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Du, Hong, Ph.D.

City University of New York, 1989

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THE ANALYSIS OF JB7-K, A TEMPERATURE SENSITIVE MUTANT OF CHINESE HAMSTER OVARY CELLS

by

Hong Du

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

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Abstract

THE ANALYSIS OF JB7-K, A TEMPERATURE SENSITIVE MUTANT OF CHINESE HAMSTER OVARY CELLS

by

HONG DU

Adviser: Professor Harvey L. Ozer

Over 200 CHO cell temperature-sensitive mutants have been isolated in the laboratory and 10% of them show a *ts*DNA phenotype. Five of them are defective in polyoma viral DNA replication at the nonpermissive temperature (NPT), whereas only one out of the five is also defective in adenovirus DNA replication at the NPT. General growth properties have been studied for those mutants selectively defective for polyoma DNA replication (JB3-O, JB7-K, JB8-D and JB11-J). One of these mutants, JB7-K, has been studied in more detail. DNA synthesis in this mutant decreased 60% after 12 h at NPT, but both RNA and protein syntheses are normal for more than 16 h at the NPT. Cell cycle analysis shows that JB7-K cell arrested predominantly in G1 at the NPT. The rate as well as level of polyoma DNA replication has been found to be reduced at the NPT. Molecular cloning of the wild type human gene that corrects the *ts* defect of JB7-K has been undertaken. Primary ts^+ transfectants (5A1) were isolated following protoplast fusion with a human genomic cosmid library (pCV103Kgpt). A secondary ts^+ transfectant (2^o5D) was isolated by DNA mediated gene transfer from 5A1. A phage genomic library of 2^o5D which contains a single copy of human *Alu* and *gpt* sequences was constructed. Unique human gene sequences (2.8 kb and 1.4 kb *EcoR* I fragments) were isolated by screening of the phage library using a human *Alu* sequence as a probe and restriction enzyme digestion of the recombinant phage DNA. This 2.8 kb sequence has been mapped to human chromosome 2 and located at 2q11-2p23 by

Southern analysis of human-rodent hybrid cells. Transfection of JB7-K by the recombinant phage results in partial correction of the *ts* phenotype. This 2.8 kb fragment has also been used as a probe to screen a human cDNA library.

This thesis is dedicated to my husband, Cong Yan, my daughter, Lily, my parents and my parents in-laws for their love and encouragment

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ABBREVIATIONS

CaP	Calcium phosphate.
CHO	Chinese hamster ovary cell.
CQ	Chloroquine.
Cz	2'-Deoxy-2'azidocytidine.
DF	1:1 mixture of DME and Ham F12 medium.
DHFR	dihydrofolate reductase.
DME	Dulbecco modified Eagle medium.
dNTP	deoxynucleoside triphosphate
EOC	Efficiency of colony formation.
EtBr	Ethidium bromide
gpt	Guanine-xanthine phosphoribosyl transferase.
HAT	Hypoxanthine (5×10^{-5} M), Aminopterin (2.5×10^{-6} M), Thymidine (10^{-5}M).
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase.
HMW	High molecular weight.
MPA	Mycophenolic Acid.
Met	Methionine.
PBS	phosphate buffered saline.
PEG	polyethylene glycol.
pfu	Plaque forming units.
Py	Polyomavirus.
SDS	Sodium dodecyl sulfate
SV40	Simian virus 40.
TdR	Thymidine.

TE	Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).
TG	Thioguanine.
X	Xanthine

Introduction

DNA replication in eukaryotes is a very intricate multienzyme process which is much less clear than that in prokaryotes. Even though the biochemistry of initiation, replication fork progression and termination is similar in eukaryotes and prokaryotes, the large amount of DNA per cell and the method of DNA packaging in eukaryotes are different from that in prokaryotes. Therefore, in general, eukaryotic DNA replication is a complex process involving a great number of specific protein-protein and protein-DNA interactions.

The eukaryotic cell goes through a proliferation cycle which can be divided into four main phases even though it consists of a continuous array of processes. These phases are: mitosis in which each cell divides into two daughter cells; gap 1 (G1) which starts at the end of mitosis and ends at the beginning of DNA synthesis; DNA synthesis (S) in which each chromosome is duplicated; and gap 2 (G2) which starts at the end of S and ends at the beginning of the next mitotic division. Mitosis begins when the chromosomes can be visualized in the light microscope as discrete units and ends when cytokinesis is completed. Mitosis can be further divided into four phases: prophase, prometaphase, metaphase and anaphase. Each phase has well defined obvious phenomena (for review see reference 1). Entrance into mitosis is regulated by mitotic factors (2) which are not species specific as evidenced by the ability of human mitotic factor to induce premature chromosome condensation in many different eukaryotic cells including amphibia (3). Injection into frog oocytes of cytoplasmic extracts from mitotic or other cell cycle phases of human (HeLa) cells shows that mitotic factors are made during phase G2, reach their peak in mitosis and disappear in G1 cells (4). Furthermore, these factors were found to be

sensitive to protease treatment, Ca^{2+} ions and basic pH. Monoclonal antibody to proteins that are present only in mitotic cells have been prepared (5).

In mammalian cells G1 is the phase in which the cell either continues to proliferate or stops growing. G1 exhibits a high variability in its length, while the other phases exhibit a reasonably constant length. Progression through G1 is affected dramatically by environmental conditions such that many types of cells in culture will arrest in G1 under starvation conditions or grown to confluence and enter a quiescent state. Differentiated mammalian cells that do not proliferate are typically arrested in G1 and aging ("senescent") cells also tend to arrest in this phase. Pardee (7) suggested that cells which have completed mitosis and have entered G1 reach a "restriction point" at which the decision whether to enter a quiescent state (G0) or to proliferate is made. In transformed cells the restriction point control is defective or lost. The RNA content in noncycling G0 cells is low and becomes increased when the cells are induced to proliferate (8). However, the synthesis of specific mRNA species and not just an increase in rRNA is required for the entry of cells into the proliferative state.

Initiation of DNA synthesis signals the beginning of S phase. DNA synthesis proceeds simultaneously at many points in different chromosomes, exhibiting a specific pattern of replication of clusters of replication (9) and synchronous replication of single replicons within each cluster. The DNA replication in S phase will be discussed later in more detail.

G2 phase is usually short. Cells prepare for mitosis during G2 phase which shows extensive chromosome condensation. The mechanism of control of the cell cycle has been studied for a long time (for review see reference 10). Here we are most interested in the process of DNA replication which is in the S phase of cell cycle.

Initiation, the control point of DNA replication, entails the recognition of origins of replication and activation so that the replication forks are constructed and set in motion. For the mammalian cell, initiation can refer to "S" phase or the start of synthesis of a nascent DNA fragment. DNA replication initiates at a specific origin in prokaryotes (for review, see reference 11). In eukaryotes, evidence is accumulating that DNA replication starts at specific origins of replication. Botchan and Dayton (12) found that replication of the sea urchin ribosomal gene repeat began in a specific region of the nontranscribed spacer about 2 kb from the 3' end of the 26 *S* gene. James and Leffak (13) showed that replication forks move in only one direction though the avian α-globin locus, consistent with replication from a unique origin. The clearest evidence for the existence of discrete origins in higher eukaryotes comes from studies on gene amplification: in the case of the amplification of the dihydrofolate reductase (DHFR) gene in CHO cells (14,15), the earliest replication portion of an amplified DHFR domain can be mapped to a single restriction fragment of 4.3 kb (16), whereas for the Chorion gene in *Drosophila* follicle cells (17, 18) the extent of amplification decreases bidirectionally from a central domain.

Additional evidence consistent with the existence of specific origins is provided by ARS (autonomously replicating sequence) elements, cis-acting segments of DNA that allow plasmids containing them to be maintained as autonomous genetic elements in eukaryotic cells. ARS elements were discovered in yeast (19) and were subsequently proposed for mammalian cells (20, 21). ARS elements are present 400 to 500 times in the yeast genome. This corresponds well with data from electron microscopic studies which suggested that there are approximately 400 replication origins per haploid yeast genome (22,23). Furthermore, *in vitro* DNA replication studies (24-26) have provided results consistent with the hypothesis that

ARS elements act as origins for bidirectional replication. Huberman (27) and Brewer (28) used two-dimensional agarose gel electrophoresis to physically map origins of replication *in vivo*. Their results showed that all detectable initiation events in early S phase in the yeast cell occur within the ARS element in both the 2 μM plasmid and a recombinant ARS 1 plasmid. The ARS sequences have been localized to sequences as short as 57 base pairs (bp) (29), possesing different primary sequences. However, a comparison of several ARS element has demonstrated an 11 base pair consensus sequence (30).

Chromosome regions replicated at a particular point in S phase are usually replicated at the same point in subsequent S phases, indicating that origins are activated in a temporally regulated fashion. The usual replication pattern for a chromosome is maintained in the presence of the normal chromosome complement; however, a change in the normal chromosome position of a gene as a consequence of chromosomal rearrangements can alter the time at which it is replicated (31). Euchromatin, in which most expressed genes are found, is GC-rich and preferentially replicated early, whereas heterochromoatin, which is AT-rich and contains satellite DNA, tends to be replicated in the later part of S phase. Initiation of DNA synthesis in mid- and late-S phase is dependent upon completion of the DNA synthesis initiated in early-S phase. Goldman et al.(32) have presented some evidence that in order for a gene to be expressed, it must be replicated early.

Replication of a segment of chromosomal DNA occurs following the essentially synchronous activation of a cluster of origins (also called a 'replication unit'). The newly synthesized DNA surrounding and including replication origins have been isolated and examined for several properties. It appears to be enriched for middle repetitive, nontranscribed sequence (33) and to contain self-complementary, inverted repeats (34).

However, some other studies argue against the conclusion that DNA replication in eukaryotes is under the control of specific origins. For example, during early embryogenesis the rapid proliferation is accomplished by the simultaneous activation of a large number of closely spaced 'origins', and it is difficult to envision that this reflects any great degree of sequence specificity. The origins active in early embryogenesis are not all active later. In the case of the two X chromosomes in mammalian cells, one is heterochromatic and replicates at a different time in S phase (35), despite their both having the same sequences and structures. Third, activated *Xenopus* oocytes have been shown to replicate any piece of duplex DNA injected into them with little evidence of specificity for the site at which synthesis is initiated (36,37).

The possible association of origins with the nuclear matrix has been investigated in several laboratories. Todorova and Russev (38) isolated DNA about 2 kb in size from cells whose DNA has been crosslinked; this DNA, which was believed to originate largely from regions around the origins, was not enriched for sequences found in firm association with the nuclear matrix. Goldberg et al.(39) characterized two DNA sequences that were strongly bound to the nuclear matrix; one had homology to the mouse B1 repetitive sequence and the other resembled the human papovavirus (BK) T antigen binding site. The authors suggested that these sequences may compose replication origins, but it was not excluded that they were bound to the nuclear matrix for other reasons. Since exposure to high salt appeared to induce an artificial association of transcribed DNA to the nuclear matrix, Jackson and Cook (40) used isotonic conditions throughout the isolation and analytical procedures, and concluded that the polymerizing complex is attached to a "nucleoskeleton" (another term for the nuclear matrix). Mirkovitch et al. (41) also showed that in the interphase nuclei of Drosophila cells a specific restriction

fragment from the histone gene repeat had a strong affinity for the nuclear matrix; it is not known if this fragment contains an origin of replication.

Studies on prokaryotic origin-recognizing proteins like the *E. coli dnaA* protein, the λO protein, and the φX174 gene A protein provide clues to the molecular events at an eukaryotic origin. In each of these cases, there is a complex interaction between multiple repeated sequences in the origin and multiple copies of the 'origin-activating' protein. Proteins enlisted include helicase and topoisomerase activities, priming and DNA polymerase activities, and possibly enzymes involved in precursor synthesis. An RNase H activity may be necessary for primer removal, and possibly for generating correct primers. The existence of a multi-protein, multi-enzyme complex ('replisome') in DNA replication is widely assumed.

Recent progress in characterizing both the eukaryotic DNA polymerase α (pol α) and primase (an enzyme capable of synthesizing short oligoribonucleotide primers) derives from improved purification procedures and from the observation that a polymerase, primase, and several other polypeptide chains can be co-purified as a multisubunit complex or "replicase" (42, 43). The replicase contains subunits of 180-182, 70-77, 55-60, and 48-50 kd. The ~180 kd subunit possesses DNA polymerase activity, and the ~50 and ~60 kd subunits appear to be responsible for primase activity. Purely biochemical studies of the eukaryotic primase have already established that it is a remarkable enzyme (44-46). It appears to operate in bursts of activity, at each burst copying six to fifteen nucleotides of a DNA template into an oligonucleotide product. Primase always initiates with a purine nucleotide, and there is usually a purine nucleotide in the template immediately preceding the template pyrimidine (47). In the absence of pol α activity, the oligonucleotide synthesized in a first burst can be extended by subsequent bursts to form much longer polynucleotides. The primase can also incorporate deoxyribonucleotides. In

the presence of active pol α and high deoxynucleoside triphosphate (dNTP) levels, however, the primase synthesizes an oligoribonucleotide primer in a single burst, and this primer is then elongated as a DNA strand. The ability of primase to synthesize DNA chains of limited length makes it unclear exactly when the transition from chain elongation by primase to chain elongation by polymerase occurs (43). Primase appears to be responsible for priming the synthesis of new Okazaki fragments on the discontinuous side of replication forks, and may also play a role in the initiation of DNA synthesis at replication origins. The latter possibility is supported by detailed mapping, at the nucleotide level, of the sites of RNA primer synthesis and of RNA-DNA transition near the replication origin of the DNA virus SV40, which utilizes the cellular primase and pol α. The data suggest that replication is initiated by synthesis of an RNA primer, six to nine residues long, in the direction of early mRNA synthesis (48). The preferred nucleotide sequences of this initial priming event are typical of those used for priming Okazaki fragments elsewhere in the SV40 genome (47); thus the same enzyme(s) are probably responsible for priming both Okazaki fragments and new strands at origins. Support for involvement of primase in initiation at origins also comes from the observation that aphidicolin (which is a strong inhibitor of DNA pol α but has no effect on primase) inhibits progression of replication forks but not initiation of limited DNA synthesis at SV40 origins, both *in vivo* (49) and *in vitro* (50).

Since DNA polymerases per se are inefficient at utilizing a base-paired duplex, it appears necessary to incorporate a helicase-like activity in the multi-enzyme 'replisome" complex. In a viral DNA replication *in vitro* system, it has been shown that the origin-binding SV40 T antigen has helicase activity (51). It requires ATP hydrolysis. Migration of T antigen along the DNA would separate the two strands so as to make available the two template strands. DNA pol α would follow close

behind synthesizing the progeny strands, both leading and lagging, a process that probably also requires ATP to maintain processivity (52). The leading strand is the one synthesized, at rates of 1-15 kb/min, in the overall 5' to 3' direction as the replication fork moves, and in principle can be synthesized in one continuous piece from origin to terminus; whether this is so is not yet known (because of the difficulty in distinguishing true nascent fragments from products of repair). Some researchers suggest that the leading strand is also synthesized by a discontinuous process (53).

The frequency with which Okazaki fragments are initiated and the exact location of the 5' terminus of the covalently attached RNA primers is regulated at several levels. In eukaryotic cells, Okazaki fragments range around 100-200 nucleotides in length 10 times shorter than their counterparts in prokaryotes. The similarity in length of DNA in nucleosomes and that of an Okazaki fragment suggests that nucleosome structure may dictate the frequency of chain initiation. Once the RNA primer has been synthesized, primase appears to add the first few deoxyribnucleotides before pol α takes over. This process may be regulated by the local deoxyribonucleotide concentration (44).

The overall movement of the DNA replication fork probably involves the coordinated separation of the base-paired strands by a putative helicase and helix-destabilizing proteins (which also facilitate DNA polymerase action on the single-strand DNA) and the unwinding of the duplex made possible by a topoisomerase. There is evidence that the parental nucleosomes are preferentially segregated with the template for leading strand synthesis. Events occurring 'downstream' of the fork must include excision of RNA primers (by RNase H?), repair of small gaps (by DNA polymerase β?) and sealing of nicks by DNA ligase. Fidelity of replication is probably maintained by a 3'-5' exonuclease (54) that may be another subunit of

the polymerase-primase holoenzyme. Other researchers report additional subunits with other activities, such as DNA topoisomerase II activity to prevent topological inhibition of the progressing replication strand (55), DNA helicase activity to separate the base-paired strand (56), and primer binding, diadenosine tetraphosphate binding, and exonuclease activities (57). However, it has generally been difficult to prove that a particular protein participates in DNA replication *in vivo*.

There is no evidence for the existence of sequence-specific signals in the DNA that stop the progression of a replication fork, though replication through certain sequences may be retarded. Instead, converging replication forks may cancel each other out. Complicating this event, however, is the helical winding present in the parental duplex. Unwinding is accomplished by topoisomerases; both type I and type II topoisomerases are widely distributed and capable of removing the positive superhelical twists (caused by the unwinding of parental duplex) that would otherwise accumulate ahead of the replication fork and those introduced into the daughter duplexes by the rewinding of the progeny DNA about a nucleosome(58). It is not known how much of the winding of the parental DNA about nucleosomes is retained as the replication fork passes. Type II, but not type I , topoisomerase activity is essential for cell survival (59,60). The type II topoisomerase acts by allowing one duplex to pass through another (61). As the two forks converge, unwinding of the DNA becomes an increasing problem, and evidence from many systems indicate that joining of adjacent replication units and of adjacent clusters of a unit are rate limiting. Although the primary 3-6 S nascent Okazaki fragments are rapidly joined by DNA polynucleotide ligase to form 10-25 S DNA molecules, the latter are slowly incorporated into 25-200 S structures. This completion of the joining process could be delayed until G2 phase (58). Finally, restoration of the

mature chromosome structure is presumably essential both for mitosis and in preparation for the next round of replication.

The small DNA tumor viruses polyoma virus (Py) and simian virus 40 (SV40) were initially studied because of their oncogenic potential. However, because these viruses can be easily grown in tissue culture and their small (5.2 kb) DNA genomes can be manipulated, these viruses are ideal for laboratory study as a simple model system for the more complex mammalian genome. These viruses contain a small double-stranded, circular genome with a single origin of DNA replication [reviewed in references (62, 63)]. The virus genome encodes only one protein that is required for replication of virus DNA, the large tumor (T) antigen (64, 65); therefore, viral DNA replication is very much dependent on the cellular chromosome DNA replication machinery. The DNA is wound around cellular histone proteins to form a structure which is similar to the cellular nucleosome. Therefore, replication of the viral genome may be similar to that of the chromosome of the host cell not only for initiation and elongation of DNA synthesis but also for the segregation of daughter DNA molecules after replication (66, 67). The genomes of these viruses can be divided into early and late regions. The late region codes for the structural proteins. The SV40 early region codes for two proteins, large T antigen and small t antigen; the polyoma early region coding for three proteins: large T antigen, middle T antigen, and small t antigen. The roles of SV40 (64) or polyoma virus large T antigens (65) in viral DNA replication were identified by studying temperature-sensitive A-gene mutants. Temperature shift experiments using these mutants led to the conclusion that SV40 or polyoma virus A-gene product is clearly required for initiation of new rounds of viral DNA replication and, possibly, for elongation of nascent replication forks (68, 69). The *ori* region of SV40 was first mapped to the sequence at or near 0.67 map units on the viral genome (70). More detailed

mapping was accomplished with *in vitro* mutagenesis (71, 72). The minimal SV40 *ori,* was identified as a 65 bp segment between nucleotides 5208 and 30 on the viral genome. Recent genetic studies indicate that the origin is quite complex, consisting of at least three functionally distinct domains (73-75). At the center of the origin are four copies of a simple 5-base pair sequence (GAGGC) organized as an inverted repeat. This sequence element is recognized by the viral initiation protein T antigen (binding site II). On one side of the T antigen binding domain is a 17-base pair segment containing exclusively A and T residues, which is probably the locus of the initial strand separation event in DNA replication. On the other side of the T antigen binding site is a 15-base pair imperfect repeat of unknown function. The polyoma virus *ori* region was initially located to the region at 0.72 map units (76). The origin includes an inverted repeat adjacent to a 14 bp A+T rich region. The origin contains multiple elements, one of which is a transcriptional enhancer sequence. Although both origins have a high homology in their minimum replication origin (*ori*-cores), and each is flanked by a T antigen binding site on its early gene side (77-80), they differ in other features (81). First, initiation of SV40 replication requires permissive monkey or human cell factors while polyoma requires permissive mouse cell factors. The permissive cell factor has been identified as associated with the polymerase α and primase complex (51). Second, the other major T antigen binding site in SV40 (site II) encompasses the *ori*-core while polyoma T antigen DNA binding sites B and C lie outside of the *ori*-core. Third, the TATA box and cap site for SV40 early mRNA synthesis are superimposed on the *ori*-core, while these elements in polyoma are outside of the *ori*-core (76). Fourth, the polyoma virus *ori* includes a transcriptional enhancer element, while the SV40 *ori* does not (82-88). Fifth, for SV40 the sites where synthesis of the two continuous strands toward opposite directions starts (defining

the origin of bidirectional replication, OBR) are coincident with each other in a 2 bp region between the *ori*-core and the T antigen binding site I. Whereas in polyoma, these OBR sites lie 16 bp away from each other in a similar region between the *ori*-core and the large T binding site (89).

The development of *in vitro* replication of SV40 and polyoma using circular DNA molecules with viral replication origins (90,91) has provided important insights into the process of initiation, fork movement, and termination. Replication in this system is dependent on a soluble extract or purified enzyme fractions from cells permissive for virus replication, a circular DNA molecule containing viral replication origin, and a high concentration of viral T antigen.

By study of SV40 *in vitro* DNA replication, Murakami et al. (51) demonstrated that the essential component provided by the permissive cell extract is the replicase (polymerase and primase complex); a primate replicase is required for SV40 replication, and a mouse replicase is required for polyoma replication. Other components of the cell extracts appear to be interchangeable. Using hamster cell extracts an *in vitro* system for polyoma DNA replication has been established in this lab by K. Lawlor (92). Wobbe et al. (93) have dissected initiation at the SV40 origin into two phases: pre-elongation, which is RNase sensitive and occurs in the absent of dNTP; and elongation, which requires dNTP. The essential products of the first phase appear to be a complex (including substrate DNA) that can be separated from most monomeric proteins by gel filtration. Since SV40 T antigen has helicase activity (94, 69), the pre-elongation complex contains DNA with parental strands partially unwound at the origin. Mastranglo et al. (95), using scanning transmission electron microscopy determined the sizes and masses of complexes formed after incubating T antigen with SV40 *ori*. They found that *in vitro* and in the presence of ATP, T antigen assembles a double hexamer, centered

on the core origin and extending beyond it by 12 bp in each direction. The assembly of this dodecamer initiates an untwisting of the duplex by 2-3 turns. In the absence of ATP, a tetrameric structure is the largest found at the core origin.

SV40 DNA replication *in vitro* also provides considerable information on the cellular proteins which are involved in viral DNA replication. Beside the DNA polymerase α-primase complex, topoisomerase I and II, other factors and proteins have been reported and purified. RF-A and CF-1C (SSI) have been isolated from the cytosol in the phosphocellulose I fraction (0.2 M) and are required in unwinding of the origin (96). RF-A has recently been purified to homogeneity (97, 98). It consists of three subunits, the largest of which binds specifically to single-stranded DNA (96). PCNA (proliferating cell nuclear antigen), RF-C, and DNA polymerase δ (pol δ) are required in elongation (96,97). Pol δ differs from pol α because it contains a readily detectable 3'-5' exonuclease activity (99) and appears to lack an associated primase activity. Also, PCNA has an effect on the activity or processivity of pol δ but not pol α. In the absence of PCNA, initiation of DNA replication at the viral origin can occur normally, but only short nascent strands, containing a maximum of a few hundred nucleotides, are synthesized (100). The observation that PCNA is required for extensive chain elongation provides strong evidence that pol δ is involved in DNA replication. A model of the two polymerases, α and δ, working together has recently become generally accepted (101). Some other proteins which are associated with the 640 kd pol α complex include accessory proteins C1 (24 kd, tetramer) and C2 (52 kd), a single-strand exonuclease (3'-5') (69 kd), an AP4A-binding protein (47 kd dimer) and a 92 kd protein of unknown function. Some other proteins involved in SV40 replication chromatograph with pol α but are not in the 640 kd complex. These include an A-T

sequence recognition protein (102), DNA-dependent ATPase, protein kinase (casein II), etc.

Biochemical analysis of polypeptides involved in viral DNA replication *in vitro* may not be equivalent to their functions in intracellular replication of DNA. In addition, some functions may only be required intracellularly. Genetic approaches can be particularly valuable in clarifying such situations.

The usefulness of conditional lethal mutants like temperature-sensitive (*ts*) mutants for the analysis of molecular mechanisms of eukaryotic chromatin duplication processes is greatly appreciated because of the successes achieved in the study of bacteria and viruses. For example, to identify the true replication enzyme in E.coli, the most crucial proof was the isolation of two clones, one markedly deficient in DNA polymerase I (103) and the other in which DNA polymerase III was temperature sensitive (104). During the last one and half decades, several groups have tried to isolate similar *ts* mutant in different mammalian cells in culture.[for review, see ref (105)]. Some of them which affect progression through G1 of the cell cycle have been studied within collections of mutants in Syrian hamster BHK cells (106, 107), rat 3Y1 fibroblast (108) and other cell lines (1, 110). Only a small number of the isolated mutants appear to have defects related to DNA replication itself, such as those in the mouse L cell line (59, 111, 112), BALB/3T3 cell line (113, 114) and FM3A cell line (115).

A *ts*DNA mutant isolated from mouse L cell line , *ts*A1S9, has been partially characterized(59, 116). DNA replication in this mutant cell lines at the nonpermissive temperature (NPT) is normal for 6-8 h after temperature shift but falls rapidly thereafter. Synthesis of Okazaki fragment DNA and their conversion to larger forms is normal at the NPT while the total DNA synthesis falls. The gene product of the mutated locus in *ts*A1S9 has been reported as required for the proper

function of DNA topoisomerase II (59), however, this has not been confirmed (Dermody and Ozer, unpublished data). Another *ts* DNA mutant from this collection is *ts*C1 (111), the *ts* defect can be corrected by sequences on the human X chromosome (117), but the function affected by the genetic lesion has not yet been identified.

A collection of *ts* mutants were isolated from mutagenized BALB/3T3 mouse cells in this laboratory (113,114). *ts*2 and *ts*20 have been studied more extensively. These two mutants cannot complement each other and both are recessive to wild type (114). Both *ts* cells were arrested in S phase at the NPT and failed to support Py DNA replication at the NPT (119,120). *In vitro* complementation of the defect in *ts*20 with extracts from wild type cells showed a more heat-labile topoisomerase I activity (119); topoisomerase II activity was unaffected (Zeng, unpublished data). Genetic analysis of *ts*2 has shown that the *ts* defect can be corrected by sequences on the human X chromosome (114). Ongoing studies are directed towards isolation of the human gene which corrects the *ts* growth defect of *ts*2 and/or *ts*20 in this laboratory.

The best characterized *ts* mutant was isolated from the mouse FM3A cell line, *ts*FT20 (121). Cell cycle analysis has shown that *ts*FT20 cells arrest in S phase at the NPT. The DNA polymerase α activity is heat-labile in the mutant indicating that *ts*FT20 possesses a genetic lesion in the gene for DNA polymerase α or a factor which is essential for its normal function. Pol α has been independently mapped to the X chromosome; however, the human chromosome which complements *ts*FT 20 has not yet been reported. The *ts* DNA mutants isolated from hamster cells like *ts*C8 (122), *ts*13A and *ts*15C (123), *ts*24 (124) have been reported but not yet characterized.

From this brief survey of the available mutants in DNA replication, most of these mutations in DNA replication were mapped to the human X chromosome, indicating a difficulty in generating mutants in autosomally encoded functions. This is undoubtedly due to the presence of a second allele which masks any recessive mutation. In an effort to remedy the deficiencies in the genetic analysis of eukaryotic DNA replication. Dr. Dermody in this laboratory isolated a collection of over 200 mutants of Chinese hamster ovary (CHO) cells which are temperature-sensitive for growth. Several considerations indicated that this cell line would be a good choice to serve as the parent of a mutant collection. First, it has favorable growth properties over a broad range of temperature (33 to 41°C), and its efficiency of colony formation approaches 100%. Second, autosomal recessive mutants dispersed throughout the genome have been isolated in CHO cells at an unusually high frequency. If genes important to DNA synthesis reside in a region of a functionally hemizygous chromosome, mutants at these loci should be easier to obtain. Third, human adenovirus (type C) and the papovaviruses SV40 and Py replicate in CHO cells. As already noted, such viruses have been well characterized genetically and biochemically. In addition, Polyoma DNA replication *in vitro* using CHO cell extracts has been established in the laboratory (92).

Although the mutants provided information about the genetics of DNA replication by somatic cell hybridization of a large collection of *ts* mutant cells and it was possible to assign an individual mutant to complementation groups, the precise gene or gene product affected was not identified. One of the approaches towards the recognition and study of *ts* DNA mutated functions is the cloning of the wild- type gene which renders *ts* DNA mutants insensitive to the restrictive temperature, thus allowing their growth. This approach has been used successfully for the cell cycle genes in yeast (125-129). The cloned genes can be transcribed and translated *in*

vitro to identify their product. The same approach had been used in mammalian cells for *ts* mutants that have defects in cell cycle progression. Several genes that complement the defect of *ts* cell cycle mutants have been identified (130,131) and isolated (132, 133, 134) following DNA -mediated gene transfer. As mentioned before, several proteins involved in mammalian cellular and viral DNA replication have been identified, but very few of them have been cloned. The DNA replication enzymes or proteins (not including histones) are generally expressed at low levels so that purification of those proteins from mammalian cultured cells usually required a considerable amount of cells and multiple steps of time consuming chromatographic separations. The instability of the protein may also cause difficulties. Therefore, cloning the wild type genes that complement the *ts* DNA mutants not only provides the identification of the genes and proteins but also permits overproduction of such proteins in appropriate expression vectors and can be very useful for the study of the eukaryotic DNA replication process in a reconstitution system. Genes related to DNA replication processes that have been cloned and reported so far encode sequences for pol α (135), topoisomerase I (136), and PCNA (137). I have adapted a gene cloning approach to identify the human gene that complements the cell defect in the *ts* DNA mutant JB7-K.

Among 200 CHO cell *ts* mutants, approximely 10% of them showed a *ts* DNA phenotype. Nine out of twenty showed a more pronounced *ts* phenotype and have been subcloned. Initial studies of these mutants were done by Drs. Dermody and Wojick in the laboratory. When I joined the laboratory, I participated in the screening of another subset of *ts* mutants isolated from V79 (Chinese Hamster lung cell line) as a parent cell line. Individual mutants were assessed by [^{3}H]-thymidine (TdR) and [^{35}S]-methionine (Met) double labelling for macromolecular synthesis. One of the mutants, called JH17-D showed a *ts* DNA phenotype. Complementation

test showed that JH17-D contained a different gene mutation from other mutants in the laboratory. Both Adenovirus and Py DNA replicated in JH17-D cells at normal levels at the NPT (39.5°C) even though cellular DNA synthesis (by means of [^{3}H]-TdR incorporation) decreased to 25% after shifting to 39.5°C for 6 h. The cell cycle analysis showed JH17-D cells are arrested at the G1 phase at the NPT. At the same time, we found that five out of nine CHO *ts* DNA mutants also restricted Py DNA replication at the NPT. Therefore I shifted my study to those mutants.

Specific Objectives

In this thesis I report the partial characterization of three *ts*DNA mutants in CHO cells that restrict Py DNA synthesis at the NPT and the further analysis of one of those mutants, JB7-K, with regard to Py DNA replication intracellularly. The molecular cloning of the human gene that corrects the *ts* cell defect has been initiated. The human genomic cosmid library developed by Kan and Lau (138) was chosen for DNA-mediated gene transfer because it carries the dominant selective marker *gpt* gene (Fig.1) which can confer resistance to the selective medium containing HAT+Xanthine+mycophenolic acid. The transfected JB7-K cells can be selected for gpt^+ in order to reduce the possibility of revertants accumulating after shift to the NPT. In addition, it carries the moderately repetitive *Alu* DNA family (139), since many human genes contain at least one copy of the *Alu* repeat (140) which can therefore serve as a natural marker for the presence of human DNA in transfected hamster cells. By identifying sequences which correct the *ts* defect in JB7-K, I propose to determine the basis of the *ts*DNA phenotype.

Methods

Tissue Culture:

The wild type and mutant CHO cells were cultured in Dulbecco modified Eagle medium (DME) supplemented with proline or an equal mixture of DME and Ham F12 medium (DF medium) with 10% newborn calf serum (M.A. Bioproducts) under conventional cell culture conditions as previously described (145). Mouse cells were cultured in DF medium with 10% newborn calf serum. Human fibroblast cells were cultured in DF medium with 10% of fetal calf serum (M.A. Bioproducts).

Individual colonies were either picked with 6-inch (15 cm) wood applicators (Thomas) or isolated by a cloning cylinder and transferred to 12-well dishes.

The cell lines used in this study are listed in Table 1.

Growth Properties:

To measure the growth rate of cells, 2×10^5 cells were seeded in a 60 mm dish at 33°C. On the next day, as the start time point, dishes were shifted to 35, 37, 38.7 and 39.5°C or continued to be incubated at 33°C. Cell numbers were determined following trypsinization with a Royco 927TC cell counter at 5, 10, 24, 48, 72, and 96, hours. Triplicate samples were counted for each time point.

The efficiency of colony formation (EOC) was determined by seeding different numbers of cells in 100 mm dishes at 33°C. Replicate dishes were shifted to 39.5°C on the next day. Two to three weeks later, the colony number was counted after staining the colonies with Crystal Violet . Triplicate samples were used for each point.

Table 1

Cell lines

Designation	Description	Reference
CHO	Chinese hamster ovary fibroblast	Dermody et al. (145)
TNT	thioguanine and ouabain resistant mutant of CHO	Radna et al.(145a)
JB7-K	*ts*DNA mutant of CHO	Dermody et al. (145)
JB3-O	*ts*DNA mutant of CHO	Dermody et al. (145)
JB8-D	*ts*DNA mutant of CHO	Dermody et al. (145)
JB11-J	*ts*DNA mutant of CHO	Dermody et al. (145)
V79	Chinese hamster lung fibroblast	Dermody et al. (145)
JH17-D	*ts*DNA mutant of a thioguanine resistant mutant of V79	Dermody et al. (145)
*ts*2	*ts*DNA mutant of mouse fibroblast 3T3	Slater et al. (113)
*ts*20	*ts*DNA mutant of mouse fibroblast 3T3	Jha et al. (114)
CV-1	monkey kidney cell line	Radna et al.(145a)
COS7	CV-1 containing an origin-defective SV40 genome (SVori$^-$ DNA)	Gluzman (145b)
HS74	human diploid fibroblast	Neufeld et al. (150a)
Cl 39	HS74 containing SVori$^-$ DNA	Neufeld et al. (150a)
HAL	HS74 containing SVori$^-$ DNA encoding a *ts*A58 mutant T antigen	Radna et al (150b)

The survival curves for *ts* mutants were determined by seeding 100 cells per 100 mm dish at 33°C. All dishes were shifted to 39.5°C after cell attachment to dishes (usually 16 h later) except for the zero time point dishes. The dishes were shifted back to 33°C after 5, 9, 16, 24, 48, 72, and 96 h and allowed to form colonies. Colonies were stained and counted after an additional two weeks.

