming progression in either direction, for these molecules. A similar analysis was performed, with similar mapping results, using replicating pSV2-neo cleaved with Bam HI. Our data indicate that the free ends of plasmid rolling circles, as do those of SV40 viral DNA, map to the SV40 origin and that such replication, once initiated, can proceed in either direction around the circle.

A rolling-circle mechanism generates dual circular plasmid molecule interconnected by a DNA bridge. When plasmids pSVOD or pSV2-neo are spread from in vitro replication mixtures, molecules can be seen consisting of two plasmid circles connected by one double-stranded DNA bridge. Several such structures are presented in Fig 15. The plasmid dual circles could represent recombination intermediates involving two separate molecules. Alternatively, they could represent a stage in the resolution of rolling-circle replication of one plasmid. Analysis of 20 such pSVOD dual circles shows that average bridge length is 3.9 kb, and 30% have DNA duplex bridges longer than 3.3 kb, monomer plasmid length. It is thus likely that the dual circles seen involve

Figure 15. Generation of dual circular plasmid molecules interconnected by a DNA bridge. Plasmids pSVOD or pSV2neo were subjected to in vitro replication conditions and spread for electron microscopy as described in methods. A-C: dual circular pSVOD molecules. D, E: dual circular pSV2neo molecules. The bars= 100 nm.


E

at least one circular molecule replicating through a rolling-circle mechanism. If the formation of a second circle represented some stage in the resolution of replication of one plasmid, one would expect that the second circle would not necessarily be of monomer size but that circles would be distributed among monomer, dimer, trimer, etc. Of the 20 dual pSVOD circles analyzed, not one involves a circle of larger than monomer size. These observations further indicate that the bridge between the dual circles is elongated via a rolling- circle mechanism.

We performed further experiments to determine whether only one or both circles of the dual structures are elongating the strand between them via a rolling circle. For this we incubated the plasmid DNA from replication mixtures under conditions that promote branch migration . Figures 15 and 16 show molecules in which migration has produced branches at junctions of circles. This is in effect a "re-rolling" of the rolling circle. (Branch migration of rolling circles can produce structures ambiguous for E.M. For our studies all structures scored as branch-migrated plasmids contain circles that deviate <10% from expected plasmid size).

Figure 16. Heterogeneous dual circular recombination forms involving plasmid molecules of different sizes. Aliquots of pSVOD and pSV2neo DNAs were added together and subjected to in vitro replication conditions for 4 hours and spread for electron microscopy. Arrows indicate junctions between the connecting bridge and circular plasmid DNA. The bars= 100nm.

A
pSV2-neo
pSVod
B
pSVod
C
pSVod
SV40
D
SV40
pSVod
E
SV40
pSVod
F
SV40
pSVod
G
SV40
pSVod

We found that branches protrude from forks at both circles of dual structures in more than 80% of such molecules recorded. Therefore, in most cases both circles are replicating to elongate the strand between them.

Next we tested the possibility that the observed dual circles are recombination forms involving two initially separate plasmid molecules. This was done by subjecting mixtures of different plasmids to in vitro replication and analyzing resulting replication intermediates by electron microscopy and gel mobility shift assays.

We also analyzed replicating mixtures of pSVOD and SV40 DNA. We find that dual circles can be formed consisting of one each of two different circular DNA molecules connected by a DNA duplex bridge. Such heterogeneous dual circles comprising either plasmids pSVOD and pSV2-neo or pSVOD and SV40 are shown in Fig 16. To see whether heterogeneous dual circle formation involves only homologous plasmids ,we tested several different plasmid mixtures as analyzed in Table 2. Plasmids pML-2 and pBR322, containing no SV40 origin, do not form dual circles. When plasmids pML-2 and pSV2-neo are incubated together, no heterogeneous dual circles are seen

Table 2. Dual circular recombination forms generated by co-incubation of different plasmids. Aliquots of DNA of the indicated plasmids were incubated together in the replication mixture. The purified products were spread for electron microscopy as in methods.

a. 6000 random plasmid molecules were scored including each plasmid type. Only molecules containing clearly identifiable circles were scored. Replicating forms were classed as single molecules.

b. Frequencies are based on the total number of molecules of all types.

c. Plasmid pSVOD was subjected to replication mixture. The purified products, plus equal amounts of plasmid pSV2neo, was incubated in a second replication mixture in the presence of 0.1 mM aphidicolin. The purified products were spread for electron microscopy as in methods.

DUAL CIRCULAR RECOMBINATION FORMS GENERATED BY COINCUBATION OF DIFFERENT PLASMIDS

	MOLECULAR FREQUENCY[a] × 10^3		
	HOMOGENEOUS DUAL CIRCLES[b]		HETEROGENEOUS DUAL CIRCLES[c]
MOL 1 MOL 2	MOL 1	MOL 2	
pML2 + pBR322	0	0	0
pML2 + pSV2–neo	0	6.0	0
pSVod + BLUR8	7.0	1.0	0
pSVod + pSV2–neo	8.0	6.0	1.5
(pSVod–2 hrs) + [(pSV2–neo) + aphidicolin]	7.5	0	0.8

and no circles involving pML-2 alone, although pSV2-neo molecules are seen as dual circles at a frequency of about 6×10^{-3}. In mixtures of plasmids pSVOD and pSV2-neo dual circles are seen at a frequency of 1.4×10^{-2} while heterogeneous dual circles are seen at 1.5×10^{-3}. There thus appears to be a preference for homology in dual circle formation. However, homologies between bacterial plasmid sequences do not contribute significantly to this formation.

These observations do not rule out the possibility that dual circles could be formed between plasmids containing non-homologous inserts at levels too low to be detected visually, but they do indicate that plasmids containing the SV40 origin recombine to form heterogeneous dual circles most readily.

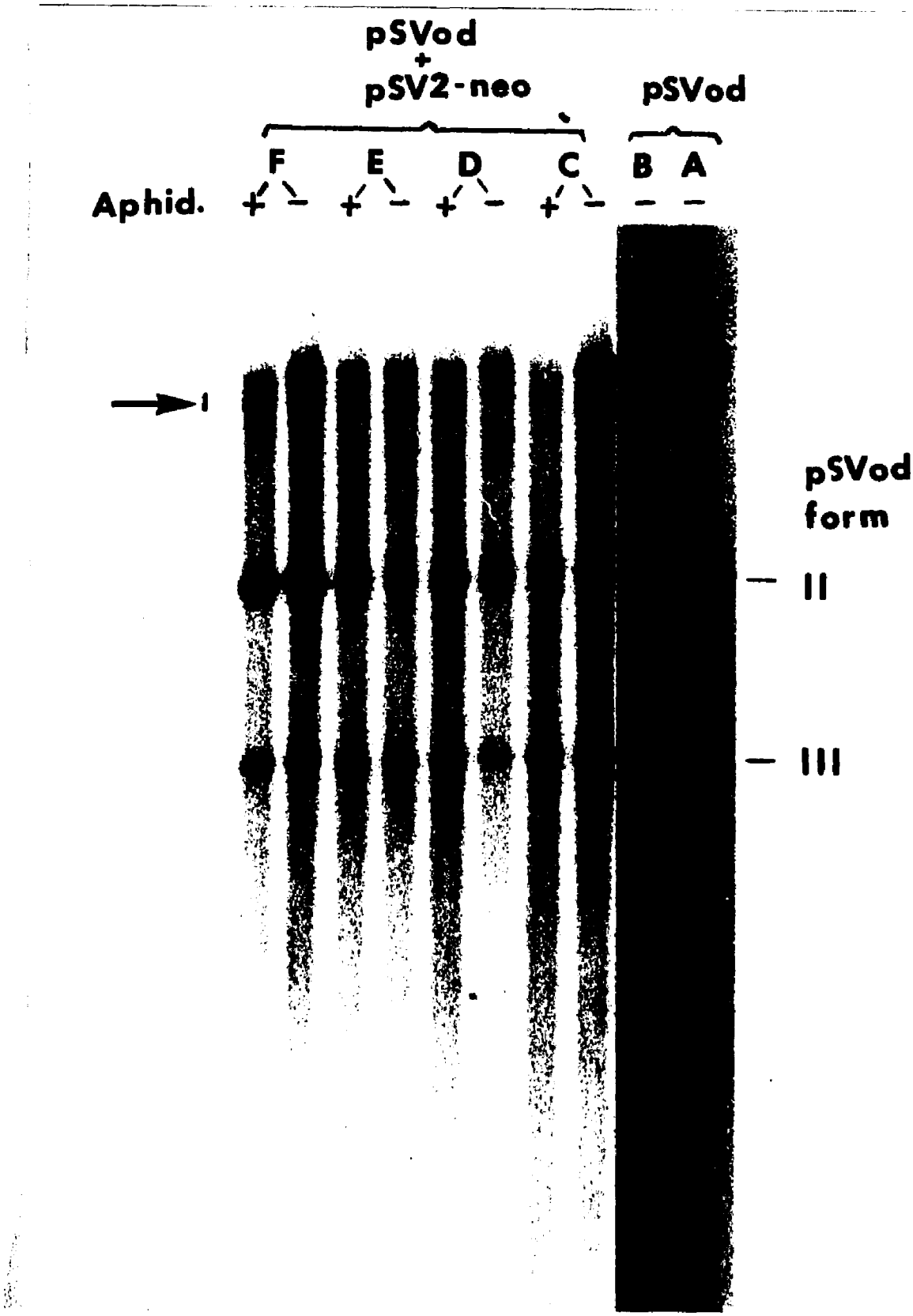
Gel mobility shift assay was used for detection of recombination intermediates. Dual circle forms could arise through synapses of a rolling circle free end on another plasmid circle opening for DNA replication. This mechanism would involve homologous base pairing at or near the origin of SV40 DNA replication. We examined conditions where mixtures of different plasmids were incubated in the presence or

absence of DNA synthesis to study any replication related recombination using aphidicolin. Aphidicolin is an inhibitor of polymerase alpha and competes with dCTP and suppresses DNA elongation. However, it does not affect the primase activity, resulting in accumulation of DNA molecules containing small bubbles of 600 bp or less (52).

Figure 17 suggest dependency of dual circle formation on DNA synthesis with involvement of short DNA segments of the replication bubbles. First we incubated plasmid pSVOD (3.3kb) in a replication mixture containing dCTP only in radioactive form to generate labeled replication intermediates and rolling circles. These were then incubated with plasmid pSV2neo (5.6) in a second reaction in the presence or absence of DNA synthesis to visualize any replication mediated recombination that occurs by the labeled molecules (however, we would not see any replication or recombination intermediates formed by pSV2neo or unlabeled pSVOD which constitute most of the input templates).

Lane A shows labeled replication intermediates of pSVOD in the presence of 0.5 um $dCT^{32}P$ after 2 hours of incubations (topoisomers are seen in darker exposures). These

Figure 17. Detection of the recombination intermediates between pSVOD and pSV2neo by gel mobility shift. The plasmid pSVOD was incubated in an in vitro replication mixture containing dCTP only in radioactive form to maximize the production of labeled replication intermediates rather than complete replication products. These were then incubated with plasmid pSV2neo in a replication reaction containing no labeled deoxynucleotides, in the presence or absence of DNA synthesis inhibitor, Aphidicolin to visualize any replication mediated recombination between the two plasmids.


pSVod
+
pSV2-neo
pSVod
F
E
D
C
B
A
Aphid.
+
−
+
−
+
−
+
−
−
−
I
pSVod
form
II
III

intermediates are then re-incubated in the presence (lanes C-F) or absence (lane B) of pSV2neo in a new reaction mixtures without any labeled deoxyCTP for 2 hours at 37 C. Lane B shows the replication of labeled molecules generated in lane A.

Lane C shows co-incubation of labeled pSVOD (lane A) and pSV2neo, where prominent labeling is at a new band which is caused by addition of pSV2neo since it is not present in lanes A or B. Lane C+ is similar to lane C- but includes inhibitory amounts of aphidicolin. Lanes D- and D+ are co-incubations of the two plasmids, which have been pre-incubated separately in the in vitro system ,in the presence and absence of aphidicolin, respectively. Comparing lanes C with D indicate a necessity for DNA synthesis (polymerase alpha activity) of the plasmids for production of dual circles. Lanes E through F suggest that preincubation of only one of the templates is sufficient for recombination intermediate formation in the presence of aphidicolin Lanes E- and E+ show co-incubation of pSV2neo with pre-incubated pSVOD (lane B) and lanes F- and F+ show co-incubation of pSVod with pre-incubated pSV2neo, in the absence or presence of aphidicolin, respectively.

