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Transformation of human diploid fibroblasts by simian virus 40

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TRANSFORMATION OF HUMAN DIPLOID FIBROBLASTS BY SIMIAN VIRUS 40

by

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Abstract

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Simian virus 40 (SV40) has been used as a model system to study tumorigenesis. It is known that SV40 large T antigen plays an important role in cell transformation. To study the transformation mechanism in human cells, transformants (HAL and AR5) have been generated by introducing origin-defective SV40 encoding heat-labile large T antigen (SVtsA58) into human diploid fibroblasts. The transformants are temperature-sensitive (ts); they proliferate at 35°C and cease to grow at 39°C.

The temperature-dependent growth was studied by focusing on the interaction between large T antigen and antioncogenes pRb and p53. At 35°C, complexes were observed between large T and pRb or p53 while at 39°C the respective complexes were greatly reduced. Mutations of pRb and p53 have been isolated in a number of tumors and overexpression of the wild-type protein can reduce the rate of cell growth in tumor cells. Thus, inactivation of pRb and p53 by complex formation may mimic mutations in tumor cells.

Cell cycle analysis of ts transformants which were immortal and those which had an extended but limited lifespan (i.e "preimmortal" transformants) was performed. Rapid appearance of a tetraploid DNA content was observed in preimmortal but not in immortal transformants at 39°C. To elucidate the tetraploid phenomenon, SV40-transformants (pRNSVtsA58dl) containing only heat-labile large T and no small t were generated. Flow cytometry showed that tetraploid DNA content was not observed in pRNSVtsA58dl. This result demonstrated that small t may contribute to the tetraploid DNA content in preimmortals.

To further address the basis for tetraploidization , I investigated the role of CENP-E, a protein localized at the centromere of chromosomes at prometaphase and shown to be involved in mitosis. CENP-E was found to accumulate to peak level at late G2 and became phosphorylated. Mutagenesis study demonstrated four consensus sites of phosphorylation for $p34^{cdc2}$ at C-terminal CENP-E. I propose that the function of CENP-E is regulated by $p34^{cdc2}$ and that interference with the expression or phosphorylation of CENP-E may deregulate its function leading to cessation of mitosis. With cells blocked in G2/M, synthesis of cellular DNA may be continued resulting in tetraploidy in preimmortal transformants.

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Chapter 1

Introduction

1.1 Normal diploid fibroblasts can be converted into a tumorigenic state through a multiple-step process

Diploid fibroblasts were among the first cells to be successfully cultured in vitro. They have a limited life span when serial cultivated (Hayflick et al., 1961; Todaro et al., 1963). After isolation from original tissue, cells will establish primary culture and have a typical elongated morphology (Phase I). Phase II starts with subcultivation of the primary culture. It is characterized by rapid cell proliferation with subsequent subcultures and lasts from 2 to 10 months. Cells enter phase III or the terminal phase after 30 to 50 population doublings. At this stage cells show a low mitotic activity and a longer period between each subculture. Finally, cells show morphological alteration and become senescent with arrest in G1 stage of the cell cycle. During subcltivation human diploid fibroblasts (HDF) do not become immortalized spontaneously whereas rodent fibroblasts have a high immortal frequency (Neufeld et al., 1987).

Fibroblasts can produce malignant fibroblastic tumors termed fibrosarcomas which consist of 0.2% of malignant human tumors (Pories

et al., 1983). The natural ability of fibroblasts to form tumors provides researchers a system to study the mechanism of tumorigenesis in vitro. Both rodent and human fibroblasts have been used extensively because they are readily cultured and can be transformed by chemical carcinogens and a subgroup of viral and cellular oncogenes.

Experiments of the last two decades suggest that malignant transformation of normal fibroblasts is a multistep process in which cells show different transformed phenotypes of true tumor cells. These in vitro observations are in good agreement with epidemiological studies which suggest that the incidence of cancer is proportional to a multiple power of elapsed lifetime or progressive pathological changes which include anaplasia, metaplasia and neoplasia during the process of tumor formation.