Determination of rates of cellular DNA, RNA and protein synthesis:

CHO cells (5×10^4) in 3 ml DF medium were seeded into flat-based culture tubes (Nunc 1409) in the prone position and incubated for 24 h at 33°C. After adding 2 ml fresh medium, the tubes were shifted to an upright position to mimic later labelling conditions for an additional 16 h. Subsequently, one set of tubes was shifted to a 39.5°C water bath, the rest of tubes remaining at 33°C. At appropriate intervals thereafter, triplicate cultures were pulse-labeled at 33°C and 39.5°C with methionine-free DME medium supplemented with proline (50 μg/ml), 10% newborn calf serum, 1 μCi/ml of [^{35}S]-methionine (800 Ci/mmol; New England Nuclear Corp.) and 1 μCi/ml of [^{3}H]-thymidine (74 Ci/mmol; New England Nuclear Corp.) for 1 h. Incorporation was stopped by washing the cells with cold PBS for three times. The cells were lysed by addition of 1 ml of lysis solution (1% sodium dodecyl sulfate, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Macromolecules were precipitated with trichloroacetic acid, collected onto GF/A filters (Whatman, Inc.), and dissolved with an NCS tissue solubilizer (Amersham Corp.). Radioactivity was determined in Liquifluor (New England Nuclear Corp.) by liquid scintillation spectroscopy after double-label correction as described previously (146).

Table 2

Plasmids

Plasmid	Description	Source and Reference
p53A6.6	Polyoma virus genome cloned at the *Bam* HI site in pAT 153	R.Kamen (141)
pJB8	Cosmid vector (Fig.1)	Y.F.Lau (138)
pCV103	pJB8 containing SV2gpt (Fig. 1)(a)	Y.F.Lau (138)
pSV2gpt	SV40 early promotor and *E.coli. gpt* gene in pBR derived vector (Fig. 2)	P.Berg (142)
pUC 19	Plasmid vector	T.Schmidt-Glenewinkel
pBlur 8	Human medium repetitive *Alu* sequence cloned into pBR322 at *Bam* HI site	W.Jelinek (143)
pS1EMBL	R6K origin plasmid vector containing Kanamycin resistance gene (Fig. 3)	H.Lehrach (144)
p4aA8	human HPRT cDNA cloned into pCD vector (Fig. 4)	H.Okayama (155)

(a) pCV103 contains the SV40 promoter regulated *gpt* gene excised from pSV2gpt at the *Bam* HI and *Pvu* II sites. The *Pvu* II site was converted to a *Bam* HI site with a synthetic *Bam* HI linker. The Bal I site of pJB8 was converted to a Bgl II site and the SVgpt fragment was inserted at the Bgl II ends of the vector.

Recombinant DNA transformation, preparation and mapping:

All the plasmid and recombinant DNAs used in this project are listed in table 2 and were handled following NIH safety guidelines.

The DNAs were digested with designated restriction enzymes following the supplier's recommended conditions. The procedures used were generally as described by Maniatis et al.(150). The DNA fragments were gel purified and eluted with glass milk (Gene Clean Kit, Bio 101). The concentration and purity of DNA were checked by gel electrophoresis and ethidium bromide (EtBr) staining with known DNA concentration and size markers (Hind III digested λ DNA or 1 kb ladder from BRL). For cloning, the designated two pieces of DNA were mixed, coprecipitated with 0.5 volume of 7.5 M NH_4Ac and three volumes of ethanol, resuspended into 4 or 8 μl of 0.1x TE, and ligated by T4 DNA ligase. The ligated mixture was used to transform competent E.coli cell strains which are suitable for recombinant plasmid DNA selection. For example, AG1 strain from Stratagene for pBR derived plasmid vectors, JM109 strain for pUC 19 vectors, XL-1 strain from Stratagene for pS1EMBL vectors. Both GM109 and XL-1 strains have a deletion in the Lac Z gene and contain the Lac Z β γ sequence to complement the Lac Z α gene in the pUC 19 and pSEMBL plasmid vectors. IPTG (isopropyl-β-D-thiogalactoside) and X-gal plates were used for indicating whether the lac Z α gene was intact or interrupted by insertion (150). The transformed, drug resistant, recombinant DNA containing colonies were picked and grown for DNA mini preparation. The correct recombinant DNAs were identified either by restriction enzyme mapping and/or by hybridization to appropriate DNA probes.

Table 3

E. coli bacterial strains

Strain	Genotype	Utility
AG-1	F^-, *rec* A1, *end* A1, *gyr* A96, *thi*, *hsd* R17 (r^-k,m^+k), *sup* E44, *rel* A1 λ-(uncharacterized mutantion improves transformation efficiency).	Used for transformation of plasmid rescue from COS7 cells fused with 2^O5D cells.
DH5α	F' *end* A1, *hsd* R17(r^-k,m^+k), *sup* E44, *thi*-1, *rec* A1, *gyr* A, *rel* A1, ϕ80 *lac* ZΔM15, Δ(*lac* ZYA- *arg* F) u169.	Host strain of pCD2 human cDNA library.
JM109	*rec* A, *end* A1, *gyr* A96, *thi*, *hsd* R17, *sup* E44, *rel* A1 [tru D36 *pro* B^+ *Lac* Iq *lac* ZΔM15].	Used to amplify the pUC19 plasmid vector and recombinants.
LE392	F^-, *hsd* R514(r^-k,m^+k), *sup* E44, *sup* F58, *Lac* Y1 or Δ(*lac* IZY)6, *gal* K2, *gal* T22, *met* B1, *trp* R55 ,λ^-.	Used to propagate bacteriophage λ vector and recombinants.
NM538	*hsd* R (r^-k,m^+k), *sup* E, $\Phi 80^r$	Used to propagate λ/EMBL 3 vector and recombinants.
NM539	NM538(P2)	Used to propagate λ/EMBL3 recombinant only.

XL-1	*rec* A1, *end* A1, *gyr* A96, *thi*, *hsd* R17 *sup* E44, *rel* A1, λ^- ,(lac), {F', *pro*AB *lac* I^q ZΔM15, Tn10 (tet^R)}	Used to amplify pS1EMBL plasmid and recombinant.
χ 1776	F^-, *ton* A53, *dap* D8, *min* A1, *gln* V44 (*sup* E42), Δ(gal-UVr B)40, λ^-, *min* B2, *rfb*-2,*gyr* A25, *thy* A142, *oms*-2, *met* C65, *oms* -1, (tte-1), Δ(*bio*H-*asd*) 29, *cyc* B2, *cyc* A1, *hsd* R2.	Host strain of pCD human cDNA library.

Determination of polyoma virus DNA replication:

Cells were seeded at 5 x 10^5 cells per 60-mm dish and transfected with 1μg of p53A6.6 DNA (141) plus 9 μg of calf thymus carrier DNA by the calcium phosphate DNA co-precipitation technique (147, 148) for 16 h or as modified for more effective uptake as described in the RESULTS section. Viral DNA was recovered by Hirt extraction, phenol and phenol-chloroform extraction, and ethanol precipitation (149). Replicated recombinant DNA was identified after digestion with *Sal* I and *Dpn* I (Promega Biotech.) and quantitated by Southern analysis with nick-translated p53A6.6 DNA.

Genetic Marker Selection:

2 x 10^6 cells seeded in a T175 flask were mutagenized with 250 μg/ml of ethyl methane sulfonate (EMS) for 16 h. Cells were washed twice with serum free medium, and fresh drug-free medium was added. After reaching confluence, cells were subcultured at 2 x 10^5 per 100 mm dish. 3 mM ouabain (Sigma) was used to select ouabain resistant (OUA^R) cells. $5x10^{-5}$ M 6-thioguanine (ICN Pharmaceuticals, Inc.) in DF medium or 10^{-5} M in DME medium was used to select thioguanine resistant (TG^r) cells. Usually after 2-3 weeks, the colonies were isolated and cultured until there were enough cells for freezing and confirming the *ts* phenotype. Thioguanine-resistant mutants were determined to be deficient in the enzyme HGPRT by their inability to form colonies in HAT medium.

Cell Fusion:

The two cell lines to be used in the experiment were trypsinized and the designated cell numbers were mixed, co-centrifuged, washed twice with DME medium, and seeded onto a 100 mm dishes at 33°C for 16-24 h. 2 ml of 50% PEG

(polyethylene glycol) 1000 in DME medium was added slowly and incubated at room temperature for 2 min. The PEG solution was then aspirated and the dishes were quickly washed with DME twice. The cultures were incubated at 33°C for 5 min in DME followed by 30 min with complete DME medium. The medium was again removed and the cultures were incubated overnight.at 33°C. The fused cells were subcultured at 10^4 or/and 10^5 cells per 100 mm dish in selection medium (HAT + 3 mM ouabain) which genetically selected for the hybrid cells and against the parental cells. In all cases, one of the parents contained two drug resistant mutations (TG^r, OUA^R) whereas the other parent was as wild type (TG^S, OUA^S).

Chromosome karyotype:

Karyotyping was done as described in Neufeld et al. (150a) with minor modifications. Briefly, a subconfluent monolayer was refed with fresh medium and incubated for 24 h. 50 µl of 10 mg/ml Colcemid (Gibco) was added to each 100 mm dish and incubated at appropriate temperature for 1-1.5 h. The dishes were tapped to loosen mitotic cells from the monolayer. The medium was collected and cells were recovered from suspension by centrifugation at 1000 g for 5 min. The cell pellet was resuspended in 8 ml of 0.075 M KCl and incubated in a 37°C water bath for 50 min. After addition of 2 ml of freshly made fixative (methanol: glacial acetic acid=3:1), the cells were centrifuged at 1000g for 5 min. The cell pellet was repeatedly resuspended in 3 ml of fixative and centrifuged at 1000g for 5 min for three times. The swollen mitotic cell suspension was dropped onto a microscope slide and heat fixed. The chromosome numbers were observed microscopically under the oil immersion lens.

Flow Microfluorimetry:

At various times, exponentially growing cells were harvested by trypsinization, washed with phosphate buffered Saline (PBS) and resuspended at 2 x 10^6 cells / ml. One milliliter of the cells was "quick dropped" to 9 ml of cold 70% ethanol for fixing. Cells were stained with propidium iodide in triton X-100, treated with RNase, and analyzed with a System 50H flow cytometer (Ortho Diagnostic) interfaced to an Ortho 2150 data analysis system by Dr. F. Traganos (Sloan Kettering Institute) as described elsewhere (161).

Protoplast fusion:

Protoplast fusion in suspension was modified in this laboratory (170). Briefly, 100 ml of plasmid or cosmid-bearing *E.coli* DH-1 were grown to a concentration of OD_{600}=0.9. Then,chloramphenicol (250 μg/ml) was added, and the cells were continued to incubate for 14 to 18 h at 37°C. The bacteria were subsequently centrifuged at 4°C at 3,000 x g for 10 min, vigorously suspended in 2.5 ml of cold 20% sucrose in 0.05 M Tris-HCl, pH8.0 and pipetted into a sterile, 50 ml shallow cone-bottomed glass tube (Bellco Glass, Inc.). Protoplasts were generated essentially as described by Sandri-Goldin et al. (149a). All solutions were filter sterilized. The bacteria were treated on ice with 0.5 ml of lysozyme (5 mg/ml in 0.25 M Tris-HCl, pH 8.0) for 5 min followed by 1 ml of 0.25 M EDTA (pH8.0) for an additional 5 min. One milliliter of chilled 0.05 M Tris-HCl,pH8.0 was then carefully added to the mixture. The suspension was incubated in a 37°C water bath for 5 min and at room temperature for 30 min or until all rods were converted to protoplasts, as observed by phase-contrast microscopy. The protoplast suspension was treated with DNase I to digest extracellular DNA. Twenty milliliters of a solution of DNase I (10 μg/ml,Worthington Diagnostics) in DME medium

supplemented with 15 mM $MgCl_2$ and 10% sucrose was very slowly pipetted into the protoplast suspension, and the mixture was incubated at room temperature for 15 min. Any remaining viscous DNA was removed with a serological pipette. The suspension could be pipetted at this point for portioning into other tubes before fusion. The protoplasts were sedimented at 800 x g for 12 min. The supernatent containing DNase was removed, and 5 ml of DME was added without disturbing the protoplast pellet. Concomitant with the centrifugation of the protoplasts, the CHO cells were trypsinized, diluted with 10 ml of DME with 10% Newborn calf serum, centrifuged, and suspended in 10 ml of DME. The cell number was determined with a Royco cell counter. Typically 10^7 cells were added per fusion. The cell suspension was then pipetted into the DME and centrifuged at 200 x g for 5 min so that the cells overlay the protoplast pellet. The suspension was aspirated, 50 µl of DME was added, and the mixed pellet was resuspended manually. Polyethylene glycol (0.4 ml of 57% Koch-Light PEG 1000 [wt/wt] in DME) was added to the mixture, and the mixture was briefly agitated (15 s) and diluted with 10 ml of DME after 1.5 to 2.0 min. The cell suspension was incubated at room temperature for 15 min and pipetted into petri dishes (10^6 cells per 150 mm dish) with preincubated complete medium. After cell attachment, the medium was replaced by complete medium which contains gentamycin (50 µg/ml) to prevent bacterial growth. Selective medium was added after 48 to 72 h of fusion.

High Molecular Weight (HMW) DNA extraction:

Confluent cell cultures were rapidly washed twice with PBS . The cells were then lysed with 4 ml of DNA lysis buffer A (100 mM NaCl, 50 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5% SDS) per 150 mm dish. The lysis mixture was collected into a 50 ml polypropylene tube with a teflon taped razor blade (VWR Scientific

Inc.), transferred quickly to a 65°C water bath for 15 min, and cooled in a 37°C water bath. Freshly made proteinase K solution (10 mg/ml) was added to the DNA lysis mixture to a final concentration of 100 μg/ml and the tubes were incubated at 55°C for 48 h with shaking at intervals. The DNA solution was extracted with an equal volume of phenol:chloroform for 3-4 times with gentle mixing. The aqueous phase was poured into a clean tube instead of pipetting to avoid shearing of HMW DNA. The DNA samples were further extracted with chloroform for 2-3 times until there was no visible protein interphase remaining. The clear DNA solution was dialyzed against a large volume of TE buffer (usually 4 liters) in the cold room with three changes of buffer. Fifteen milliliters of the HMW DNA solution were aliquoted into a 50 ml sterile tube, 1 ml of 5M NaCl was added to a final concentration of 330 mM and mixed well, followed by addition of 2 volumes of 100% ethanol. The DNA precipitated out immediately upon mixing and was picked up by a hooked pasteur pipet, washed with 70% ethanol and dissolved in 1.5 ml of TE. The HMW DNA solution was left at room temperature overnight in order to let the DNA dissolve in solution completely, then stored at 4°C. The concentration of DNA was determined by UV spectrophotometry (O.D.260). The quality of HMW DNA was judged by gel electrophoresis in 0.3% agarose with intact linear λ DNA (50 kb), T5 phage DNA (99 kb) and T4 phage DNA (160 kb) as markers.

Construction of genomic DNA library in λ phage vector:

To prepare a genomic DNA library, HMW DNA from a secondary *ts*$^+$ transfectant (7K2^{o}5D) was partially digested with *Sau3A* I (0.015 μ/μg DNA) at 37°C for 1 h. To ensure good mixing of the DNA and enzyme, the reaction was performed in multiple tubes (20 μg DNA per 2 ml conical Eppendorf tube). The reactions were stopped by adding 0.5 M EDTA to a final concentration of 20 mM

EDTA. The DNA samples were loaded on a linear 5-25% NaCl gradient with 100 µg DNA per tube and centrifuged in a SW41 rotor at 37,000 rpm for 4.5 h at 20°C. Fractions of 0.25 ml were collected by a Density Gradient Fractionator (ISCO Model 185). 10 µl aliquots of each even number fraction plus 30 µl of H_2O and 8 µl of loading dye were mixed well and analyzed in a 0.4% agarose gel by electrophoresis for 24 h at 40 V in TPE buffer (0.08M Tris-phosphate, 2 mM EDTA). DNA size marker (HMW DNA marker from BRL) was run in parallel with corresponding salt concentrations. DNA fractions in the 15-21 kb size range were pooled and ethanol precipitated directly. The DNA pellets were washed with 70% ethanol twice and resuspended in 0.1X TE at 0.25 µg/ml.

Alkaline phosphatase treated λ EMBL3 /Bam HI arms (obtained from Promega) were preannealed at the *cos* sticky end sites in 10 mM $MgSO_4$ buffer at 42°C for 1 h (151). To construct a library, 1:1 and 2:1 molar ratios of the fractionated DNA to preannealed arms were ligated at 16°C for 20 h. The self ligated λ arms served as a negative control and pSD8 plus λ arms as a positive control (see RESULTS III, section 3, for reference). Aliquots of 1 µl were taken from each reaction tube before and after ligation and analyzed on 0.8% agarose gel by Field Inversion electrophoresis program number 4 (MJ Devices PPI-100 with swither chip version 100.3) in 0.5X TBE buffer (1X TBE: 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) to check the quality of ligation. Thereafter, the ligation mixtures were packaged into phage particles *in vitro* for 2 h at room temperature using a commereical packaging extract (from Promega). The packaged libraries were titered on E.coli LE392 strain on NZYCM plates (151) (NZ amine: 10 g, NaCl: 5 g, yeast extract: 5 g, casamine: 1 g, $MgSO_4 \cdot 7H_2O$: 2 g, pH 7.5, per liter), and P2 lysogen strain NM539 on BBL plates (152) (BBL Trypticase peptone:10 g, NaCl: 5 g, agar: 10 g. pH 7.2, per liter supplemented with 10 mM $MgSO_4$).

Titration and Amplification of phage library:

Host strain NM539 "plating" cells were prepared as described elsewhere (150). Briefly, cells were grown overnight in LB medium supplemented with 10 mM $MgSO_4$ and 0.2% Maltose at 37°C. A 1:50 dilution of the overnight culture was inoculated into the same medium and grown to A_{600}=0.5. The cells were centrifuged at 2000 rpm for 15 min and resuspended into a half volume of 10 mM $MgSO_4$ solution. 100 µl of the "plating" cells plus 100 µl of the phage library at different dilutions (phage dilution buffer: 0.1 M NaCl, 20 mM Tris-HCl, pH 7.4, 10 mM $MgSO_4$)were incubated at 37°C for 30 min. Three ml of top agar (0.5% agar in BBL medium) was added, mixed by vortexing and poured on the 100 mm BBL agar plate. The plates were incubated overnight at 37°C and the plaque numbers were determined.

The packaged phage library was amplified in P2 lysogen strain NM539 which genetically selects against wild type λ phages as described elsewhere (151, 152). $2x10^4$ phage were added to 0.5 ml of freshly prepared NM539 "plating" cells and incubated at 37°C for 15 min. 6.5 ml of top layer agarose was added to each tube and poured into a 150 mm BBL agar plate. Plates were incubated at 37°C for 16 h and removed to 4°C. 10 ml of cold SM (NaCl: 5.8 g, $MgSO_4$:7 H_2O: 2 g, 50 ml of 1 M Tris-HCl, pH 7.5, and 5 ml of 2% gelatin per liter) was added to each plate. The plates were left at 4°C overnight on a level surface. The SM solution was collected and the plates were washed with an additional 5 ml of SM solution for each 5 plates. Bacterial debris was spun down at 5000 rpm for 15 min at 4°C and the supernatent was saved. Chloroform was added to the supernatent to 5% and stored in teflon capped glass tubes (153).

Screening of the phage library:

500 µl of freshly prepared "plating" NM 539 cells (8 x 10^8cells/ml) infected with 50,000 phage of the genomic library were plated onto a BBL agarose layer in a single 150 mm plate; a total of 1.25 x 10^6 plaques were screened. The plates were incubated at 37°C for 6.5 hours and then left in the cold room for at least one hour. Replica filters were lifted on the plates: 10 min for the first one and 20 min for the second one. The same orientation for duplicate filters and master plate was made by needle. The master plates were saved in the cold room. The replica filters were denatured with 0.2 N NaOH/1.5 M NaCl for 2 min, then renatured with 0.4 M Tris-HCl, pH 7.6/2X SSC (1X SSC: 150 mM NaCl, 15 mM Na Citrate) for 2 min and 2X SSC alone for another 2 min. The filters were baked at 80°C in a vacuum oven for 90 min and wet individually with hybridization solution (5X SSC, 48% formamide, 1X Denhart, 10% dextran sulfate, 0.1% SDS, 50 µg/ml salmon sperm DNA). The filters were hybridized for 40 h with a human *Alu* sequence DNA probe (labelled with ^{32}P by the nick-translation method), washed with 2X SSC at room temperature for 15 min twice and with 0.2X SSC /0.1% SDS at 50°C for 20 min for three times. The filters were air dried, the orientation hole marked by pen and exposed to X-ray film.

Growth of phage, isolation and analysis of recombinant phage DNA:

Phage lysates were prepared from individual plaques and phage DNAs were isolated as described by Grossberger (154). Briefly, 200 µl of phage suspension from a single plaque was used to infect 200 µl of "plating" cells (NM539) in 15 ml NZYCM medium containing 0.2% maltose at 37°C overnight. The tubes were put at 45 degree position. For lysis, chloroform was added to 5% and the overnight culture shaken at 37°C for an additional 15 min. The bacterial debris was pelleted

by centrifugation at 2000 rpm for 10 min. The supernatent was collected and centrifuged in a SW41 rotor (Beckman) at 30,000 rpm for 30 min at 4°C The phage particle pellet was resuspended in 200 µl of SM buffer and transferred to a 1.5 ml microfuge tube. 200 µl of freshly made proteinase K (1 mg/ml) was added to each phage suspension and incubated for 2 h at 37°C. The digested solution was extracted once with phenol and once with phenol/chloroform. The DNA was precipitated with 100 µl of 7.5 M NH_4Ac and 1 ml of 100% ethanol. After microcentrifugation for 15 min, the pellet was washed with 100% ethanol, dried in the air and dissolved in 100 µl TE.

Recombinant phage DNAs isolated from primary, secondary, and tertiary screened positive plaques were digested with different restriction enzymes (5u/µg DNA for 3 h). Three aliquots were made from each enzyme digested sample and analyzed on three 0.8% agarose gels electrophoresis. One of the gels was stained with EtBr and photographed under UV light. All the gels were transferred to Nytran membranes. The three filters were hybridized to three different ^{32}P-labeled DNA probes (i.e. *Hind* III digested λ DNA, pBR322 plasmid DNA, and total human DNA which detects multiple repetitive but not single copy sequence). The bands detected by different probes were compared to the bands on the EtBr stained gel picture to identify the bands that did not hybridize to any of the three probes.

EcoR I digested 2.8 and 1.4 kb fragments from recombinant phage λa14 were gel purified and individually subcloned into pS1EMBL (144) at the unique EcoR I site.

Southern blot and Northern blot hybridization:

The Southern blot procedure was performed as described elsewhere (150). Total cytoplasmic RNA was prepared by using the RNA Preparation Kit (5 prime--

3 prime). Electrophoresis, transfer and hybridization were performed as described elsewhere (150).

cDNA library screen.

Two human cDNA libraries have been screened. The first one contains cDNA prepared from mRNA extracted from a SV40-transformed human fibroblast (GM 639), ligated with pCD vector (Fig. 33A)and transformed into E. coli strain X1776 (155). The second one contains cDNA prepared from mRNA extracted from a normal human fibroblast, ligated with pCDneo vector (which has a covalently linked dominant selectable marker neo^R, see Fig. 33B) and transformed into E.coli αDH5 strain (156).

Bacteria containing a human cDNA plasmid library were grown in proper medium specific for each host strain (X medium for X1776 strain, LB medium for αDH5 strain). X medium per liter: Bacto-tryptone (25 g), Bacto-yeast extract (7.5 g), 1 M Tris-HCl, pH7.5 (20 ml), supplemented with 1M $MgCl_2$ (5 ml), 1% diaminopimelic acid (10 ml), 0.4 % thymidine (10 ml), 20 % glucose (25 ml). The cell numbers were determined by both spectrophotometry (O.D.600) and colony formation. The bacteria are grown to O.D.600=0.1. The titer of the X1776 strain in X medium with ampicillin is usually consistent with the O.D. reading only when they are plated on the HTAF filter from Millipore. Nitrocellulose filters from another company (S&S) causes a 20 fold reduction in the expected colony count. The titer of αDH5 strain in LB medium with ampicillin on HTAF filter is 20 fold less than predicted from the O.D. reading.

10^4 bacteria containing cDNA plasmid (provided by Dr. H. Okayama, NIH) were mixed with 20 ml of X medium, and evenly applied onto prewet 82 mm nitrocellulose filters using a sintered glass support 90 mm filter holder apparatus

(Model FG 90, Whatman). A total of 3 x 10^5 cells were plated per screen. The filters were incubated on the100 mm X plates at 37°C for 14 h, then transferred to the cold room until ready to prepare replicas. All the filters were prewet on the agar plates. Two replica filters were made from each master filter by pressing the filters together with glass plates. The replica filters were placed on X plates and incubated at 37°C overnight. Thereafter the master filters were put back on the X plates and left at room temperature overnight, sealed and stored at 4°C. The replica filters were transferred to LB plates containing chloramphenicol (170 mg/ml) for αDH5 strain (157) or to X plates containing spectromycin (250 mg/ml) for X1776 strain (158), and incubated overnight at 37°C. The filters were denatured on 0.5 N NaOH solution saturated 3 MM paper for 5 min, renatured by sequential exposure to 3 MM paper saturated with 1 M Tris-HCl, pH7.5 and 1.5 M NaCl: 0.5 M Tris-HCl pH7.5 for 5 min each, and baked in a vacuum oven at 80°C for 90 min. The filters were wet individually in hybridization solution (5X SSC, 4X Denhart, 10 mM Tris-HCl, pH7.5, 0.1% SDS, 35% formamide, 50 μg/ml ss DNA), prehybridized overnight at 42°C, and hybridized for 48 h at 42°C with nick-translated ^{32}P labeled human unique sequence 2.8 kb fragment DNA as a probe (gel purified). The filters were washed, dried, marked and exposed to x-ray film under the same conditions as described in the phage library screening method.

Results

Chapter I: Polyoma viral DNA replication in CHO *ts* mutants

1. Polyoma virus DNA replication in CHO ts mutants

Over 200 CHO cell mutants temperature sensitive (*ts*) for growth had been isolated in this laboratory. 10% of them showed a DNA replication *ts* phenotype (145). After further study of these mutants by more detailed time course of the rates of DNA and protein synthesis at both the permissive and nonpermissive temperature, nine of them that showed the most pronounced *ts* phenotype in terms of cellular DNA replication were subcloned. All these nine mutants have been examined to determine whether the cellular *ts* gene products were involved in adenovirus DNA replication by B. Wojick (159) and polyoma virus DNA replication by me and J. Dermody. Even though polyoma virus cannot infect the CHO cell directly, presumably because of a defect in virus adsorption and /or penetration, Dr. LaBella in this laboratory had shown that viral DNA can replicate in the CHO cell and produce infectious viral particles if the viral DNA was transfected into the cells by DNA calcium-phosphate (CaP) coprecipitation (147). The replicated viral DNA can be detected at 40 h post transfection by *Dpn* I assay (160). Since the input recombinant viral p53A6.6 DNA (an intact polyoma virus genome cloned in the pAT153 plasmid vector at Bam HI site) was prepared in dam^+ E.coli, the DNA was methylated on the N^6-adenine and was *Dpn* I sensitive. The restriction enzyme *Dpn* I only digests the methylated DNA sequence, not the nonmethylated or semimethylated sequence. The DNA replicated in CHO cells is

nonmethylated or semimethylated so it is *Dpn* I resistant. The replicated DNA was linearized by the single cut enzyme *Sal* I for easy comparison and quantitation. For all mutants, the transfection condition was the same as described in the methods. A quarter of each duplicate DNA sample prepared at different times post transfection was digested with *Dpn* I and *Sal* I, electrophoresed on a 1% agarose gel, transferred to nitrocellulose and hybridized to a ^{32}P-labeled p53A6.6 DNA probe. Cultures were incubated at either 33°C or 39.5°C for 1 to 4 days. Some cultures were also incubated initially at 33°C for 1 or 2 days followed by a subsequent shift to 39.5°C. Newly replicated p53A6.6 DNA was not detectable at one day post transfection at either 33°C or 39.5°C in any cell line tested. However, all the mutants subsequently showed polyoma DNA replication at 33°C. Five of the mutants (JB3-O, JB3-B, JB 7-K, JB8-D and JB11-J) do not support polyoma DNA replication at 39.5°C. An example of one of those (JB7-K) is shown in Fig. 5. Viral DNA is evident at 48 h at 33°C and thereafter in the high molecular weight region as a single band at 8.9 kilobase (kb). The multiple smaller fragments represent *Dpn* I-digested (input) sequences. All five showed little or no detectable level of polyoma DNA replication when incubated for 48 h at 39.5°C (lanes 5 and 6). Moreover, they also showed deminished accumulation of viral DNA after shift of culture from 33°C to 39.5°C as can be seen by comparison of lanes 1 and 2 (48 h at 33°C) with lanes 3 and 4 (24 h at 33°C plus 24 h at 39.5°C), and lanes 7 and 8 (72 h at 33°C) with lanes 9 an 10 (48 h at 33°C plus 24 h at 39.5°C). Six other mutants, four isolated here (JB1-C, JB5-G, JB10-O JB6-N), and two isolated elsewhere (*ts*C8, *ts*13A) showed results similar to wild type CHO K1 cells in that there was no effect on polyoma DNA replication at the nonpermissive temperature The result with one such mutant (JB10-O) is shown in Fig. 6. The level of viral DNA increased between 48 and 72 h at 33°C (compare lanes 1 and 2 with lanes 7

and 8). As expected, viral DNA synthesis was enhanced at 39.5°C; cells incubated at 39.5°C for 48 h (lanes 5 and 6) or 24 h at 33°C followed by 24 h at 39.5°C (lanes 3 and 4) had more viral DNA than those incubated continuously at 33°C. The results with all the other mutants are shown in Appendix I.

2. Growth properties of the *ts*DNA mutants

As a first step in the further characterization of these *ts*DNA mutants, the general cell growth properties of three of them have been studied. JB3-B has been previously described by Wojcik (159).

Growth curves were performed on each mutant and wild type cells. Cells were plated at 33°C and duplicate cultures were shifted to 39°C after 16 h. The time of shift was designated T=0. Cell numbers were counted as described in the methods at T=5, 10, 24, 48 h. The growth rate for wild type cells (CHO K1) at 39.5°C is higher than that at 33°C as expected (Fig. 7A). The cell number doubling time in log phase for CHO K1 cells is 12.5 h at 39.5°C and 16-18 h at 33°C. All the mutants show poor growth at 39.5°C. Cell growth rate for JB 7-K cells at 39.5°C is markedly slowed as compared to that at 33°C, and appears not to increase significantly after 12-24 h at 39°C.(Fig. 7B). For JB3-O cells (Fig. 7C), there appears to be an initial period of growth following shift to 39.5°C for 7 to 10 h, however, there is less than a 2-fold increase over 48 h at 39.5°C. The cell doubling time at 33°C is 24 h. The cell growth rate for JB8-D at 39.5°C is similar to that at 33°C during the first 24 h after shift to 39.5°C (Fig.7D). But the total cell number is lower in 39.5°C than in 33°C at T=48 h. A subclone of JB8-D (JB8D-TG^{I}) also shows growth at 39.5°C at a lower level than that at 33°C (data not show).

We had previously observed that *ts*DNA mutants often lose viability after incubation at nonpermissive temperature (113,118). We therefore determined the

degree of remaining colony forming ability after sparse cultures are shifted to 39.5°C and then returned to 33°C (a survival curve experiment as described in the methods). The results are shown on Table 4. The efficiency of colony formation for the non-shifted culture (maintained at 33°C) was greater than 50 per 100 cells plated for all three cell lines. It is considered as 100% (T=0) and percent survival was calculated relative to that value. After a 24 h period at 39.5°C, JB3-O had 60% cells surviving,.JB7-K had 82% cells, JB8-D had 80% cell. After 48 h period at 39.5°C, survival falls markedly for JB3-O (7.1%) and JB8-D (7.8%). However, JB7-K is relatively slow to die at 39.5°C (62% survival). These results also suggested that the temperature shift experiment for those mutants should be designed such that the incubation times at the nonpermissive temperature is less than 48 h for JB7-K and less than 36 h for JB8-D and JB3-O.

The efficiency of colony formation (EOC) at different temperatures (i.e. 33, 35, 37, 38.7 and 39.5°C) for all three mutants JB7-K, JB8-D and JB3-O were also studied. The results are shown on Table 5. All three showed a marked drop in EOC at 38.7°C; no discrete colonies were observed at 39.5°C. In the case of JB7-K, cultures seeded at 5 x 10^5 cells in a 100 mm dish showed persistent survival and/or growth so that there were areas of confluence.

3. Cellular DNA and protein synthesis

Wild type and mutant cells were pulse labelled for 1 h with [^{3}H]-TdR and [^{35}S]-Met as described in the methods to assay the rate of macromolecular synthesis under different growth condition to verify the original *ts*DNA phenotype. [^{3}H]-TdR incorporation measures the rate of DNA synthesis whereas [^{35}S]-Met incorporation measures the rate of protein synthesis. The results are shown in Fig. 8. Wild type CHO cells showed increased [^{3}H]-TdR and [^{35}S]-Met incorporation at both 33°C

Table 4

Survival of colony formation after incubation at 39.5°C [a]

Cell line	Hours at 39.5°C							
	0	4-7	24-26	38-40	48-50	54	72	96
JB3-O	80[b]	70	48	ND[d]	6	4	0	0
	100%[c]	87.5%	60.7%	ND	7.1%	5.4%	<1%	<1%
JB7-K	92	83	75	69	57	48	41	5
	100%	90.9%	81.8%	74.9%	62.5%	52.4%	44.7%	5.5%
JB8-D	64	68	52	41	5	ND	0	0
	100%	100%	80.5%	64%	7.8%	ND	<1%	<1%

(a) one hundred cells were plated at 33 °C for 16 h and shifted to 39.5 °C for the indicated periods of time. They were then returned to 33 °C for an additional two weeks for colonies to appear. Numbers are the average of triplicate dishes.

(b) number of colonies observed.

(c) percent of survived cells.

(d) Not determined.

Table 5

Efficiency of colony formation at different temperatures (a)

	Temperature (degrees centigrade)				
Cell lines	33	35	37	38.7	39.5
JB3-O	87	45	37	0.029	$<10^{-4}$
JB7-K	58	54	28	ND(b)	$<10^{-3}$
JB8-D	62	74	80	0.033	$<10^{-4}$

(a) Different numbers of cells were seeded at 33°C overnight, i.e. 50, 100 cells per dish at 33°C, 35°C; 100, 250 cells per dish at 37°C; 10^4 and 10^5 cells at 38.7°C and 39.5°C. Dishes were shifted to each temperature and observed for 16 days for colonies to appear. Numbers are the average of percentage of colonies formation in triplicate dishes.

(b) Not determined.

and 39.5°C (Fig.8A). As expected, the rates at 39.5°C are higher than that at 33°C. In all the mutants, the DNA and protein synthesis at 33°C increase over the experiment because the total cell number in each culture tube is increased. But each mutant shows slightly different rates of DNA and protein synthesis at 39.5°C. JB3-O (Fig. 8B) shows a somewhat strange pattern of both [^{3}H]-TdR and [^{35}S]-Met incorporation at 39.5°C as compared to the other mutant's incorporation, they first increase a little and then both decrease in parallel. In all cases TdR incorporation at 39.5°C was most severely affected. An independent experiment showed the same pattern; it involved the cultures utilized for the growth curve data (see Fig. 7C). In that experiment, the cell number increased after shift to 39.5°C for 7-12 h, was stable for 24 h and then decreased. A subclone of JB3-O (i.e.JB3O-THO) is included for compareson as well (Fig. 8F). It also showed a preferential inhibition of DNA synthesis at 15 h at 39.5°C. However, it did not show the unusual pattern seen with JB3-O. JB7-K (Fig. 8C) showed a 60% decrease in the rate of DNA synthesis after 12 h at 39.5°C as compared to the rate at shift time T=0, it fell further to 10% at 16 h at 39.5°C. TdR incorporation at 33°C increased throughout. The protein synthesis rate was increased dramatically at 39.5°C and was elevated even at 24 h at 39.5°C as compared to 33°C. JB8-D (Fig. 8D) showed 56% decrease of the rate of DNA synthesis at 39.5°C and fell to 10% after 16 h at 39.5°C. The "overall " pattern is quite similar to that of JB7-K despite the difference in the growth curve. A fourth mutant JB11-J (Fig. 8E) showed a 50% decrease of the rate of DNA synthesis at 39.5°C after 5 h and slowly fell to 20% incorporation after 14 h at 39.5°C. The rate of protein synthesis slowly increased at 39.5°C until 14 h and then decreased. In all cases, incorporation at 39.5°C was less than at 33°C.