These results suggest that in vitro SV40 DNA synthesis produces DNA molecules that can recombine with one another through a polymerase alpha independent mechanism.

Visualization by electron microscopy confirmed these results. We incubated plasmid pSVOD in the in vitro replication mixture for two hours to generate rolling circles. We then purified and precipitated the pSVOD DNA and added to it plasmid pSV2-neo circles in another aliquot of COS7 cell extract with added aphidicolin. After further two hours of incubation, we isolated and spread the DNA for electron microscopy. To insure stastical significance of recorded observations, at least 6,000 circular molecules were examined for each sample. (That number represents the contents of 1-2 grids, and their distinct shape facilitates dual circle identification).

Results are presented in table 2. Heterogeneous dual circles are seen although pSV2-neo homogeneous dual circles are not. This indicates that our extract catalyzes strand exchange between the free end of a pSVOD rolling circle and an intact pSV2-neo circular plasmid in a polymerase a independent step. Similar results were detected when either

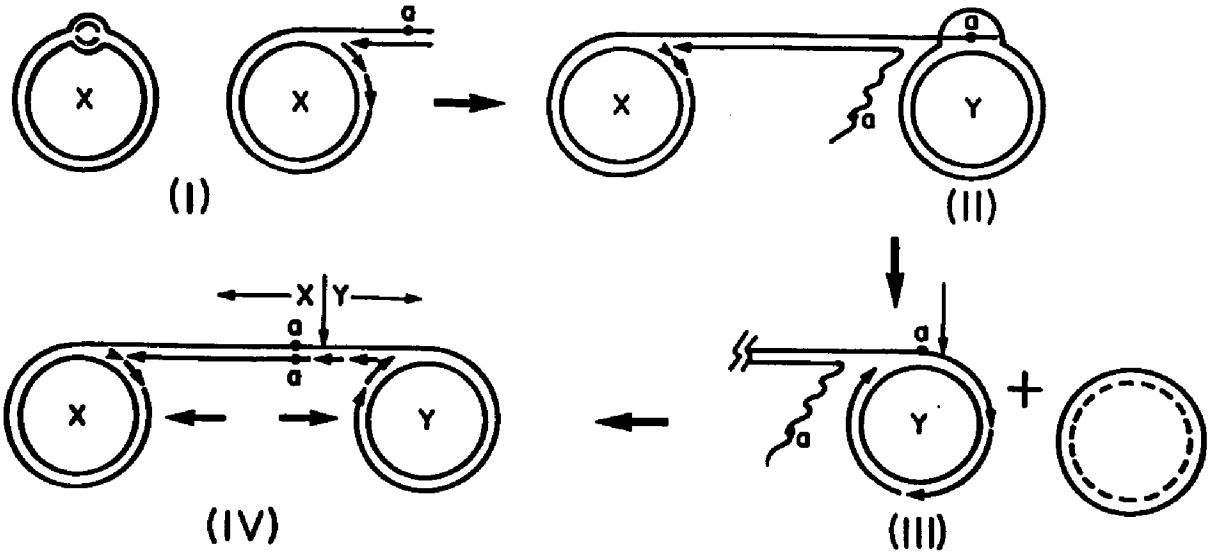
plasmids was co-incubated with SV40 DNA.

The results in tables 1 and 2 and figures 15 and 16 indicate a replication mediated strand transfer between plasmids containing the SV40 origin sequences. The structure of the resulting recombinant molecules suggest the insertion of rolling circle tails onto homologous sequences of a second replicating molecule.

Figure 18 depicts a simple model ,where, strand transfer by a single stranded tail of a newly replication initiated sigma molecule to complimentary single strands of a nascent bubbles (a D loop). After the transfer, the rolling circles can continue DNA synthesis elongating the bridge between the two molecules. Continuation of the bi-directional replication, however, would yield theta structures connected to a sigma molecule .

This mechanism may involve strand invasion and strand displacement resulting in an excluded DNA strand. The structures visualized by electron microscopy also display duplex branches, which could form from the excluded strands.

Figure 18 . A mechanism for formation of dual circular recombination forms involving strand displacement by a rolling circle free end. I, Initiation of DNA synthesis on plasmid molecule X and formation of a rolling circle free end. II, Strand invasion of plasmid molecule Y by the free end of molecule X (displacement of the 5' end is depicted here). III, Continuation of DNA synthesis and release of a circular molecule of Y. Extension of the interconnecting DNA bridge by rolling circle DNA synthesis in both directions. A point on plasmid molecule X is indicated by (a). The recombination junction is indicated by a vertical arrow.


X
X
a
X
a
Y
(I)
(II)
X
Y
a
a
X
Y
a
Y
+
(IV)
(III)

In vitro replication of eukaryotic sequences

Plasmid BLUR8 is a pBR322 derivative containing 265 bp human Alu family insert (106). The insert has two segments that share homology to a number of origin sequences including that of SV40. A number of SV40 T antigen binding sites are present in this sequence and have been shown to bind to the protein (7). T antigen is a multifunctional protein that binds near the SV40 origin of replication and with a DNA helicase activity initiates SV40 DNA replication(49).

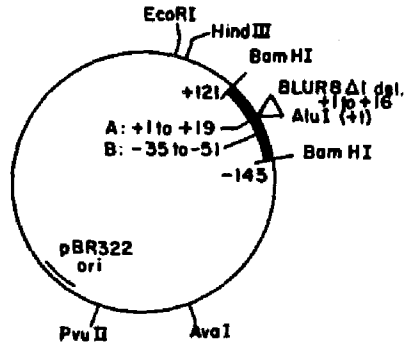
We studied the replication of plasmid BLUR8 in cellular extracts of monkey cells supplemented with purified T antigen.

Figure 19 shows the restriction map of map of BLUR8 as well as the homologous sequences to other origin sequences. The preserved consensus sequences between these origins and ALU family repeats, further widely dispersion of these sequences in the eukaryotic genomes suggest the close involvement of ALU repeats in the chromosomal DNA

Figure 19. Blur 8 map: the restriction map of BLUR 8 and its homology in A and B regions to other origin sequences.

REGION A:

	-50 -40	
BLUR 8 Alu	CTGAAAATACAAAAATT	
	20	
SV40 ori A-T domain	AAATAAAAAAAATT	11/14
Yeast ARS consensus	AAAATACAAAT	10/11
Tet. rDNA ori	AAAAAACAAAAAT	12/13
Phy. rDNA C.S.	CTGAAAAAAT	8/9



BLUR 8
4.6Kb

REGION B:

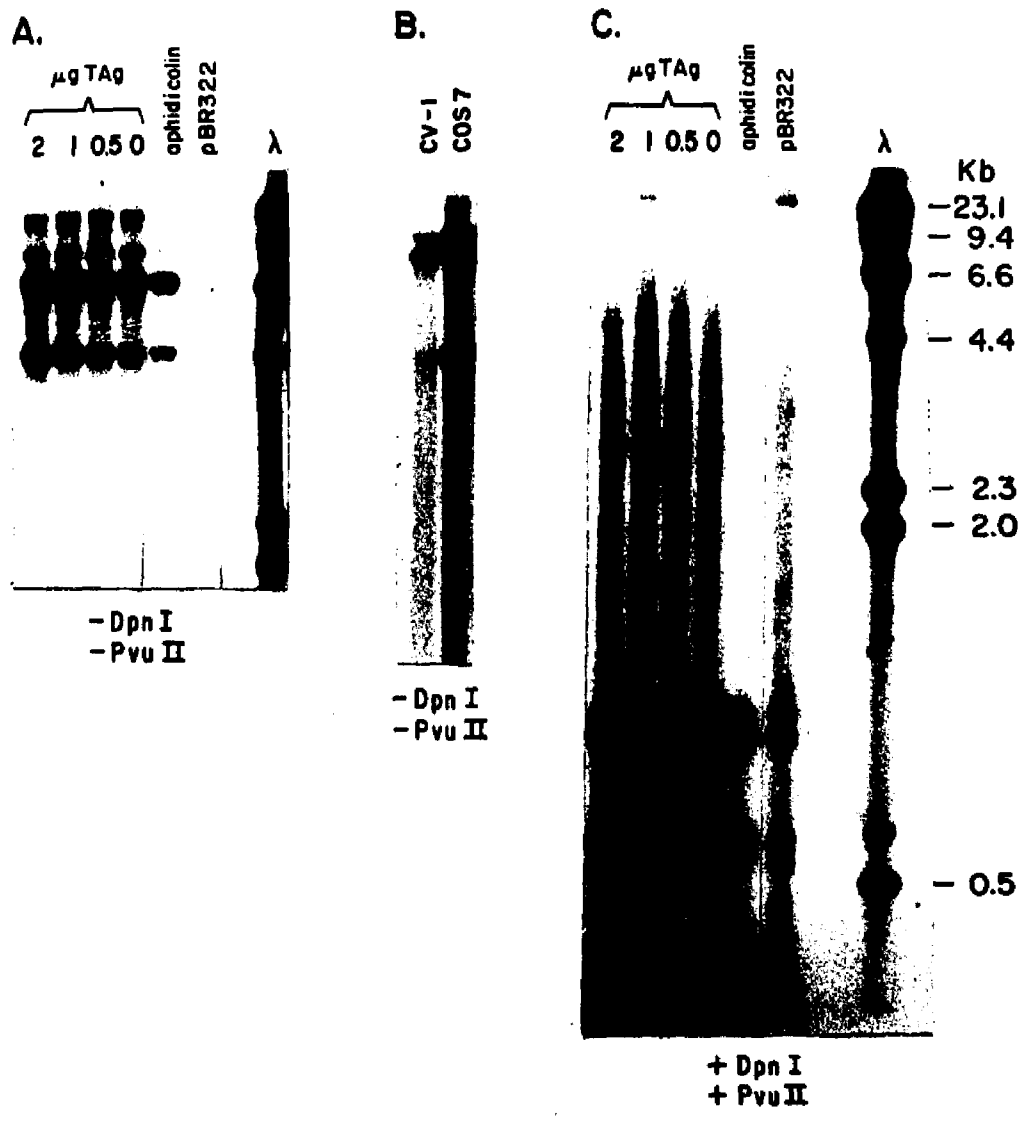
	+1 +10	
BLUR 8 Alu	CTACTTAGGAGGCTGAGAC	
	5215 5220	
SV40 early palindrome	CTACTTCTGGAA	6/6
	5235 5240	
SV40 TAg binding	GAGGCCGAGGC	9/11
Tet. rDNA ori	ACTTAGAGAA	6/6
Tet. TopoI consensus	ACTTAGAGAA	6/6

replication.

Figure 20.I A shows the extensive labeling of BLUR8 in COS7 cell extracts with varied concentrations of added T antigen. In comparison to pSVOD similar amounts of labeled dCTP is incorporated into BLUR8 . However, after DpnI digestion, only a small percentage of BLUR8 is detected as fully replicated monomer size molecules (figure 20.IC). In the absence of DpnI Figure 20.IA, replication intermediates such as catenated dimers or theta molecules and replication products such as monomeric topoisomers are detected, indication of DNA synthesis labeling. These are further inhibited with Aphidicolin, a polymerase alpha (and delta) inhibitor, confirming the replication mediated labeling.

Furthermore , this replication is dependent on the SV40 T antigen , peaking at 0.5-1.0 ug / reaction. Some labeling is observed in COS7 cells (that produce T antigen constitutively) and increases with additional purified T antigen. Control experiments using CV1 or HL60 extracts without added T antigen, yielded only the background labeling observed with control plasmids, pML2 and pBR322 (figure 20.I B). Furthermore, a 16 bp deletion from the BLUR8

Figure 20.I A) In vitro replication of BLUR8, B) comparison of CV1 and COS7 extracts on BLUR8 replication and C) DpnI digestion of BLUR8. BLUR8 was labeled in the replication mixture in the absence or presence of T antigen. Aliquots of the same reactions were linearized before digestion with DpnI. Aphidicolin was added to 0.1 mM. The marker is lambda DNA cut with HindIII.