In vitro tumorigenic study has been focusing on investigating the mechanism of transformation either by chemical carcinogens or cellular and viral oncogenes. These different agents result in cells with different aspects of the transformed phenotype. Namba studied transformation of human fibroblasts to infinite lifespan by using chemical and physical carcinogens (Namba et al., 1978, 1980, 1981 1985, 1988). More than 260 attempts were made to induce cells to infinite lifespan but only two immortal cell lines were obtained after repeatedly treating normal human fibroblasts either with 4-nitroquinoline 1-oxide (4-NQO) or ^{60}Co gamma rays. Besides the immortal phenotype, both cell lines show altered cell morphology, abnormal karyotype and one of them has showed diminished requirement for growth factor (Namba et al., 1984). However, the two cell lines show little anchorage-independence and form no transplantable

tumors in nude mice. These characteristics indicate that only a partially transformed phenotype has been achieved by treating normal fibroblast cells with 4-NQO and Co-60. To study the effects of retroviral oncogenes on these immortal cells, Namba et al. (1988) introduced c-Ha-*ras* or v-Ha-*ras* into the cell lines through protoplast fusion. The results showed further transformation of these immortal cells with enhanced clonability in soft agar and progressively growing tumor in athymic mice. These findings demonstrated that tumorigenicity is a multiple, cooperating process in which immortalization is induced by chemical and physical carcinogens for initiation of tumor while other agents will extend immortal phenotype to fully transformed state.

The v-*sis* , PDGF2/c-*sis* and T24 H-*ras* oncogenes were examined by Fry et al. (1988) and Hurlin et al. (1987) for their transformation roles on normal human fibroblasts. They found that foci and colonies in soft agar were formed after transfecting cells with either *sis* or H-*ras* oncogenes. However, spindle-shaped morphology is retained only in *sis*-transformed cells. The transformants of both *sis* and *ras* oncogenes undergo senescence and remain non-tumorigenic. These results further support the model of human cell transformation which involves a multiple, independent steps.

The hypothesis of tumorigenicity of human cells also applies to the transformation of rodent cells, which has been studied in considerably greater detail. Ruley (1983) and Land et al. (1983) used primary rat embryo fibroblasts to investigate the transformation roles of polyoma antigens, E1A, E1B, *myc* and H-*ras* oncogenes. The results of these

experiments indicated a cooperation between different oncogenes in tumorigenic transformation of primary cells. According to their functions, these viral and cellular oncogenes can be classified into two groups. Oncogenes of group A can immortalize primary embryo cells and include *myc*, adenovirus E1A, the E7 gene of human papillomavirus and polyoma large T antigen (Phelps, et al., 1988; Jat et al., 1987). Whereas those of group B, such as *ras*, adenovirus E1B and polyoma middle T, are responsible for alteration of morphology and growth properties of normal cells. The only oncogene which has both group A and B functions is the large T antigen of simian virus 40. Apparently these oncogenes are involved in different steps required for cellular transformation and together they can produce fully transformed rodent tumor cells.

1.2 Transformation of mammalian diploid fibroblasts by simian virus 40 and possible cellular targets for DNA tumor viruses

Animal viruses can interact with cells of different species. During interactions viruses encoded regulatory proteins alter the cellular processes to induce transformed phenotypes or promote their own replication. These activities provide us a tool to understand key pathways of cellular metabolism, differentiation and growth controls and identify important cellular regulatory proteins. Simian virus 40 is one of such viruses which has been used extensively for the above purposes. The virus has a small DNA genome and encodes a multifunctional regulatory protein termed large T antigen which controls the viral infection and cellular transformation.