4. Complementation analysis

After the initial biological study of all the mutants that restrict polyoma DNA replication at the NPT, I wished to choose one of them to study in more detail. Before I made any decision, I felt that it was important to determine whether the defects in those mutants involved the same gene or different genes; that is, were they able to complement each other at the nonpermissive temperature?

In order to study the mutant defects by gene complementation, each of the two parent cell lines should have its own selectable dominant genetic marker. For example, one cell line is resistant to ouabain (OUA^R) and thioguanine (TG^r =$HPRT^-$) and another cell line is OUA^S and $HPRT^+$ (TG^S). This allows one to isolate hybrids without using temperature as a primary selection marker. Several ts mutants and wild type cell lines were available to me in which these drug resistant markers had already been introduced. Such cell lines are designated by the symbol THO. JB7-K, JB8-D, JB3-O and two mouse cell *ts* DNA mutants *ts*2 and *ts*20 which were previously isolated in the laboratory (113, 114) were fused to each other and examined as to whether the *ts* defect gene in two different *ts* cells could be complemented. Each *ts* mutant which has a THO (TG^r and OUA^R) genotype was fused to wild type cells as a positive control; JB7-K was fused to TNT cell [a wild type CHO cell which has a THO genotype (160a)]. Fusion involving the same mutant with the *ts* THO genotype and regular *ts* cell was used as a negative control. The hybrid cells were selected in HAT+ouabain medium. The list of cell fusions is showed on Table 6. The result of *ts* complementation has been verified by growing up the hybrid colonies picked in selection medium at 39.5°C or 33°C and comparing the efficiency of colony formation at 39.5°C and 33°C (Data not shown). To exclude the possibility of *ts* revertant or selectable marker revertant, the ts^+ hybrid cells were also examined for chromosome number by karyotyping in

Table 6

Complementation Analysis[a]

	$TG^{r}OUA^{R}$ Cell		
	JB3-O	JB8-D	TNT
$TG^{s}OUA^{s}$ cell			
ts 2	+[b]	+	ND
ts 20	+	+	ND
JB3-O	-	+	+
JB7-K	+	-[c]	+
JB8-D	+	-	+
CHO-K1	+	+	ND

(a) Cells were fused, subcultured as described in the methods. The hybrid cells were selected in HAT+OUA(3 mM) medium at both 33°C and 39.5°C. The origin of the cell lines is listed in Table 1.

(b) Hybrid cells grow at 39.5°C.

(c) Hybrid cells do not grow at 39.5°C.

selected cases. Every *ts* mutant (JB7-K, JB8-D and JB3-O) can be complemented by ts^+ CHO cell (CHO-K1 and TNT, see Table 1), which means that they are all recessive mutants. JB8-D can complement JB3-O, *ts*2 and *ts*20; JB3-O can complement JB7-K, JB8-D *ts*2 and *ts*20; JB7-K can complement JB3-O, *ts*2 and *ts*20, However, JB8-D and JB7-K do not complement each other. No colonies were obtained in HAT+ouabain at 39.5°C. Several JB8-D and JB7-K hybrid colonies (named Hy8D-7K) were picked from 33°C dishes, grown up and seeded at different densities to test the EOC at 39.5°C. There were no colonies observed even when the hybrid cell was seeded at $5x10^5$ cell per 100 mm dish. A karyotype supports the interpretation that the Hy8D-7K cell is a true hybrid cell, because it contains 42 chromosomes and JB7-K and JB8-D each contains 21 chromosomes. I conclude therefore that these two mutants are in the same complementation group but it is different from JB3-O and the mouse *ts*DNA mutants (*ts*2 and *ts*20).

5. Further studies with JB7-K

In view of the complementation data and other aspects of the ts cell phenotype to be discussed later, I elected to study JB7-K in detail. As a prelude to such investigations, I did a further series of preliminary studies.

a. Cellular RNA synthesis

Although the data in figure 8 and elsewhere documented a preferential fall in thymidine incorporation as compared to methionine incorporation, it would be useful to verify that RNA synthesis was not affected as would be expected for a ts DNA mutant. Accordingly JB7-K and JB11-J (for comparison) were pulse-labelled with [^{3}H]-uridine and [^{35}S]-Met for 1 h at the same time interval as [^{3}H]-TdR and [^{35}S]-Met labelling at both 33°C and 39.5°C. Both of them showed no primary

inhibition of uridine incorporation as a measure of overall RNA synthesis (essentially ribosomal RNA) at the nonpermissive temperature as shown in Fig. 9. JB11-J showed some decrease of ^{3}H-UR incorporation.

b.Cell cycle analysis of JB7-K at 39.5°C

A mutant cell arrested at S phase of the cell cycle would be considered as a DNA synthesis defective mutant, but not all DNA synthesis defective mutants are arrested at S phase at the NPT. Cells defective in some other cell cycle functions could also show a decrease in TdR incorporation. I would like to know where JB7-K cells are arrested at the NPT. Cell cycle analysis was performed for JB7-K cells in collaboration with Dr. Traganos. JB7-K cells were seeded at low density (8 x 10^4 cells/100mm dish) for two days; some of the cultures were shifted to 39.5°C and some remained at 33°C. Cultures were withdrawn at T=1, 3, 5, 10, 24 and 36 h from 39.5°C and also from 33°C at T=1, 10, 24, and 36 h as a control. Cells were prepared as described by Dr. Traganos (161) Briefly, cells were trypsinized, washed with PBS and resuspended at 2 x 10^6 cells/ml in PBS. One ml of each cell suspension was added to 9 ml of cold 70% ethanol by a "quick push" from a pasteur pipette. Once fixed in ethanol, this cell suspension can be left at 4°C for weeks before staining and determination of DNA content as described in the Methods. The DNA content measurement was done in collaboration with Dr. F. Traganos (161 a). The distributions of the cell populations are shown in Fig. 10. Calculation of the percentage of cells in each cell cycle phase was determined by conventional procedures, based on the DNA content per cell, and is shown in Table 7. When cultures were shifted to 39.5°C for 5 h, the percentage of G1 cells increased from 57% to 67% and gradually increased futher to 76% at 36 h. The number of cells in S phase decreased; however there was only a 50% decrease in

Table 7

Cell cycle distribution of JB7-K

33°C	Time(hours)	G1	S	G2M
	1	56.8	26.8	16.4
	10	57.7	27.7	14.6
	24	57.8	28.2	14.0
	36	57.3	30.2	12.5
39.5°C	1	57.4	27.2	15.4
	3	57.4	26.5	16.1
	5	66.9	20.6	12.5
	10	69.5	18.1	12.4
	24	71.5	16.4	12.1
	36	76.2	14.3	9.5

the percent of S phase cells even after 36 h at the NPT. A growth curve is provided. It appears that JB7-K cells are preferentially arrested in G1 phase.

c. Isolation of a thioguanine-resistant cell line of JB7-K.

Thioguanine resistant ($HGPRT^-$) mutants were isolated from JB7-K as a prelude for later gene transfer studies. There were eight TG^r colonies isolated from 2×10^6 mutagen-treated JB7-K cells as described in the methods and named JB7K-TG1-8; one TG^r colony isolated from 2×10^6 JB7-K cells by spontaneous mutation was named JB7K-TG-s. Four of these nine TG^r colonies showed a *ts* phenotype characteristic of JB7-K in terms of EOC at both 33°C and 39.5°C(Data not shown). These four JB7K-TG cells were further examined for their *ts*DNA phenotype by [^{3}H]-TdR and [^{35}S]-Met double pulse-labelling at both 33°C and 39.5°C (Fig. 11). Two of them (JB7K-TG4 and JB7K-TG8) showed a DNA *ts* phenotype similar to JB7-K. Both of them also restricted polyoma virus DNA synthesis when the cells were transfected with p53A6.6 DNA and analyzed for the polyoma DNA in the same way as described early (data not shown).

Chapter II. Polyoma virus DNA replication in JB7-K cell

Using small viruses as models to study host cellular factors and proteins involved in DNA replication has provided significant contributions to the understanding of DNA replication in prokaryotic systems. Here we would like to study the polyoma virus (Py) DNA replication phenotype in the *ts*DNA mutants instead of the effect on the cellular DNA replication directly because of the greater information available for viral DNA synthesis. JB7-K was chosen for further study of its temperature sensitive defect in supporting Py DNA replication because of its interesting genetic properties (see RESULTS Chapters I and III). The Southern hybridization results in Chapter I showed that the accumulation of replicated Py DNA at 39.5°C after 2 days is about ten fold lower than that at 33°C. As a first step, it was necessary to determine whether the results were due to low rate of Py DNA replication in JB7-K at 39.5°C or due to fewer transfected cells attached to the dish at 39.5°C at the time that viral DNA was harvested. Determination of synthesis of replicating viral DNA by [^{3}H]-TdR pulse labelling at certain times post transfection at both 33°C and 39.5°C would answer that question. In order to be able to detect the replicating viral DNA by [^{3}H]-TdR pulse labelling, I tried different approaches to increase the calcium phosphate (CaP) DNA transfection efficiency as described in the following section.

1. The optimal transfection condition for JB7-K cell

DNA mediated gene transfer has become a popular technique since the DNA and CaP co-precipitation technique was developed by Graham and Van der Eb (147). However, the frequency of DNA transfection into mammalian cells by the

CaP technique varies widely. CHO cells have been rather poor DNA recipients by comparison with mouse L cells and NIH 3T3 cells (162-167). How competent the cell membrane is to uptake of the DNA precipitate may be one of the rate limiting steps. The naked DNA once in the cell also faces degradation by nucleases before its expression and/or replication in the nucleus. Mammalian cells contain many nucleases capable of degrading nucleic acids (166,167). A report from Strain and Wyllie showed that after 2 h exposure to [^{3}H]-labeled SV40 DNA CaP co-precipitate under basal conditions, up to 7% of the input DNA became cell-associated, with approx 4% reaching the nuclear fraction. Substantial degradation of SV40 DNA occurred within a further 4 h, apparently in both nucleus and cytoplasm. Less than 0.5% of the total transfected DNA which reached the nucleus was protected from nuclease attack (168).

Several reagents have been reported to increase the efficiency of transfection. Amphotericin B, a membrane active reagent, has been reported to alter the cell during transfection to increase the uptake of the CaP DNA co-precipitate in mouse cell (165). Chloroquine (CQ), which can change the local pH in the lysosome to prevent intracellular nuclease release or activation from lysosomes, has been reported to increase the efficiency of DNA transfection in mouse cells (169). In preliminary studies, amphotericin B was used by J. Dermody and M. Orlian of this laboratory for enhancement of CaP mediated DNA transfection of CHO cells and optimal concentration of Amphotericin B and time of exposure were determined (unpublished data). The optimal concentration was found to be 5 μg/ml. Chloroquine was tested in one of the CHO *ts* mutants, JB3-O, by me. Different concentrations of CQ (100 μM and 200 μM) were used in calf thymus DNA transfection by the CaP technique for 2, 4, and 16 h. The number of residual cells were counted at 48 h post transfection to examine the toxicity of CQ to the cell.

Results are shown in Table 8. The culture treated with 100 μM CQ for 2 h had 26.5% less cells than the culture without treatment. There was approximately 50% loss of cells after 5 or 16 h treatment. If the CQ concentration was increased to 200 μM, the cell number was further decreased after 2 h but not 5 h treatment. To minimize the toxicity of CQ, I selected 100 μM for 2 hours.

Chloroquine has also been tested with regard to the efficiency of long term gene expression. A selectable plasmid pRSVgpt DNA (1 μg) was co-transfected into JB3O-TG (HPRT$^-$) cells with 9 μg of calf thymus DNA by the CaP technique. CQ was added during cell exposure to CaP for different times as shown in Table 9. At 48 h post transfection, the cultures were subcultured at 2 x 10^6 cells per 150 mm dish in selective HAT medium and *gpt*$^+$ colonies were stained and counted two weeks later. The highest efficiency was the sample of cells exposed to CaP-DNA and 100 mM CQ for 2 h. It was about 3 fold increased by comparison with the sample exposed to CaP for 2 h without CQ. Another point that should be noted from this experiment is that the short exposure time of cells to CaP (2 h) actually has higher efficiency than long exposure time (16 h), presumably because the JB3O-TG cell is sensitive to the CaP-DNA suspension. There was 60% loss of cells during 16 h of exposure time with or without CQ.

The effect of CQ on the replication of polyoma DNA was also checked in JB7-K cell by *Dpn* I-assay and Southern analysis. p53A6.6 DNA (5 μg) was transfected into 8 x 10^5 JB7-K cells at 33°C with different exposures to CaP-DNA and chloroquine. There were four different conditions. In condition A, the monolayer cells were exposed to the CaP-DNA coprecipitate for 4 h without chloroquine; for condition B, the cells were exposed to the CaP-DNA suspension for 4 h, chloroquine was added after 2 h of addition of CaP-DNA and kept for 2 h; for condition C, the cells were exposed to the CaP-DNA for 2 h, refed and then

Table 8

Toxicity of Chloroquine to CHO cells [a]

Time of exposure		2 h		5 h		16 h
CQ concentration	0	100 μM	200 μM	100 μM	200 μM	100 μM
Cell number ($x10^{-5}$)	11.9	8.9	6.8	6.08	5.8	5.7
Percent remaining(%)	100	74.7	57.1	51.1	49.1	47.9

(a) 5 x 10^5 JB3-O cells were seeded onto a 60 mm dish one day before. 10μg calf thymus DNA was transfected in each dish by the CaP co-precipitation technique. The cells were exposed to chloroquine (CQ) (100 μM or 200 μM) at the same time that they were exposed to the CaP-DNA co-precipitate. After the indicated time (2,5,16 h), the medium was removed and replaced with standard DF medium. Cell numbers in triplicate were counted at 48 h post transfection as described in methods.

Table 9

Effect of Chloroquine on transfection efficiency [a]

Exposure to CaP-DNA (h)	2	2	5	16	16
Exposure to CQ (h)	0	2	5	0	16
cell number at 48h post transfection ($x10^{-6}$)	10.2	9.2	7.9	4.0	7.2
Transfection efficiency (colonies/μg pRSV gpt DNA)	375	1018	963	168	102

(a) 2 x 10^6 cells (JB3O-TG) were seeded in 100 mm dishes one day before transfection by pRSVgpt (1μg) and 9 μg calf thymus DNA per dish. Cultures were split into 2 x 10^6 per 150 mm dish in selection (HAT) medium at 48 h post transfection. The *gpt*$^+$ colonies were stained and counted two weeks later. The transfection efficiency results represent the average of five dishes.

exposed to chloroquine for 2 h; for condition D, the cells were exposed to CaP-DNA and chloroquine for 4 h together. Cells were incubated for a total of 48 h. A one tenth aliquot of each DNA sample was digested with *Dpn* I and *Sal* I .The results are shown in Fig. 12. The cells exposed to CaP-DNA and chloroquine at the same time for 4 h (condition D) show the highest amount of residual input DNA (Dpn I sensitive); cells exposed to CaP-DNA for 4 h and chloroquine for 2 h is second in terms of input DNA. Cells exposed to CaP-DNA for 2 h, refed, and then exposed to chloroquine for another 2 h had the lowest amount of input DNA. For replication of DNA, conditions B, C, D, had similar amounts of Dpn I resistant DNA, and condition A which did not involve exposure to chloroquine is lower than all others. In an effect to combine the efficiency increase by chloroquine and minimize toxicity to the cell, I concluded that the optimal conditions for JB7-K would be to use 100 μM chloroquine for 2 h during CaP-DNA transfection.

2. The minimal pulse label period and optimal time to start pulse label

To find out the minimal pulse label period, p53A6.6 DNA was transfected into JB7-K cells as described above. At 40 h post transfection at 33°C, cultures were pulse labelled with 100 μCi/ml [^{3}H]-TdR in duplicate for 15 min, 30 min, 45 min and 60 min and chased with 0.2 mM non-radiative TdR and 0.01 mM CdR for 30 min. Cells were washed with cold PBS twice and lysed with Hirt extraction buffer. The viral DNA was purified, and 1/3 of each sample was analyzed on 0.7% agarose gel by electrophoresis under 50 V for 16 h in 1X TPE buffer. The gel was treated with EnHancer for 3 h , precipitated in cold water containing 5% acetic acid for one h, dried in the vacuum gel dryer and autoradiographed with X-ray film. The results are shown in Fig. 13. The samples pulse-labelled for 30 min and chased for 30 min

have a strong enough signal to be detected as form I and form II DNA. Form II DNA is presumably due to nicking in the process of DNA extraction.

The optimal time to start pulse labelling is the time at which the replication rate of polyoma DNA is increasing or stable instead of decreasing or close to decreasing. An experiment was done with an one h pulse label of [^{3}H]-TdR at different times post transfection. The result is shown in Fig. 14. Viral DNA synthesis increases from 40 h to 64 h post transfection at 33°C. Even though the rate at post transfection 64 h is higher than that at 48 h, the 40 h of post transfection was chosen to facilitate subsequent studies of temperature shift.

3. The rate of polyoma virus DNA replication at 33°C and 39.5°C

The JB7-K and wild type cells were transfected with p53A6.6 by the modified CaP transfection. Post 40 h transfection, dishes were shifted to 39.5°C for 6 h. Cultures were pulse labelled at both 33°C and 39.5°C as described before. One third of the DNA samples from each dish was analyzed by agarose gel electrophoresis. The fluorography result is shown in Fig. 15A. The polyoma virus DNA form I, II and III is indicated in the marker lane which is p53A6.6 DNA labeled with [^{3}H]-TdR during replication in *E.coli*. In JB7-K cells, the polyoma DNA replication rate at 33°C is similar for T=0 and T=6 (compare lanes 3 and 4 in Fig.15A); but the viral DNA replication rate is decreased about two fold when cells were incubated in 39.5°C for 6 h. Southern analysis of aliquots of the same samples (Fig. 15B) shows that there was little difference in the level of replicated DNA among the three samples (compare lanes 2 and 3 in Fig.15B). A temperature shift experiment was also done with the wild type CHO-K1 cell as a control and is shown in Fig. 16. The same transfection protocol was used for CHO-K1 cells except that the cell number seeded was 3 x 10^5 per 60 mm dish instead of the 8 x

10^5 cells for JB7-K, because of the more rapid growth of the CHO-K1 cells. Since transfection conditions were optimized for JB7-K cell, it may not be optimal for CHO-K1 cells. The incorporation of [^{3}H]-TdR into polyoma virus DNA at 40 h post transfection (T = 0) in CHO-K1 cell is not as good as in JB7-K (compare lanes 1 and 2 in Fig. 16 to lane 3 in Fig. 15A). But in the CHO-K1 cell, the rate of replication of polyoma DNA at 39.5°C is 4 fold higher than that in 33°C at T=16 (lanes11,12 and lanes 9,10). The rate at T=6 is too low at 33°C to permit accurate comparison. So the rate of replication of polyoma DNA in JB7-K at 39.5°C is several fold less than a comparable culture of wild type cells. Therefore, the results shown previously by Southern analysis in which the replicated viral DNA was very low in JB7-K at 39.5°C after 2 days are due to a decrease in the actual rate of viral DNA replication instead of lower (infected) cell number.

4. Is there any accumulated viral DNA replicative intermediate form?

We next sought to determine which step of polyoma DNA replication was blocked in JB7-K cells? Is the block in initiation , elongation, or termination? It is very difficult to measure the viral DNA initiation step intracellularly; however, it is possible to examine the replication elongation step intracellularly by pulse chase experiments and measurement of changes of replicative intermediate forms in the gel autoradiograph. JB7-K cells were transfected and the DNA analysed under conditions that provide good resolution in the gel autoradiograph (0.5% agarose gel and long electrophoresis time). A band exists in the polyoma DNA transfected sample in the position of a possible replicative intermediate form as indicated (Fig. 17, see the arrow indication). No other discrete higher molecular weight bands were observed only in the infected cell (i.e. not in lane 1). In view of the location of the band, it could also be a dimer form of the plasmid DNA. To resolve these

alternatives, aliquots of the same sample were further analyzed by Southern hybridization using p53A6.6 as a probe. The Southern analysis result showed that there were two bands above the form II DNA in the samples from the culture at both 33°C and 39.5°C (Fig. 18). Treatment with 2'-Deoxy-2'azidocytidine (Cz), a nucleotide analog that inhibits the initiation of polyoma DNA replication *in vivo* (169a), would be expected to result in decreased accumulation of replicative intermediates. When transfected cells were treated with Cz (2 mM) for 4 h before [^{3}H]-TdR pulse labelling, no change was observed in the pattern of the bands above form II DNA (data not shown) supporting the possibility that the two bands may not be replicative intermediate forms but dimers. When the same aliquot samples were digested with *Dpn* I and analyzed by Southern hybridization, the two bands above form II DNA were sensitive to *Dpn* I digestion (data not shown). Thus, the bands represent methylated DNA sequences in the input DNA sample and did not replicate after transfection.

In order to get dimer-free p53A6.6 DNA , 150 μg of p53A6.6 DNA were loaded on a 5-20% Sucrose gradient (1 M NaCl, 10 mM Tris-HCl, 5 mM EDTA, pH 8.0), and centrifuged at 22,000 rpm for 16 h at 4°C. The fractions (#27, 28, and 29) that contain only monomer form I and form II DNA and those containing detectable dimer (#22, 23 and 24)were separately pooled, dialysed, precipitated and resuspended in TE buffer. Both preparations of p53A6.6 DNA were used in transfection experiments and result is shown in Fig. 19. The samples with a 60 min chase have more labeled polyoma DNA than the sample without a chase. In all cases, there was no obvious difference in any other DNA forms, i.e. replicative forms (RF), between the samples with chase and without chase. Therefore, the *ts* defect may not be related to DNA elongation but rather preferentially involved initiation. Duplicate samples from the same transfection were analyzed further by

gel electrophoresis (Data not shown). Half of the gel was transferred to Nytran and hybridized to ^{32}P-labeled p53A6.6 DNA; another half of the gel was dried down to autoradiograph directly. The putative RF form observed on the Southern blot is in virtually the same position as CHO cellular DNA (probably mitochondrial DNA) on the [^{3}H]-TdR labelling gel autoradiograph. Furthermore, the Southern analysis shows that it is present in cells transfected with dimer-free DNA as well and at both temperatures, suggesting that it formed subsequent to transfection due to intracellular recombination since all the putative dimer was previously found to be Dpn I sensitive. Therefore, we concluded that it would be very difficult to study the replicative intermediate forms directly by these techniques. However, the results do not support accumulation of replicative intermediate at the non-permissive temperature as might be expected for a defect in strand elongation.

Chapter III: Molecular cloning of the human gene that corrects the *ts* phenotype in JB7-K

1. Primary gene transfer in JB3-O, JB7-K, and JB8-D

An initial gene transfer experiment was done with all three mutants JB7-K, JB3-B and JB8-D. A human genomic cosmid library, pCV103Kgpt, that contains the *E. coli* derived dominant selective genetic marker *gpt* gene under the SV40 early promoter, was the source of human genes. The protoplast fusion technique (170) was chosen to transfer the cosmid library into the *ts* cells, because extraction of the cosmid library DNA and large amplication of library necessary for preparation of the cosmid DNA for CaP DNA co-precipitation techniques may result in a less representative total population of the library. The bacteria were grown to OD600=0.9. Protoplast fusion in suspension was performed as developed in this laboratory and described in the Methods. The ratio is 10^4 protoplasts to one mammalian cell in the fusion mixture, and a total 10^7 mammalian cells were fused. 10^6 fused cells were seeded per 150 mm dish and incubated in nonselection medium at 33°C, after 72 h incubation, the cells were refed with selection medium (HAT+Xanthine +Mycophenolic Acid) and incubation continued at 33°C. After ten days, gpt^+ colonies were counted. The results are shown in Table 10. JB7-K is the best recipient cell for protoplast fusion among the three mutants, which had 62 gpt^+ colonies per 10^6 cell (one per 1.6 x 10^4 cells). JB8-D had 8 gpt^+ colonies per 10^6 cells and JB3-O had less than one gpt^+ colony per 10^6 cells.

Table 10

Gene transfer in JB3-O, JB7-K and JB8-D [a]

	Fused cell number	Number of *gpt*+ colonies/dish	Total *gpt*+ colonies (33°C)
JB3-O	10^7	<1[b]	6
JB7-K	10^7	62	620
JB8-D	10^7	8	80

(a) 10^7 cells of each mutants were fused with pCV103Kgpt containing protoplasts. 10^6 of fused cells were seeded on 150 mm dish and refed with gentamycin at 24 h post fusion and with selection medium (HAT+X+MPA) at 72 h post fusion. The *gpt*+ colonies appeared 12 days later.

(b) numbers are the average of gpt^+ colonies in 4 counted dishes. For JB7-K and JB8-D, the other 6 dishes have similar number of colonies as the counted four dishes. For JB3-O, only three out of ten dishes had colonies.

2. Primary transfection of JB7-K

Since the *ts* cell complementation experiment showed that JB7-K and JB8-D did not complement each other, the defect may be in the same gene (see Chapter I). Therefore I chose one of them to do molecular cloning of the human gene that corrects the *ts* defect in JB7-K, because it is the better recipient of the two mutants for gene transfer. 10^8 JB7-K cells were fused with pCV103Kgpt-containing protoplast at 33°C. The fused cells were seeded at 10^6 per 150 mm dishes. Cultures were refed with gentamycin at 24 h postfusion and with selection medium (HAT+X+MPA) at 72 h postfusion. There were about 4600 gpt^+ positive colonies visible after two weeks in selection medium, and all dishes were shifted to 39.5°C. Only 9 colonies survived after 10 days incubation at 39.5°C. All nine colonies were transferred to 12-well dishes employing cloning cylinders and incubated at 33°C in the selection medium. After the colonies were grown up, the ts^+ phenotype of these nine colonies were examined by efficiency of colony formation (EOC) at both 33°C and 39.5°C (Table 11). Four out of nine, 3A, 5A, 9A and 10A, were considered good candidates of primary transfectants of JB7-K since all formed colonies when 10^3 cells were seeded; 9A and 10A have low EOC at 33°C (13.3% and 24.0%) so their growth phenotype at 39.5°C is relatively better than other primary transfectants. They and 5A have been further analyzed in their growth property in regular DF medium and *gpt* selection medium to verify the presence of the *gpt* sequences. K2/8 cells which is a CHO ($HPRT^-$) cell line transfected with pSV2gpt were used as a positive control in both mass culture level and low density colony formation level. (Table 12). 5A showed most pronounced concordance between gpt^+ and ts^+ phenotype, and has been subcloned as 5A1, 5A3 and 5A4. The subcloned 5A1 showed much better growth in HAT+X+MPA medium (see Table 13).The ts+ phentype was not determined at the colony level.

Table 11

Efficiency of colonies formation of primary transfectants in DF medium[a]

	33°C			39°C			
	number of cells seeded						
	50	10^2	10^3	10^2	10^3	10^4	10^5
7K2A	48[b]	86	716	-	-	++[c]	++++
7K3A	38	57	560	-	12[d]	++	++++
7K4A	37	70	420	-	-	+/-	++++
7K5A	30	67	528	-	+/-	+++	++++
7K6A	40	91	502	-	-	(300) +/-	++++
7K7A	39	52	ND	-	-	-	(36) /+
7K8A	37	64	652	-	-	(>7)+/-	++++
7K9A	7	11	151	-	3	(>45)+/-	++++
7K10A	9	17	356	-	+	++	++++

Table 11 legend

(a) Different numbers of cells were initially seeded in DF medium in 60 mm dish at 33°C for 16 h and maintained at 33°C or shifted to 39.5°C. The cultures were observed after one week and colonies were stained two weeks later.

(b) Colonies represent the average of three dishes at each indicated seeding number.

(c) For culture at 39.5°C, there were areas of generalized cell growth. They were scored as following:+/-: 5% of the culture in the center growing, +: 20% of the culture are growing, ++: 20-50% of the culture are growing, +++:50-70% of the culture are growing, ++++: 70-90% subconfluent culture.

(d) Individual colonies were also determined when possible.

Table 12

Primary transfectant growth in different medium [a]

	At low density level						At mass culture level	
	DF			HAT+ X+MPA			DF	HAT+ X+MPA
Cell number:	50	10^2	10^3	10^2	10^3	10^4	$5x10^5$	$5x10^5$
K2/8	19[b]	42	ND[e]	ND	160	TNT[f]	++++[c]	++++
5A	21	46	350	0	3	14	++++	++++
9A	7	15	118	0	0	0	++++	+[d]
10A	29	69	512	2	0	3	++++	+/++

(a) K2/8 and three primary transfectant cell lines were grown in DF medium or HAT+Xanthine+Mycophenolic Acid medium either at low density (see the cell seeding number) or in mass culture level ($5x10^5$ cells per 60 mm dish). The cultures were grown at 33°C. At low density level, the colonies were stained and counted two weeks later. At mass culture level, the culture were observed 4 days and 10 days after seeding. Results shown is observation after 10 days.

(b) Colonies represent average of triplicate dishes in each seeding number.

(c) well grown mass culture, 70-90% confluent.

(d) poorly grown mass culture, 20-30% confluent.

(e) Not determined.

(f) too numerous to count.

Table 13

Growth properties of 5A subclones in HAT+X+MPA medium [a]

	33°C				39°C
	$5x10^3$	10^4	$5x10^4$	10^5	10^5
5A1	133	244	TNT	TNT	++++
5A3	112	252	TNT	TNT	++++
5A4	182	456	TNT	TNT	+++

(a) The subcloned cultures were seeded in HAT+X+MPA medium in 60 mm dishes at the indicated cell number at 33°C. The dishes with 10^5 cells were shifted to 39°C next day. Colonies were stained and counted two weeks later. Other conditions were as in Table 12.

The genomic DNAs from 5A1 and 5A3 digested with Eco RI were analyzed by Southern hybridization. A gel purified human *Alu* fragment (released from pBlur 8 by Bam HI) and pSV_2gpt were used as probes (Fig.20). *Alu* fragment can detect 9 bands and pSV2gpt can detect one major and 4 minor bands.These results suggested that multiple cosmids may be integrated into the 5A primary transfectant.

3. Secondary transfection

To further verify whether the ts^+ phenotype in the primary transfectant is due to the human gene expression and also to eliminate non-specific human sequences existing in 5A1 and 5A3, it was necessary to perform a secondary transfection. HMW DNA was prepared from 5A1 as described in the Methods section and twenty micrograms of HMW DNA were transfected into $2x10^5$ JB7K-TG4 cells in 100 mm dishes by the CaP co-precipitation technique for 16 h. A total of 50 dishes were transfected. Seventy two hours later each dish was split into two 150 mm dishes in HAT medium. There were 300 gpt^+ colonies evident per dish 10 days later. All the dishes were shifted to 39.5°C. Sixteen putative gpt^+ and ts^+ colonies were picked from 39.5°C. Each colony was split into two, one half was seeded at 33°C and the other half at 39.5°C, in 12 well dishes. Only one of them showed ts^+gpt^+ phenotype, and was designated 2^o5D. Genomic DNA from the secondary transfectant was digested with different restriction enzymes and analyzed by Southern hybridization. The gel purified human *Alu* sequences and *gpt* (*Bgl* II-*EcoR* V) fragment were used as probes (Fig. 21). Hybridization of *Alu* and *gpt* with different restriction enzyme digested DNA from 2^o5D showed only a single band in each case. This was considered strong evidence that the secondary transfectant contains only a single cosmid insertion.

The polyoma DNA replication was also checked in this *gpt*$^+$ and *ts*$^+$secondary transfectant 2^{o}5D. p53A6.6 (1μg of plasmid DNA and 9 μg of calf thymus DNA per 60 mm dish) was transfected into the 2^{o}5D cell and wild type CHO K1 cells. The viral DNA was recovered by Hirt extraction at 2 days and 3 days post transfection at 33°C and 39.5°C and analyzed as described in the methods. The Southern analysis using p53A6.6 as a probe revealed that polyoma virus DNA replication in 2^{o}5D cell at 39.5°C is similar to the wild type CHO-K1 cell (Fig. 22A). The replicated viral DNA in 2^{o}5D (Fig. 22B) for 2 days at 39.5°C was more than one day at 33°C plus one day at 39.5°C (compare lanes 5 and 6 to 3 and 4) and at least as great as at 2 days at 33°C (compare lanes 5 and 6 to 1 and 2).Thus the human sequences restored the ability of JB7-K to synthesise viral DNA.

Since the secondary transfection recipient cell is JB7K-TG cell (HGPRT$^-$) and the secondary transfectant is *gpt*$^+$ (HGPRT$^+$) there is an opportunity to do back selection for TG^r cells. Since the *gpt* gene is covalently linked to the human gene, the selection against *gpt*$^+$ (in 6-thioguanine medium) could theoretically lead to loss (segregation) of the entire cosmid including the human gene. Thus the back selected cell which is *gpt*$^-$ should also reacquire the *ts* phenotype. Alternatively, loss of *gpt* function could be associated with a mutation in the *gpt* sequence, in which case, the human sequence should not be lost and the cells would be expected to remain *ts*$^+$. In a 2^{o}5D back selection experiment , cells were passaged 1:10 in non-selection medium for eight times. 5 x 10^6 cells in 50 (100 mm) dishes were treated with 6-thioguanine (5 x 10^{-5} M in DF medium) and TG resistant colonies were picked by the wooden dowel method. Two of them B6 and B10 (*gpt*$^-$) were analyzed by EOC and genomic Southern hybridization. The Southern result (Fig 23) showed that the *gpt* sequence is still there and human repetitive sequence is also present (see lanes 4, 5, 6 and 7 in both panel A and B of Fig. 23). The EOC result showed that B6

and B10 were still *ts* $^{+}$ phenotype. This combination of results suggests that the colony's growth in TG-containing medium (*gpt*$^{-}$ phenotype) is due to a mutation in the *gpt* gene instead of whole gene deletion.

4. Human sequence rescue from secondary transfectant

Several stratagies are available to rescue the human sequence from transfectants. Rescue of even a small human unique sequence is sometime enough since such sequence can then be used as a probe to screen a human cDNA library to isolate the whole coding sequence of the human gene. Such a strategy was used initially. When COS 7 cells are fused with 2^{o}5D, the T antigen constitutively expressed in COS 7 cells can bind to the SV40 origin in the cosmid vector which is integrated into the genome of 2^{o}5D. Replication of the SV40 ori intergrated into a chromosome leads to an onion skin-type DNA structure which frequenty excises and forms a closed circular DNA (171, 172). This extra-chromosomal form can then be recovered by Hirt extraction and amplified by transforming into competent E.coli. The ampicillin resistant bacterial colonies should contain the rescued plasmid. Different ratios of COS 7 cell to 2^{o}5D (5:1, 2:1 and 1:1) were fused by 50% PEG 1000 as described in the Methods. After 24, 48, and 72 h of fusion, the low MW DNA was recovered by Hirt extraction, followed by organic solvent extraction, ethanol precipitation and resuspension in TE buffer. Five out of ten microliters of the 5:1 fused cell DNA were used to transform the competent E.coli cells and selected with ampicillin. There were 13 colonies in eight plates. All of them contained plasmid DNA with approximate size of 7.5 to 9.2 kb (Data not shown). The two largest plasmids (pSD8 and pSD 11) were grown up for large scale preparation of plasmid DNA. The nine digestion patterns with different

restriction enzymes of pSD8 and pSD11 were the same. The uncut plasmid pSD8 and pSD11 also showed the same size so pSD 8 was chosen for further study. The restriction map was determined by double digestion (Fig 24). *Bam* HI, *Sal* I, *Bgl* II and *Kpn* I. have single cutting sites. The human repetitive *Alu* sequence was located in the *Hind* III - *Sal* I fragment by Southern blot of the mapping gel and hybridization to the gel purified *Alu* fragment. The *EcoR* I - *Hind* III fragment was derived from the human sequence because it is between the site of insertion of the human sequence in the cosmid vector and the *Alu* sequence. However it was very difficult to identify the real size of fragment from *EcoR* I to *Hind* III. When pSD8 was double digested with Hind III + Bam HI and Hind III + EcoR I, the second double digestion results with a total fragment size 1 kb less than predicted based on the first double digestion. Sequential digestion with one enzyme first, then extraction with phenol:chloroform, ethanol precipitation, followed by digestion with second enzyme, show the same result as double digestion. Also the order of addition of enzyme did not affect the digestion (Data not shown).