A.
μg TAg
2 1 0.5 0
aphidicolin
pBR322
λ
- Dpn I
- Pvu II
B.
CV-1
COS 7
- Dpn I
- Pvu II
C.
μg TAg
2 1 0.5 0
aphidicolin
pBR322
λ
Kb
23.1
9.4
6.6
4.4
2.3
2.0
0.5
+ Dpn I
+ Pvu II

insert in the T antigen binding sites eliminated the in vitro labeling, confirming the results obtained in vivo (7, 108).

The majority of the labeled BLUR8 is however, sensitive to DpnI digestion as shown in figure 20.IC. We attribute the BLUR8 labeling to the following possibilities :

I. DpnI sensitive , labeled molecules ,may arise from elongation of DNA molecules primed by spuriously annealing RNA transcripts similar to the ones observed in yeast cells. Alu transcripts are present as small RNA molecules with unknown functions but have been suggested to be used as primers for cellular DNA synthesis. Their bindings to multiple "origin" sequences in the plasmid and further elongation by the replicase machinery, would yield segments of the plasmids containing newly synthesized labeled fragments. Since the plasmid contains many DpnI sites (>10), it is likely that the DNA molecules ,labeled by this method, remain DpnI sensitive. Preliminary experiments, using RNase A and RNase H , to prevent annealing of the ALU transcripts to the template plasmid, failed to prevent labeling. Thus, this result may rule out the above possibility in the labeling of plasmid BLUR8 (71).

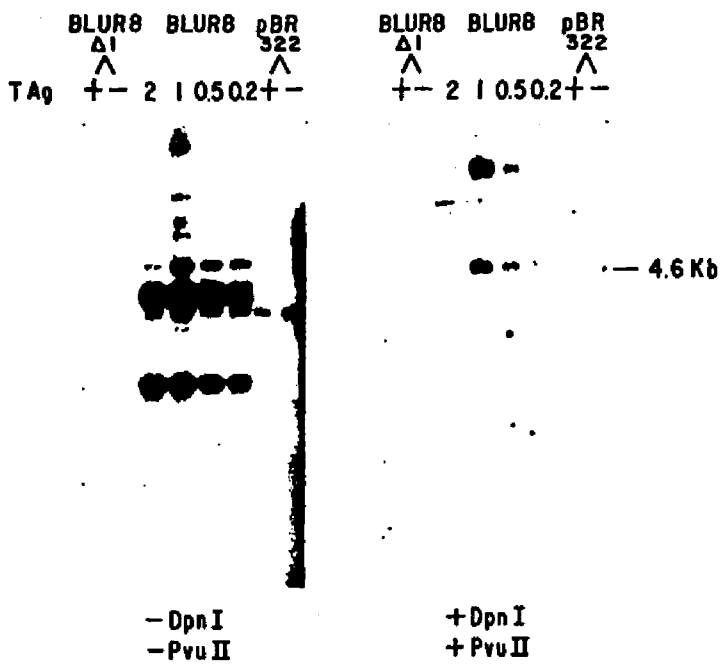
II. A second mechanism would involve sensitivity of hemimethylated plasmids (one round of replication) to DpnI digestion. It has been observed that salt concentrations of less than 200 mM in the DpnI digestion mixture, increases the sensitivity of hemimethylated DNA molecules to this enzyme (personal observations and 209). This possibility was tested in figure 20.II under similar conditions as figure 20.I except that salt concentrations of higher than 200 mM were used in DpnI digestions. A several fold increase in the DpnI resistance BLUR 8 linear form is detected. Control plasmids, pBR322 and BLUR8 delta-1 , show no replication as expected.

This may indicate that most of the ,DpnI sensitive, labeled BLUR8 molecules in figure 20.I may have gone through one round of DNA replication, resulting in hemimethylated plasmid molecules. This is further tested by two dimensional gel electrophoresis and electron microscopy.

Repair labeling by editing functions of polymerase delta, mismatch repairs by polymerase beta, or otherwise topoisomerase-replicase labeling similar to the ones observed ,at lesser extends, in control plasmids, also can contribute to BLUR8 labeling.

Figure 20.II. In vitro replication of plasmids BLUR 8 and BLUR 8 delta-1. The plasmids were incubated in the replication mixture as in figure 20.I. The purified products were linearized with Pvu2 prior to digestion with DpnI in a high salt buffer as described in the methods.

Deletion of 15bp Abolishes BLUR8 Labeling



To determine the types and amounts of labeling of replication or repair intermediates , we subjected labeled BLUR8 DNA to a series of two dimensional agarose gels and electron microscopy.

Figure 21 A shows a neutral - alkaline gel electrophoresis of labeled BLUR8. The plasmid was labeled in the replication mixture and the purified products were run in a first neutral dimension. The lane corresponding to BLUR8 was excised and submerged into an alkaline gel and run in a second alkaline dimension as described in the methods. Products of bi-directional DNA synthesis are shown as monomeric topoisomers in the first dimension . when denatured these run the positions of CCC , denatured covalently closed double stranded circles; l, linear single stranded DNAs and C, circular single stranded molecules. d, includes catenated dimers, and sigma structures. Dark spots indicate denatured, covalently closed catenated dimers, as well as nicked catenated dimers that separate under alkaline conditions and run at the monomer size right below.

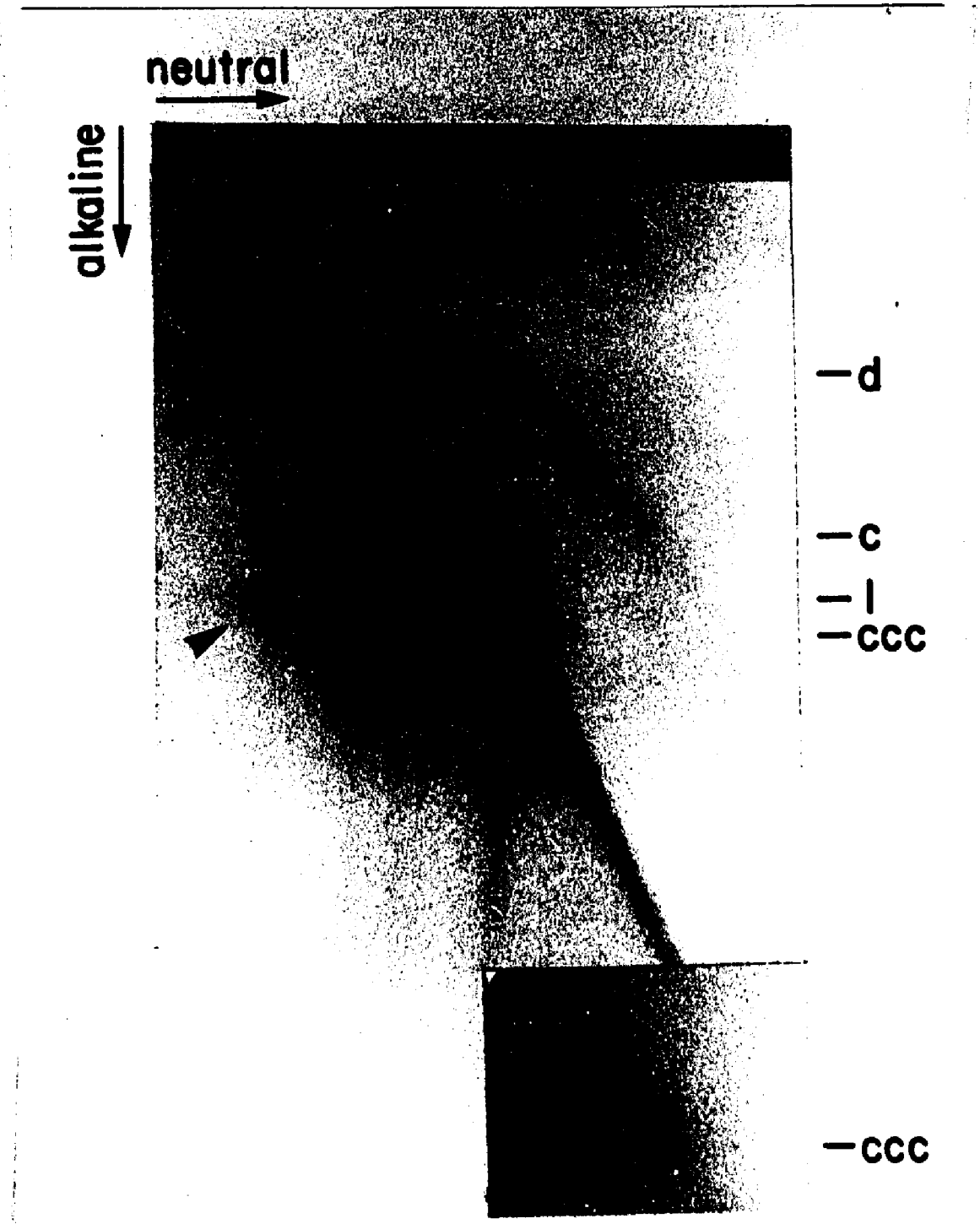
To further indicate that the labeled molecules are completely replicated, another neutral-alkaline gel was made

as follows : BLUR8 was labeled and run in the first dimension. The lane corresponding to the plasmid was excised and digested with excess Pvu2 to linearize the molecules inside the gel. This was run in a second alkaline condition. Figure 21 B shows the result . Completely replicated molecules appear at monomer linear size. Repair synthesis appears as vertical smears right below each DNA forms.

These results indicate the extensive replication of BLUR8 plasmid in the labeled forms of topoisomeric monomers and slow migrating products . Repair synthesis contributes minimally in this figure as vertical smears.

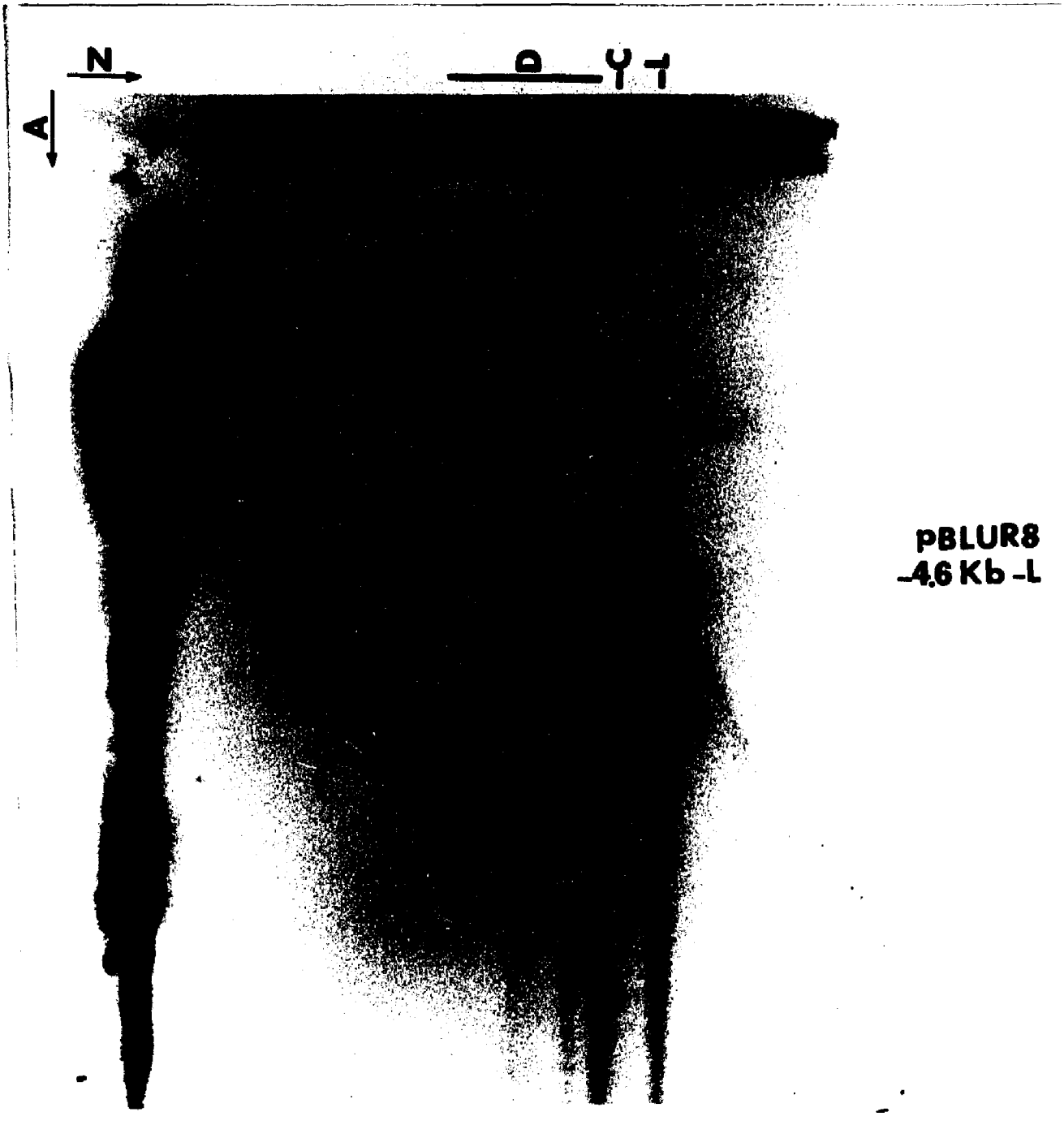
We conclude that most of the BLUR 8 molecules replicate only once leading to hemimethylated plasmids which are cleaved into small fragments by DpnI under the digestion conditions. Small amount of DpnI resistant bands present in figure 20 C, may therefore indicate multiple rounds of replication that result in unmethylation of the plasmid at the DpnI sites. Chromosomal replication normally occurs once in the S phase of the cell cycle under precise constraints, perhaps controlled by DNA modifications (such as methylation at specific sites). If Alu sequences are used as origins of DNA