The genome of SV40 is a circular double-stranded DNA with 5243 base pairs (Fiers et al., 1978; Reddy et al., 1978). Through its origin of DNA replication, the genome is almost equally divided in size to early and late genetic regions which have opposite direction for transcriptions. After infection of permissive simian cells and semipermissive human cells, the early region of the virus is transcribed immediately and its mRNA is spliced into two products which are translated to two early proteins termed large T antigen and small t antigen. Large T antigen is a phosphoprotein and is composed of 708 amino acid with an apparent molecular weight of 90-100 kD (Reddy et al., 1978). This protein is important during lytic infection of its permissive and semipermissive cells. Large T antigen binds to origin of DNA replication to serve as the DNA replication initiator to amplify the viral DNA. Through the interaction with the genomic control region, large T autoregulates its own expression and activates the viral late genes. The late region of the genome codes for three capsid proteins which build viral shells for its progeny (Tooze, 1981). Finally, the viral particles are released from infected cells with newly made viral DNAs packaged in their protein shells. However, in non-permissive mouse cells, the viral DNA is not replicated and the late genes are not expressed.

The effects on host cells after lytic infection of permissive or semipermissive cells by the virus are related to the progression of cells from a resting stage (G0) through G1 and entering DNA synthesis phase (S) of the cell cycle. To prepare the cells for viral and cellular DNA synthesis, the activities of a variety of enzymes, such as deoxypyrimidine kinases (Postel and Levine, 1976), DNA ligase and DNA polymerase

(Sambrook and Shatkin, 1969; Kit et al., 1967), are increased as well as the enzymes involved in the biosynthesis of pyrimidine deoxyribonucleotides (Tooze, 1973). Tjian et al. (1978) demonstrated that large T antigen is responsible for the induction of cellular DNA synthesis by microinjecting T antigen into quiescent monkey cells. T antigen not only induces the expression of cellular protein-encoding genes, but also stimulates the transcription of large rRNA species and some small mRNAs. The control of expression of both viral and cellular transcription is through modulating cellular general transcription factors, such as AP1, AP2 and SP1, by large T antigen.

Eddy et al. (1961) injected newborn hamsters with rhesus monkey kidney cell extracts and malignant tumors were induced in the animals. Simian virus 40 was identified as the oncogenic substance in the kidney cell extracts which induced tumors in the hamsters (Eddy et al., 1962). Although the virus is responsible for induction of tumors in hamsters, it has no tumorigenic ability in mice, humans and its natural host-rhesus monkeys (Allison et al., 1967). However, in vitro SV40 can transform rodent and human cells which are non-permissive and semipermissive for the viral infection, respectively.

The abilities of transformation of normal cells and induction of tumor in susceptible animals are correlated with a normal functioning of large T antigen. To determine the viral protein which is responsible for transformation, Kimura and Dulbecco (1972) used a temperature-sensitive mutant of SV40 T antigen to transform cells and the transformation phenotype of the cells is affected by temperature shift.

After infection of non-permissive rodent cells, stably transformed cells are generated at a higher frequency than that obtained in infection of semipermissive human cells (Tooze, 1980). Moreover, immortalization is almost 100% in SV40-transformed rodent cells whereas the transformed human cells rarely become immortalized (Gotoh et al., 1979; Huschtscha et al., 1979; Neufeld et al., 1987). Compared to their normal diploid cells, the lifespan of SV40-transformed human fibroblasts become extended about 20 more population doublings before these cells enter a stage termed crisis (Ide et al., 1984; Stein, 1985). At about 80 population doublings, SV40-transformed cells enter crisis during which cell division is balanced with cell death. This stage can last for several months before loss of the culture; immortalized cells may emerge at any time during the crisis stage. Although some researchers consider crisis as the same process as normal cellular senescence, they do behave differently at their end stage. Normal human fibroblasts enter a viable, G1 arrested senescent state while SV40-transformed cells continue to attempt to divide and die if they do not overcome senescence (Stein, 1985).