In order to clarify this anmolous result, the fragment from *Pst* I to *Hind* III, which contains the *EcoR* I - *Hind* III fragment was subcloned into the vector pUC 19. The *Pst* I to *Hind* III fragment was purified by sequential digestion and sequential gel purification. First, pSD8 was digested with *Pst* I, the large fragment (5.35 kb) was eluted from agarose gel by glass milk and then digested with *Hind* III. The smaller fragment (2.1 kb) was eluted in the same way. This fragment served as a control. The original vector DNA is a dimer. Therefore, the recombinant (called P_1H_S) was directly subcloned into the*Pst* I and *Hind* III site of the pUC 19 vector. The white colonies in the ampicillin and X-gal plate were picked and the correct subclone was verified by *Pst* I and *Hind* III digestion of mini preparation

plasmid DNA followed gel electrophoresis. One of the recombinant plasmid, pPH1, was grown up for large preparation.

Analysis of pPH1 with restriction enzymes showed there were multiple possible bands in the 0.35 region. The problem was resolved by electrophoresis in 4% NuSiev agarose gel (FMC Bioproducts,). The result is shown in Fig 25. The ethidum bromide stain of the 0.35 area in the regular agarose gel actually contain at least four fragments: one 0.29 kb and a triplet of 0.35 kb bands which total 1.34 kb, consistent with the size of the missing 1.05 kb fragment from pSD 8 digested with *EcoR* I + *Hind* III or *EcoR* I + *Bam* HI. The complete map of the rescued plasmid pSD8 is shown in Fig. 26. The 0.35 and 0.29 kb fragments were gel purified from pPH1 by 4% NuSiev agarose gel electrophoresis and ^{32}P random primer labeled as probes to hybridize with DNA from JB7-K, 2^{o}5D and human cellular DNA digested with *Bgl* II in Southern analysis. The result showed in Fig. 27 that both the 0.35 kb and 0.29 kb are human α-repetitive like sequences (compare lane 5 in panel A and B to lane 5 in panel C). The panel C is hybridized to a cloned human α-repetitive sequence αpE1(a gift from Macoska and Henderson) (Fig. 27) (173,174). The longer exposure of panel C lane 5 showed almost identical repetitive pattern as lane 5 of panel A and B. The 0.35 and 0.29 fragment probes have much stronger signals in total human DNA than cloned αpE1 because the probes of 0.35 and 0.29kb are gel purified fragments whereas the probe of αpE1 contains the plasmid sequence, which also reacts with the plasmid DNA in the primary transfectant 5A1 (lane 2 in panel C or D). Since the random primer labeling makes a very high specific radioactive probe, a small amount of cross contamination in the fragment purification process would cause the reaction with the same band in the blot by two different probes. The 0.35 and 0.29 kb fragments were therefore checked by Southern analysis. The DNAs were electrophoresed in

NuSiev gel, the gel was blotted and hybridized with 0.29 kb as a probe. The result (data not shown) suggest no evidence of cross contamination between 0.35 and 0.29 kb fragments. Both of them are the same α-repetitive like sequences. Thus the *EcoR* I-*Hind* III fragment from pSD8 contains human α-repetitive sequences only. The fragment *Hind* III-*Sal* I (for reference see Fig. 27) has been digested with frequent cut enzymes like *Sau3A* I, *Alu* I, *Taq* I, *Rsa* I, *Dpn* I and *Cfo* I. The digested DNA analyzed by electrophoresis on 1.5% agarose gel showed fragments from 0.05 kb to 0.7 kb. The gel was transferred and the blot was hybridized with total human DNA as a probe. All the bands shown on the ethidium bromide stained gel were detected by probe DNA (data not shown). Therefore, this rescued plasmid pSD8 cannot be used to find human unique sequences. I then chose to construct a phage genomic DNA library from the secondary transfectant to rescue the human sequence.

5. Construction and screening of the genomic DNA phage library from secondary transfectant

Sau3A I partial digested HMW DNA from the secondary transfectant was size fractionated in a NaCl gradient as described in the Methods, ligated to λEMBL 3 arms, and packaged into λ particles *in vitro*. The titer of the library was determined by infection of the P2 lysogen strain NM539 which genetically selects against the parental λ/EMBL3. The titer was 7.5 x 10^5 per μg vector DNA. The library was amplified and stored at 4°C with 0.3% chloroform and at -70°C with 0.7% DMSO. Some important experimental conditions are described in the following sections.

a. Partial digestion of genomic DNA

There are two methods usually used for partial digestion. One is to dilute the enzyme with the DNA; another is to dilute enzyme first, then add different amounts of diluted enzyme to the DNA solution. I found the second method is better than the first one in terms of enzyme concentration corresponding to the size distribution of the cleaved DNA. The maximal distribution of DNA from 15 -22 kb is the sample digested with 0.03 units *Sau3A* I per μg DNA , so the optimal amount of enzyme should be 0.015 u/μg DNA (151). I then determined how much DNA in one tube will still give the same pattern of partial digestion when using 0.015 units *Sau3A* I per μg DNA. The scaled up partial digestion reaction was tested with 2 μg, 20 μg and 100 μg DNA per tube reaction. The sample with the largest amount of DNA (100 μg /tube) had much less digestion compared to the small amount DNA (2 μg/tube). The 20 μg DNA /tube showed a similar yield of partial digestion as 2 μg /tube. I therefore used a scale up partial digestion with 20 μg DNA per tube in 30 tubes with 0.015 units *Sau3A* I per μg DNA.

The phage library was constructed as described in the Methods. pSD8 was used as a positive control to construct λpSD8.

b. Choosing the correct probe and right hybridization conditions to screen the library

Different hybridization conditions of temperature (37, 40, 43°C) and probes (gel purified human *Alu* fragment, purified α-repetitive fragment EE.35, and pSD8) have been tested with the positive control phage λ pSD8 at different plaque densities in NM539 strain. Results showed almost no difference with different temperatures. But the different probes did make a lot of difference. The result with a low density of plaques in the plate is shown in Table 13. 100% of λpSD8 plaques showed on

the blot if pSD8 was used as a probe; 34% of plaques were detected when the α-repetitive sequence was used as a probe and 28% of plaques when the *Alu* fragment was used as a probe. Even though the λpSD8 contain both α-repetitive sequence (in four tandems) and *Alu* sequence (unknown copy number), the percentage of plaques which react with the probes is still different; the longer the probe, the higher the percentage of plaques with a positive signal. However, in the real library screening experiment, I chose to use the *Alu* fragment as a probe because the rescued plasmid pSD8 map showed *Alu* sequence located closer to the human unique sequence than the α repetitive sequence (Fig.26).

c. Positive signal versus phage incubation time

Since most of regular phage screen protocols emphasize that the phage plaque size should be as small as pinpoint. I evaluated plaque size (different incubation time) versus positive signal in λpSD8. 200 pfu were used to infect NM539 in 100 mm plates. The plates were left in the incubator for 5, 6, 7, 8, and 16 h. Phage growth was stopped by shift to 4°C. Nitrocellulose filters were lifted from each plate and hybridized with α-repetitive probe EE.35 and *Alu* sequence respectively. The incubation time for plaque formation should be at least 6 h to see a positive signal by EE.35 probe and 7 h by *Alu* probe. The efficiency of visible plaque formation changed from 45% for 5 h to 77.5% for 7 h (gel purified human *Alu* fragment, purified α-repetitive fragment EE.35, and pSD8) and 84% for 16 h (Table 14). Considering the density of library to be screened, the incubation time should be at least 6.5 h.

Table 14

Hybridization activity with different probes (a)

		Hybridization probes		
Temperature of hybridization	Plaques on plate	Alu	EE.35	pSD8
37°C	99(b)	27(c)	34	99
40°C	99	28	32	99
45°C	87	26	35	87

(a) 100 pfu of λpSD8 were used to infect NM539 strain cells and poured on 100 mm plate. Three plates were poured. Triplicate filters were lifted from each plate. The first set were hybridized to *Alu* fragment as a probe; the second set were hybridized to EE.35 fragment as a probe; the third set were hybridized to pSD8 as a probe.

(b) number of plaques on each plate after overnight incubation at 37°C.

(c) number of positive signal showed on the blot by different probes

Table 15

Hybridization and duration of phage infection [a]

Infection time (h)		5	6	7	8	16
Plaque number/plate		91[b]	131	155	161	168
Exposure time to see positive signal (hours)	Alu	>72[c]	>72	72	24	24
	EE.35	72	48	24	5	5

(a) 200 pfu of λpSD8 were used to infect NM539 cell. Plates were incubated at 37°C for the indicated time and then moved to 4°C. The visible plaques were counted. Duplicate filters were lifted from each plate. One set of the filters were hybridized to Alu fragment and another set of the filters were hybridized to EE.35 fragment.

(b) Numbers of plaques on the plates after incubation for the indicated time are the average of triplicate plates.

(c) The minimal exposure time to x-ray film to detect the positive plaque hybridization.

D. Phage library screen

1.25 x 10^6 phage were screened using the *Alu* fragment as a probe. 30 putative positives were picked. 14 of them which remained strongly positive were further purified as single plaques by two additional rounds of hybridization screening. DNAs were isolated from mini lysis of seven individual plaque and checked by digestion with *Sal* I, *Sal* I +*EcoR* I, and *Sal* I + *Bam* HI. All of them showed an identical pattern (Fig 28). Further study was focused on λa14 only.

E. Restriction mapping of the recombinant phage

Recombinant phage were double digested with *Sal* I plus different restriction enzymes (Fig.29). Three sets of samples were analyzed by gel electrophoresis on 0.6% agarose. The gels were transferred to nytran filters. Filter A was hybridized with ^{32}P labeled λ/*Hind* III digested DNA; filter B was hybridized with plasmid DNA (pSV_2gpt); filter C was hybridized with sheared total human DNA as a probe which detected highly and medium repetitive sequences. By comparison of the bands seen on the EtBr stained gel and bands positive in different blots, the map of the recombinant phage was resolved (Fig. 30). The orientation of inserted DNA was decided by single digestion with *Xho* I or *EcoR* I which gives the predicted size. Since the insertion fragment has *Alu* reactive sequence on the left and plasmid reactive sequence on the right, the sequence in between must be human instead of CHO cell DNA. The fragments seen on the EtBr stain gel and missing from all three blots that hybridized with different probes were presumed to be human unique sequences. For example, there were six bands after digestion with *Sal* I and *EcoR* I in the EtBr stained gel: 19.3, 9.2, 6.9, 3.9, 2.8, 1.4 kb. The first two bands (19.3 and 9.2 kb) were reacted with λ/*Hind* III probe DNA, the 6.9 kb band was reacted with total human DNA probe, and 3.9 kb band was reacted with pBR probe DNA.

The two fragments 2.8 and 1.4 kb which did not react with any of these probes were assumed as human unique sequence. There were several restriction enzymes that generated these kinds of human unique sequences; for example, 5.8 kb from *Hind* III+ *Sal* I (lane 2); 3.4 kb from *Bgl* II+*Sal* I(lane 7); 2.6 and 3.3 kb from *Pvu* II+*Sal* I (lane 9). For easy of purification and separation from other bands, the human unique sequences generated by *EcoR* I+*Sal* I, 2.8 kb and 1.4 kb, were chosen for further study.

6. Subcloning of the two unique sequences

Since the gel purified fragment is hard to resolve from the other fragment run on the same gel, the 2.8 and 1.4 kb fragments were first gel purified, then subcloned into pS1EMBL which is a plasmid vector containing the R6K origin of DNA replication and kanamycin resistance gene. Therefore, the sequence of the pS1EMBL vector has no homology to any pBR derived plasmid sequences (Fig. 3). The 2.8 and 1.4 kb fragments were each cloned into the unique EcoR I site of the vector. Recombinant white colonies in IPTG X-gal plates were picked and screened by digestion of plasmid minipreparation DNA with EcoR I. The subclones of 1.4 and 2.8 kb fragments were named pS1.4 and pS2.8.respectively.

The 2.8 and 1.4 kb human unique sequences were verified by using them as radioactive probes to hybridize with human fibroblast cell DNA digested with EcoR I . The Southern result showed single bands of 1.4 and 2.8 kb respectively, by pS1.4 and pS2.8 probes (Fig.31), which suggested three points. First, both 1.4 and 2.8 kb represent different human gene unique sequences. Second, the subcloned pS1.4 and pS2.8 are totally free of repetitive sequence. Third, there is no evidence for sequence rearrangement during the cosmid library transfection, secondary transfection, and construction of genomic library.

7. Is λa14 able to rescue the *ts* phenotype of JB7-K?

Since the recombinant phage λa14 contains a 15 kilobase insertion fragment which has *Alu* positive sequence on it's left end and plasmid positive sequence on right end, it is possible that this recombinant phage may contain the intact human gene which can correct the JB7-K *ts* phenotype. The λa14 DNA was co-transfected with p4aA8 ($hprt^+$ plasmid) and pSV_2gpt into JB7K-TG4 and JB7-K cells, respectively, by the CaP transfection techniques described in the methods. Twenty micrograms of λa14 DNA plus 0.5 μg of the selectible plasmid DNA were transfected to $5x10^5$ cell in 100 mm dish. The molar ratio is approximately 4:1. JB7K-TG4 cells were also transfected with the same amount of p4aA8 plus calf thymus DNA (20 μg) as a control. The dishes were split one to four at 48 h post transfection in the selection medium (HAT medium for JB7K-TG cell with p4aA8 co-transfection, HAT+X+MPA medium for JB7-K cell with pSV_2gpt co-transfection). One of each set of dishes was stained for colony number after two weeks in the selection medium at 33°C. All the other dishes were then shifted to 39.5°C and the colony number determined one week later. The results arepresented in Table 16. There was a big difference in the ratio of colonies at 39.5°C versus 33°C for the cells co-transfected with and without λa14. The p4aA8 DNA plus calf thymus DNA transfected sample showed a background of surviving colonies. The colonies which survived at 39.5°C for three weeks for λa14 co-transfected cells and one week for calf thymus transfected cells were picked and subcultured in selection medium at 33°C until there were enough cells for assay and frozen storage. To determine if the *ts* rescued phenotype actually corresponds to recombinant λa14 DNA integration, cellular DNA was extracted from propagated cells of individual colonies digested with *EcoR* I or *Bam* HI+*Sal* I, and analyzed by Southern

Table 16

λa14 DNA transfection in JB7-K and JB7K-TG cell [a]

Receipt cell	JB7K-TG	JB7K-TG	JB7-K
Transfected DNA	p4aA8+CT DNA	p4aA8+λa14	pSV2gpt+λa14
Selection medium	HAT	HAT	HAT+X+MPA
Colonies number per dish at 33°C	1184[b]	1920	1638
Colonies number per dish at 39°C	8[c]	368	240
Ratio of colonies number at 39°C/33°C	1:148	1:5.2	1:6.8

(a) 20 μg of λa14 DNA plus 0.5 μg of p4aA8 and/or pSV2gpt were transfected into JB7K-TG and/or JB7-K cells by CaP coprecipitation techinique at 33°C. 0.5 μg of p4aA8 plus 20 μg of calf thymus (CT) DNA were transfected into JB7K-TG cell as a control. Dishes were split 1:4 at 48 h post transfection in each indicated selection medium at 33°C. All the dishes were incubated at 33°C for two weeks and then shifted to 39°C. HAT^+ and/or gpt^+ colonies were counted at 33°C just before shift and also after shift 10 days at 39°C.

(b) number of HAT^+ and/or gpt^+ colonies per dish at 33°C at two weeks.

(c) number of HAT^+ts^+ and/or gpt^+ts^+ colonies per dish after shift to 39.5°C for 10 days.

Table 17

EOC of λa14 DNA transfected JB7K-TG and JB7-K cells [a]

	33°C		39°C		
Number of cells incubated	$2X10^2$	10^3	10^3	10^4	10^5
7KTG-M3	161[b]	733	0	0	0
7K-λ3	167	767	0	>20	++++[c]
7K-λ8	152	707	0	>20	++++
7KTG-λ3	162	752	0	0	4
7KTG-λ5	138	623	0	4	67

(a) JB7K-TG and JB7-K cells were co-transfected with λa14 plus p4aA8 and/or pSV2gpt as described in the result. $HPRT^+ts^+$ (7K-TGλ3 and 7K-TGλ5) and gpt^+ts^+ (7K-λ3 and 7K-λ8) colonies were picked after incubation in the selection medium for 10 days at 33°C plus three weeks at 39°C. 7K-M3 was picked from the control transfection dish (JB7K-TG4 transfected with p4aA8 DNA plus calf thymus DNA) after 10 days at 33°C and one week at 39°C. Cells were seeded at different densities at 33°C in 100 mm dishes. After 16 h, the desired dishes were shifted to 39°C. Colonies were stained two weeks later. JB7-K and JB7K-TG would not be expected to form colonies or show mass growth at 39.5°C at these cell densities.

(b) Number of colonies at indicated seeding density. Numbers represent the average of triplicate cultures.

(c) Cells grow as a mass culure two weeks after seeding.

hybridization with pS2.8. The results are shown in Fig. 32. The control transfection (p4aA8+calf thymus DNA) is negative for λa14 DNA, as expected. The rest of the colonies picked in the selection medium after three weeks at 39.5°C contain the λa14 DNA sequence in both JB7-K and JB7K-TG4 recipient cells. There was a 15 kb band in each sample digested with *Sal* I which means that every colony has at least one copy of λa14 in which the integration site is not inside of the 15 kb recombinant DNA insertion fragment. There were also some other bands bigger or smaller than 15 kb in *Sal* I digested samples which means that there are different integration sites and some of them are inside of 15 kb insertion. There were a major 2.8 kb band and some other larger size bands found with EcoR I digested sample. Most integration occured outside of 2.8 kb sequence; a small fraction of them were integrated within the 2.8 kb sequence. It should be noted that the 2.8 probe did not react with the DNA from Chinese hamster cells. The cultures grown from individual colonies were also tested for efficiency of colony formation at 33°C and 39.5°C. (Table 17). In some of them like 7-Kλ8 and 7Kλ3 their *ts* phenotype were partially corrected. In other such as 7-KTGλ5, its *ts* phenotype is less corrected. No correction was observed with the survivor picked from that co-transfected with the calf thymus DNA (7K-M3).

Discussion

DNA replication in mammalian cells has been studied from different aspects and different strategies. The isolation of temperature sensitive mutants and the use of simple (viral) model systems to characterize the *ts* mutant has been widely used in the study of prokaryotic DNA replication. The same strategies were used here in the study of eukaryotic DNA replication. DNA viruses have been chosen as molecular probes to characterize the cellular *ts* defect because of the ability of CHO cells to support an appreciable level of viral DNA replication for both adenovirus and polyoma virus. For adenovirus DNA replication, the identified cellular factors include at least nuclear factor I, a single strand DNA binding protein (175); nuclear factor II, the topoisomerase I (176); nuclear factor III, an octomer binding protein which is also a ubiquitous transcription factor called OTF I, oct 1 etc. (177); nuclear factor IV, a cellular protein of unknown function (178) and those steps required for deoxynucleotide metabolism. For polyoma virus DNA replication, only one viral coded protein is required for viral replication: large T antigen. Viral DNA replication is, therefore, very much dependent on the cellular DNA replication machinery. Therefore the *ts* DNA cell mutan*ts* that also affect polyoma virus DNA replication are potentially more interesting to study, and would give even more broad range of information on the molecular mechanism of the eukaryotic DNA replication.

There were five mutants (JB3-B, JB3-O, JB7-K, JB8-D and JB11-J) defective in polyoma DNA replication at the nonpermissive temperature and all of them except JB3-B support adenovirus DNA replication at 39.5°C. Therefore several possible *ts* defects could be excluded from those four mutants. Both adenovirus and polyoma virus DNA replication need cellular deoxynucleoside triphosphate

(dNTP) pools, therefore these mutants are not defective in enzymes required for deoxynucleotide synthesis. The defects in these mutants are also unlikely to be due to a block in cell cycle progression rather than S phase. Although papovavirus infection of synchronized cells shows that the start of viral DNA synthesis depends on the infected cell proceeding through G1 into S, subsequent syntheses of viral replicons is not cell cycle dependent. Here polyoma DNA synthesis was inhibited even when transfected cultures were maintained at 33°C for 24 h and then shifted to 39.5°C.

One of the common features of all these mutants are that they grow slower than wild type CHO-K1 cells even at the permissive temperature (33°C). Cell doubling time for mutants is 24-30 h at 33°C and 16-18 h for wild type cells. The mutants studied here were isolated by either one long selection (for JB3-O, JB7-K), multiple short selections (for JB8-D) of mutagenized cells at restrictive temperature followed by bromodeoxyuridine (BudR) treatment and expousure to UV light or by non-selective replica plating of mutagenized cells (for JB11J) (145). The mutagen and selective method may have an effect on cell growth properties which may or may not relate to the *ts*DNA phenotype.

Among those four mutants, JB7-K was chosen for further analysis. Complementation analysis indicates that JB7-K is complemented by several previously or simultaneously isolated *ts* DNA mutants except JB8-D. When JB7-K and JB8-D cells were fused, there were no cell hybrids observed at 39.5°C. When hybrid cells grown at 33°C were replated and shifted to 39.5°C, the EOC is less than one in 10^5. That hybrid cells formed between JB7-K and JB 8-D is not only confirmed by selection in HAT plus ouabain medium but also by karyotyping. The hybrid cell Hi8D-7K contains 42 chromosomes, and JB7-K and JB8-D each contain 21 chromosomes. The kinetics of DNA and protein synthesis of both

mutants at both 33°C and 39.5°C showed a similar pattern. Therefore, JB7-K possesses the same genetic function as JB8-D and one for which a *ts*DNA mutant has not previously been isolated. The initial cosmid library gene transfer experiments showed that JB7-K is the best recipient cell among JB3-O, JB8-D and JB7-K. Both cell fusion and protoplast fusion experiment show JB3-O cell is sensitive to PEG toxicity. Since JB7-K and JB8-D may be defective in the same gene, study of one of them will give information about the other.

I attempted to identify possible basis for the *ts* defect in JB7-K. As noted earlier, the differential effect on polyoma and adenovirus DNA synthesis ruled out a defect in synthesis of dNTP; similarly there does not appear to be a major defect in ribonucleotide triphosphate synthesis since incorporation of [^{3}H]-uridine is not temperature-dependent. Polyoma requires RNA primase; adenovirus does not. Analysis of cell cycle kinetics upon shift of JB7-K to the non-permissive temperature shows a gradual increase in G1 cells, comparable with a defect in the initiation of S phase or of DNA replicons. The latter occurs throughout S phase; however, it might be expected that cells already in S phase may have sufficient "initiation factor" to complete that S phase. Therefore, most cells would arrest at the G1/S border rather than within S phase. This interpretation is also consistent with studies on polyoma DNA synthesis which also support a defect in replicon initiation.

The ability to use [^{3}H]-TdR pulse labelling to investigate polyoma virus DNA replication in CHO cells has not been previously reported in the literature. For JB7-K there appears to be at least a 5 fold decrease in the replication rate of polyoma DNA after 6 h at 39.5°C as compared to the sample at 33°C, which provides further evidence that the defect in JB7-K is indeed in DNA synthesis.Whether the defect is in a step of elongation has been studied by looking for changes of replicative

intermediate forms in the pulse label samples with or without cold TdR chase. It has been difficult to define the polyoma DNA largest replicative intermediate form (i.e.concatenated dimer) since the recombinant DNA p53A6.6 monomer size is 8.9 kb and the CHO cellular mitochondrial DNA is 17-20 kb, and the latter is a dominant [^{3}H]-TdR labeled band in the autoradiograph. Use of dimer free p53A6.6 DNA in the transfection and comparison of the different forms of polyoma DNA in parallel gel autoradiographs and Southern hybridization show that the DNA dimer form is very close to the position of cellular mitochondrial DNA band. Further analysis of this mutant biochemically is currently being studied by Ken Lawlor with an *in vitro* polyoma virus DNA replication system using cell extracts prepared from JB7-K cells grown at 33°C and 39.5°C. That may provide evidence for a defect involved in the initiation or elongation step. However, the failure to observe a differential increase in labelled viral DNA form I after a chase at 39.5°C (as compared to 33°C) suggests that the defect is in initiation rather than a slowing of strand elongation. The availability of several known proteins required for the *in vitro* system for papovaviruses DNA replication (96) could allow us to study *ts*DNA mutants by *in vitro* complementation. Another approach to characterize the *ts* defect in JB7-K involves molecular cloning.

There are very few genes required in DNA synthesis that have been cloned. In each case, a molecular probe for the gene was already available and all used a similar strategy. I elected to use an alternate approach based on a functional approach for gene isolation. A human genomic cosmid library containing a covalently linked dominant selectable marker was used as a source to correct the primary *ts* defect. This provides the advantage of reducing the population which could contain a *ts* revertant. This is especially relevant for JB7-K since it also shows a density dependent phenomenon at 39.5°C. For example, when $5x10^5$ cells

were seeded in 100 mm dish at 33°C for 16 h and then shifted to 39.5°C, the cells will continue to grow and/or survive and approach confluence. If 10^5 cells were seeded in the same size dish and then shifted to 39.5°C, none of the cells would grow to form colonies. Therefore, use of a selectable genetic marker in human gene transfer for two-step selection is a critical step in molecular cloning of the gene which corrects the *ts* defect in JB7-K. Interestingly, the thioguanine subline (i.e. JB7K-TG4) does not show as much of a density phenomenon so that it dose not appear to be an obligatory aspect of the *ts* mutation but may rather be a reflection of the selection method for *ts* mutants initially used.

The primary transfectant 5A1 isolated from 4000 gpt^+ colonies contains 8 copies of human medium repetitive *Alu* sequence and also contains multiple copies of the *gpt* gene which is not surprising since the ratio used for protoplast to cell fusion is 10^4:1. The secondary transfectant contains a single copy of human *Alu* sequence and a single copy of *gpt* gene by different restriction enzyme digestion. So the secondary transfection not only verified that the ts^+ phenotype corresponds to a specific human genomic cosmid DNA but also eliminated the other human genomic DNA.

The human sequence rescued by cell fusion between COS 7 cells and secondary transfectant 2°5D gave a rapid way to recover the vector and adjacent sequences due to the presence of an SV40 origin. The excised plasmid usually is small (172). In this case the rescued plasmid is 9.2 kb. Besides the cosmid vector sequence required for replication (SV40 ori), amplification in E.coli (plasmid origin) and the antibiotic resistant gene (Amp^r), it also contains about 3.35 kb of sequence linked to the vector. Use of frequent-cutting restriction enzymes (i.e. *Taq* I, *Alu* I, *Sau3A* I and *Rsa* I) and Southern analysis using total human DNA as a probe reveals that 2.3 kb of them contains *Alu*-like repetitive sequence, the other 1.05 kb contains α-

like highly repetitive sequence. It was, therefore, not useful for identifying a unique sequence corresponding to the human gene.

I subsequently turned to an alternate approach: λ cloning of genomic DNA isolated from $2^{o}5D$. Good quality of HMW DNA is needed to construct the genomic library. I found that prolonged Proteinase K digestion (for 48 h instead of the usual 24 h) results in much less interphase during phenol and phenol:chloroform extraction and is easier to handle. After *Sau 3A* I partial digestion, I used a 5-25% NaCl gradient to isolate 15-22 kb DNA in preference to sucrose gradients since the DNA could be ethanol precipitated directly. This procedure avoids a time consuming dialysis step which could also damage the ends of the DNA fragment. The λ/EMBL-3 arm was preannealed at 42°C for 1 h in 10 mM $MgSO_4$ which increases the amount of concatamer recombinant phage after ligation. The ligation of λ/EMBL-3 arm to library insertion DNA gives the best result when the ratio is 1:2. The ligation mixture is packaged by Promega packaging extract which gives the wild type concatamer λ DNA titer as high as 10^9 pfu/μg DNA. Field inversion electrophoresis showed that the ligated recombinant DNA is mostly dimer, trimer and monomer, and some of it was too big to resolve well in the gel and remain in the well. The titer of the packaged library was determined in NM539 P2 strain which replicate the recombinant phage and not the parent wild type λ because of Spi^{+} phenotype. The library was also amplified in the NM539 strain.

The genomic phage library constructed from the secondary transfectant has been screened by using human repetitive sequence *Alu* as a probe. The human unique sequences (2.8 and 1.4 kb) isolated from the positive recombinant phage as described in the results of Chapter III were verified by hybridization to total human DNA digested with EcoR I. The results show that both 1.4 and 2.8 kb fragments

are human unique sequence and the subcloned fragments are free of any kind of human repetitive sequence. The gel purified 2.8 fragment was subcloned and used as a probe to hybridize to human-mouse hybrid cell DNA and human-hamster hybrid cell DNA in collaboration with Dr. Wes McBride at NIH. There is a strong 2.8 kb band signal when hybridized to EcoR I digested human DNA, a weak well-resolved band at 17 kb to mouse DNA. When the 2.8 kb fragment hybridized to EcoR I digested human-hamster hybrid DNA , there is only one 2.8 kb band and no hamster DNA hybridized to the probe at the relatively high stringency. The human chromosome localization of this gene was determined by use of the 2.8 kb fragment as a probe to hybridize to human-rodent hybrid cell DNA which had been previously shown to contain few, one or even part of human chromosomes. The human gene that corrects the JB7-K *ts* phenotype is located on human chromosome number 2; there was no case of discordance seen (Table 18). Using known chromosome 2 translocations, it was concluded that the gene is located between 2q11-2p23. I would propose that it is quite near to the centromere in fact since the original cosmid which corrected the defect also contains human α-like sequences which are typically centromeric. Furthermore,the α-like repetitive sequence rescued in pSD8 which contain multiple EcoR I fragments is 2-3 kb upstream from the human repetitive *Alu* sequence, and the *Alu* sequence is about 6-8 kb upstream from the human unique fragment 2.8 kb. Southern analysis of secondary transfectant cellular DNA using *Alu* as a probe shows that there is only one copy of human *Alu* sequence in 2^{o}5D cells. So the α-repetitive sequence is about 8-11 kb away from the 2.8 kb sequence if no DNA rearrangement occurred between the COS-7 cell and 2^{o}5D cell fusion and 2^{o}5D cell genomic library construction. The α–repetitive EcoR I family has been found in human chromosome 13 and 21 (173,

174) and almost all chromosome centromeres. This location near the chromosome centromere may be a useful property for other type of genetic analyses.

Since the recombinant phage contain an 15 kb insert, it is possible that the intact human gene that corrects the JB7-K *ts* phenotype is present. The co-transfection of λa14 and p4aA8 (human cDNA for HPRT) into JB7K-TG cells (HPRT $^-$) show 20% of the HAT resistant colonies are also *ts* $^+$. In the control experiment in which calf-thymus DNA is transfected with p4aA8, 0.7% of HAT resistant colonies survived at non-permissive temperature. 15 colonies were picked from co-transfection dishes and 3 colonies were picked from control dishes, the Southern analysis of cellular DNA show that every one from the co-transfection dishes contain the λa14 using 2.8 kb as a probe. The *EcoR* I digested samples in the blot show there is a major 2.8 kb band and a few copies of other sized bands, which means that most of integration recombination is outside of the 2.8 kb sequence. An intact *Sal* I fragment is also present, consistent with a functional human gene.

As mentioned early, JB7-K and JB8-D do not complement each other, and they have a similar pattern of cellular DNA synthesis kinetics by [^{3}H]-TdR pulse-labelling. But their growth curve patterns are different. Another feature existing in JB7-K but not in JB8-D is density dependent growth at NPT. This may reflect the difference of subline of parent cell (JB7-K isolated from CHO-S cells, JB8-D isolated from CHO-K1 cells [145]) or multiple mutation exist in JB7-K. The latter might also explain that the *ts* correction phenotype on JB7-K transfectants is not quite complete.

Further studies on JB7-K will be to sequence the gene and identify the gene product and it's function. To facilitate such sequencing, I have initiated screening of a human cDNA library (see Fig. 2) using the 2.8kb sequence as a probe. Three

Table 18

Segregation of the 2.8 kb fragment with human chromosome 2[a]

Human chromosome	Gene/Chromosome +/+	+/-	-/+	-/-	% Discordance
1	17	5	14	59	20
2	22	0	0	73	0[b]
3	12	10	23	50	35
4	21	1	37	36	40
5	10	12	15	58	28
6	15	7	33	40	42
7	13	9	24	49	35
8	14	8	22	51	32
9	15	7	17	56	25
10	8	14	11	62	26
11	13	8	25	59	24
12	14	8	25	48	35
13	14	8	21	52	31
14	8	14	34	39	51
15	14	8	31	42	41
16	8	14	27	46	43
17	20	2	37	36	41
18	15	7	35	38	44
19	12	10	16	57	27
20	15	7	25	48	34
21	10	12	52	21	67
22	9	13	18	55	33
X	9	13	39	34	55

(a) The 2.8 kb fragment contain gene was detected as a 2.8 kb hybridizing band in EcoR I-digested human-rodent somatic cell hybrid DNAs after Southern blot

Table 18 legend

hybridization with a 2.8 kb EcoR I insert fragment subcloned in a pS1EMBL vector. This band was well-resolved from a weakly hybridizing 17.5 kb band in mouse DNA and no cross-hybridizing hamster sequence was found. Detection of the human band is correlated with the the presence of at least one human chromosome in the group of the somatic cell hybrid. The presence or absence of the 2.8 kb band was correlated with the presence or absence of each specific chromosome in the hybrid cell used to prepare the DNA in each lane, based on the previously determined chromosome composition of the cell hybrid. Discordancy represents the presence of the gene in the absence of a specific chromosome(+/-) or absence of the gene despite the presence of that chromosome (-/+), and the sum of these numbers divided by total hybrids examined (x100) represents percent discordancy. The human-hamster hybrids consisted of 28 primary clones and 14 subclones (5 positive of 42 total) and the human-mouse hybrids represented 13 primary clones and 40 subclones (17 positive of 53 total).

colonies have been isolated from duplicated filter from a pCDNeo library but they have not yet been characterized. Oligopeptides of 10-15 amino acids length from the N-terminal, C-terminal, or middle region can be synthesized based on the cDNA sequence and used as immunogens. The specific antibody (coupled with sepharose A) can be used to purify the polypeptide. The purified protein can be then studied directly.

Since the human unique sequence (2.8 kb) has only weak hybridization to mouse cell DNA and no hybridization to hamster cell DNA, it will be interesting to compare the cDNA sequence of the gene to other known sequences by computer search and study the conservation of those genes involved in DNA replication. One would have expected better evidence for conservation in different species. Either the 2.8 kb fragment is outside of conserved regions of the gene between human and hamster or the gene has low conservation but the coded proteins have a similar three dimensional structure so functional complementation can occur between human and hamster cells. Another possibility is that the genes may have many 3rd position base changes such that protein sequence might be highly conserved while nucleotide sequence might be divergent. Evidently many interesting questions remain to be answered and analyszed further.

Fig. 1. Map of pJB8 and pCV103Kgpt.