Figure 21. A) 2-dimensional neutral-alkaline gel electrophoresis of BLUR8: plasmid BLUR8 was replicated in the reaction plus 0.5 Ug of T antigen and run in the first neutral dimension . The gel lane corresponding to BLUR8 was removed and placed in a second alkaline gel as described in the methods. CCC= denatured, covalently closed, double stranded circles; l= linear, single stranded DNA; C= circular, single stranded DNA; d= catenated dimers.



BLUR 8 2 hrs

Figure 21. B) 2-dimensional neutral-alkaline gel electrophoresis of BLUR8: Aliquots of the reaction from figure 21 A were run in a first neutral dimension and the BLUR8 lane was removed and digested with excess Pvu2 to linearize the plasmids inside the gel. Completely replicated molecules appear at the linear size. Repair synthesis is shown as a smear below each DNA forms. This DNA was then run in a second alkaline conditions. The marker is lambda-HindIII.


N
A
pBLUR8
-4.6 Kb -L

replication , they would be under these cellular control mechanisms which may also be present in the in vitro replication extracts that allow only one round of DNA replication to take place.

Clonal selection of autonomously replicating sequences

To characterize chromosomal regions that initiate DNA replication for DNA synthesis or gene amplification, we have developed a cloning method that selects chromosomal DNA sequences for their replication efficiency in cellular extracts, using an in vitro replication system plus a restriction endonuclease assay .

Endonuclease DpnI digests our vector plasmids at many sites (GmethATC) only if the adenosine residues are methylated at both strands. By propagating the plasmids in a Dam+ strain of E.Coli, these residues become methylated at both strands. Mammalian cells however, do not methylate this sequence upon each round of DNA replication, resulting to molecules which are resistant to DpnI digestion.

We subject plasmids containing eukaryotic chromosomal sequences to in vitro replication conditions and digest the products with excess DpnI. Unreplicated parental molecules are fragmented into small segments, leaving intact circular products of DNA replication. These replicated molecules are then propagated (methylated) in HB101 and are subjected to

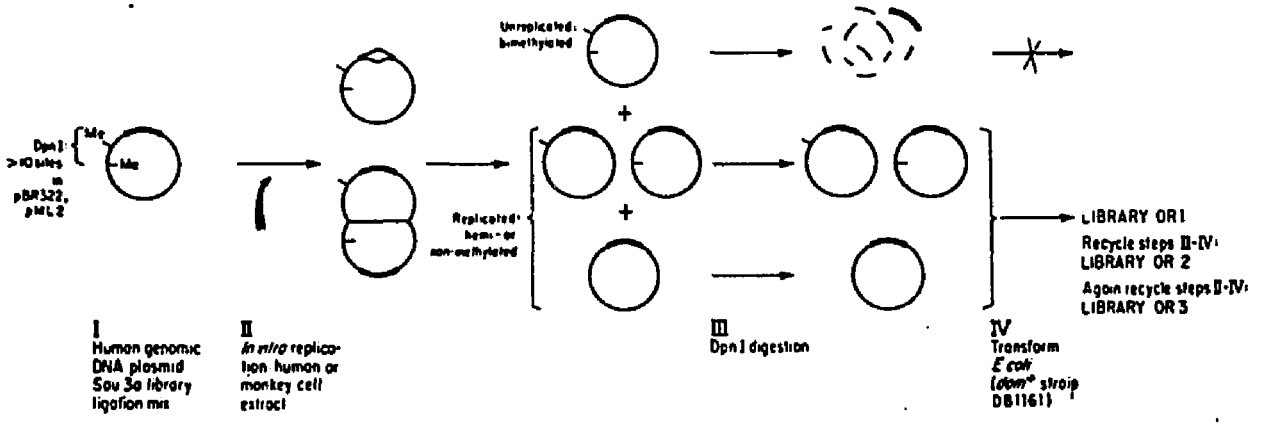
replication - digestion selection again. After several cycles, most efficiently replicating clones are isolated (figure 22).

Libraries of HL60 DNA are prepared by partially digesting purified HL60 DNA with the restriction endonuclease BamHI and inserting them onto BamHI site of plasmid pML2, a derivative of pBR322 from which sequences inhibitory to mammalian replication have been removed. The fragmented HL60 DNA is separated in a 1% agarose gel and bands corresponding from 0.5 to 4 kbps are cut out. The DNA fragments are electroeluted out of the gel and are ligated to pML2 plasmids as described in the methods. The library is then subjected to in vitro replication, DpnI digested and bacterial amplification as described in methods. to select the most efficiently replicating clones. Control experiments using plasmid pML2 plus library mixtures or pML2 alone in the replication assay showed no detectable pML2 clones at the end of the first cycle.

Four genomic libraries were isolated after three cycles of replication and named RO 1-4(replication origin). The insert sizes of the libraries range from 0.5->6 kbps (larger

Figure 22. Outlined clonal selection method: clonal selection of plasmids containing autonomously replicating sequences.

CLONAL SELECTION OF
HUMAN DNA SEQUENCES INITIATING REPLICATION *IN VITRO*

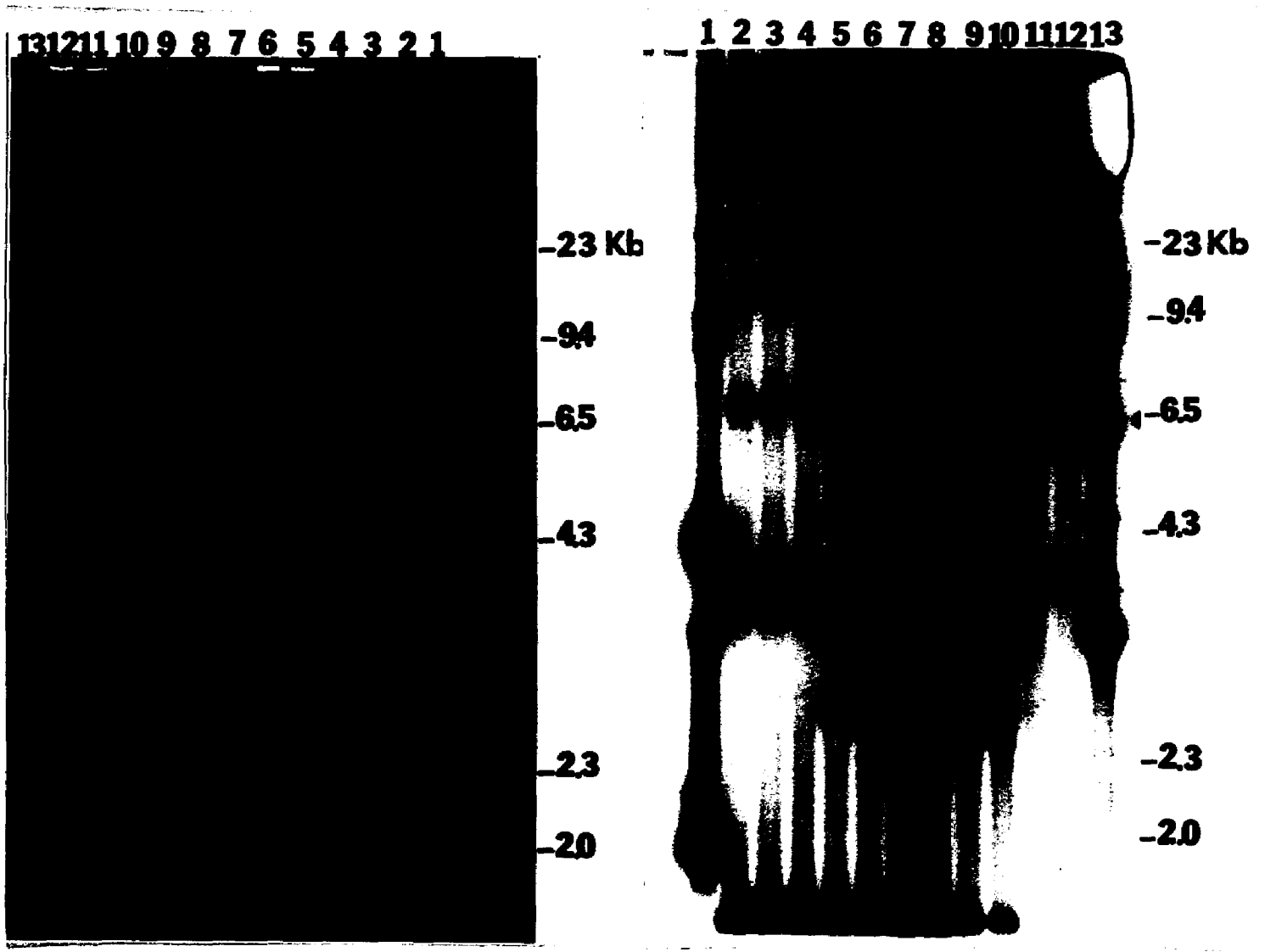


molecules are present but they may contain concatemers of smaller inserts) .

Figure 23 identifies the libraries by their hybridization to pML2 . The result indicates that the libraries seem to contain larger than average size inserts (mostly > 2 kbps) considering the sizes of 0.5-4kbps as the input genomic DNA fragments. This may be surprising since in DpnI digestion and bacterial amplification , smaller molecules have more survival advantage over the larger ones. Therefore, our results indicate the selection of larger inserts in the in vitro replication part of the replication-DpnI digestion-bacterial amplification cycle. This selection may arise from clones containing multiple origin sequences or one origin plus positively regulating elements which enhance replication in these extracts.

Auxiliary components that regulate core origin sequences are present in the genomes of animal viruses ,such as SV40 and polyoma ,that replicate autonomously using their hosts ' replication machinery. Similar components are also thought to be present in higher eukaryotic organisms(56). Further work is needed to bring down the sizes of the

Figure 23. Hybridization of plasmid pML2 to genomic libraries ORI 1, 2 and 3: libraries ORI 1, 2 and 3 were run in a 1.5% agarose gel and hybridized to plasmid pML2. A) gel picture; B) autoradiograph; lane 1, pM12; lanes 2-4, ORI 1 after another cycle of replication selection; lanes 5-7, ORI 1; lanes 8-10, ORI 2 ; lanes 11-13 ORI 3. Lanes 7, 10 and 13 were digested partially with EcoRI to linearized the plasmids.


13 12 11 10 9 8 7 6 5 4 3 2 1
-23 Kb
-9.4
-6.5
-4.3
-2.3
-2.0
1 2 3 4 5 6 7 8 9 10 11 12 13
-23 Kb
-9.4
-6.5
-4.3
-2.3
-2.0

A

B

131

inserts to core origin sequences.