Wright et al. (1989) have postulated a two-stage model to interpret the phenomena by using an experimental system in which expression of SV-40 large T antigen is controlled through adding or removing of a steroid. In their model, normal cellular senescence and cell death in crisis stage are designated as mortality stage 1 (M1) and 2 (M2), respectively. SV40-transformed fibroblasts will bypass but not inactivate M1 and have a M2 death if immortalization does not occur in the transformants. To overcome M2 death and produce immortal cells, T antigen has to be present to

promote a cellular change to indirectly inactivate M2. Losing T antigen expression, the transformed cells will experience M1 death for both precrisis and postcrisis cells. However, the cellular change in M2 is a rare event and probably a mutation in the human genome. The cellular control of M2 acts in dominant manner, since the hybrids between normal diploid cells and immortal transformants also undergo crisis (Pereira-Smith and Smith, 1987). Therefore, immortalization requires inactivation of both copies of the gene which is responsible for M2.

The discrepancies in transformation and immortalization between rodent and human fibroblasts may reflect the different viral and cellular interactions. One such interactions is that there is no viral DNA synthesis and progeny virus production in non-permissive rodent cells, therefore no cell death is curred after viral infection. After infection of semipermissive human cells, some of the cells support viral DNA replication, release viral progeny and die. Others behave like non-permissive cells and survive the infection to give rise to transformed human cells at a lower frequency comparing to rodent cells. To increase the transformation efficiency, Small et al. (1982) showed that the highest efficiency of transformation is obtained with origin-defective SV40 which is incompetent in viral DNA replication. This result implies that the possible cell death before or after cellular transformation is at least partially responsible for the reduced transformation frequency.

The roles T antigen plays in lytic infection and tumorigenicity are closely related to its biochemical properties. T antigen can form complexes with at least six cellular proteins which include the product of the

retinoblastoma susceptibility gene (RB) (DeCaprio et al., 1988) and a RB related protein known as p107 (Dyson et al., 1989 a), p53 (McCormick and Harlow, 1980; McCormick et al.,1981; Schmieg and Simmons, 1988), DNA polymerase alpha (Gannon and Lane, 1987), a heat-shock-like protein (Sawai and Butal, 1989) and the enhancer-binding protein AP2 (Mitchell et al., 1987). In addition to the property of protein interaction, T antigen can unwind DNA (Stahl et al., 1986; Dean et al., 1987) and RNA (Scheffner et al., 1989) through a helicase activity. It also has ATPase activity and hydrolyzes ATP or dATP to unwind DNA and RNA during DNA synthesis and RNA transcription processes (Clark et al., 1981; Stahl et al., 1986).

Of these effects, the interaction of SV40 and some other DNA tumor virus proteins with anti-oncogenes of retinoblastoma susceptible gene product (pRB) and p53 are particularly important in the corresponding viral transformed cells. The interaction between them alters the normal functions of these anti-oncogenes. This effect may be equivalent to the mutational inactivation of anti-oncogenes which is observed in some tumor cells.

Retinoblastoma is manifested as sporadic and familial forms (Weinberg, 1990). Children of sporadic retinoblastoma have no family history and usually have one eye affected whereas the familial form involves both eyes and is multifocal. Knudson postulated that somatic mutations are responsible for both forms of the diseases while for familial disease only one genetic lesion is needed to convert retinal cells to malignant state since one of the alleles has already been altered in fertilized

egg (Knudson, 1971). This hypothesis is supported by Yunis and Ramsay's finding (1978). In retinoblastoma cells, they found that occasional interstitial deletions affecting the chromosomes 13q14 band which encodes the RB protein. Similar observations have also been documented in some other tumors. Altered or missing RB protein is detected in small lung carcinoma, breast carcinoma, bladder carcinomas, various sarcoma and leukemias (Weinberg, 1991). To study the RB function in cell growth control, Huang et al. (1988) and Takahashi et al. (1991) introduced normal RB gene into RB negative retinoblastoma, osteosarcoma and human bladder carcinoma cells. They found that expression of exogenous RB gene in these tumors can reduce the growth rate of tumor cells, and decrease soft agar colony formation and tumorigenicity in nude mice. These findings indicate that normal RB function is responsible for suppression of the neoplastic phenotype and loss of both normal RB genes is related to the uncontrolled cell proliferation of the tumors.