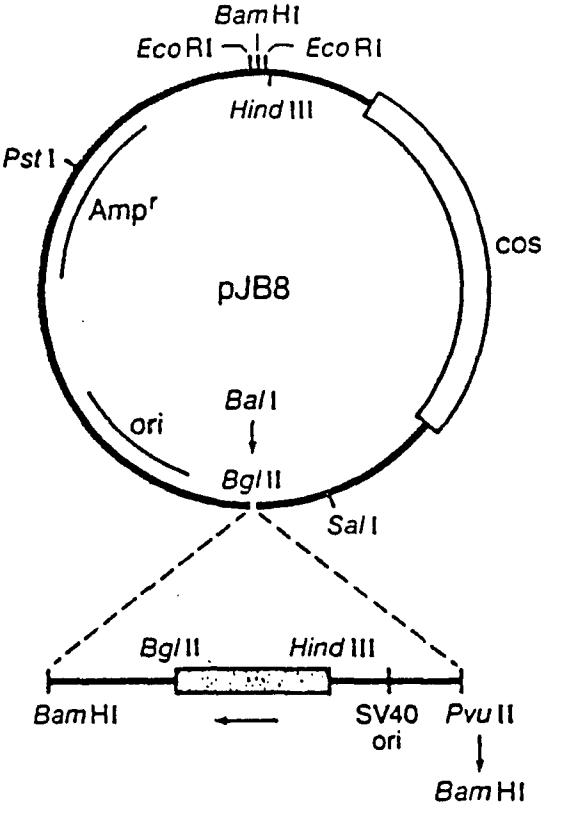
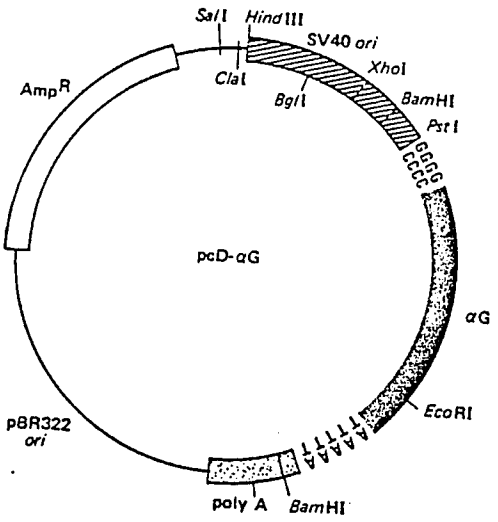

BamHI
EcoRI
EcoRI
Hind III
Pst I
Amp^r
cos
pJB8
Bal I
ori
Bgl II
Sal I
Bgl II
Hind III
BamHI
SV40
ori
Pvu II
BamHI

Fig. 2. Map of cDNA library vectors.

A


Sal I
Hind III
SV40 ori
Xho I
Cla I
Bgl I
BamHI
Pst I
Amp^R
pcD-αG
αG
EcoRI
pBR322 ori
poly A
BamHI

B

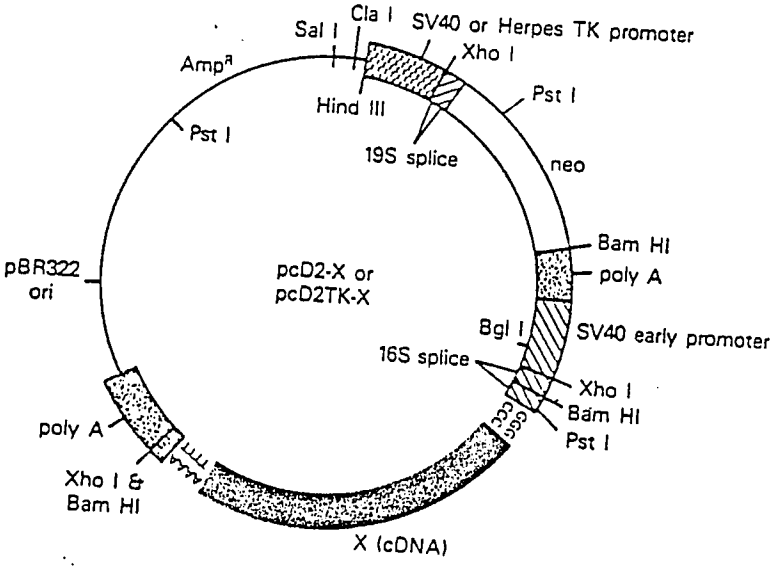

Sal I
Cla I
SV40 or Herpes TK promoter
Xho I
Amp^R
Hind III
Pst I
Pst I
19S splice
neo
Bam HI
poly A
pBR322 ori
pcD2-X or pcD2TK-X
Bgl I
SV40 early promoter
16S splice
Xho I
Bam HI
Pst I
poly A
Xho I & Bam HI
X (cDNA)

Fig. 3. Map of pS1EMBL.

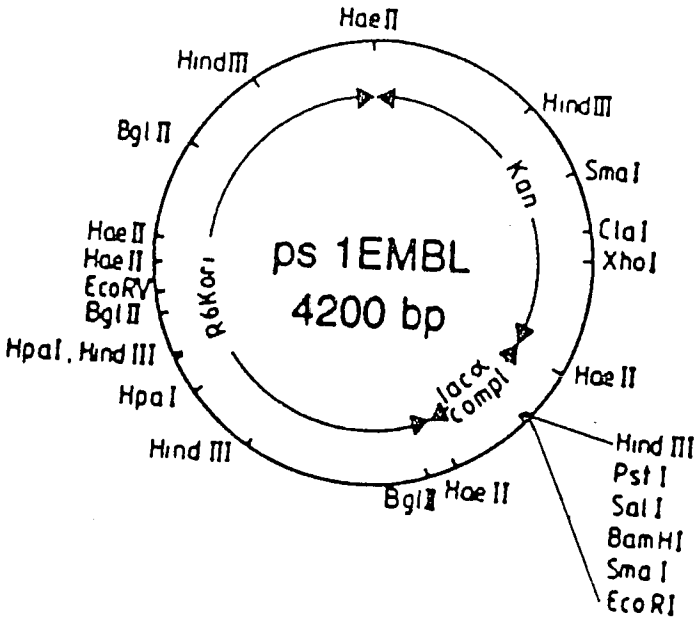

Hae II
Hind III
Hind III
Bgl II
Sma I
Kan
Cla I
Xho I
Hae II
Hae II
EcoRV
Bgl II
ps 1EMBL
4200 bp
R6Kori
Hpa I, Hind III
Hae II
lacα
compl
Hpa I
Hind III
Hind III
Pst I
Sal I
BamHI
Sma I
EcoRI
Bgl II
Hae II

Fig. 4. Map of p4aA8.

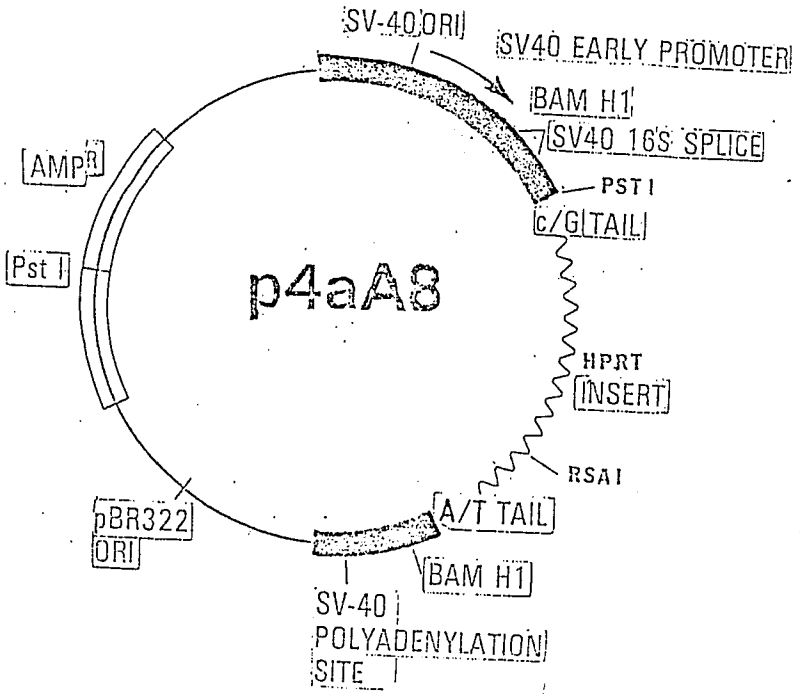

SV-40 ORI
SV40 EARLY PROMOTER
BAM H1
SV40 16S SPLICE
PST I
c/G TAIL
HPRT
INSERT
RSA I
A/T TAIL
BAM H1
SV-40
POLYADENYLATION
SITE
pBR322
ORI
Pst I
AMP R
p4aA8

Fig. 5. Polyoma DNA replication in a temperature sensitive mutant restrictive for viral DNA synthesis. JB7-K cells were transfected with p53A6.6 and viral DNA was extracted and quantitated by Southern analysis with nick-translated p53A6.6 DNA as described in Methods. The arrow corresponds to linear unit-length p53A6.6. Lanes: 1 and 2, infected for 48 h at 33°C; 3 and 4, infected for 24 h at 33°C followed by 24 h at 39.5°C; 5 and 6, infected for 48 h at 39.5°C; 7 and 8, infected for 72 h at 33°C; 9 and 10, infected for 48 at 33°C followed by 24 h at 39.5°C; 11 and 12, infected for 24 h at 33°C followed by 48 h at 39.5°C. Adjacent lanes correspond to duplicate transfected cultures.

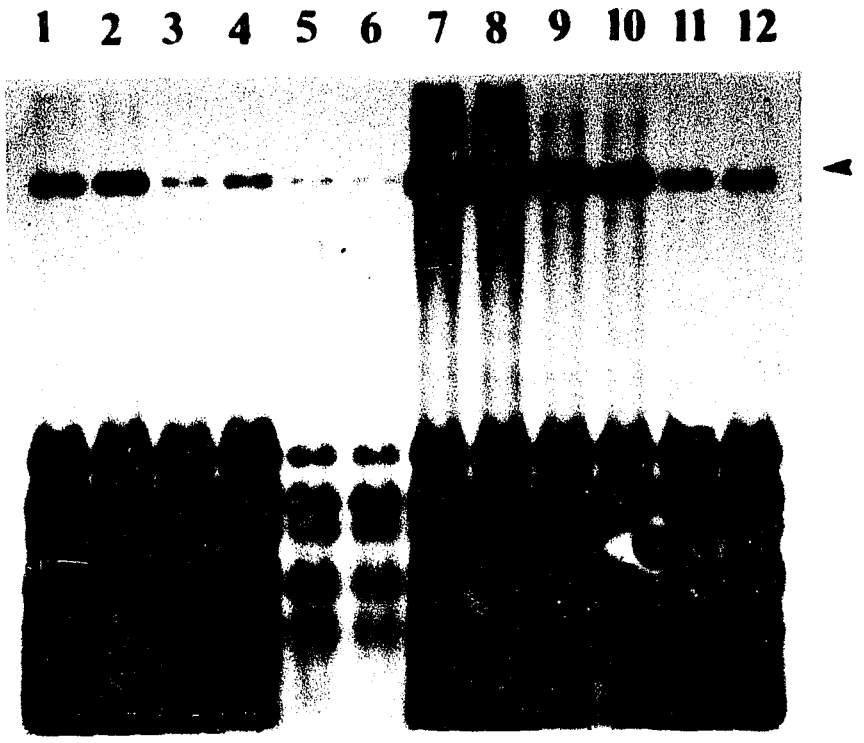

1 2 3 4 5 6 7 8 9 10 11 12

Fig. 6. Polyoma DNA replication in a temperature-sensitive mutant nonrestrictive for viral DNA synthesis. JB10-O cells were transfected and analysis was performed as described in the legend to Fig. 5.

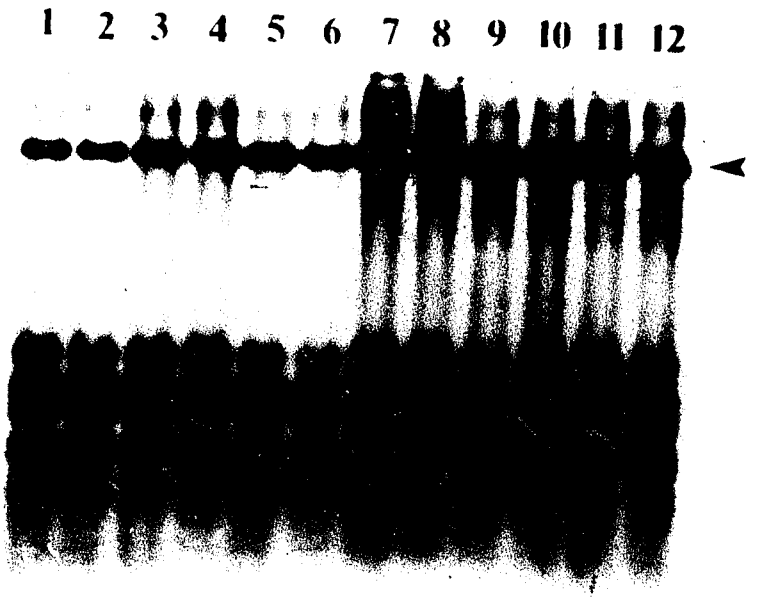
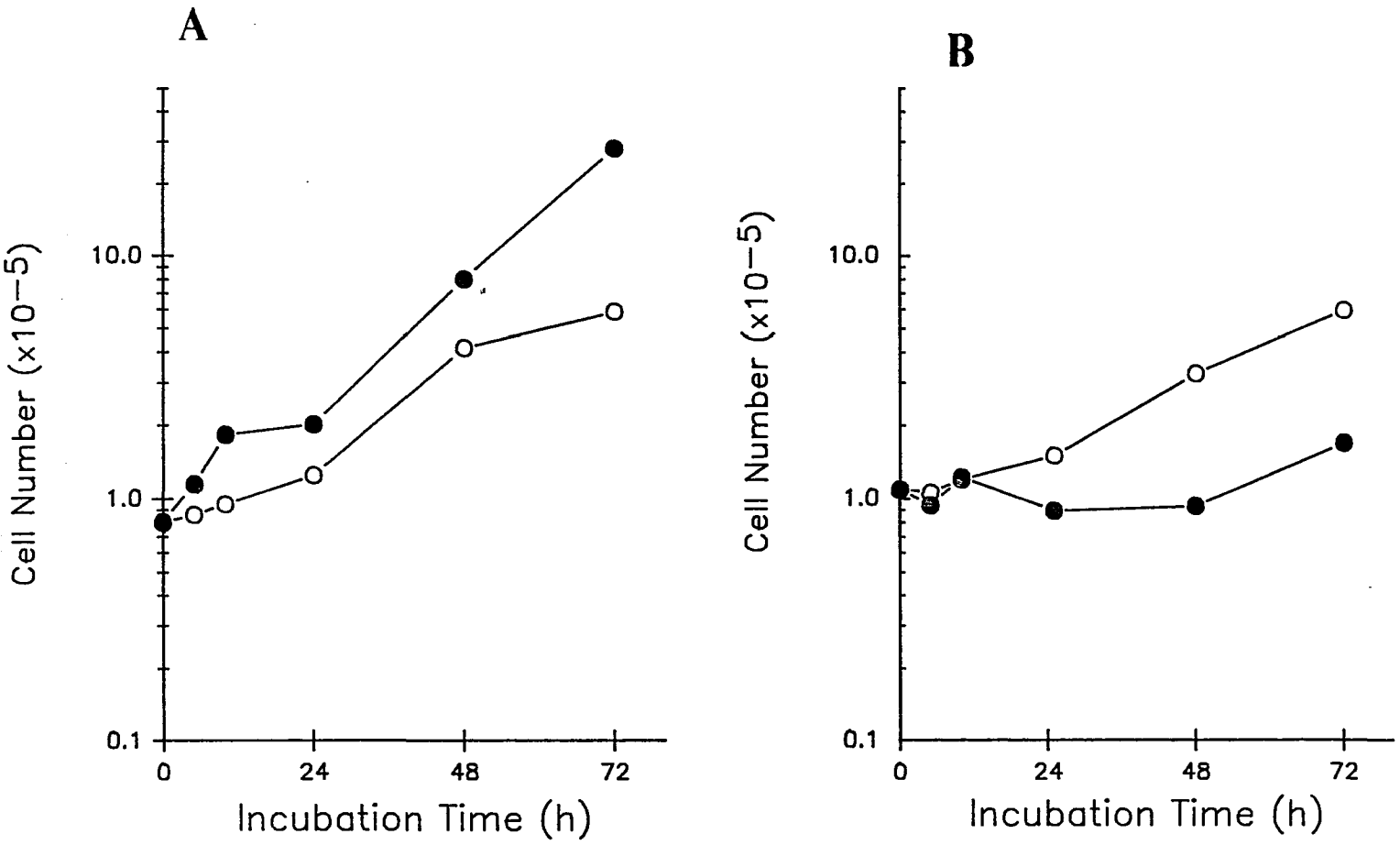

1 2 3 4 5 6 7 8 9 10 11 12

Fig. 7. Growth curves of wild type CHO cell and *ts* mutant CHO cells. $5x10^4$ cells were seeded in 60 mm dish at 33°C for 16 h. Half of dishes were shifted to 39.5°C and rest of dishes were continually incubated at 33°C. Cell numbers were determined as described in Methods. Numbers are the average of triplicate dishes. O---O: cell numbers at 33°C; ● --- ● : cell numbers at 39.5°C. Growth curve of wild type cell (3A); JB7-K (3B); JB3-O (3C); JB8-D (3D).


A
B
Cell Number (x10−5)
10.0
1.0
0.1
0
24
48
72
Incubation Time (h)

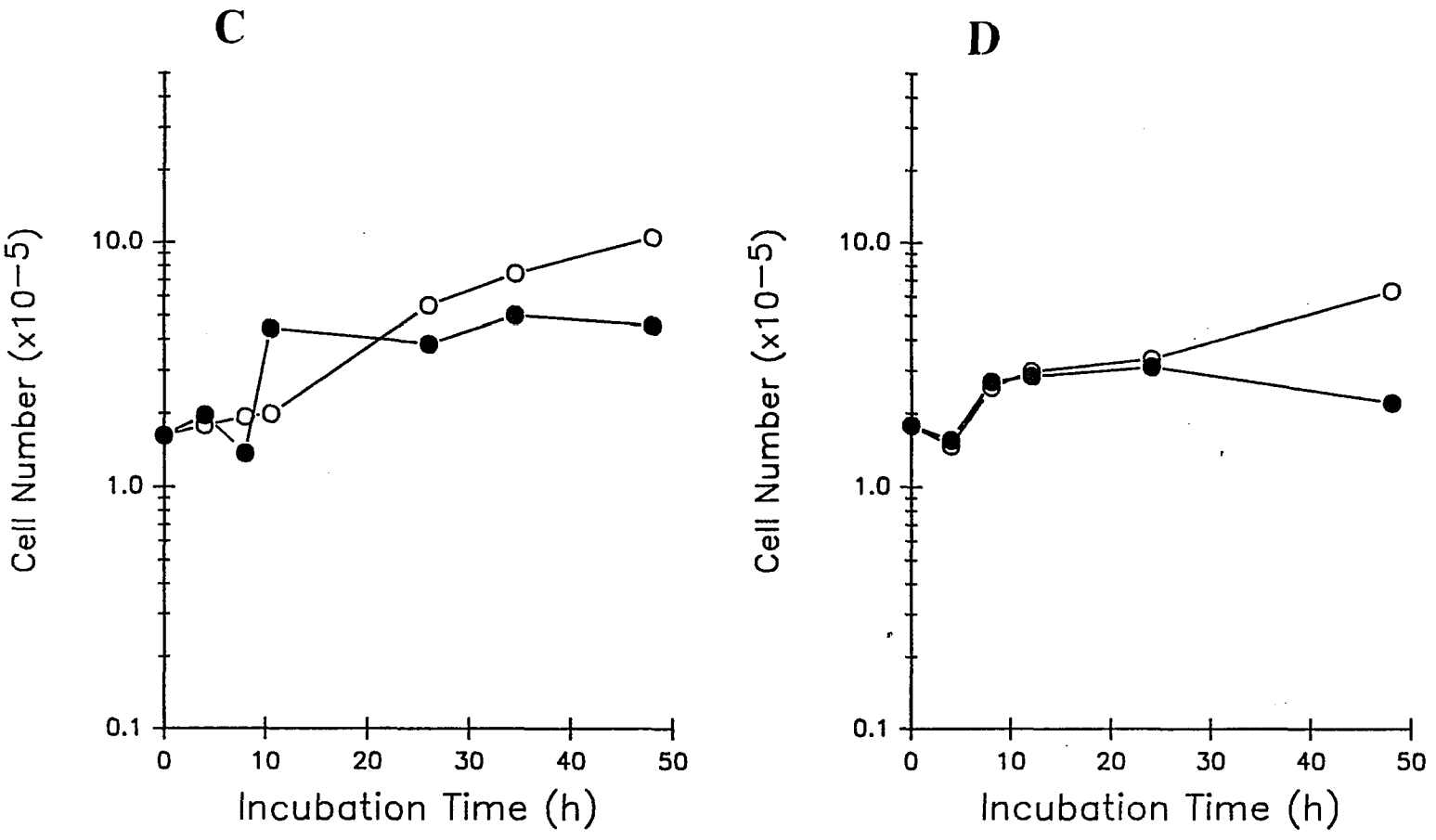
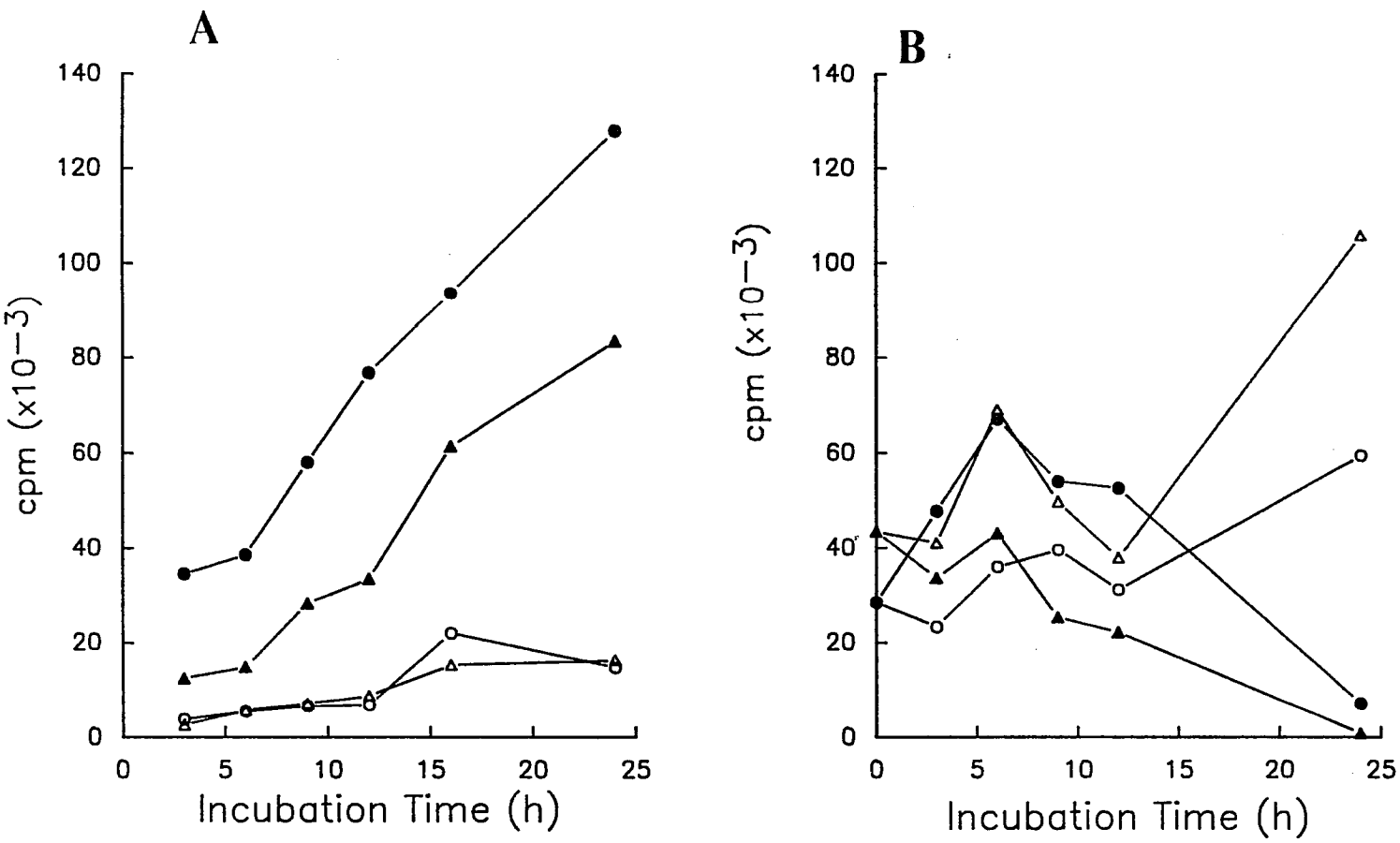
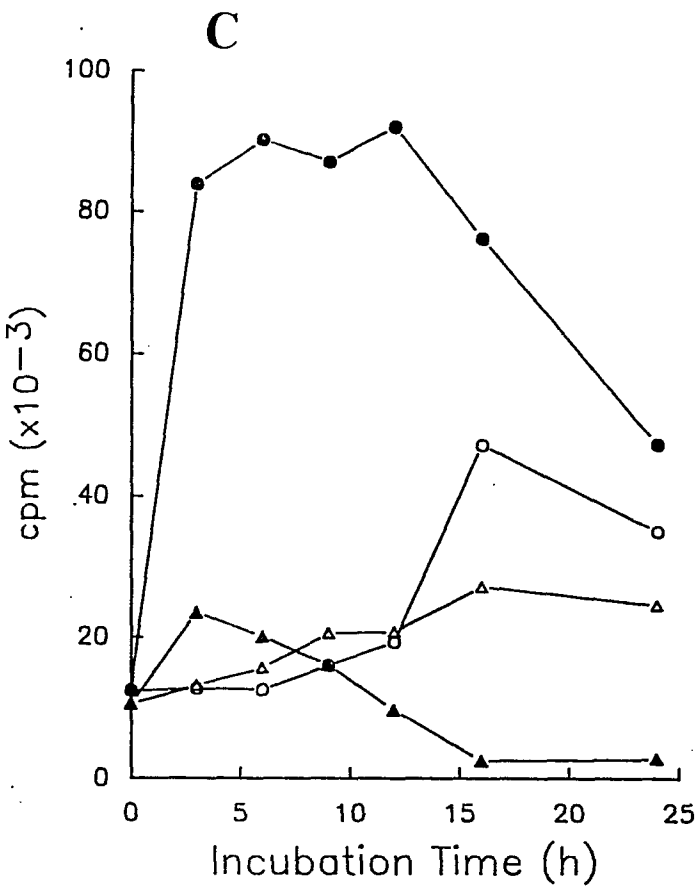
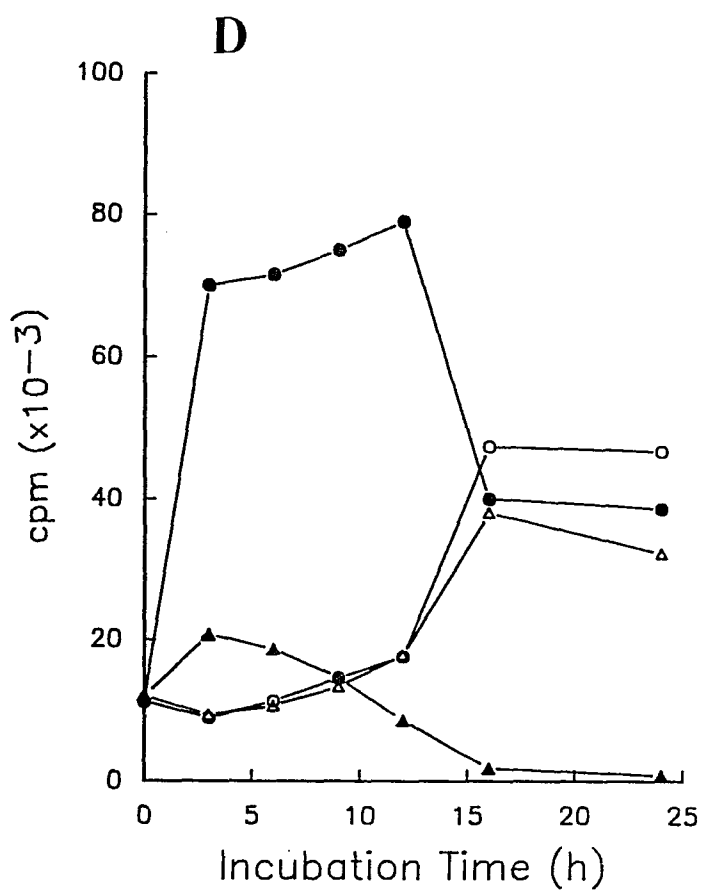
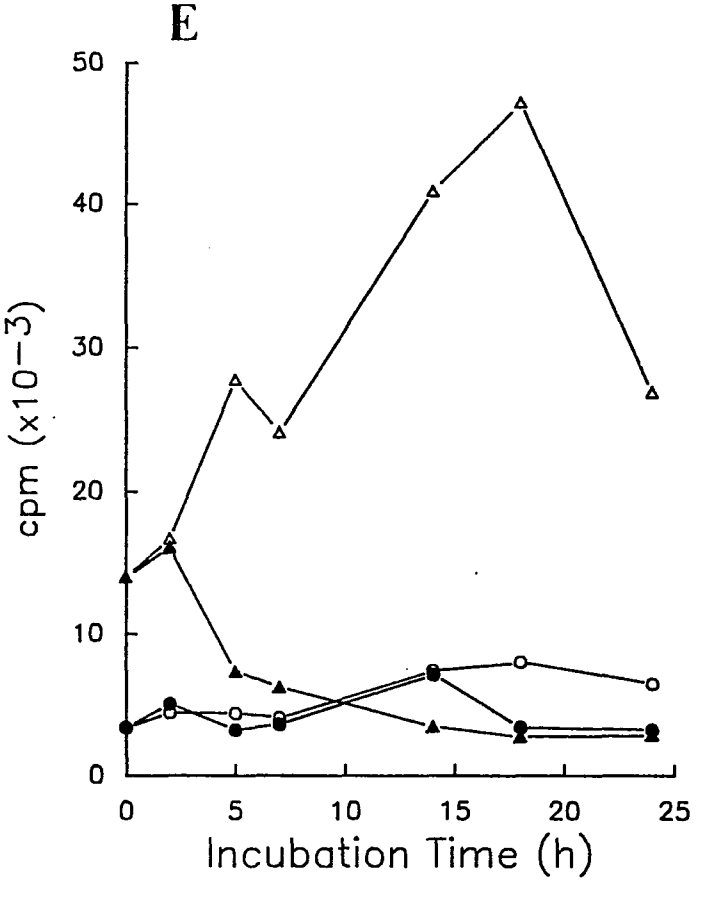

C
D
Cell Number (x10−5)
10.0
1.0
0.1
0
10
20
30
40
50
Incubation Time (h)

Fig. 8. DNA and protein synthesis in temperature-sensitive mutants. Cultures of each cell line were seeded and incubated at 33°C for 40 h, half were shifted to a 39.5°C water bath at time 0. Cultures were simultaneously pulse-labeled with [^{3}H]-TdR and [^{35}S]-Met at appropriate intervals and analyzed for radioactivity as described in Methods. Each point represents the average number of three cultures. O---O: [^{35}S]-Met incorporation at 33°C; ● --- ● : [^{35}S]-Met incorporation at 39.5°C; Δ---Δ: [^{3}H]-TdR incorporation at 33°C; ▲---▲: [^{3}H]-TdR incorporation at 39.5°C. Wild type CHO-K1 (A); JB3-O (B); JB7-K (C); JB8-D (D); JB11-J (E); JB3O-THO (F).


A
B
cpm (x10−3)
Incubation Time (h)
0
20
40
60
80
100
120
140
0
5
10
15
20
25


C
100
80
60
40
20
0
cpm (x10−3)
0
5
10
15
20
25
Incubation Time (h)


D
100
80
60
40
20
0
cpm (x10−3)
0
5
10
15
20
25
Incubation Time (h)


E
cpm (x10−3)
50
40
30
20
10
0
0
5
10
15
20
25
Incubation Time (h)

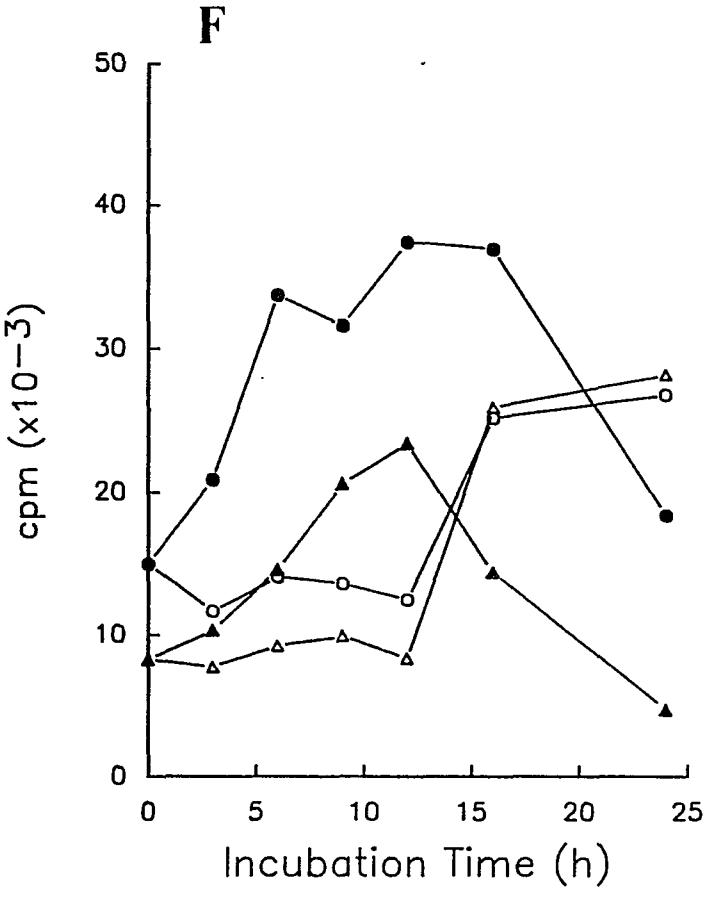

F
cpm (x10−3)
50
40
30
20
10
0
0
5
10
15
20
25
Incubation Time (h)

Fig. 9. RNA and protein synthesis in JB7-K and JB11-J. Culture of each cell line were seeded and incubated at 33°C for 40 h, half were shifted to 39.5°C water bath at time 0. Culture were pulse-labeled with [^{3}H]-UR and [^{35}S]-Met at appropriate intervals and analyzed for radioactivity as described for Fig. 8. Each point represents the average of three cultures. O---O: [^{35}S]-Met incorporation at 33°C; ● --- ● : [^{35}S]-Met incorporation at 39.5°C; □---□: [^{3}H]-UR incorporation at 33°C and ■---■: [^{3}H]-UR incorporation at 39.5°C. Analysis of JB7-K (A) and JB11-J (B).