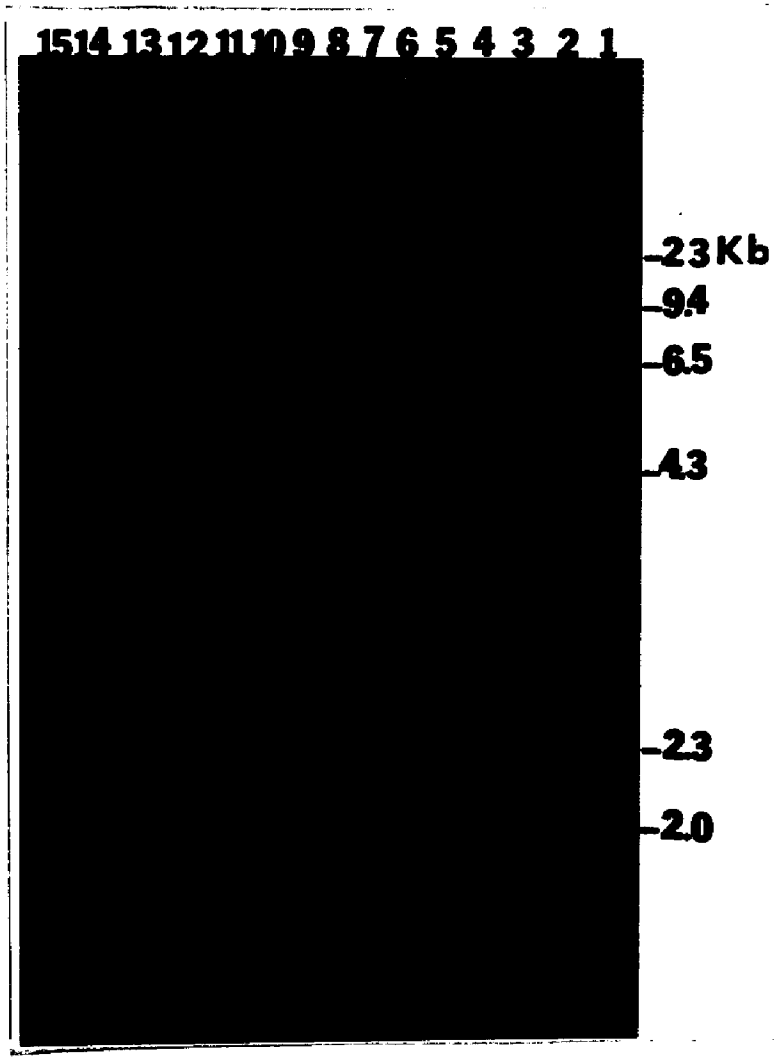
After three rounds of replication, two individual clones were selected from RO 1 and RO 4 libraries and used for further analysis (named pREP 1 and pREP 4 respectively).

PREP 1 has an insert of 2.3 kbp which slightly hybridizes to HL60 genomic DNA, indicating a low copy number of this sequence in the genome. However , it cross hybridizes extensively to many clones of libraries RO 1 and RO 3, suggesting a preferential selection of this sequence in the replication assays (Figure 24).

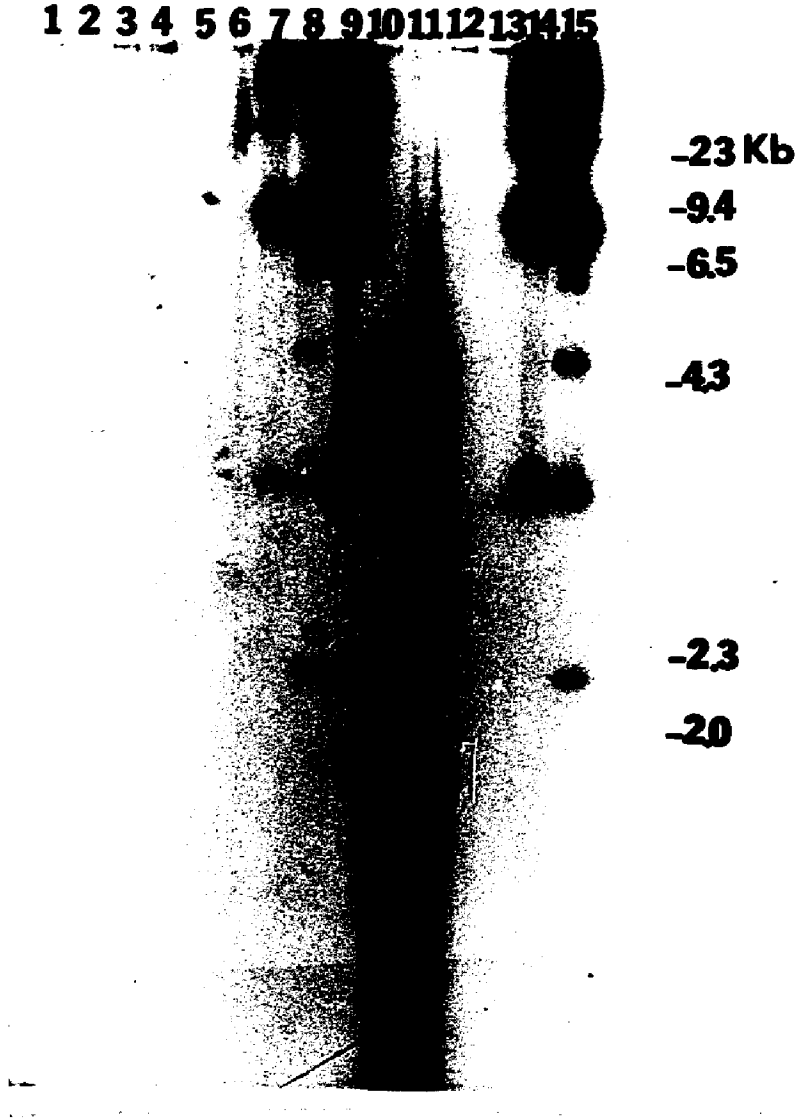
Alu sequences have been suggested to be good candidates for origin of replication in higher eukaryotes since their repetitious number in the genome is enough to account for the hypothesized number of genomic origins and further they share homologous sequences to the origins of some animal viruses, including SV40. Hybridization of an Alu probe (Dr. Jelinek of NYU) to the libraries RO 1, RO 2 and RO 3 indicate minute homology between the repetitive sequences and the genomic inserts.

PREP 4 has an insert of 2.0 kbp and shows extensive hybridization to genomic DNAs of monkey (COS1) and human

Figure 24. Hybridization of pREP1 to genomic libraries ORI 1, 2 and 3: The libraries and control DNA were run and hybridized to pREP1 insert as described in the methods. A) gel picture; B) autoradiograph. Lane 1, lambda-HindIII; lane 2, pBR322; lane 3, pML2; lane 4, BLUR8; lane 5, bacterial DNA (HB101) digested with BamHI; lane 6, HL60 genomic DNA cut with BamHI; lane 7, uncut ORI 1; lane 8, BamHI cut ORI 1; lane 9, uncut pREP1; lane 10, pREP1 cut with BamHI; lane 11, pREP1 cut with EcoRI; lane 12, uncut ORI 2; lane 13, BamHI cut ORI 2; lane 14, uncut ORI 3; lane 15, BamHI cut ORI 3. The marker is Lambda DNA cut with HindIII.


15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
23 Kb
9.4
6.5
4.3
2.3
2.0

A


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
23 Kb
9.4
6.5
4.3
2.3
2.0

B

(HeLa, HL60) cells.

Genomic distribution of pREP4. To see if the pREP4 sequences are dispersed through out the genome, or otherwise are spaced close to each other at specific locations, genomic DNAs of HL60, HeLa and COS1 cells were purified and digested with one of five different restriction endonucleases : BamHI(lane 1), BglI(lane 2), EcoRI (lane 3), PstI (lane 4) and Pvu2(lane 5). The DNAs are separated on 1% agarose gel and hybridize to the pREP 4 insert (figure 25).

At short exposures, a series of several prominent bands are visible in each lane, corresponding to oligomeric units present as tandemly repeated regions that are digested with the restriction endonucleases into single prominent bands. However, on longer exposures, DNA smears appear in each lane which indicate moderately dispersed flanking sequences. These results are summarized in table 3 showing the sizes of fragments at short exposures.

The results in figure 25 suggest that the pREP 4 insert is a moderately repeated sequence that is present mainly as oligomeric units however, at different chromosomal locations in the HL60, HeLa and COS1 genomes. Figure 25 also suggest

Figure 25. Genomic distributions of plasmid pREP4 insert: Genomic DNAs from COS1, HeLa and HL60 cells were purified and digested with five different enzymes, ran on a 1% agarose gel and hybridized to pREP4 insert. Lanes numbered 1 are digested with BamHI; lanes numbered 2 are digested with BglI; lanes numbered 3 are digested with EcoRI; lanes numbered 4 are digested with PstI; lanes numbered 5 are DNAs digested with Pvu2. The marker is Lambda DNA cut with HindIII.

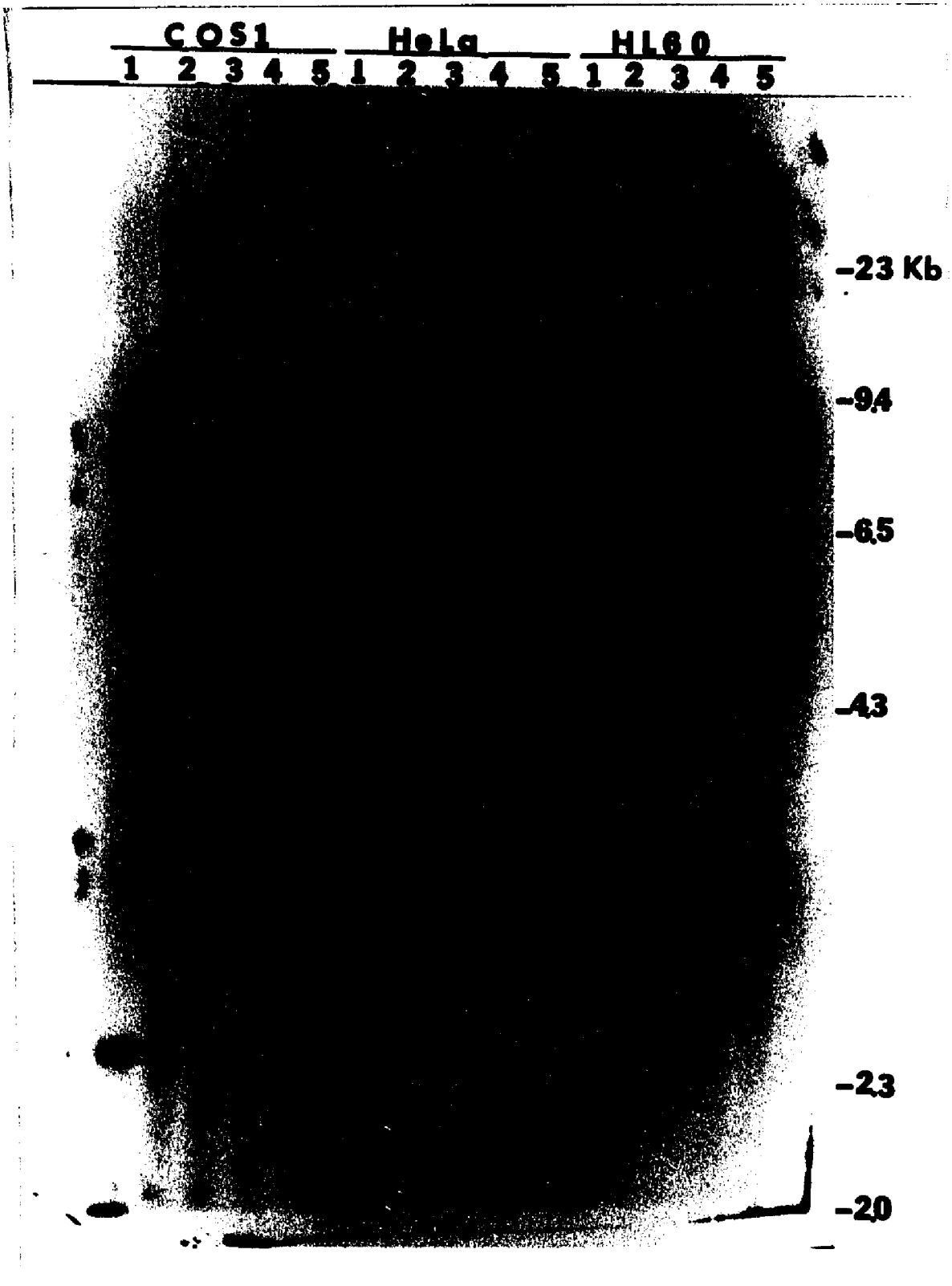

COS1
HeLa
HL60
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
-23 Kb
-9.4
-6.5
-4.3
-2.3
-2.0

Table 3. Genomic distributions of plasmid pREP 4. purified genomic DNAs from COS1, HeLa and HL60 cells were digested with one of five different restriction endonucleases, ran on a 1% agarose gel and hybridized to pREP 4 insert as in methods. Short exposures of the film revealed prominent bands as shown.