RB gene product is a 105-kD nuclear phosphoprotein with 928 amino acid (Lee et al., 1987). Besides its DNA binding ability, pRB can forms complexes with viral proteins in DNA tumor virus-transformed cells. This physical association is demonstrated for the SV40 and polyoma large T antigens (DeCaprio et al., 1988; Dyson et al., 1990), the adenovirus E1A protein (Whyte, 1989) and the human papilloma virus E7 protein (Dyson et al., 1989b). These viral oncoproteins could thus inactivate pRB functions at the protein level in the viral transformed cells. Thereby, sequestered or inactivated pRB mimics the altered or missing pRB state seen in the tumor cells. To study the transformation mechanism of polyoma virus, Larose et

al. (1991) demonstrated that the large T mutants which cannot bind to pRB are defective in producing mouse immortal cells. Mutations involved in pRB binding region of E1A and SV40 large T also lose their transformation function. These results indicate a possible mechanism for viral transformation and lend further support for Knudson's hypothesis.

Inactivation of RB gene or loss of pRB function in tumorigenic cells suggests that growth constrains of normal cells is imposed by pRB. It has been showed that mutations of pRB commonly occur at oncoprotein-binding pocket for adenovirus E1A, SV40 large T and papilloma E7 proteins and the mutant pRB proteins, thus, blocking pRB's ability to bind to viral oncoproteins (Horowitz et al., 1989; Hu et al., 1990; Huang et al. 1990). On the other hand, the viral oncoproteins share a motif which has structural similarity and is involved in binding of pRB (Kaelin et al., 1990). Moreover, this motif is important for viral transformation (Whyte et al., 1988). It is conceivable that the oncoprotein-binding pocket in pRB is normally occupied by cellular protein or proteins to control cell growth. To study the cellular counterparts of viral oncoproteins, Huang et al. (1991), Chittenden et al. (1991) and Kaelin et al. (1992) have successfully used recombinant fusion proteins containing the pRB pocket to identify a number of specifically associated cellular proteins. Among these cellular proteins, E2F-1 which can regulate cellular transcription is the best-characterized RB-binding protein. Chellappan et al. (1991) have showed that E1A protein can dissociate the RB-E2F complex and the sequence required for the dissociation is also important for transformation activity of E1A. These observations indicate that an important region for normal function of pRB is targeted by viral oncoproteins and in transformed cells

the oncoproteins can displace some cellular proteins which are captured by pRB to control the cell proliferation.

pRB exists in hypophosphorylated and hyperphosphorylated forms. The switches between the two forms occur in a cell cycle-specific manner. pRB become heavily phosphorylated in late G1 phase of the cell cycle and remains phosphorylated in S, G2 and most of M phase. Before M-G1 transition hyperposphorylated pRB is dephosphorylated and this form of pRB is found in the G0 and G1 phases of the cell cycle (DeCaprio et al., 1989; Chen et al., 1989; Mihara et al., 1989; Buchkovich et al., 1989). The correlation between the phosphorylation state of pRB and the cell cycle progression suggests that pRB may control the cell cycle through changing its phosphorylation forms. This hypothesis is supported by several observations. First, Ludlow et al. (1989) have discovered that large T antigen of SV40 preferentially associates with underphosphorylated retinoblastoma gene product in transformed cells. Second, Stein et al. (1990) studied the ability of senescent and quiescent human diploid fibroblasts to enter S phase and the state of phosphorylation of pRB after fresh serum stimulation. They have found that quiescent cells can enter S phase after serum stimulation and this transition is correlated to the changing of hypophosphorylated to hyperhosphorylated form. On the other hand, senescent cells do not enter S phase and there is no phosphorylation of pRB. Third, it is known that fusion of cells containing DNA viral oncogenes such as SV40 T antigen, E1A and E7 with either senescent or quiescent cells can induce DNA synthesis in the cell hybrid (Stein et al., 1982 and 1986). Finally, phosphorylation of pRB in late G1 causes release of transcription factors such as E2F which may up- or

down-regulate cellular proteins and permit cell cycle progression. These results suggest that pRB becomes inactivated through phosphorylation or complex formation with viral oncogenes and hypophosphorylated form of pRB is responsible for suppression of cell growth.