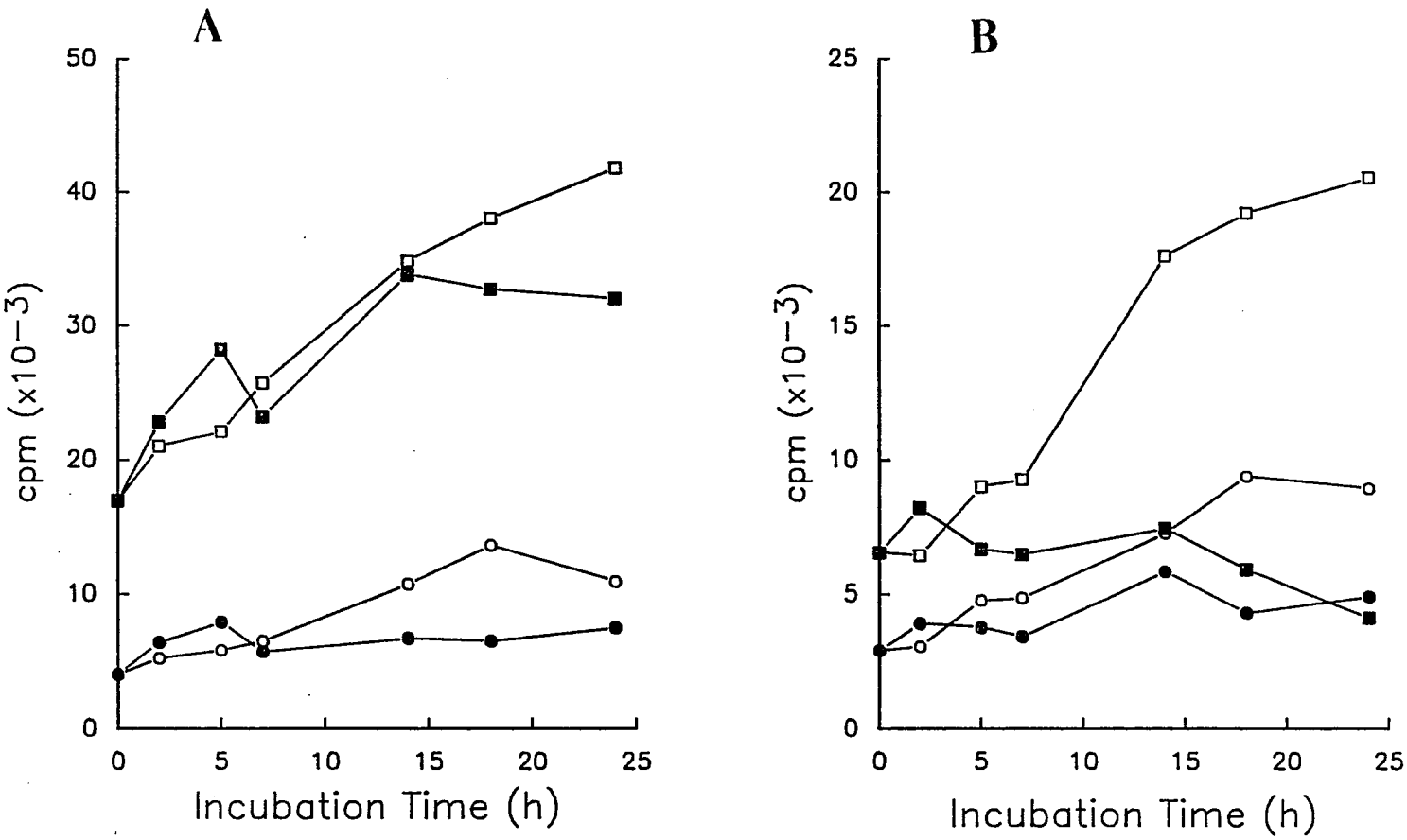
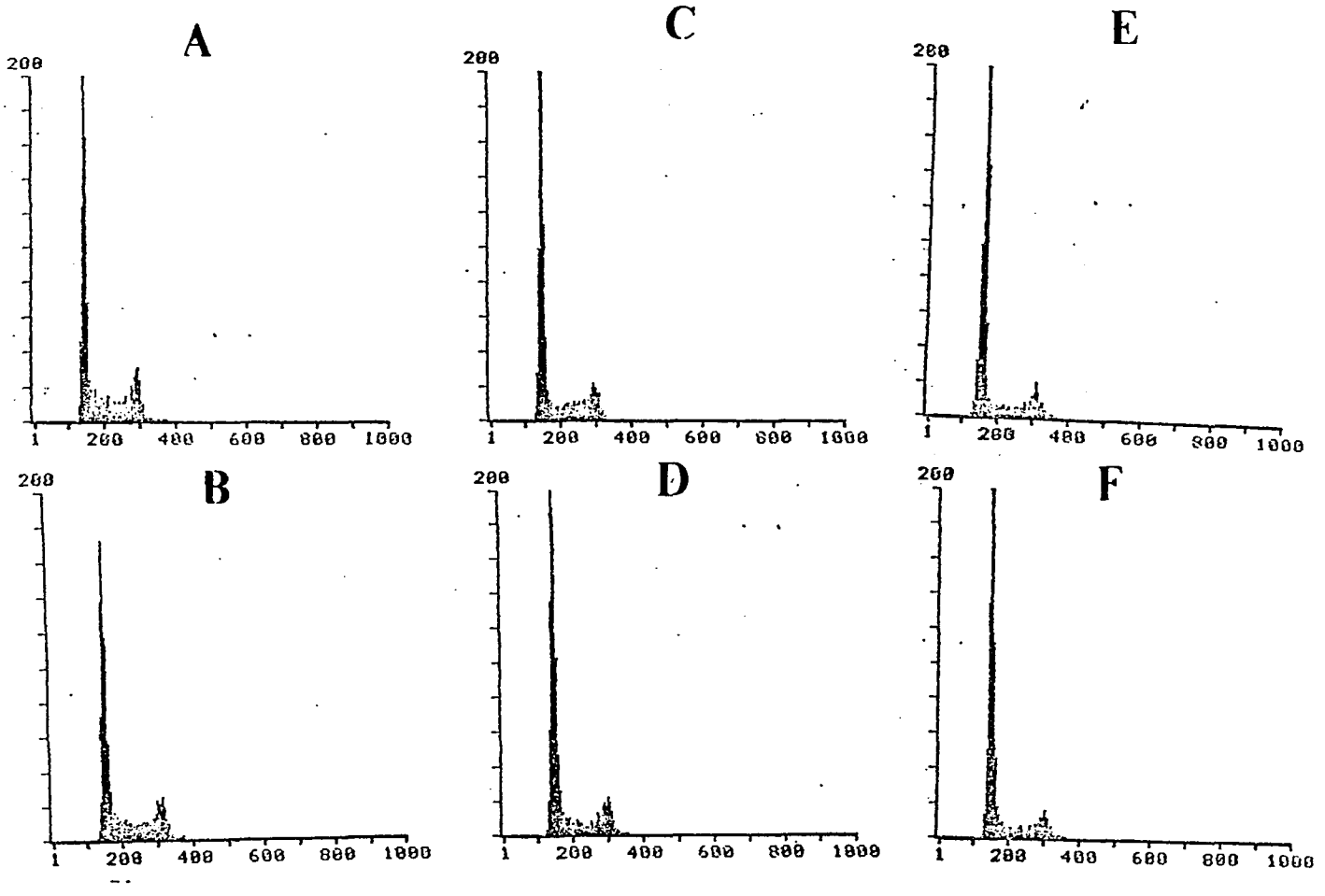

A
B
cpm (x10−3)
Incubation Time (h)
0
5
10
15
20
25
30
40
50

Fig. 10. Flow cytometry analysis of JB7-K at 33°C and 39.5°C. At the times indicated replicate cultures were trypsinized for cell count and fixed for FMF as described in the text and Methods. A: T=1 h , B: T=3 h, C: T=5 h, D: T=10 h, E: T=24 h, F: T=36 h. X-axis: Fluorescence. Y-axis: Cell number. A'-F': Cultures from 33°C, A-F: cultures from 39.5°C. The cell numbers of corresponding time points are also provided. O---O: 33°C, ● --- ● : 39.5°C.


A
200
1
200
400
600
800
1000
C
200
1
200
400
600
800
1000
E
200
1
200
400
600
800
1000
B
200
1
200
400
600
800
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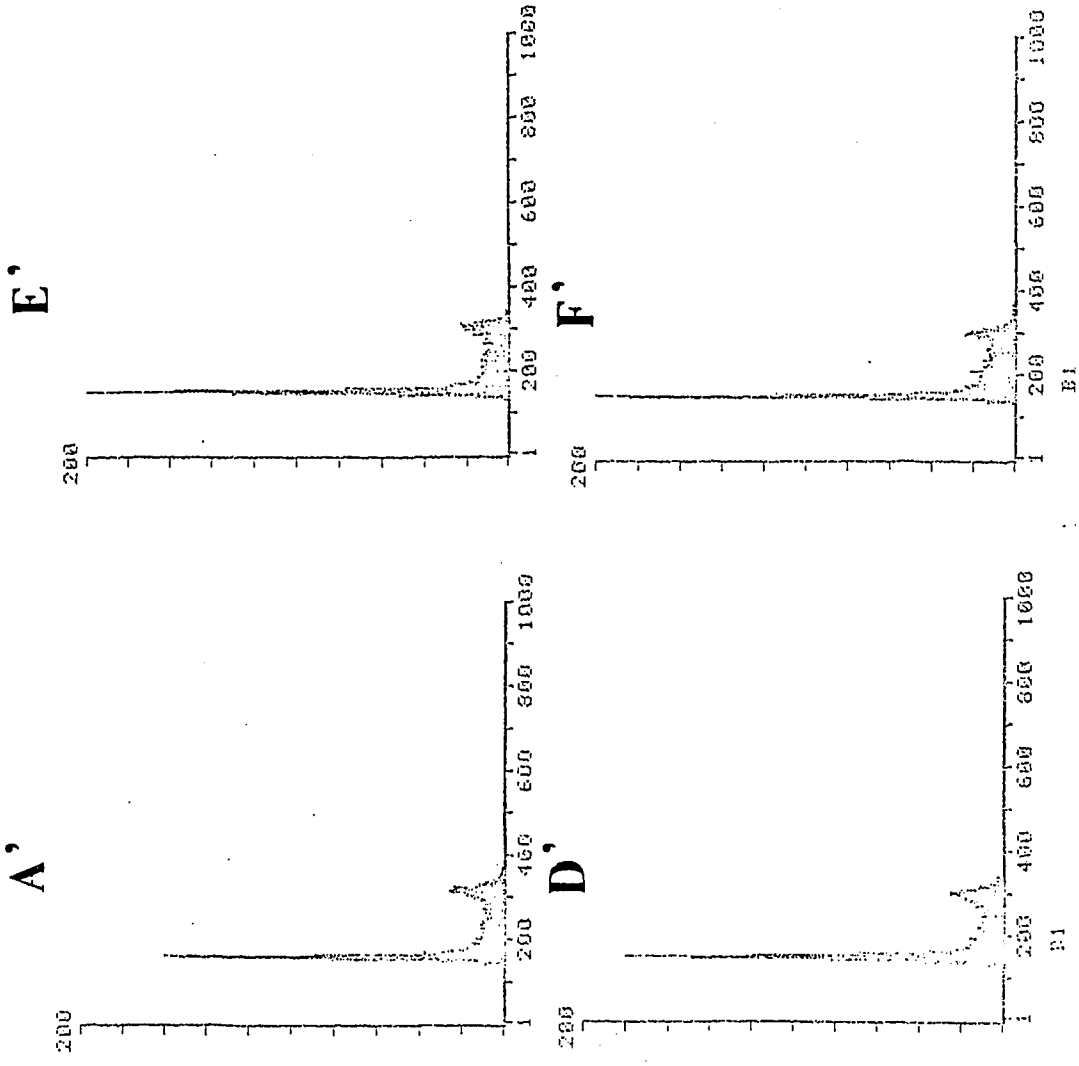
D
200
1
200
400
600
800
1000
F
200
1
200
400
600
800
1000


A'
E'
D'
F'
200
1
200
400
600
800
1000
E1

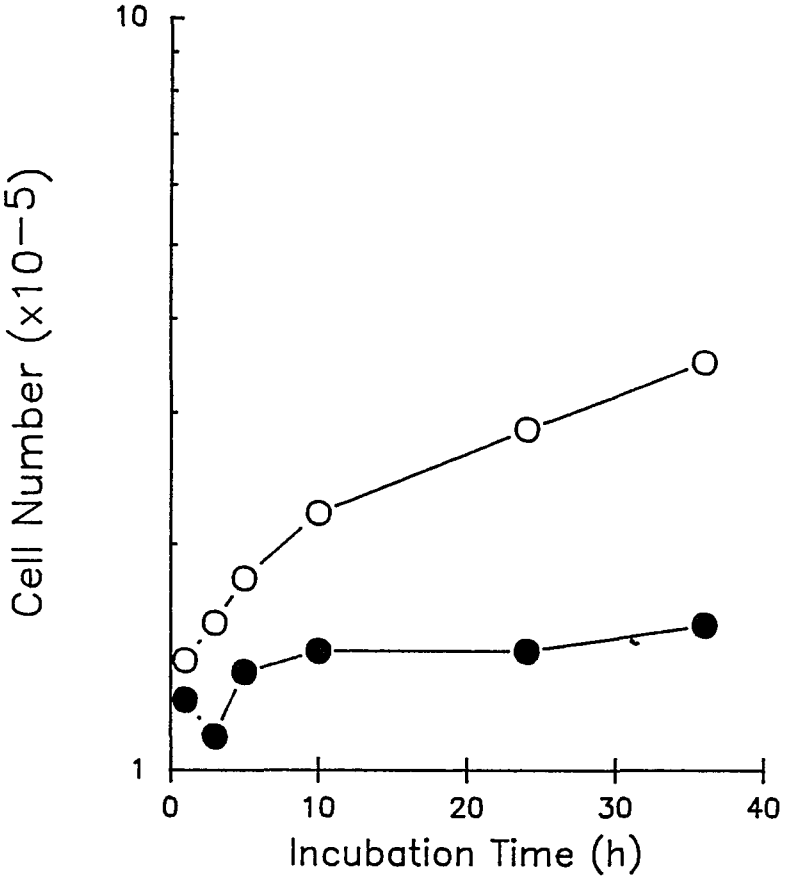
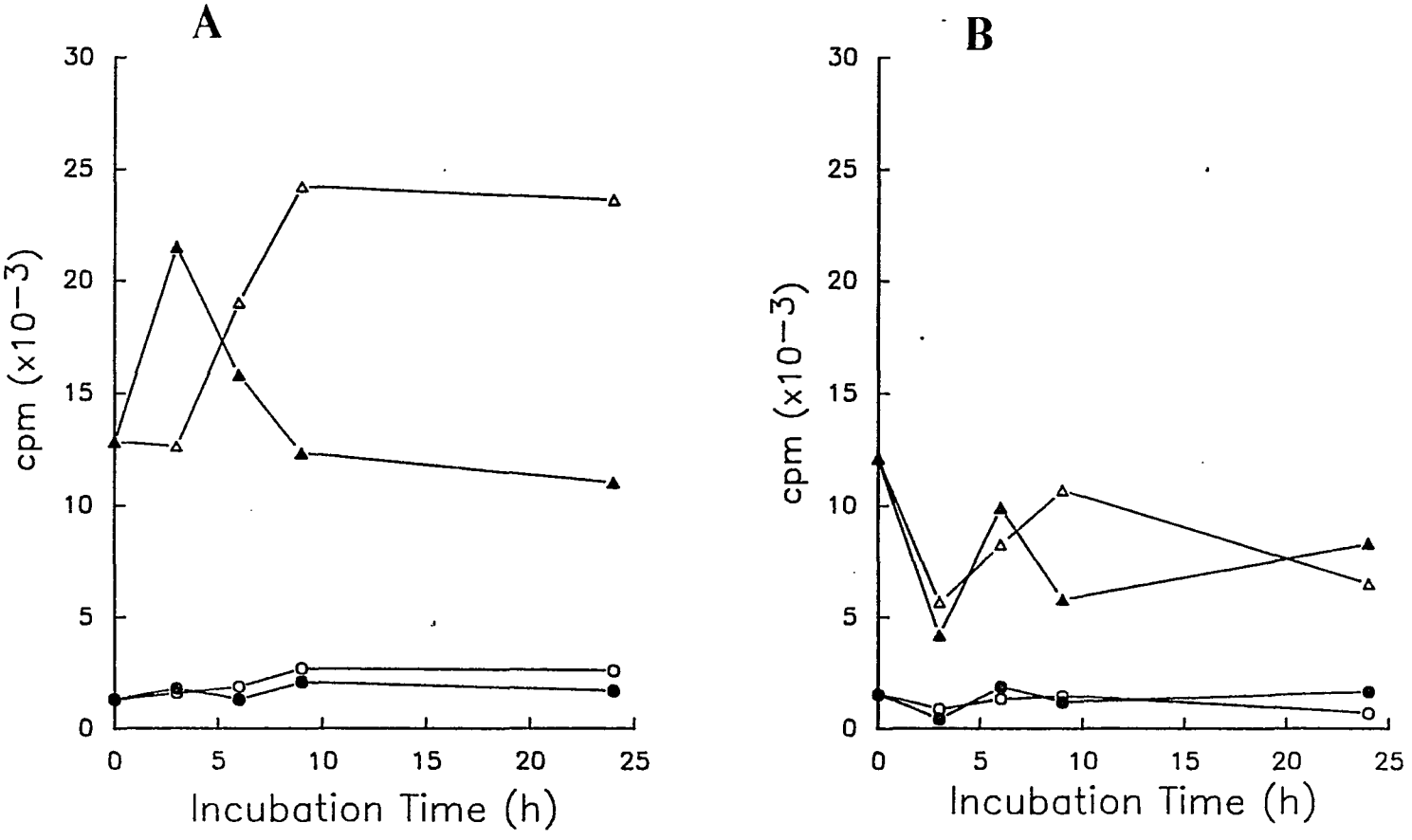
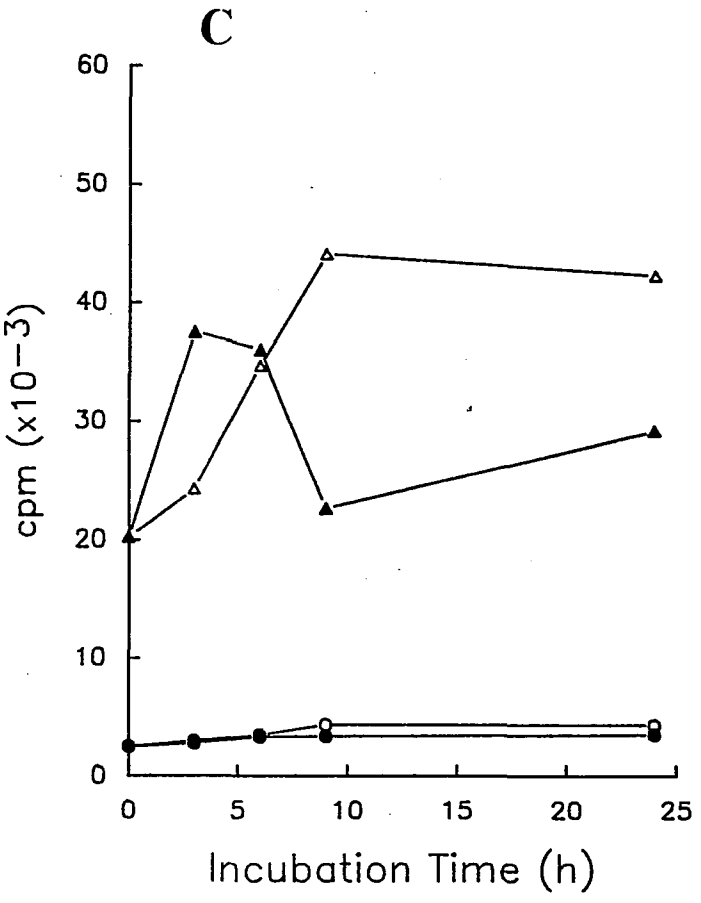
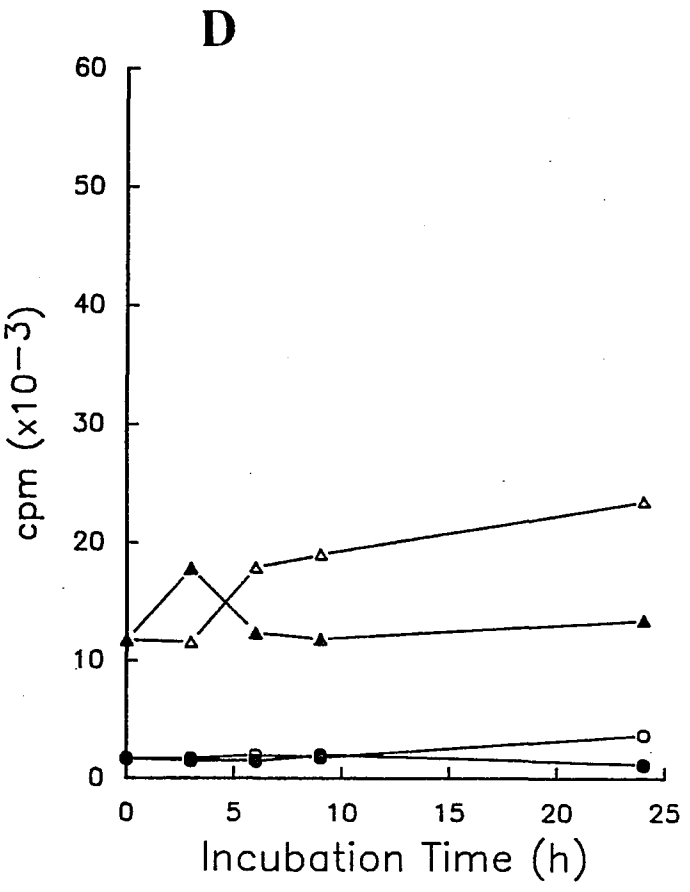

10
1
Cell Number (x10−5)
0
10
20
30
40
Incubation Time (h)

Fig. 11. DNA and protein synthesis rate in JB7K-TG cells. Cultures were seeded, incubated at 33°C and shifted to 39.5°C and pulse-labeled as described in legend for Fig.8. O---O: [^{35}S]-Met incorporation at 33°C; ● --- ● : [^{35}S]-Met incorporation at 39.5°C; Δ---Δ: [^{3}H]-TdR incorporation at 33°C; ▲---- ▲:[^{3}H]-TdR incorporation at 39.5°C. JB7-K (A); JB7K-TGs (B); JB7K-TG4 (C); JB7K-TG3 (D); JB7K-TG8 (E).


A
B
cpm (x10−3)
Incubation Time (h)
0
5
10
15
20
25
30


C
60
50
40
30
20
10
0
cpm (x10−3)
0
5
10
15
20
25
Incubation Time (h)


D
60
50
40
30
20
10
0
cpm (x10−3)
0
5
10
15
20
25
Incubation Time (h)

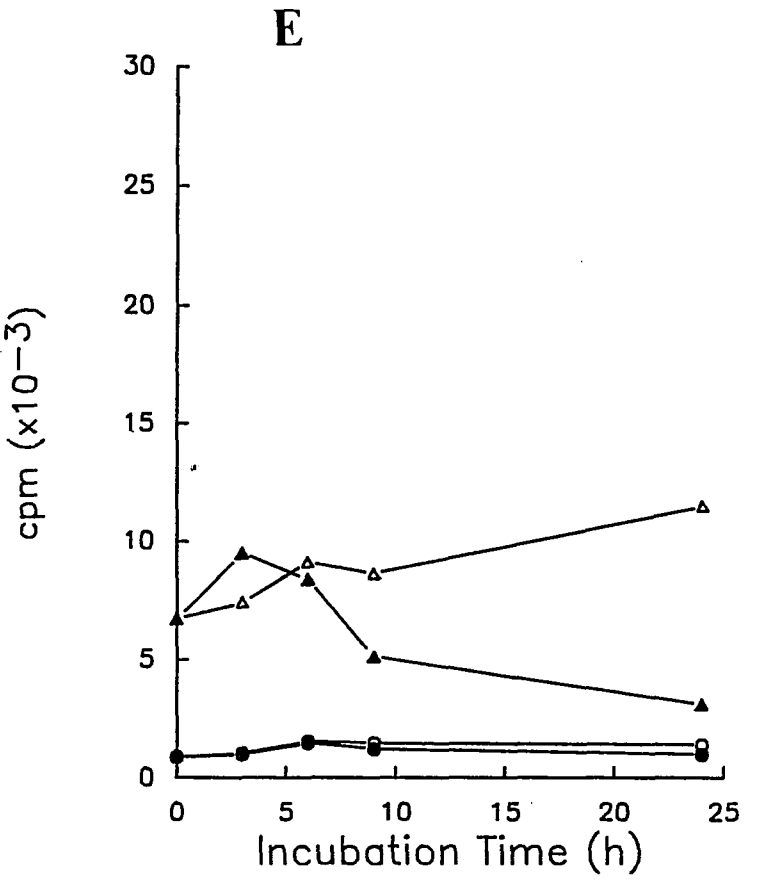

E
30
25
20
15
10
5
0
cpm (x10−3)
0
5
10
15
20
25
Incubation Time (h)

Fig. 12 Effect of Chloroquine (CQ) treatment on polyoma DNA transfection into JB7-K cell. p53A6.6 DNA was transfected by CaP co-precipitation at 33°C. The precipitate was kept on monolayer cell for different times with or without addition of 100 μM of CQ. Viral DNA was analyzed by Southern hybridization with nick-translated p53A6.6 DNA as a probe. (A): Transfection for 4 h without CQ (lanes 1 and 2); (B): transfection for 4 h with CQ for 2 h (lanes 3 and 4); (C): transfection for 2 h refed with CQ for 2 h (lanes 5 and 6); (D): transfection for 4 h with CQ for 4 h (lane 7 and 8).

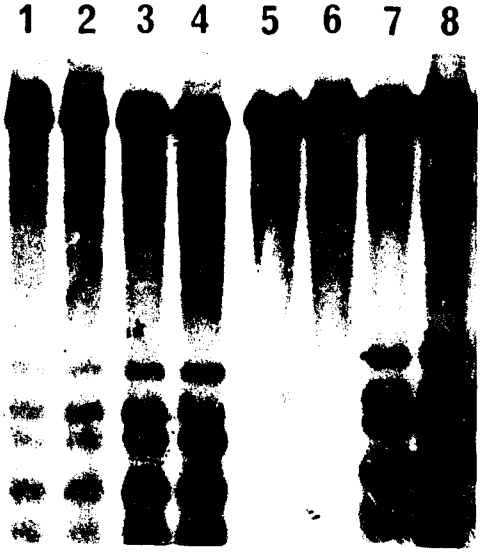
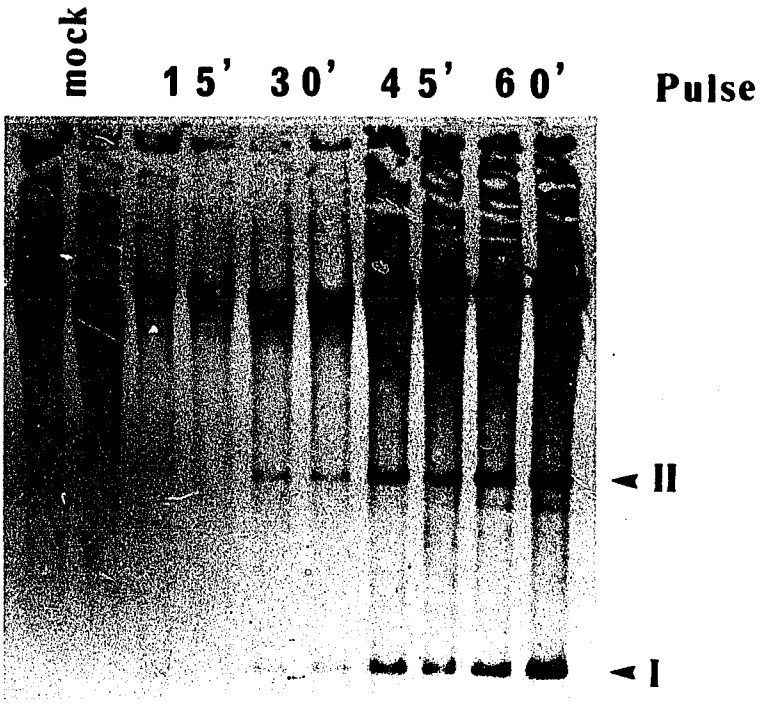

1 2 3 4 5 6 7 8

Fig. 13. Polyoma DNA replication rate in JB7-K cells. P53A6.6 (10 μg) was transfected into JB7-K ($8x10^5$ cell/60 mm dish) by modified CaP co-precipitation for 4 h at 33°C. The cultures were treated with Amphotericin B (5 μg/ml) for 4 h and CQ (100 mM) for 2 h during the transfection. 10 μg of calf thymus DNA was used in the mock transfection dish (lane mock). At 40 h post transfection, dishes were pulse-labeled with [^{3}H]-TdR (100 μCi/ml) in 1 ml medium for 15 min, 30 min, 45 min and 60 min as indicated following by 30 min chase with TdR (0.2 mM) and CdR (0.01 mM) in 5 ml medium. Viral DNA was recovered as described in methods. 1/3 of each sample was analyzed by 0.7% agarose gel electrophoresis for 16 h under 50 V in 1X TPE. The gel was treated with EnHance solution, dried and exposed to X-ray film for a week in panel A. The viral DNA forms I and II are indicated. Adjacent lanes correspond to duplicate transfections. The densitometric intensity of the sum of Py DNA form I and II (average of the duplicate samples) is plotted in panel B.


mock
15'
30'
45'
60'
Pulse
II
I

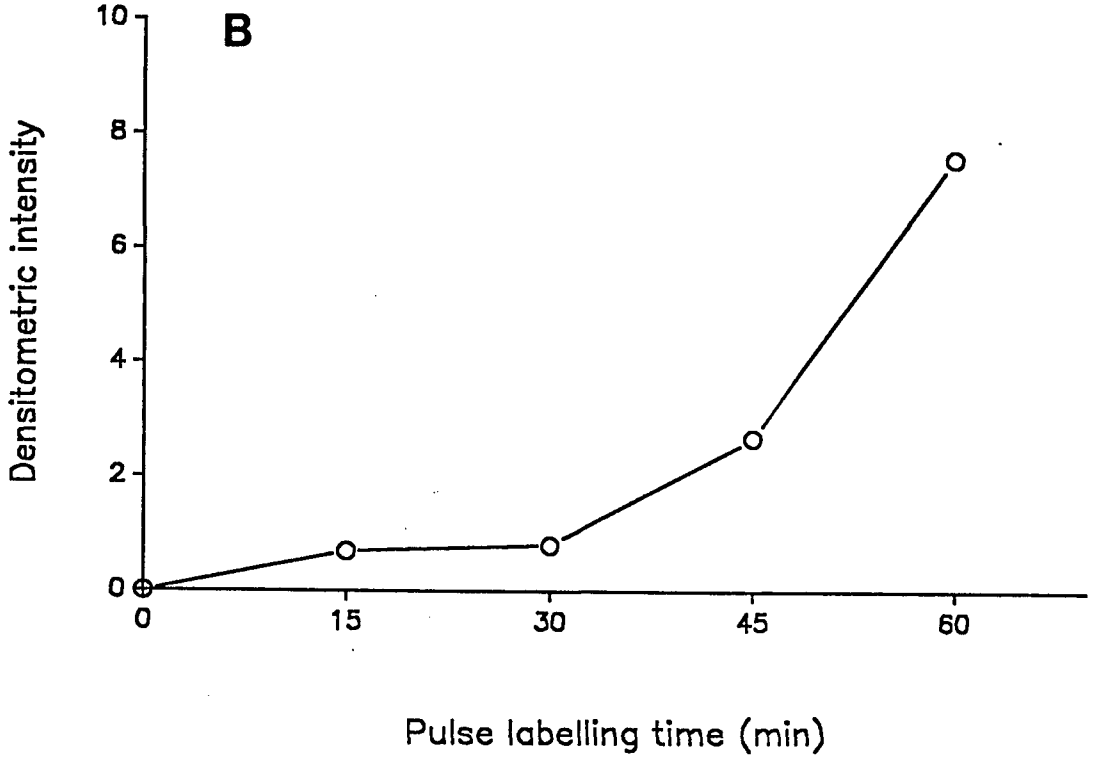
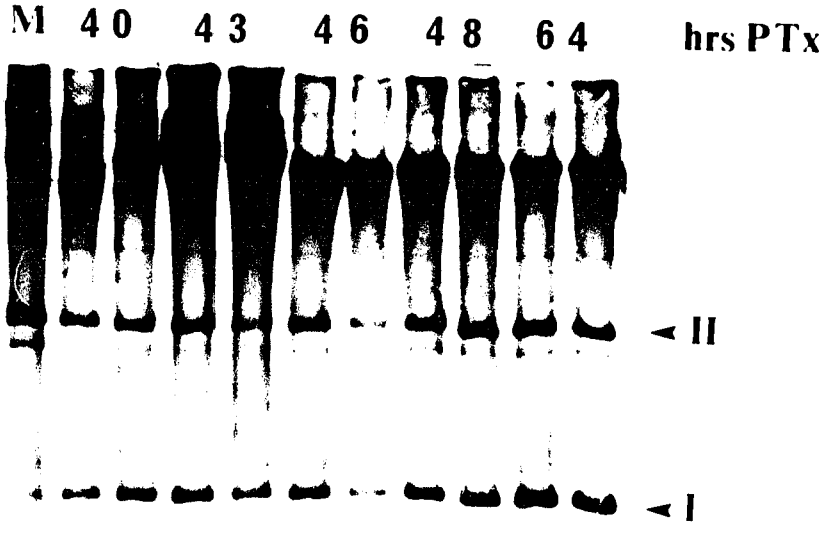

B
Densitometric intensity
10
8
6
4
2
0
0
15
30
45
60
Pulse labelling time (min)

Fig. 14. Polyoma DNA replication rates at different times post transfection. JB7-K cells were transfected with p53A6.6 DNA at 33°C and analysis was performed as described in legend to Fig. 13. All the cultures were pulse labeled with 100 μCi/ml [^{3}H]-TdR for 60 min followed by a 30 min chase at different times post transfection as indicated. Adjacent lanes correspond to duplicate transfected cultures. Densitometric intensity of the sum of Py DNA form I and form II (average of the duplicate samples) is plotted in panel B.