Restriction enzyme	genomic DNAs from cells		
	COS1	HeLa	HL60
BamHI	2.0, 2.5, 8	2.0, 2.5	2.0,3.3,7.0,8.0
BglI	3.8, 5.2, 8.0	3.8, 5.2, 8.0	3.6, 6.6, 8.0
EcoRI	3.0, 3.8, 5.2, 8.0	3.8, 5.2, 8.0	3.3, 3.8, 8.0
PstI	3.0, 3.3, 8.0	3.0, 8.0	3.2, 3.3, 8.0
Pvu2	8.0	8.0	3.3, 8.0

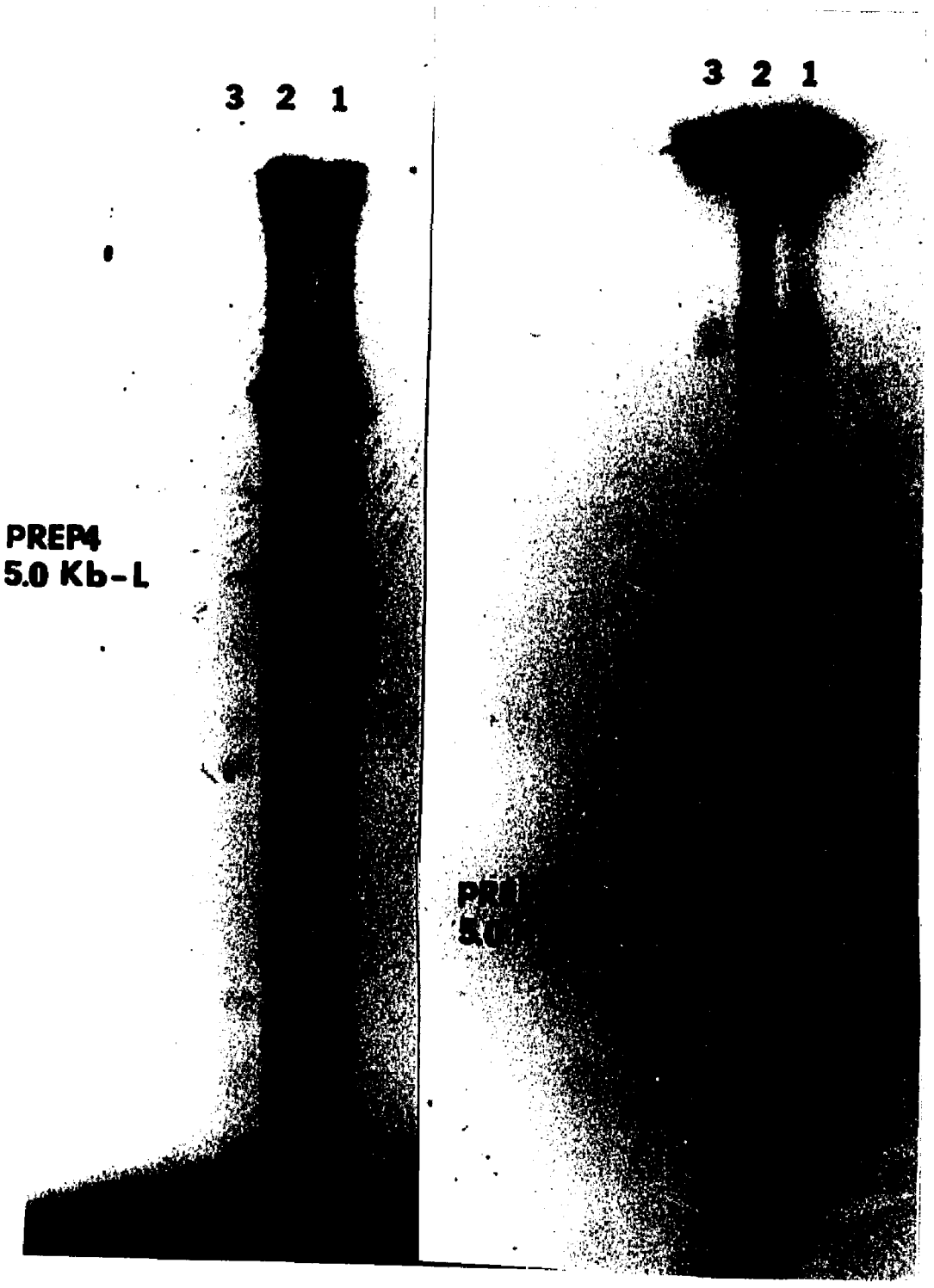
that pREP 4 locations in HL60 genome are different from COS1 and HeLa genomes which may be contributed to the facts that, HL60 cells are the donor cells for pREP 4 genomic insert and further express C-myc gene in high levels due to a chromosomal translocation . However , further sequencing of the insert could specifically relate this sequence to other replication elements such as oncogenes or viral origins.

Plasmid pREP 4 was further used as template for DNA replication experiments in cellular extracts and in transient transfections to test its replication efficiency in vitro and in vivo.

Figure 26 shows in vitro replication of pREP 4 in HL60 cellular extracts (A) with incubations of 40 minutes and 2 hours (lanes 1,2) or 2 hours plus inhibitory amounts of Aphidicolin (lane 3). Slowly - migrating replication intermediates and catenated dimers are visible as well as supercoiled monomeric size topoisomer end products of bi-directional fork movement.

This result is confirmed by DpnI assay (B) : the products of replication mixtures were purified and linearized by BamHI and then subjected to excess digestion with DpnI that

Figure 26. In vitro replication of pREP4: PREP4 was incubated in the replication mixture containing p^{32}dCTP plus or minus Aphidicolin.The gel is 1.5% agarose and the purified DNA was linearized with BamHl prior to digestion with DpnI. A) uncut replication products; B) BamHI and DpnI digestion of replication products. Lane 1, replication incubation of 40 minutes ; lane 2, replication incubation of 2 hours; lane 3, replication incubation of 2 hours plus inhibitory amounts of Aphidicolin (0.1 mM). L= linear size plasmid, 5.0 kb. Lambda-HindIII is run as the marker.


3 2 1
PREP4
5.0 Kb-L
3 2 1

cleaves unreplicated molecules to many small fragments. A prominent linear band (L= 5.0 kb) is visible in lanes 1 and 2 but not in lane 3, indicating polymerase alpha (and/or delta) dependent DNA synthesis.

Control pML2 ,without the insert , incorporates labeled deoxyCTP slightly which is further degraded into small fragments by DpnI, indicative of repair labeling (fig 8 B).

PREP4 replication was further studied by electron microscopy . It was incubated in the replication mixture and the purified products were spread for electron microscopy as described in the methods. Figure 27 , I) depicts molecules from subsequent stages of bi-directional DNA replication (A through D). Figure 27, II) shows broken theta structures resembling rolling circles (A, B, and C) ; D and E show plasmid molecules simultaneously initiating DNA replication at multiple sites.

PREP 4 was further transfected into human HeLa and HL60 cells to determine its ability to initiate replication in vivo. Figure 28 shows plasmid DNA isolated according to Hirt and purified prior to gel electrophoresis. Aliquots of the isolated DNAs were linearized with BamHI and digested with

Figure 27. Electron microscopic visualization of replicative pREP4. pREP4 was incubated in the replication mixture and the purified DNA was spread for electron microscopy as described in the methods. I) bi-directional replication intermediates : A , B : replication bubbles; C: Cairn structure; D : catenated dimers. II) A, B, C : broken theta structures or rolling circles; D, E : initiation of replication at multiple sites. The bar=100 nm.