The cell cycle-dependent phosphorylation of pRB implies that cyclins and cyclin-dependent kinases (cdks) may be responsible for pRB phosphorylation since the expression of the cyclins and related kinase activities are regulated in a cell-cycle fashion. The level of particular cyclins such as A, B, D, or E become elevated at specific time during each cell cycle. These cyclins form complexes with cdks and determine their substrate specificities.

Among cyclin-dependent kinases, $p34^{cdc2}$ kinase, M-phase-promoting factor (MPF), is the prototypic form of the cdk family. This serine/threonine kinase was original recognized as a kinase which is essential for G1/S and G2/M transitions in fission yeast (Nurse et al., 1976). Later, Lee and Nurse (1987) isolated a human cdc2 homologue which can substitute for the yeast gene function at G1/S and G2/M transitions. The level of $p34^{cdc2}$ at the G2 and M transition is constant throughout the cell cycle, but its catalytic activity is dependent on forming complexes with cyclin B and dephosphorylation of its threonine-14 and tyrosine-15. Tyrosine-15 is phosphorylated by a tyrosine kinase called Wee1 (McGowan and Russell, 1993) and dephosphorylated by cdc25 phosphatase (Sebastian et al., 1993). Mammalian cells microinjected with antibody against $p34^{cdc2}$ kinase and a mouse cell line containing temperature sensitive cdc2 gene protein has shown that $p34^{cdc2}$ is essential

for progression through mitosis (Riabowol et al., 1989; Th'ng et al., 1990). Cells entering mitosis are characterized by chromosome condensation, reorganizing of microtubules to a mitotic spindle apparatus, disassembly of the nuclear lamina and breakdown of nuclear envelope. Among these events disassembly of the nuclear lamina is best understood process in which lamins are phosphorylated by $p34^{cdc2}$/cyclin B resulting in disassembly of lamina and nuclear envelope breakdown.

Besides its function at G2/M transition, $p34^{cdc2}$ can complex with cyclin A and this complex may be responsible for cells in progression through S phase (Furukawa et al., 1990; D'Urso et al., 1990 and Marraccino et al., 1992). Microinjection of antisense cyclin A cDNA or affinity-purified anti-cyclin antibody into G1 cells can inhibit DNA synthesis in S phase (Girard et al., 1991). Although $p34^{cdc2}$ controls cell entering mitosis in all eukaryotes and may regulate S phase functions, controls of G1 and S phase of higher eukaryotes may involve some other distinct, structurally related kinases such as cdk2, cdk4 and cdk5 (Meyerson et al., 1992). Expression of cyclin D and E in G1 phase of the cell cycle contributes to the activation of related cdks(Sherr, 1993).

At the present time it is equivocal which cdks and cyclins are responsible for pRB phosphorylation. To determine whether cdc2 kinase is responsible for pRB phosphorylation, Lees et al. (1991) and Lin et al. (1991) tested the ability of several protein kinases to phosphorylate pRB and found that only $p34^{cdc2}$ kinase phosphorylated pRB efficiently. Immune depletion of $p34^{cdc2}$ kinase from mitotic cell extracts abolishes RB kinase activity of the cell extract. By using tryptic phosphopeptide

mapping, they showed that pRB is phosphorylated at multiple threonine and serine site, and both in vivo and in vitro mapping give rise to very similar pattern. However, when Ewen et al. (1993) coexpressed cdk4 and D-type cyclins in insect cells, RB kinase activity is also generated. Moreover, introducing exogenous cyclin A, E or D with pRB into RB-negative human osteosarcoma cells can overcome cell growth arrest caused by pRB (Hinds et al., 1992; Ewen et al., 1993). Dowdy and colleagues (1993) showed that cyclin D2 and D3 bind to pRB directly and this interaction is competed by oncoproteins. These observations suggest that pRB is phosphorylated either by cdc2 or its related cdks. The mammalian G1 cyclins such as E and D may very well be responsible for specific pRB kinase activity.