M 40 43 46 48 64 hrs PTx
II
I

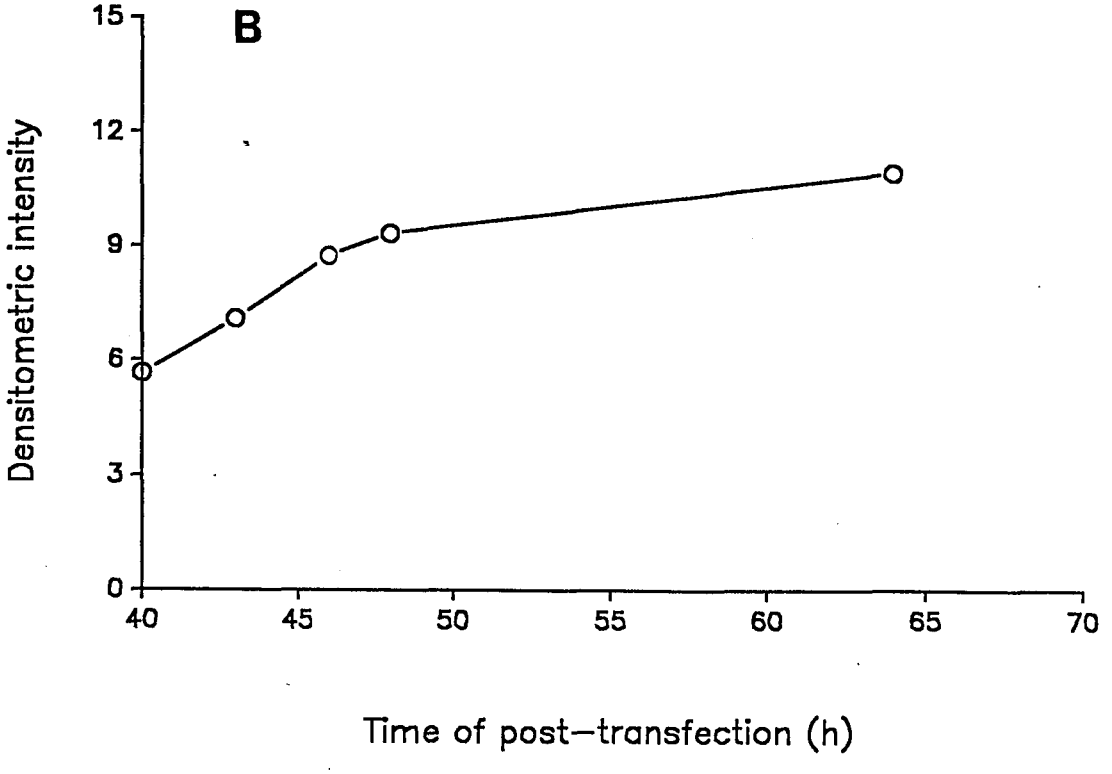
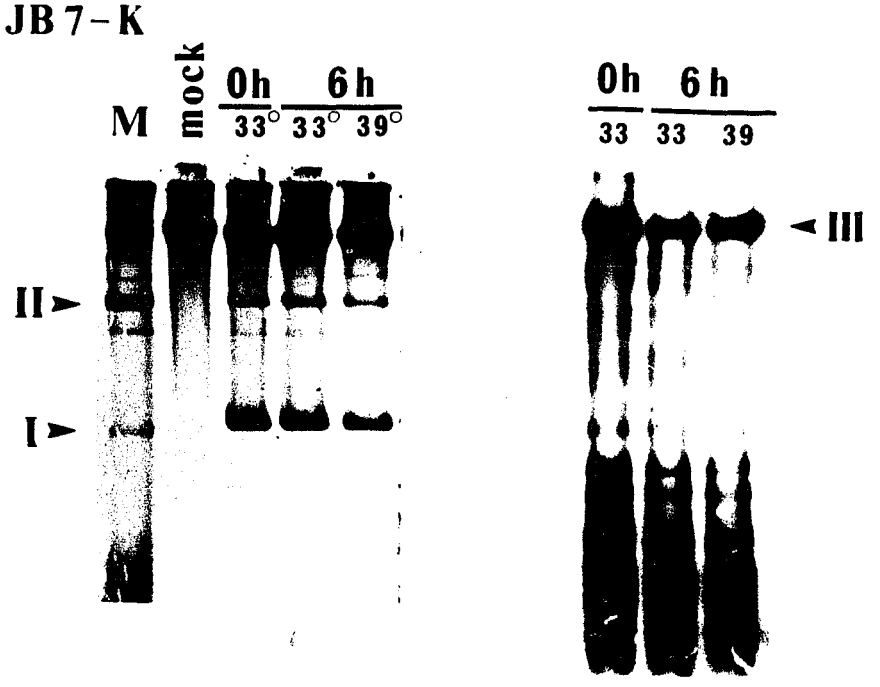

B
Densitometric intensity
15
12
9
6
3
0
40
45
50
55
60
65
70
Time of post—transfection (h)

Fig. 15. Polyoma virus DNA replication rate in JB7-K at 33°C and 39.5°C. JB7-K cells were transfected with p53A6.6 DNA at 33°C as described in legend to Fig. 13. At 40 h post transfection (T=0), half of the cultures were shifted to 39.5°C. Cultures were pulse labeled with [^{3}H]-TdR (100 μCi/ml) for 60 min at T=0 at 33°C and T=6 at both 33°C and 39.5°C and chased for 30 min. The mock transfection dishes were labeled at T=0. In part A, 1/3 of each sample was electrophoresed on 0.7% agarose gel under 50 v for 16 h, dried and autoradiographed for 2 days. Lane M corresponds to marker DNA. In part B, 1/10 aliquot of the same sample was digested with *Dpn* I and *Sal* I, and analyzed by the Southern procedure on a 1% agarose gel and hybridization with a p53A6.6 DNA probe. The arrow corresponds to replicated DNA (Dpn I resistant) form III linearized by *Sal* I. In panel C, the densitometric intensity of the sum of Py DNA form I and form II of panel A is plotted.


JB 7-K
M
mock
0h
6h
33°
33°
39°
II
I
0h
6h
33
33
39
III

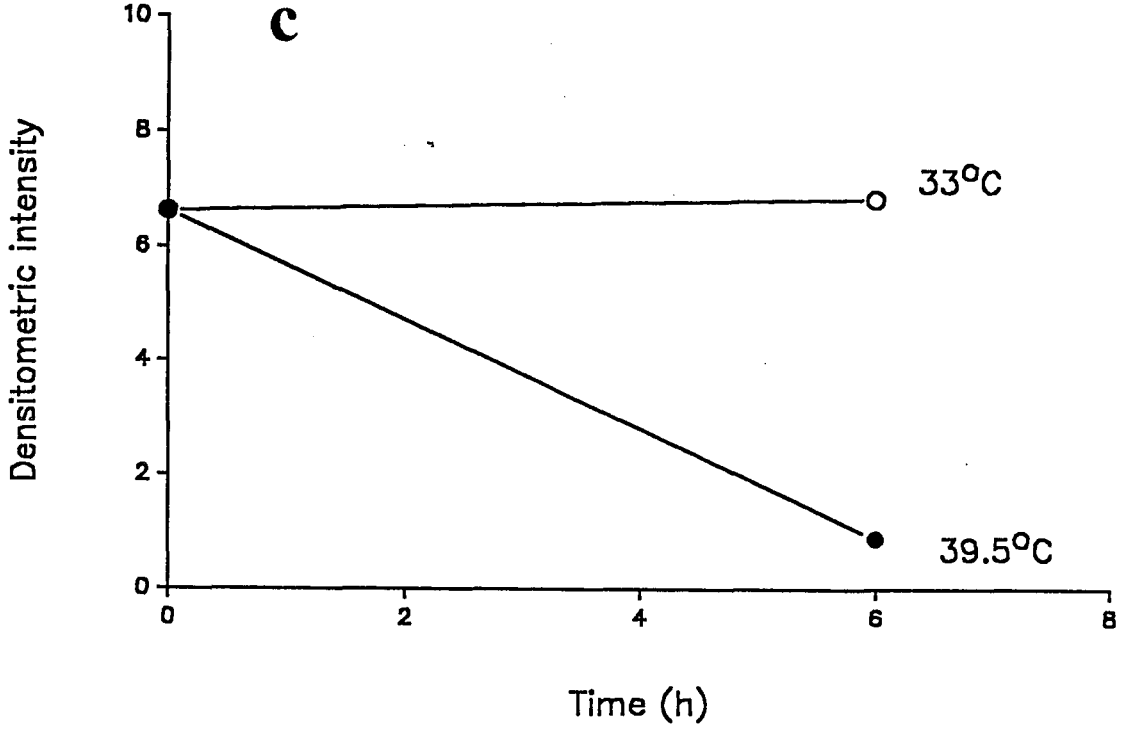

c
Densitometric intensity
10
8
6
4
2
0
0
2
4
6
8
33°C
39.5°C
Time (h)

Fig.16. Polyoma DNA replication in wild type CHO-K1 cells. p53A6.6 DNA was transfected into $3x10^5$ cells per 60 mm dish. Cultures were shifted and pulse labeled as described in the legend to Fig.15. Mock transfection sample pulse labeled at 40 h post transfection at 33°C equivalent to T=0 (lanes 1 and 2). Transfected samples pulse labeled at T=0 h (lanes 3 and 4); T=6 h at 33°C (lanes 5 and 6); T=6 h at 39.5°C (lanes 7 and 8); T=16 h at 33°C (lanes 9 and 10); T=16 h at 39.5°C,(lanes 11 and 12). The gel was exposed for 2.5 days.

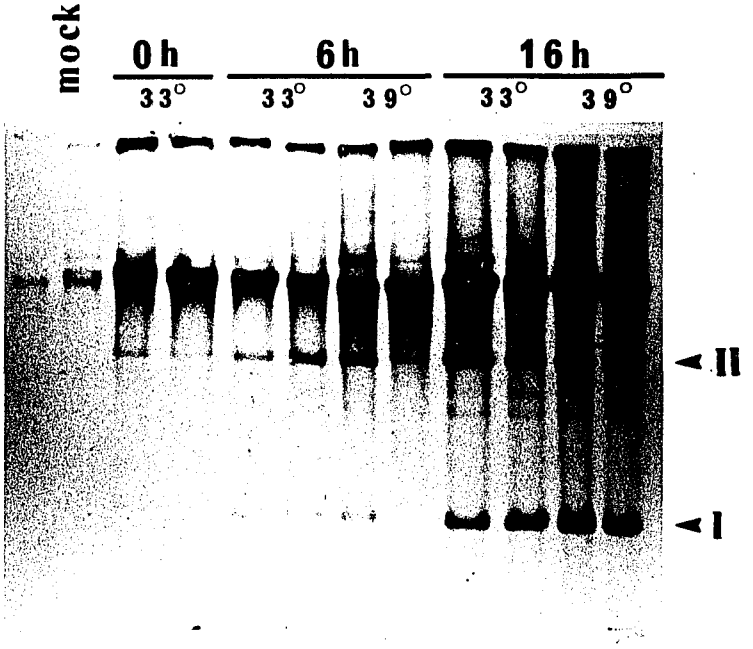

mock
0h
6h
16h
33°
33°
39°
33°
39°
II
I

Fig. 17. Pulse labeled polyoma DNA in JB7-K cell. p53A6.6 DNA was transfected into JB7-K cells as described in the legend to Fig. 15A for 24 h. Cultures were then shifted to 39.5°C for 16 h or remained at 33°C. The transfected cultures were pulse-labeled at 40 h post transfection for 45 min with chase for 2 h (lanes 4, 5, 8 and 9) and without chase (lanes 2, 3, 6, and 7) as described in the Methods. DNA samples prepared from 33°C cultures are in lanes 1, 2, 3, 4 and 5, and from cultures shifted to 39.5°C are in lanes 6, 7, 8 and 9. Lane 1 is a mock transfection sample.

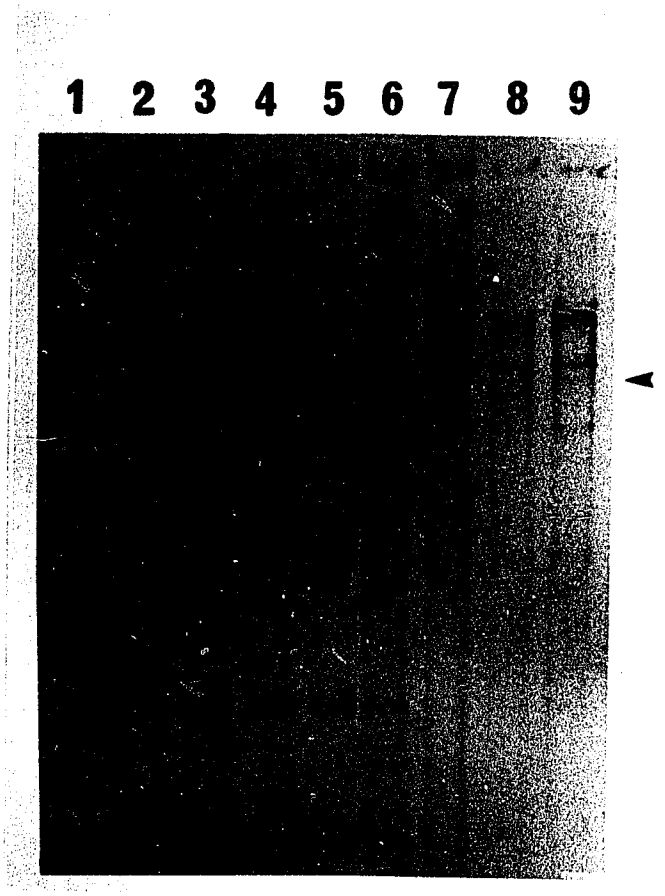

1 2 3 4 5 6 7 8 9

Fig. 18. Southern analysis of transfected p53A6.6 DNA in JB7-K cell. Aliquots of DNA samples used in Fig. 17 (lanes 2-9) were analyzed by 0.7% agarose gel electrophoresis under 40 V for 16 h. The gel was stained with EtBr and the portion which is just below the form II p53A6.6 DNA was removed. The gel was transferred to Nytran membrane and hybridized with ^{32}P-labeled p53A6.6 DNA probe. The form II and form III of plasmid are indicated. Samples from pulse-labeled culture with chase are in lanes 1, 2, 3 and 4, or without chase in lanes 5, 6, 7 and 8. Samples from 33°C culture are in lanes 1,2, 5 and 6 and from 39.5°C (see Fig. 17) culture are in lanes 3, 4, 7 and 8.

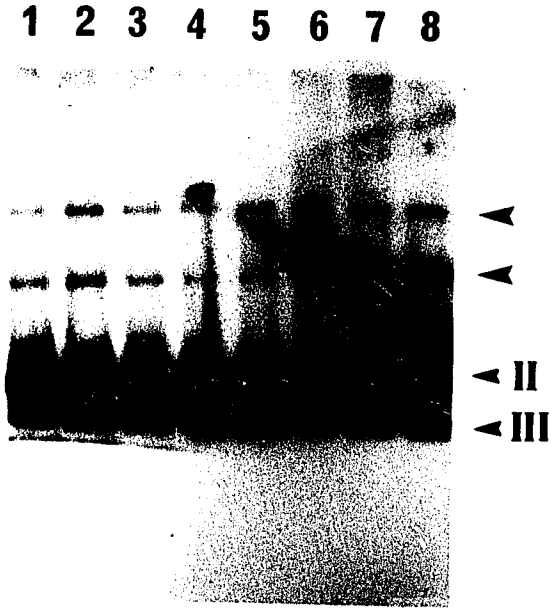

1 2 3 4 5 6 7 8
II
III

Fig. 19. Comparison of dimer-free and dimer-containing polyoma DNA replication in JB7-K cell . p53A6.6 DNAs prepared from sucrose gradient were transfected into JB7-K cell as described in legen for Fig. 17. Cultures were shifted to 39.5°C at 40 h post transfection (T=0) for 6 h or remained at 33°C. Duplicate samples were pulse-labeled for 45 min, one of the duplicate was chased for 1 h and another one is without chase. Lanes 1, 3, and 5 are the samples from dimer-containing DNA transfection cultures, lanes 2, 4, and 6 are the samples from dimer-free DNA transfection cultures. Lanes 1, 2, 3, and 4 are the samples from 33°C and lanes 5 and 6 are the samples from 39.5°C. The DNA form I, II, and III are indicated.

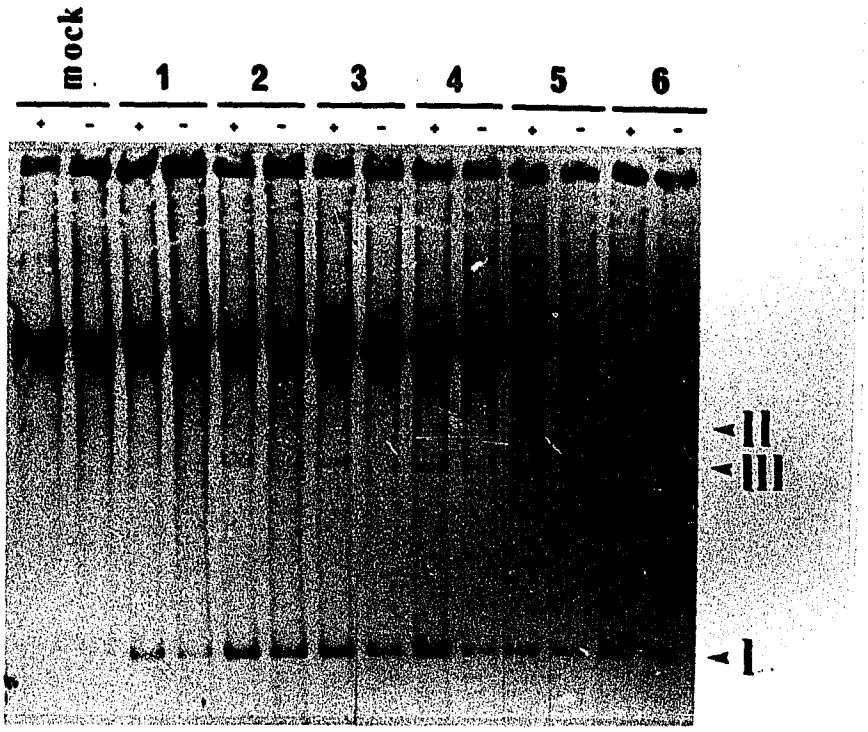

mock
1
2
3
4
5
6
+ - + - + - + - + - + -
II
III
I

Fig. 20. Southern analysis of primary transfectant. 10 and 20 μg of high molecular weight (HMW) DNA extracted from primary transfectants 5A1 and 5A3 were digested with EcoR I and analyzed with Southern procedure using nick-translated DNA as probes. In panel A, the filter was hybridized to a gel purified *Alu* fragment (*Bam* HI- *Bam* HI) from pBlur 8 and in panel B, the filter was hybridized with pSV2gpt.

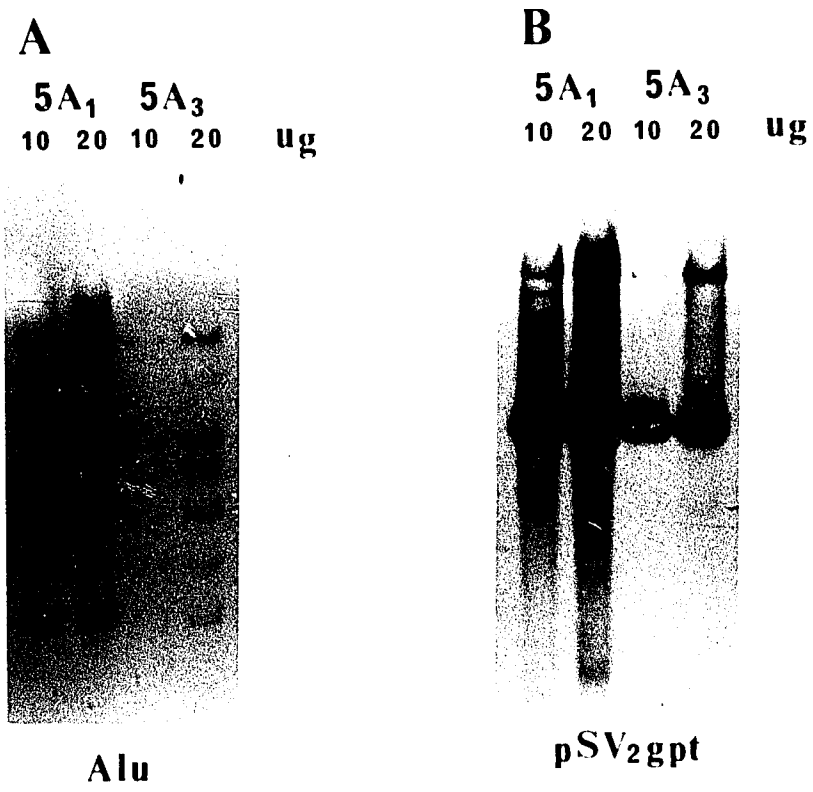

A
5A1 5A3
10 20 10 20 ug
Alu
B
5A1 5A3
10 20 10 20 ug
pSV2gpt

Fig. 21. Southern analysis of secondary transfectant. 10 μg HMW DNA extracted from JB7-K (lane 5), JB7K-TG4 (lane 6) and the secondary transfectant (lanes 1 to 4) were digested with *EcoR* I (lanes 1, 5 and 6); with *Sal* I (lane 2); with *Pst* I (lane 3) or with *Bgl* II (lane 4). Samples were electrophoresed under the same condition as legend to Fig. 21. The blot was hybridized to Alu fragment (*Bam* HI -*Bam* HI from pBlur 8) in part A and with the *gpt* fragment (*Bgl* II- *EcoR* V from pSV2gpt) in part B.

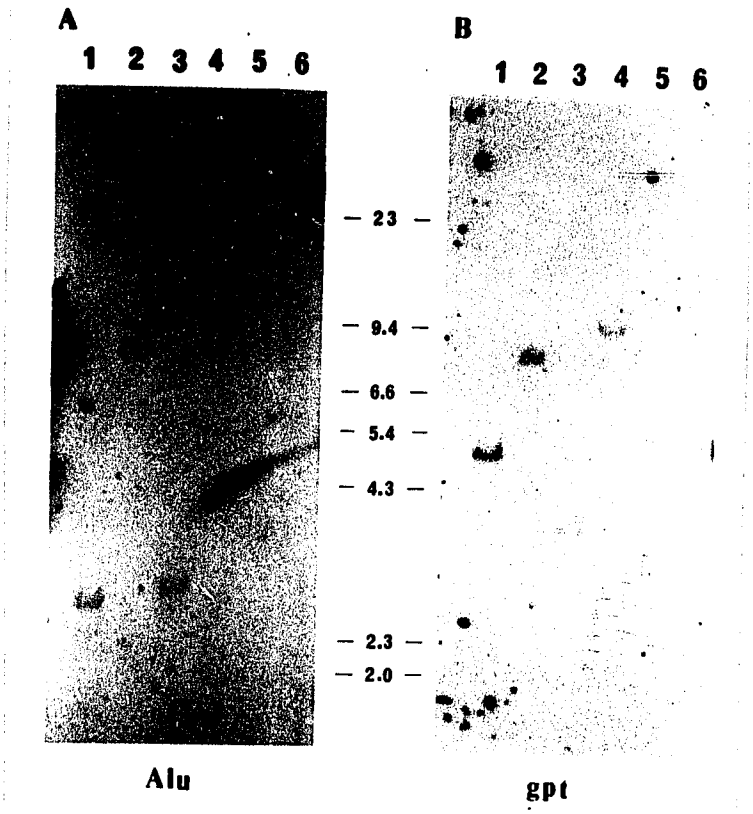

A
1 2 3 4 5 6
B
1 2 3 4 5 6
23
9.4
6.6
5.4
4.3
2.3
2.0
Alu
gpt

Fig. 22. Polyoma virus DNA replication in secondary transfectant and wild type cell. p53A6.6 DNA was transfected into 2°5D and wild type K1 cell as described in the Methods. The analysis was performed as described in the legend to Fig. 5. Samples from CHO-K1 cell in panel A and samples from 2°5D cell in panel B. Lanes 1 and 2 (2 days at 33°C); Lanes 3 and 4 (1 day at 33°C plus 1 day at 39.5°C); Lanes 5 and 6 (2 days at 39.5°C). The arrow indicates the position for linearized p53A6.6 DNA.

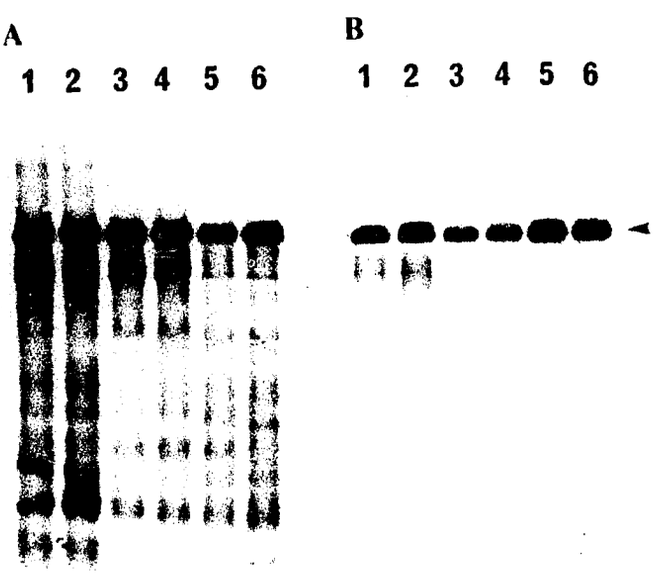

A
1 2 3 4 5 6
B
1 2 3 4 5 6

Fig. 23. Southern analysis of back selected cells. HMW DNA extracted from JB7-K (lanes 3 and 4); primary transfectant 5A1 (lanes 1 and 2); back selection cell B6 (lanes 5 and 6) and B10 (lanes 7 and 8) were digested with *EcoR* I (lanes 1, 3, 5 and 7) or *Pst* I (lanes 2, 4, 6 and 8), and electrophoresed on a 0.8% agarose gel under 40 v for 18 h. The gel was transferred and hybridized with gel purified Alu fragment (*Bam* HI-*Bam* HI from pBlur 8) in panel A; with gel purified *gpt* fragment (*Bgl* II- *Bam* HI from pSV2gpt) in panel B.

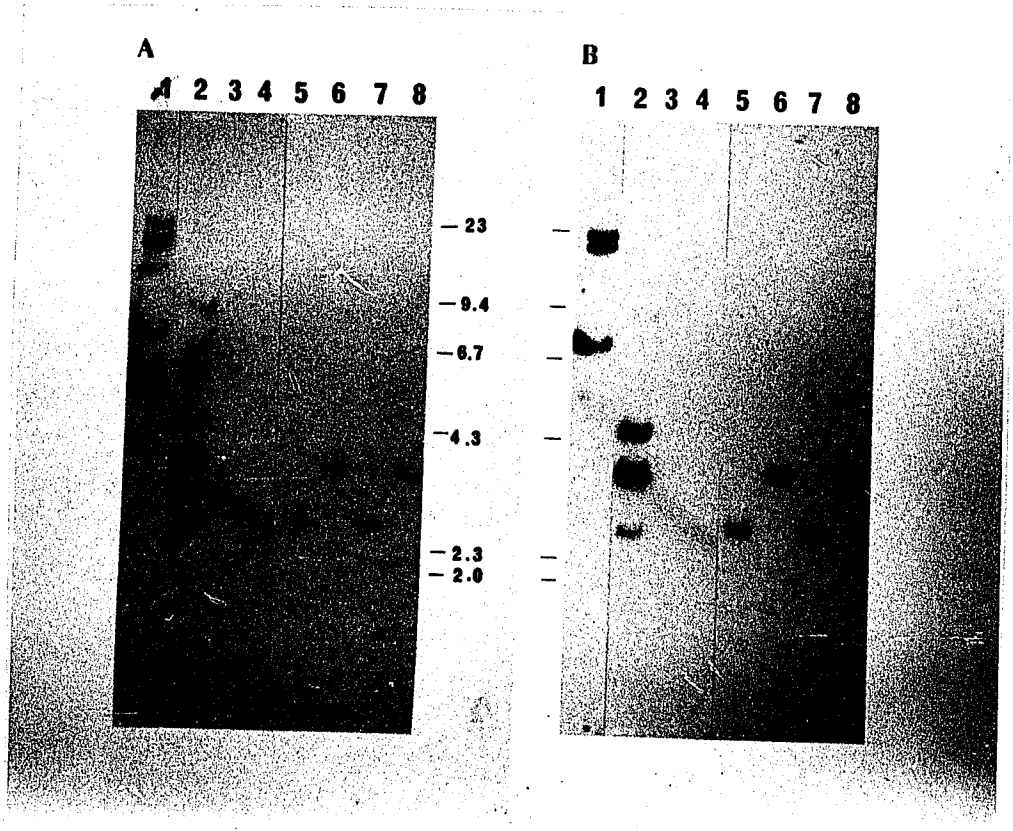

A
1 2 3 4 5 6 7 8
23
9.4
6.7
4.3
2.3
2.0
B
1 2 3 4 5 6 7 8

Fig. 24. Restriction mapping of pSD8. pSD8 DNA digested with different restriction enzyme and electrophoresed on a 1% agarose gel under 40 V for 10 h. λ*Hind* III DNA (lanes 1 and 16), pSD8 uncut DNA (lane 2), pSD8 digested with *EcoR* I (lane 3), *Hind* III (lane 4), *Sal* I (lane 5), *Pst* I (lane 6) *Pvu* II (lane 7), *Ban* III (lane 8), *Bgl* I (lane 9), *EcoR* I+ *Hind* III (lane 10), *EcoR* I + *Bam* HI (lane 11), *Pst* I *Sal* I (lane 12), *Hind* III + *Sal* I (lane 13), *Bgl* II + *Sal* I (lane 14), *Hind* III + *Pst* I (lane 15).

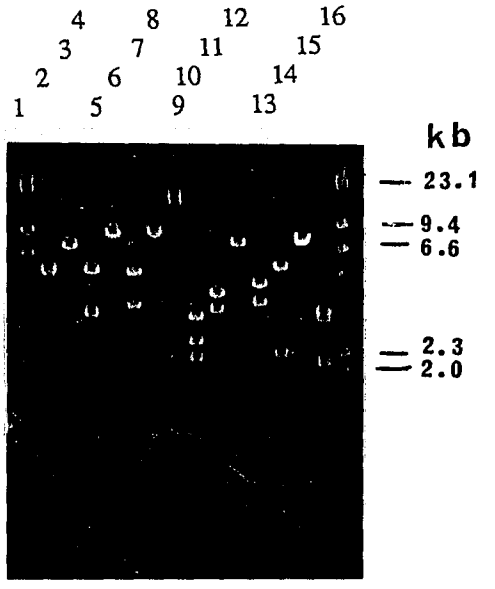

4 8 12 16
3 7 11 15
2 6 10 14
1 5 9 13
kb
23.1
9.4
6.6
2.3
2.0

Fig. 25. High resolution NuSiev agarose gel. pPH1 DNA was digested with restriction enzyme and analyzed in 4% NuSiev agarose gel. DNA size marker (1 kb ladder) in lane 1; pPH1 DNA digested with *EcoR* I in lanes 2 and 3; with *EcoR* I+*Hind* III in lane 4; with *Pst* I+ *Hind* III in lane 5.

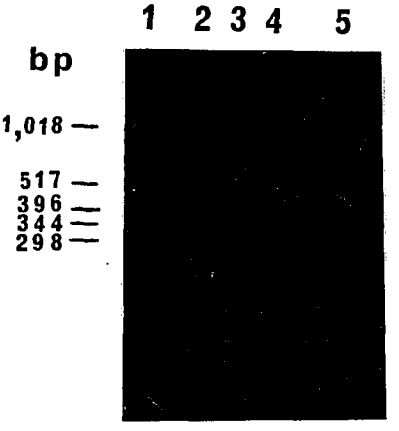

1 2 3 4 5
bp
1,018
517
396
344
298

Fig. 26. Restriction map of rescued plasmid pSD8. RI: *EcoR* I, H: *Hind* III, P: *Pst* I.

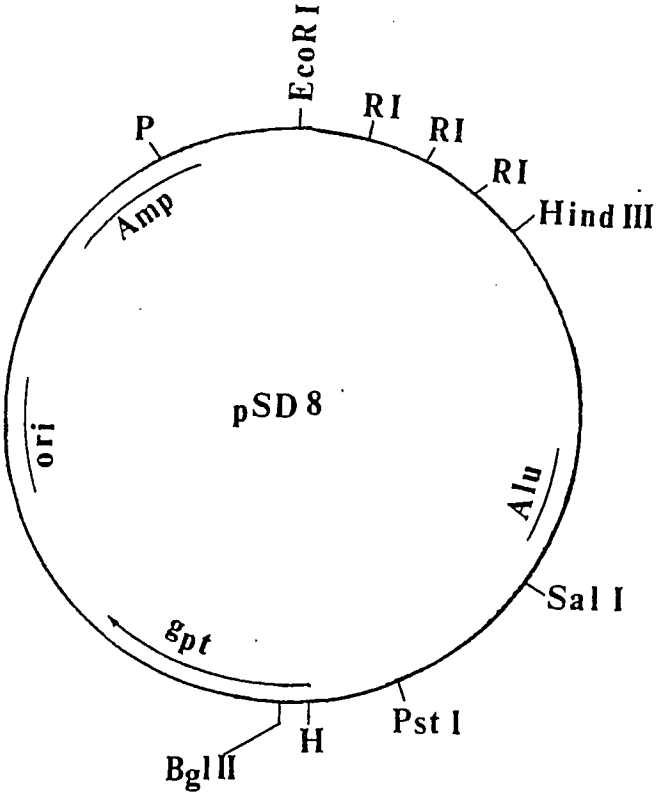

EcoR I
RI
RI
RI
Hind III
Sal I
Pst I
H
Bgl II
P
Amp
ori
pSD 8
Alu
gpt

Fig. 27. Southern analysis for α-repetitive DNA. Ten micrograms of cellular DNA from JB7-K (lane 1), primary transfectant 5A1(lane 2), secondary transfectant (lanes 3 and 4) and human fibroblast HS74 (lane 5) were digested with *Bgl* II and analyzed by Southern percesure. Filter A was hybridized with EE.29 DNA fragment, filter B was hybridized with EE.35 DNA fragment, filter C was hybridized with cloned α-repetitive DNA αpE1. The filters were exposued for 7 h. A longer exposure of panel C is shown in panel D.

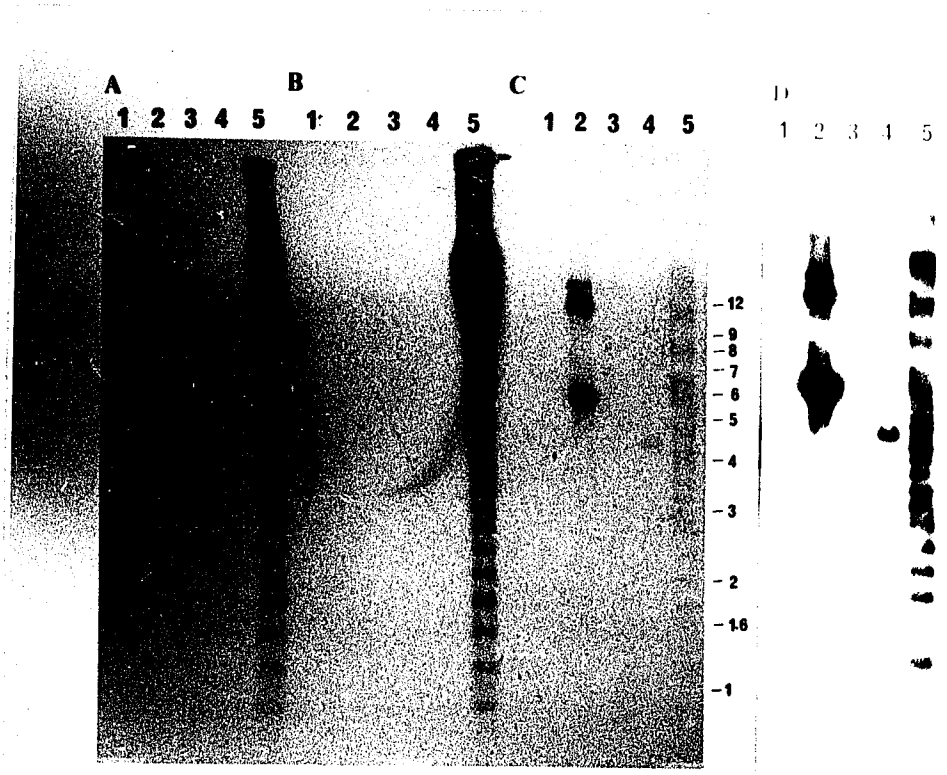

A
B
C
D
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
12
9
8
7
6
5
4
3
2
1.6
1

Fig. 28. Preliminary mapping of recombinant phage DNA. DNA was digested with *Sal* I (lane 1) , with *Sal* I +*EcoR* I (lane 2), with *Sal* I +*Bam* HI (lane 3). markers were λ/EMBL3 digested with *Bam* HI + *EcoR* I (lane 4), purified left arm and right arm (lane 5), DNA size marker (lane 6). The size of fragments result from *Sal* I and *Sal* I + *EcoR* I digestion are indicated.

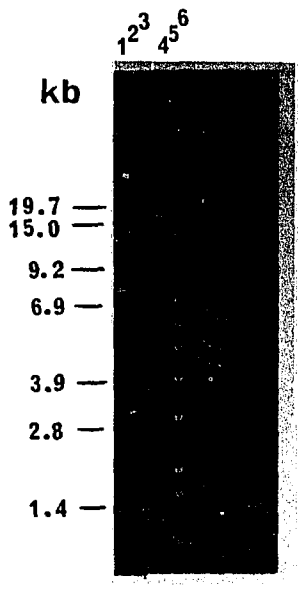

1 2 3 4 5 6
kb
19.7
15.0
9.2
6.9
3.9
2.8
1.4

Fig. 29. Restriction mapping of recombinant phage λa14. The λa14 DNA were double digested with *Sal* I plus different enzymes: +*EcoR* I(1), +*Hind* III (2), +*Ava* I (3), +*Pst* I (4), +*Xho* I (5), +*Xba* I (6), +*Bgl* II (7), +*Nae* I(8), + *Pvu* II (9). The digested DNAs were electrophoresed on three 0.6% agarose gels and transferred to Nytran. Filter A was hybridized with λ/*Hind* III DNA; filter B was hybridized with plasmid DNA ; filter C was hybridized with total human cellular DNA. The size of fragments from the *Sal* I + *EcoR* I digestion are indicated for the EtBr stain gel (panel D) and the corresponding fragments detected with the different probes.