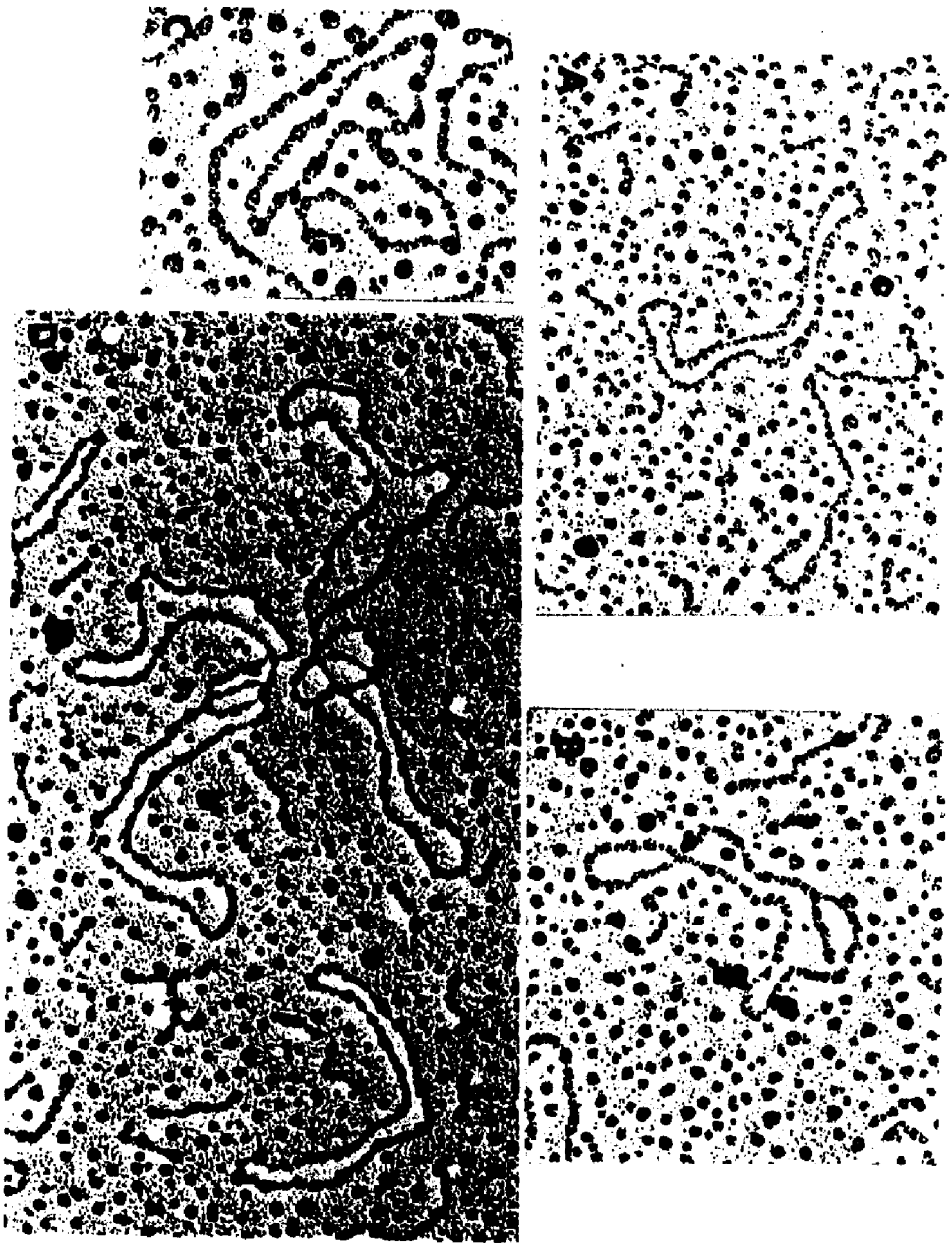
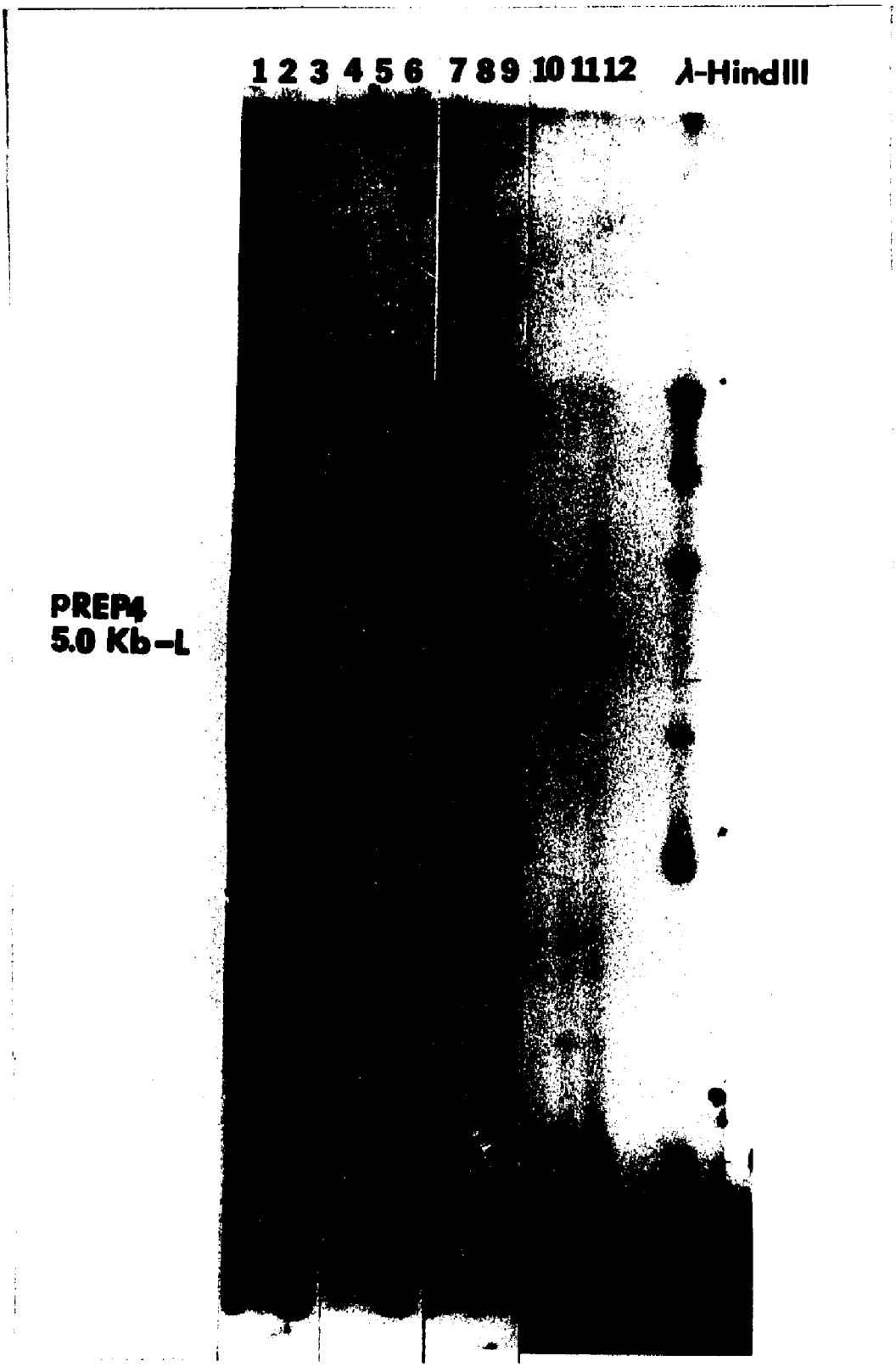


Figure 28. In vivo replication of pREP4: human HeLa and HL60 cells were transfected with 10 Ug of pREP4 plasmid according to the methods. Hirt supernatant was isolated and purified prior to digestion with BamHI and DpnI, and hybridized to the pREP4 insert. Lanes 1-3, uncut Hirt supernatant isolated at 24, 48 and 72 hours post transfection from HeLa cells; lanes 4-6 , Hirt supernatant isolated at 24, 48 and 72 hours post transfection from HeLa cells linearized with BamHI and digested with DpnI; lanes 7-9, uncut Hirt supernatant at 24, 48 and 72 hours post transfection from HL60 cells; lanes 10-12, Hirt supernatant isolated at 24, 48 and 72 hours post transfection from HL60 cells, linearized with BamHI and digested with DpnI. L= linear size plasmid, 5.0 kb. Lambda-HindIII is the marker.


1 2 3 4 5 6 7 8 9 10 11 12 λ-HindIII
PREP4
5.0 Kb–L

DpnI to segregate replicated molecules from total input plasmids. The filter was hybridized to the pREP 4 insert. Lanes 1-3 , uncut DNAs , and lanes 4-6 are DNAs linearized with BamHI and digested with DpnI, from Hirt supernatant isolated at 24, 48, and 72 hours post transfection from HeLa cells. Similarly, lanes 7-9, uncut DNAs, and lanes 10-12 are DNAs linearized with BamHI prior to digestion with DpnI, from Hirt supernatant isolated at 24, 48, and 72 hours post transfection from HL60 cells.

Undigested products (lanes 1-3) contain all three forms of the plasmid pREP 4 plus some slow migrating molecules perhaps indicative of plasmid dimers formed in the cells, or otherwise a contaminating plasmid, as well as replication intermediates such as catenated dimers. These however, disappear when digested with DpnI (lanes 4-6) and serve as internal controls.

In HeLa cells there is an increasing amount of DpnI resistant band (L= 5.0 kb) with increasing post-transfection period , peaking at 72 hours post-transfection, indicating semi-conservative replication.

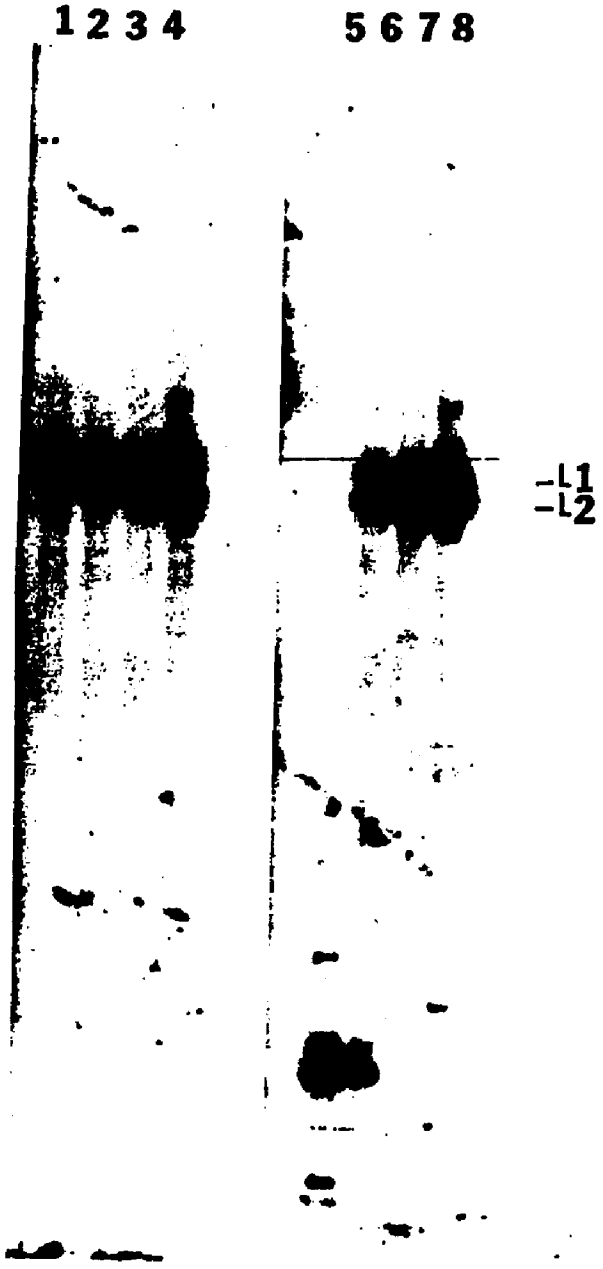
In HL60 cells, however, pREP 4 shows an increase of linear plasmid after DpnI digestion at 48 hours post transfection which decreases afterwards, suggesting the degradation of the plasmid by the cells. Smaller DNA fragments , indicative of degradation of unreplicated parental plasmids are also visible.

These results suggest that pREP 4 replicates in a higher copy number in HL60 cells than in HeLa cells; however, it degrades sooner in HL60 cells than in HeLa cells.

To examine replication efficiency of pREP 4 compared to SV40-origin-containing plasmids in vivo, we transfected an equal mixture of pREP 4 and plasmid pSV2neo into COS1 cells that produce T antigen constitutively and can support autonomous replication of pSV2neo.

Figure 29 shows the Hirt supernatant isolated from COS1 cells at 0, 24, 48 and 72 hours post transfections either linearized with BamHI (lanes 1-4) or cut with BamHI plus DpnI (lanes 5-8) . The filter was hybridized to pML2. DpnI resistant linear bands at 5.6 kbps (pSV2neo) and 5.0 kbps (pREP 4) indicate a similar replication efficiency between the two co-transfected plasmids, which is much higher than

Figure 29. In vivo replication of pREP4 and pSV2neo in COS1 cells. COS1 cells were co-transfected with 5 Ug of pREP4 plus 5 Ug of pSV2neo as described in the methods. Hirt supernatant was isolated at 0, 24, 48 and 72 hours post transfection and digested with BamHI and DpnI and hybridized to the plasmid pML2. Lanes 1-4, plasmid DNAs isolated at 0, 24, 48 and 72 hours post transfection from COS1 cells and linearized with BamHI; lanes 5-8, plasmid DNAs isolated from COS1 cells at 0, 24, 48 and 72 hours post transfection and linearized with BamHI prior to digestion with DpnI. L1: linear pSv2neo= 5.3 kbp; L2: linear pREP4= 5 kbp.


1 2 3 4
5 6 7 8
L1
L2

pREP 4 replication in HeLa or HL60 cells.

This high copy replication of pREP 4 in COS1 cells may reflect an enhancement of replication by T antigen that is produced in these cells. Interestingly, recently Ariga and co-workers have shown that SV40 origin containing plasmids are capable of autonomous replication in HL60 cells (though less efficiently than in COS cells) that produce high levels of the C-myc protein (101, 102). Thus ,If the C-myc protein has similar regulatory affects on DNA replication as does T antigen (though to a lower degree) , then the enhancement of replication of pREP 4 in COS1 cells would justify since pREP 4 originates from HL60 cells under over expression of the C-myc gene.