In summary, retinoblastoma susceptible gene product suppresses cell proliferation in its normal hypophosphorylated form. Inactivation of pRB through hyperphosphorylation (possibly by Cdk's) and mutations or formation of complexes with viral oncogenes could render cells in controlled proliferation or uncontrolled tumorigenic state.

The other cellular protein which may play an important role in tumorigenesis is p53. This protein was originally identified as a 53kD nuclear phosphoprotein which is co-immunoprecipitated with SV40 large T antigen in the viral infected cells (Lane and Crawford, 1979). p53 was thought to be an oncogene since the level of the protein is higher in many transformed and rapidly growing cells than in stationary cells. Indeed, early data reported that p53 could produce transformed foci when rat embryo fibroblasts were co-transfected with p53 and *ras* oncogene.

In 1989, Hinds et al. discovered that all p53 used in the transformation experiments were mutant and mutation of p53 was required to produce transformed foci in the co-transfection experiments. Soon after Baker et al. (1989) demonstrated that both deletions of chromosome 17p, the region containing the gene for p53, and point mutations within the conserved region of p53 gene itself occured in two colorectal carcinomas they studied. This result was consistent with the previous studies in which 75% of colorectal carcinomas showed the short arm deletions of chromosome 17. It is now recognized that mutations of p53 gene are the most commonly observed genetic change in human tumors (Hollstein et al., 1991).

These findings suggest that normal p53 may function like pRB as a tumor suppressor in cells. This theory is supported by the studies of Baker et al. (1990) and Diller et al. (1990) who respectively transfected colorectal carcinoma and osteosarcoma cells containing mutant p53 with a construct which overexpresses wild-type p53. They found that these tumor cells have reduced colony forming efficiency and failed to incorporate thymidine into DNA of wild-type p53 transfected cells. In vitro, viral tumorigenesis studies also imply the involvement of p53 in the process of cellular transformation. In DNA viral transformed cells, p53 can form complex with viral protein such as large T antigen of SV40, E1B of adenovirus and E6 of human papillomavirus 16 which is highly associated with human anogenital cancers (Sarnow et al., 1982; Werness et al., 1990). Furthermore, Srinivasan et al. (1989) showed that mutated large T antigen which does not bind p53 is unable to transform most cells efficiently,

indicating the importance of complex formation between large T antigen of SV40 and p53.

The mechanisms of cell growth control by p53 is suggested by the observations in which the level of p53 is increased after exposure of cells to ionizing radiation and this increase is temporally correlated with G1 arrest of irradiated or DNA damaged cells (Kastan et al., 1991; Kuerbitz et al., 1992). In their studies, they showed that after gamma irradiation cells lacking endogenous p53 genes continued to progress through S phase and transfection of wild-type p53 into these cells results in G1 arrest. They also found that p53 may function in a 'dominant negative' manner because introduction of mutant p53 into cells containing endogenous wild-type p53 can abrogate G1 arrest of the cells. In addition, cells from cancer-prone patients of ataxia telangiectasia were shown to have no induced increase in p53 level after irradiation. These studies demonstrated that p53 functions as a cell-cycle-checkpoint determinant after DNA damage. Loss of this function in normal cells could increase genetic errors in their progeny and accumulate genomic changes necessary for neoplastic transformation to occur since transient G1 arrest permits cells to repair DNA damages before replication of DNA to prevent propagation of mutagenic lesions.