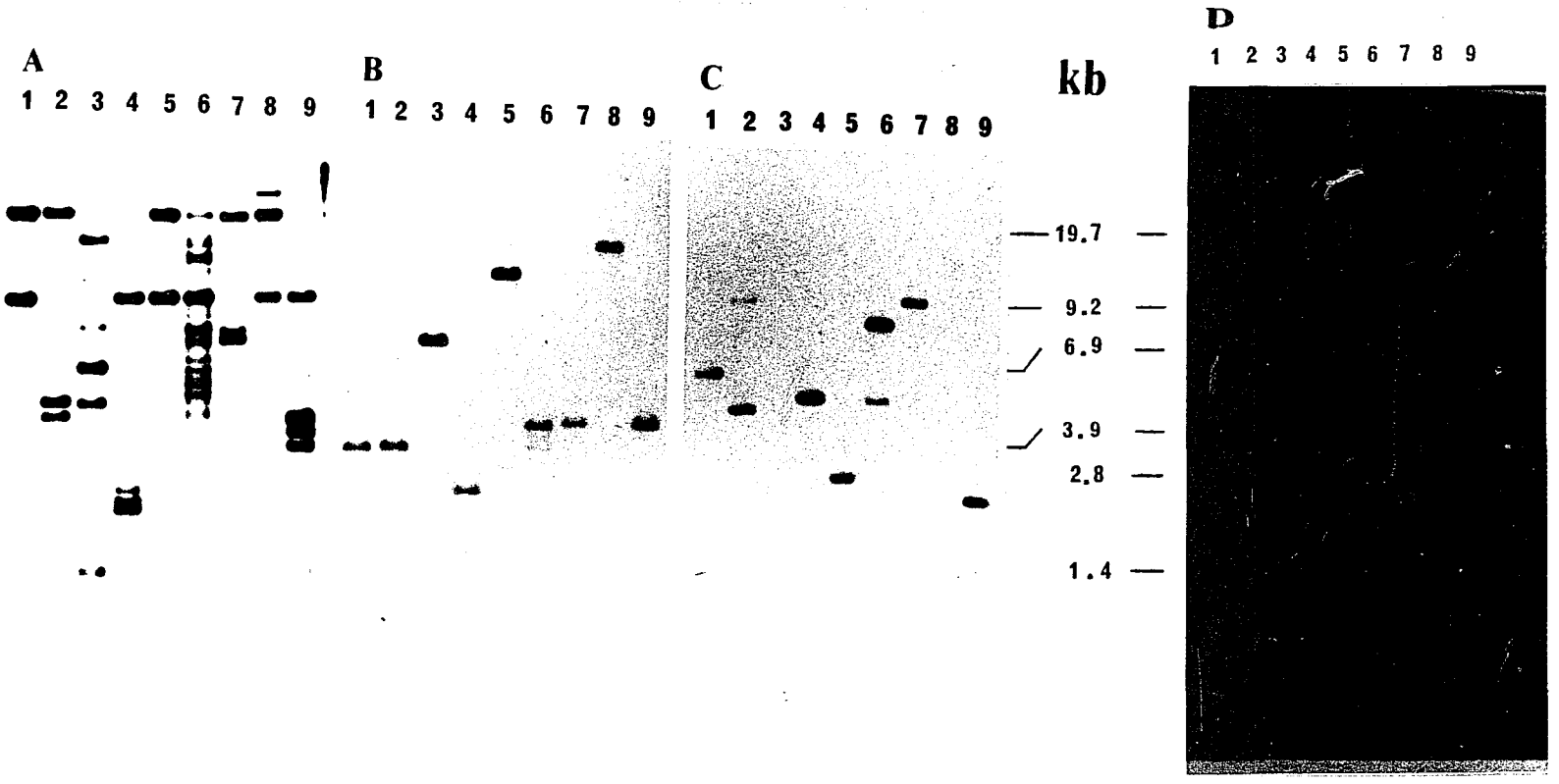

A
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9
C
1 2 3 4 5 6 7 8 9
kb
19.7
9.2
6.9
3.9
2.8
1.4
D
1 2 3 4 5 6 7 8 9

Fig. 30. Restriction map of λa14. The λ/EMBL 3 left arm is 19.3 kb and right arm is 9.2 kb. The insertion fragment is 15 kb. Pv: *Pvu* II, X: *Xho* I, H: *Hind* III, R: *EcoR* I, Bg: *Bgl* II. The fragments which hybridized to human *Alu* DNA and pBR sequence are indicated. The human unique sequence 2.8 and 1.4 kb are also indicated.

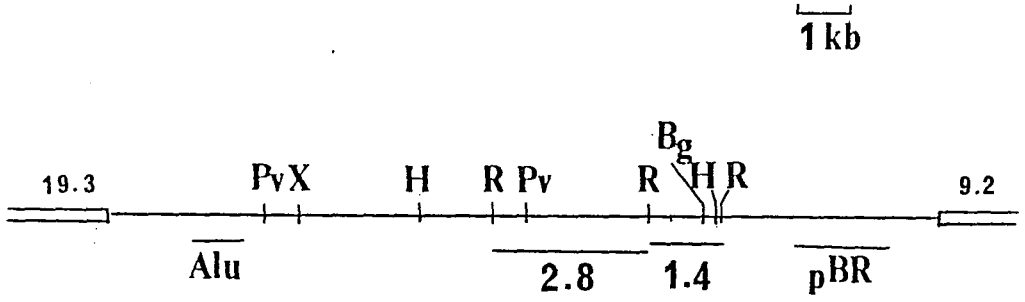

1 kb
Bg
19.3
PvX
H
R Pv
R
H R
9.2
Alu
2.8
1.4
pBR

Fig. 31. Southern analysis of human DNA unique sequence. Ten microgram of human fibroblast cellular DNA were digested with *EcoR* I and analyzed as previously. Lane a was hybridized with pS1.4 and lane b was hybridized with pS2.8 DNA

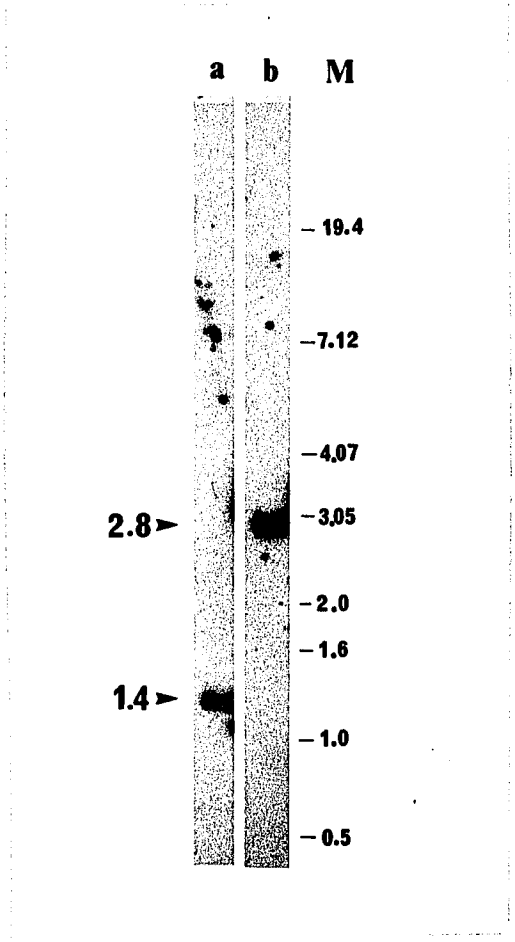
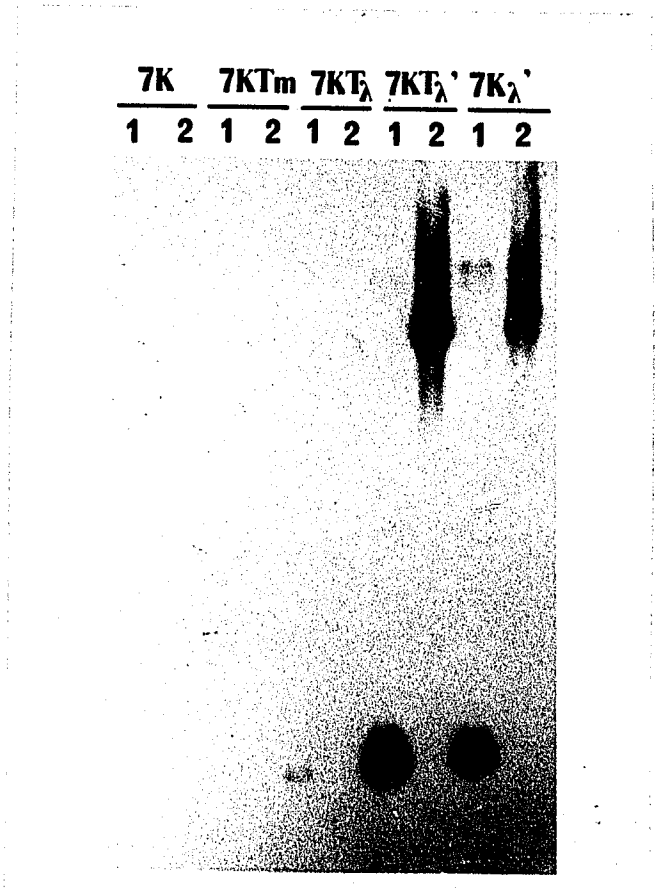

a
b
M
19.4
7.12
4.07
3.05
2.0
1.6
1.0
0.5
2.8
1.4

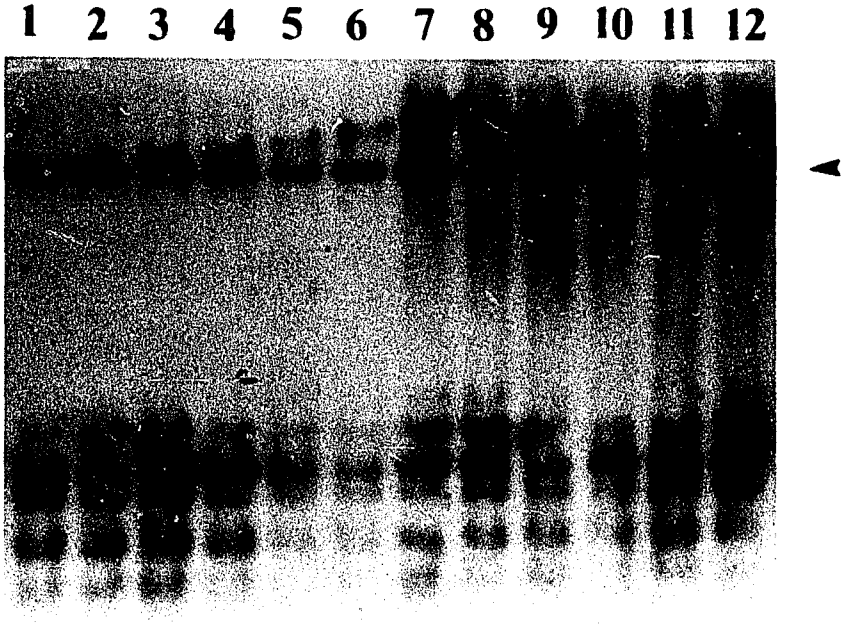
Fig. 32. Southern analysis of cellular DNA for λa14 sequences. 10 μg of HMW DNA were digested with EcoR I (lane 1) or Sal I (lane 2), analyzed with Southern procedure, and hybridized with ^{32}P-labelled gel purified 2.8 kb fragment. JB7-K (7K), Calf thymus DNA transfected JB7K-TG (7KTm), DNA from individual colonies co-transfected with λa14(7KTλ) or from pooled colonies co-transfected with λa14 (7KTλ and 7Kλ, corresponding to JB7K-TG and JB7-K recipient cell lines respectively).


7K
7KTm
7KTλ
7KTλ'
7Kλ'
1 2 1 2 1 2 1 2 1 2

Appendix I

Polyoma DNA replication in tsDNA mutants

Fig. A. Py DNA replication in CHO-K1 cells. Cells were transfected with p53A6.6 and viral DNA was extracted and quantitated by Southern analysis with nick-translated p53A6.6 DNA as described in Method. The arrow corresponds to linear unit-length p53A6.6. Lanes: 1 and 2, infected for 48 h at 33°C; 3 and 4, infected for 24 h at 33°C followed by 24 h at 39.5°C; 5 and 6, infected for 48 h at 39.5°C; 7 and 8, infected for 72 h at 33°C; 9 and 10, infected for 48 at 33°C followed by 24 h at 39.5°C; 11 and 12, infected for 24 h at 33°C followed by 48 h at 39.5°C. Adjacent lanes correspond to duplicate transfected cultures.


1 2 3 4 5 6 7 8 9 10 11 12

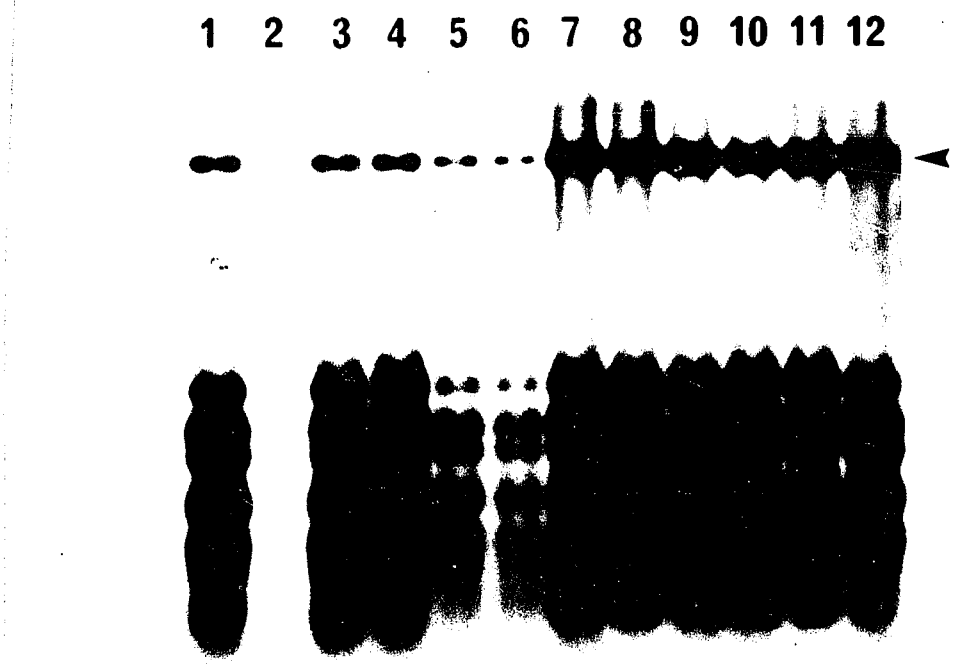


Fig. B. Py DNA replication in JB1-C.

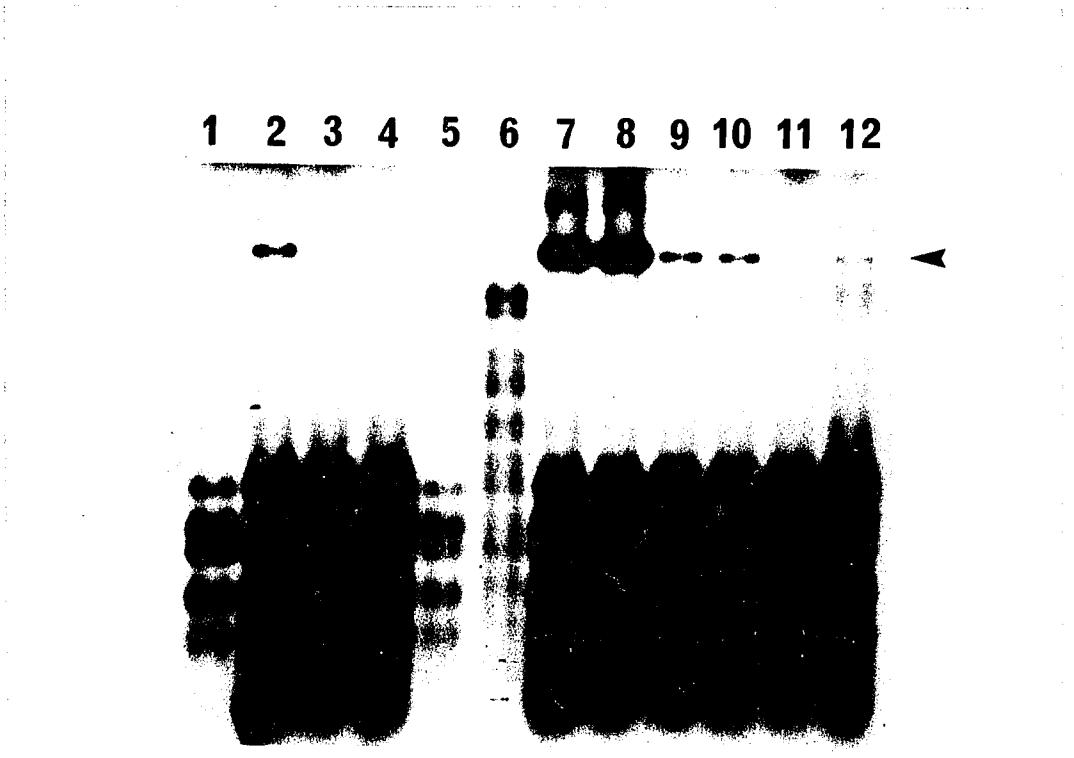


Fig. C. Py DNA replication in JB3-B.

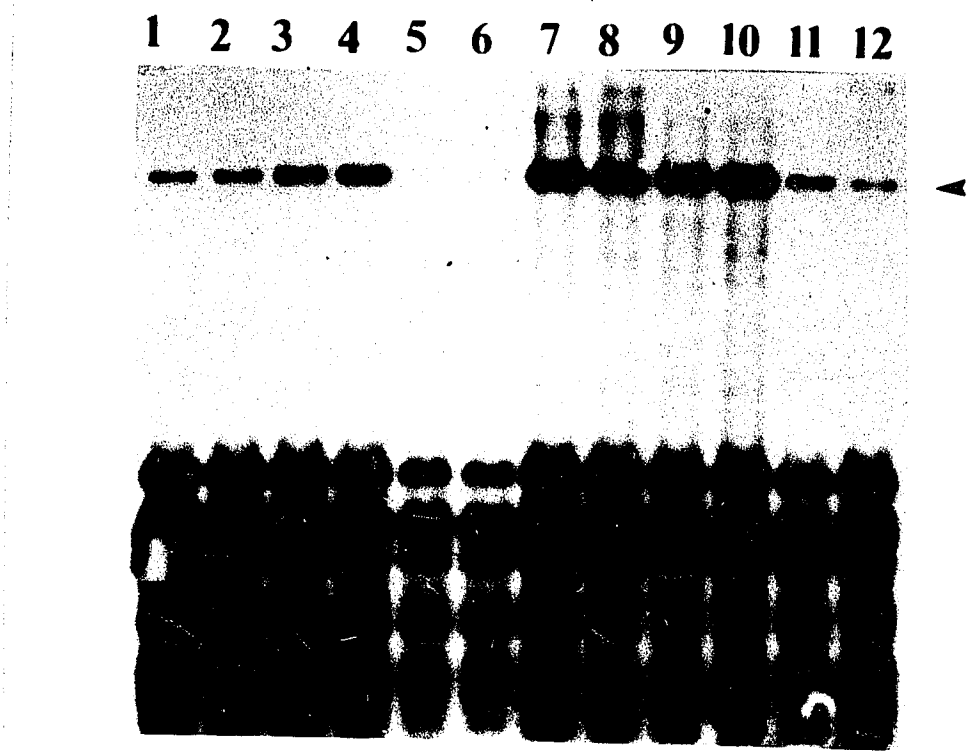


Fig. D. Py DNA replication in JB3-O.

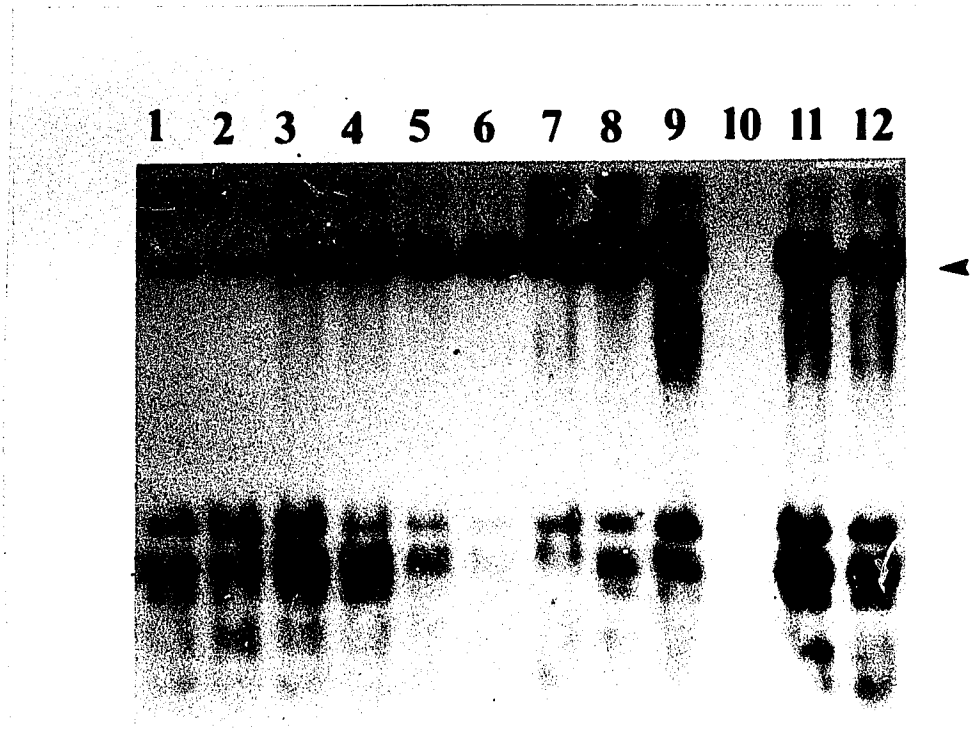


Fig. E. Py DNA replication in JB5-G

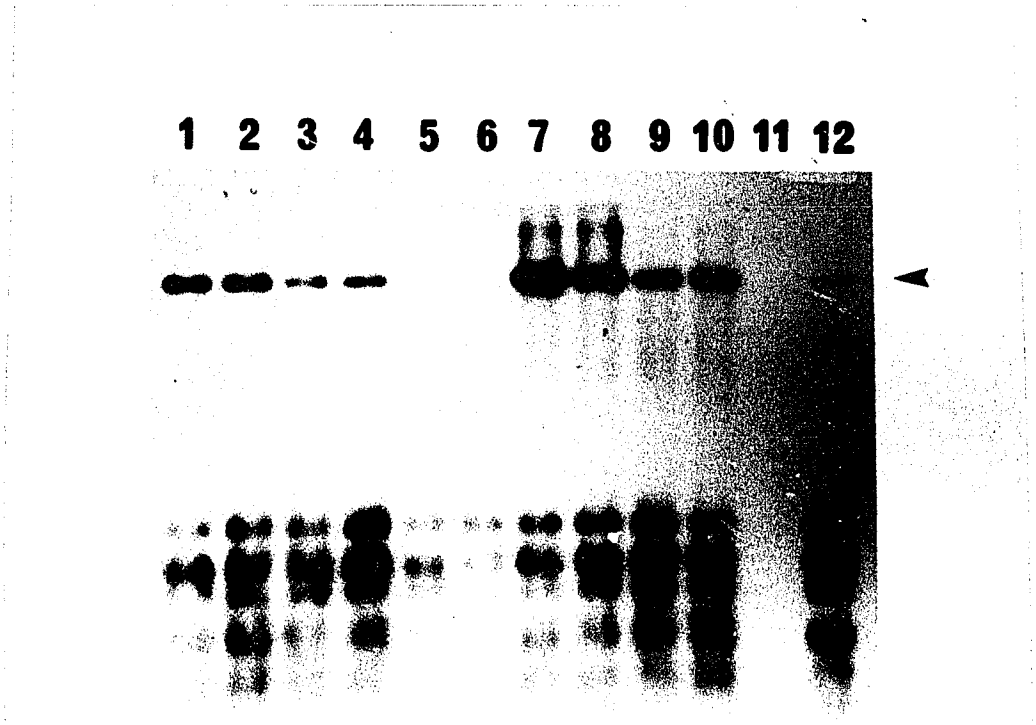


Fig. F. Py DNA replication in JB8-D.

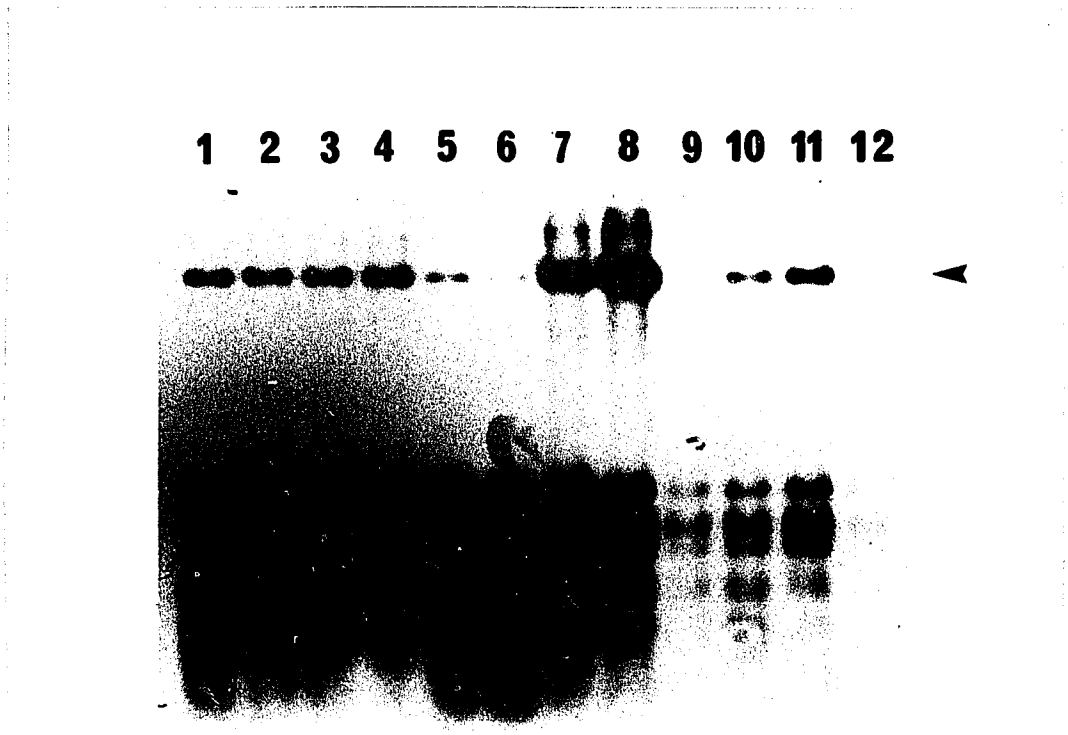


Fig. G. Py DNA replication in JB11-J.

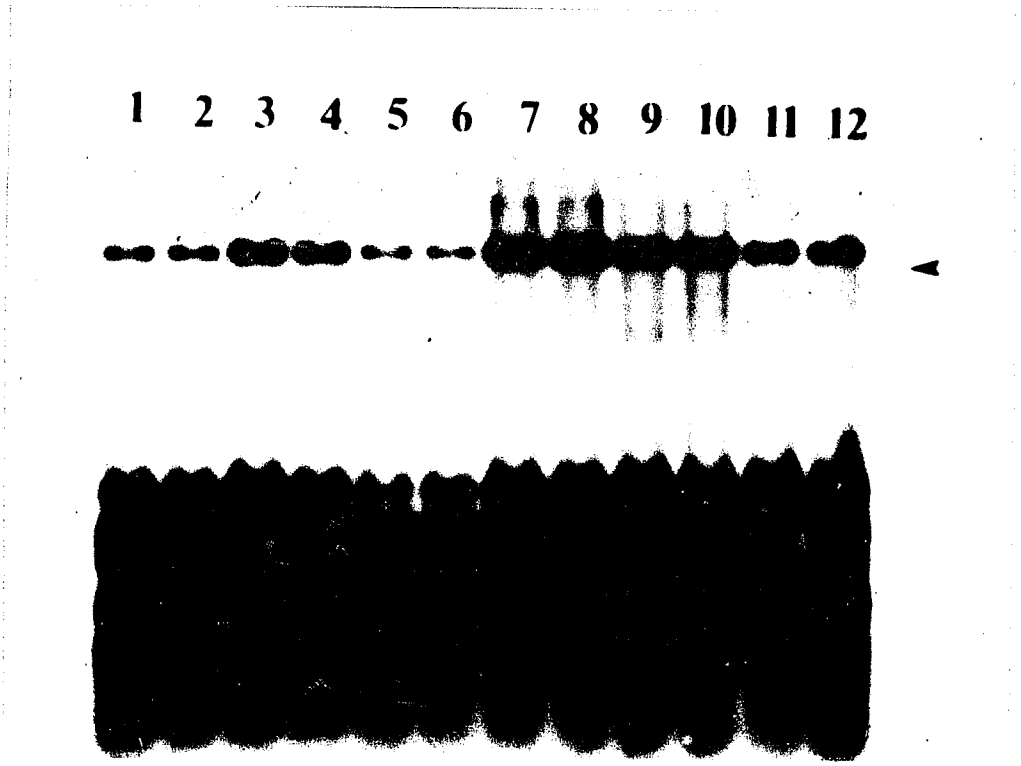


Fig. H. Py DNA replication in *ts*13A.

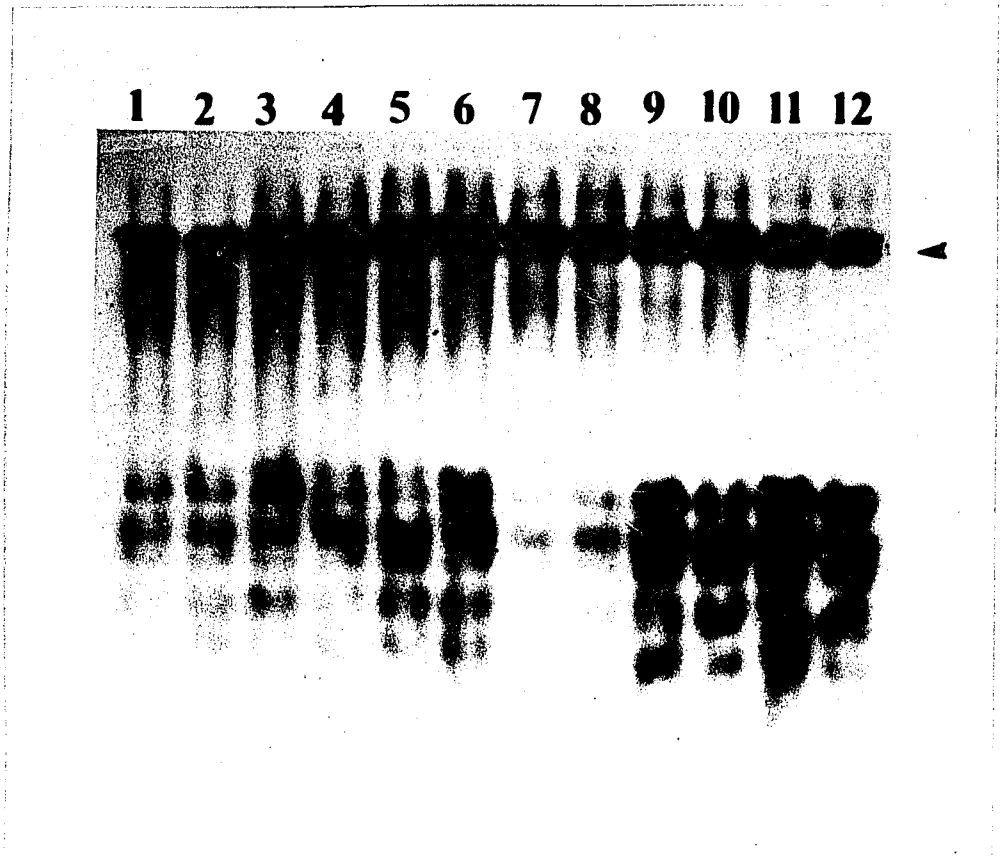


Fig. I. Py DNA replication in *ts*8C.

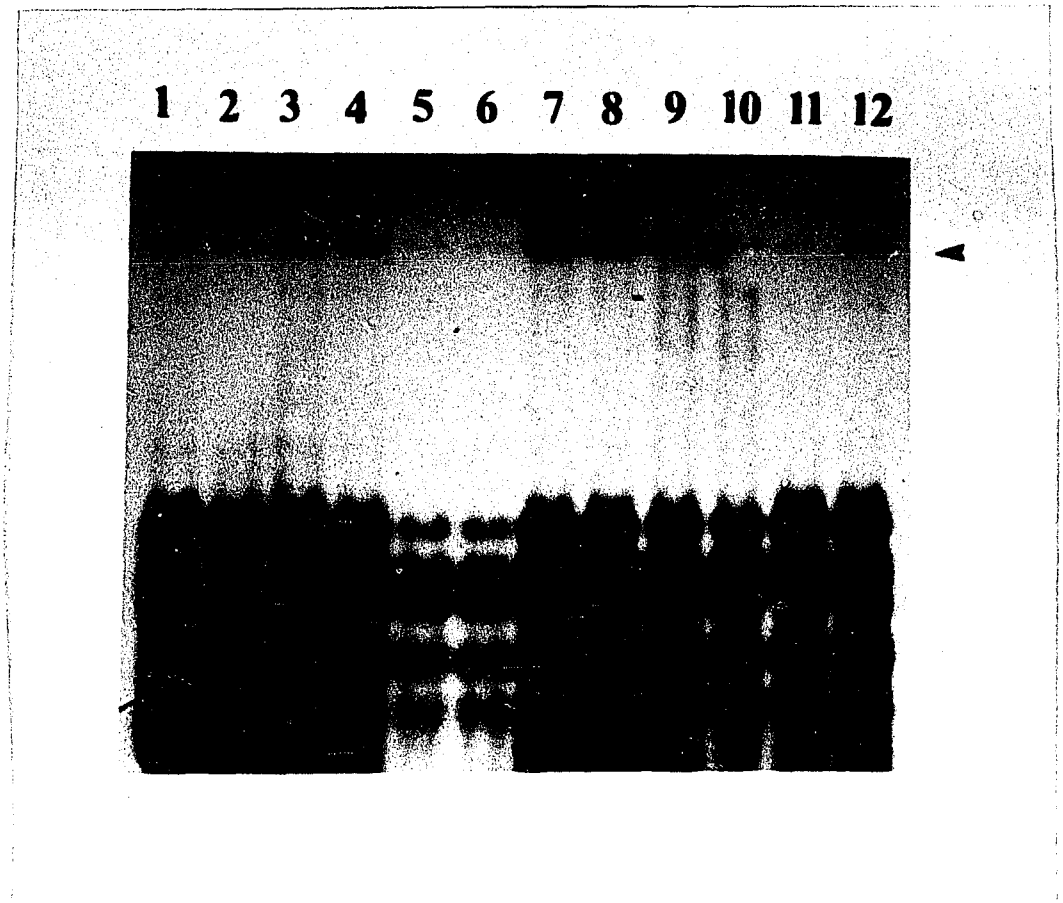


Fig. J. Py DNA replication in JB3-B R1.

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