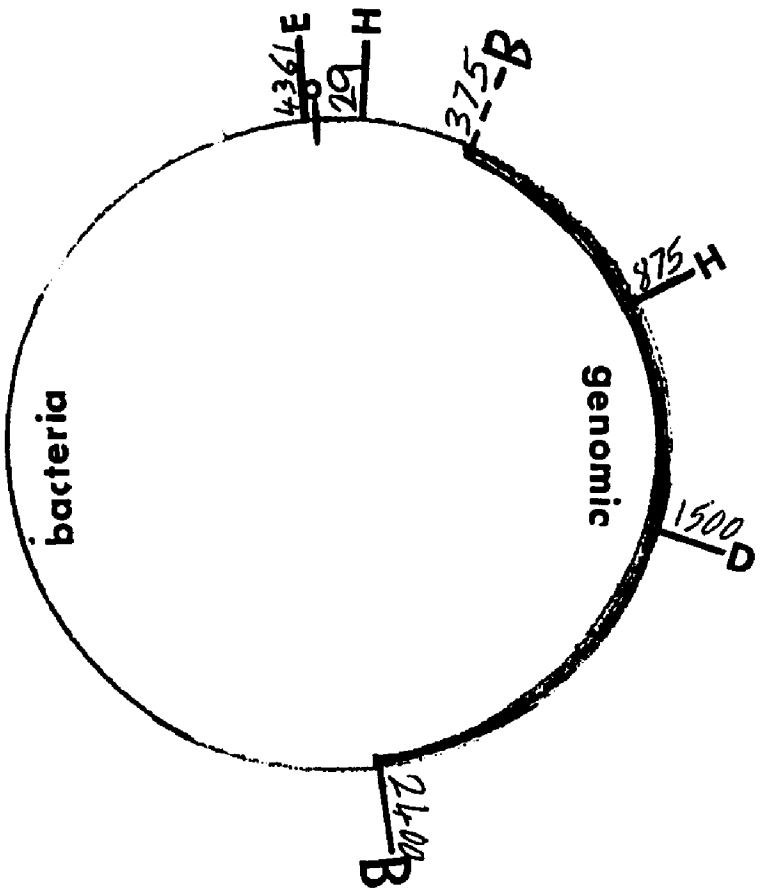
However , Further in vitro replication experiments are needed to identify the roles of specific proteins and sequences involved in initiation, elongation and termination of eukaryotic DNA synthesis.

Here we describe the isolation of sequences that initiate replication autonomously in vitro and when transfected into certain cell lines , as measured by DpnI resistance. The liability of the system depends on the

availability of in vitro replication systems and DpnI assays and is confirmed by electron microscopy and by using eukaryotic DNA replication inhibitors such as aphidicolin . Control template plasmids such as pBR322 and pML2 do not support replication in the cellular extracts .

The presence of a reliable in vitro replication system will enhance isolation of negative or positive regulatory sequences and further would facilitate further studies of the complex eukaryotic DNA synthesis machinery. Ultimately, we may recognize the causes and elements responsible for uncontrolled replication and gene amplification that characterizes tumorgenicity in many cells.

Figure 30. Restriction map of plasmid pREP4: The plasmid was digested with restriction enzymes BamHI, BglI, EcoRI, HindIII or DpnI. There are no BamHI, BglI, EcoRI or Pvu2 sites in the insert. There is one HindIII site and one DpnI site at the shown positions. B= BamHI, D= DpnI, E= EcoRI, H=HindIII.



pREP 4 - 5.0 kb

DISCUSSION

We have used an in vitro replication system which initiates DNA synthesis using the SV40 origin of replication, SV40 T antigen and cellular replication machinery to study mechanisms and intermediates of SV40 DNA replication and further to extend this system towards isolating chromosomal DNA fragments that can initiate plasmid replication in vivo.

Few studies are available for isolation of chromosomal sequences that initiate replication autonomously. Further there is no general method or study of isolating DNA replication origins in vitro. Nascent DNA fragments have been cloned into pBR322 from serum starved, replication inhibited cell lines when added serum (220); and from chromosomal DNAs cross linked with trioxlin to inhibit replication fork movement (3). Other studies include insertion of specific genes into random positions in the genome of cell lines that are deficient for that gene and isolation of resistant cells caused by replication dependent amplification of the gene

(35, 221).

There are no SV40 replication -based systems that are used for selection of chromosomal initiating sequences. Most of the available studies include identification of cellular and viral proteins that are involved in the replication of SV40 sequences rather than chromosomal DNAs (131,184,209). Here I describe the initiation of an SV40 in vitro replication system that lead me to select and isolate clones containing autonomously replicating sequences.

First I studied intermediates and products of plasmid molecules, containing the SV40 origin , replicating in monkey cellular extracts ; which initiate specifically at the SV40 origin by the help of T antigen with unidirectional or bi-directional fork movements (figures 12, and 13). Control plasmids containing only the bacterial origin with or without sequences inhibitory to replication in mammalian cells and electron microscopy confirmed the initiation of replication at the SV40 sequences (figure 14). Similar to in vivo SV40 replication , bi-directional replication intermediates from newly-initiated bubbles through catenated dimers ending to monomer topoisomers are visible in this system. Full

supercoiling of these circles into form I plasmid, reportedly depends on inclusion of nuclear extract in the reaction mixture.

Two dimensional gel analysis and electron microscopy observations revealed a second class of intermediates , depicting a sigma shape molecule , that are the result of unidirectional fork movement (figures 10, 12, 13). Rolling circle replication is thought to initiate at a nick in a replication bubble (or in a theta structure) and elongate unidirectional to synthesize an ever lasting tail which is incised into monomer progeny viral DNA molecules by unknown mechanisms. The production of sigma molecules in the in vitro replication mixtures, further resembles this system to in vivo replication of SV40.

A third class of DNA replication intermediates were recognized when plasmids pSVOD or pSV2neo were incubated in the extracts. (figure 15) They resemble dual circles, connected by a double strand DNA bridge. The length of the bridge varies from few hundred to few thousand base pairs, averaging at 3.9 kbps; all of the circles are however the same size as that of the input template. Extensive electron

microscopic experiments, lead We to hypothesize the dual circles to be the result of homologous strand transfer between two replicating SV40 sequences with one replicating as a rolling circle . To distinguish between recombination or replication machinery for dual circle formation, we subjected a mixture of different sized plasmids, pSVOD 3.3 kbp and pSV2neo 5.6kbp , or SV40 DNA 5.3 kbps, to the in vitro system.

We detected heterogeneous dual circle formation between the incubating plasmids, indicating a recombination dependent mechanism involving two initially separate molecules . Therefore, the dual circles are not replication intermediates of one molecule. heterogeneous dual circles containing dimers, trimers or larger molecules would be expected in a replication dependent process; however, we have never seen such structures (we have seen dual circles where one circle is connected to a replicating theta structure) . Aphidicolin inhibited the dual circle production; however, after a preincubation step in the replication mixture, it has no effects, suggesting the involvement of replication intermediates in a polymerase - independent strand transfer.

Rolling circle replication of papovaviruses has been reported to produce highly recombinogenic intermediates (53) however, there are no known mechanisms or sequences on this subject. In our system, rolling circle formation occurs mostly in prolonged incubations of plasmid DNAs in the replication mixture which is also true for dual circle formation. Our results suggest that SV40 rolling circles readily form in vitro by the DNA synthesis machinery and unidirectional replication may be another pathway for viral progeny production. Since there is no termination sites in the rolling circle mode of replication (unlike that of bi-directional DNA replication) these recombinogenic molecules , are capable of producing replication-dependent recombination forms,the dual circles. However, many experiments have to be done to understand the initiation , the elongation and the termination mechanisms of SV40 rolling circle replication.

The in vitro SV40 replication assay has allowed us to dissect the roles of individual proteins on replicating molecules at different stages of the DNA synthesis; which will certainly enhance purification of unknown replication and recombination proteins and sequences.

With the establishment of the in vitro SV40 replication system, we set up to first, test the replication ability of eukaryotic origin candidates such as repetitive sequences, the Alu family and second, isolate autonomously replicating sequences from human cell lines. The human genome is organized mostly as interspersion of repeated DNA sequences with several kbp of single copy sequences. Alu family represent a majority of the repeated elements. They have been sequenced and a representative sequence has been cloned into pBR322, called BLUR8. Alu elements have been found in gamma, beta, and sigma globin gene clusters as well as near the human insulin gene and many other genes. Even though,there are no known functions for these elements, the consensus sequence shares great homologies to many replication origins, including SV40 (figure 19 , 105, 107).

BLUR8 has been shown to replicate in vivo,when transfected into COS 7 monkey cells that produce T antigen (needed for SV40 replication) constitutively. Transfections of an Alu repeat into a rodent cell line, however, failed to show any replication, further indicating that T antigen is needed for BLUR8 plasmid replication (92, 108). In vitro replication

studies have been controversial. Ariga and co- workers have observed the BLUR8 replication in their SV40 replication system while others have failed (7, 131).

In our in vitro replication assay, the plasmid BLUR8 is highly labeled through a T antigen dependent process. DpnI resistant molecules however, constitute only a small fraction of this labeling which peaks at 0.5-1.0 ug of T antigen per reaction. Electron microscopy observations and two dimensional gel electrophoresis contradicted the DpnI assay, indicating that most of the labeled BLUR 8 plasmids have replicated in the extracts. Theta and sigma forms of replication intermediates were observed by electron microscopy (figures 11, 12 and 20) . A 16 bp BLUR8 deletion at the sequences that are homologous to the SV40 T antigen binding sites, abolishes this labeling, indicating a T antigen dependent replication of plasmid BLUR 8.

SV40 T antigen has been recently shown to have a helicase activity . It binds to the sequence GAGGC and opens up the SV40 DNA sequences near the replication origin and initiates viral DNA replication(49, 50).It may function similarly on the plasmid BLUR8,suggesting the presence of functionally

analogous proteins (perhaps helicases) in the eukaryotic cells that bind to the consensus sequence GAGGC and initiate chromosomal DNA replication (perhaps at the Alu sequences).

The restriction endonuclease DpnI digests plasmid molecules that are methylated at the adenosine residues of the sequence GATC at both strands; unmethylated plasmids are resistant to DpnI digestion, presenting a simple method for segregating replicated plasmid molecules from total DNA templates. DpnI resistance of hemimethylated plasmid molecules is however, inconsistent and depends on incubation conditions such as salt concentrations and spermidine content.

Labeled BLUR8 molecules that are shown by electron microscopy and two dimensional gel electrophoresis to be the products of DNA synthesis, are observed to be sensitive to DpnI digestion, indicating the hemimethylation of BLUR 8.

These results suggest that most of the BLUR8 molecules replicate only once under cellular constraints similar to chromosomal replication during the S phase of the cell cycle. They thus, support a role for Alu sequences as autonomously replicating sequences in mammalian cells (though, in the presence of T antigen). However, only isolation and sequencing

of natural initiation sequences will illuminate this point.

Next we set up to isolate human DNA sequences that would carry the replication initiation in human cellular extracts. Genomic libraries from human promyelocytic leukemia cells (HL60) were prepared in pML2 and incubated in the replication mixtures. DpnI digestion which eliminated the parental plasmids, allowed us to isolate chromosomal sequences that can replicate the plasmids in vitro.

The insert sizes of the plasmids after three cycles of replication indicate ,interestingly that plasmids containing larger than average input inserts (>2 kbps) are selected over smaller ones. This preference occurs during DNA replication in the cellular extracts since bacterial amplification and DpnI digestion select smaller plasmids.

The preferred plasmids may contain multiple origin sequences or cis-acting regulatory elements that enhance replication. The later are present in the genome of many viruses and are thought to exist in chromosomal as well. If this is our case, the preferred insert sizes of 2-3 kbps indicate that the cis acting elements work best when positioned within few kbps from the core origin sequences.

From 4 different libraries , two individual clones of similar sizes were selected . One contained moderately reiterated sequences while the other one appeared to be present only scarcely in the HL60 genome. When pREP 4 (the former) was hybridized to digested chromosomal DNAs, several prominent bands were observed in light exposures, indicating that the insert of pREP 4 is located as tandemly repeated DNA fragments at multiple locations. Darker exposures, showed smears around the bands, suggesting the dispersion of the insert in other locations (figure 25).

Plasmid pREP 4 replicated efficiently in cellular extracts of HL60 cells as tested by DpnI assays and electron microscopic observations (figures 26, 27). Using transient transfections, we also showed that plasmid pREP 4 is capable of autonomously replicating in HeLa, HL60 and COS1 cells, indicating that it contains sequences necessary for plasmid replication in these cells. However, the replication efficiency seems to differ between COS1 cells and HeLa or HL60 cells. When HeLa or HL60 cells were transfected with pREP 4 only very small amount of the input plasmid replicated , indicating a highly regulated process of perhaps one

replication per cell cycle (figure 28). However, pREP 4 replicated in a high copy number and out of cell control in COS1 cells (figure 29). These cells produce T antigen that is needed for SV40 initiation of replication and may also play a role in amplification of the hosts ' chromosomal DNAs.

The in vitro replication assay, not only will lead to isolation of DNA replication initiation sequences as well as cis acting regulatory elements, but will also ultimately allow identification of the specific roles of proteins involved in DNA replication, recombination and gene amplification.

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