The biochemical studies of p53 showed that this protein functions at least in part as a transcription factor. Fields and Jang (1990) found that p53 has a transcription activating sequence by using hybrid protein which contains the NH_2-terminal 73 residues of p53 and DNA binding domain of yeast GAL4. The hybrid protein can activate the transcription of reporter gene in both yeast and mammalian cells. Then, the specific DNA-binding

ability of p53 was shown by Bargonetti et al. (1991) and Funk et al.(1992), and a genomic consensus sequence was identified. Recently, it was found that expression of two cellular proteins, *GADD* 45 and *WAF* 1, is activated by p53 (Kastan et al., 1992; El-Deiry et al., 1993). These investigations demonstrate that p53 can activate cellular genes and relay its signals to some other cellular proteins to gain its cell proliferation control.

The in vivo and in vitro studies of anti-oncogenes pRB and p53 provide clues as to how cells become tumorigenic from their normal state and the likely cellular targets that are altered by DNA tumor viruses during transformation processes. To transform cells and inactivate anti-oncogenes, large T antigen of SV40 is the only viral oncogenic protein which can form complexes with both pRB and p53. Whereas transformants of adenovirus and human papillomavirus, pRB is complexed with viral protein E1A and E7 respectively while p53 is bound to E1B and E6. Inactivation of anti-oncogenes through complex formation between the tumor suppresors and viral oncogenes mimics the mutation state of antioncogenes in tumorigenic cells. By studying of viral transformants, cellular components involved in the pathway of cell growth control may be elucidated eventually.

The next two sections of this chapter are intended to provide some basic information on the study of tetraploid DNA content observed in SV40-transformed cells and on a centromere associated protein (CENP-E) which may be directly or indirectly responsible for this tetraploid phenomenon. The reason to put these sections here is that tetraploid DNA content was observed in characterizing HDF transformants by

temperature-sensitive mutant of SV40 at nonpermissive temperature. Therefore, the study was extended to explore the possible mechanism for this observation.

1.3 DNA tetraploidy of SV40 infected or transformed cells

Infection of permissive, semipermissive or nonpermissive cells by SV40 can induce tetraploid populations (Friedrich et al., 1993). In nonpermissive cells, 10-20% of infected cells becomes tetraploid whereas 80% of infected permissive cells have tetraploid DNA content. In SV40-transformed cells, tetraploid population are also observed and these cells have the ability to proliferate (Lehman and Defendi, 1970). The induction of tetraploidy is due to two rounds of DNA synthesis without mitosis. This phenomenology implies that somehow SV40 overrides the normal cell cycle controls which are responsible for mitotic checkpoint control. To study the phenomenon, Laffin et al. (1989) demonstrated that the appearance of tetraploid DNA content was concomitant with the expression of large T antigen and p53 in precrisis cells. The level of these proteins was higher in tetraploid cells than in diploid cells. Similarly, Rinehart et al (1992) used a temperature-sensitive mutant of large T antigen to transform human endometrial stromal cells and found that tetraploid phenotype was observed in 35% of transformed clonal populations and diploid transformants continuously give rise to a tetraploid population at permissive temperature. Tetraploid DNA content can be accelerated by shifting of transformants from non-permissive to permissive temperature after transformants were incubated at 39^{o}C for

three days. These results demonstrate that the tetraploid population is correlated with the function of large T antigen.

As previous reviewed, large T antigen of SV40 can stimulate DNA synthesis in quiescent cells and induce G1 phase cells into S phase (Pipas et al.,1983; Soprano et al., 1983). The induction of cells entering S phase appears to be related to the abrogating of the blocking effects by tumor suppressors pRB and p53 through binding to large T antigen and a novel function of DNA-binding domain of the large T antigen (Dobbelstein et al., 1992). A second round of DNA synthesis without entering mitosis is dependent on a distinct function of T antigen other than those functions needed for initiating DNA synthesis for G1 phase cells (Friedrich et al., 1992). In their study, a temperature mutant of large T antigen was used to infect quiescent CV-1 cell. They found that infected cells were only induced into S phase with a G2 DNA content while the second round S phase and tetraploid DNA content were not observed at nonpermissive temperature. This finding further supports the involvement of large T antigen in inducing tetraploid DNA content. Most importantly, it indicated that the function of large T to start a second round S phase is a function distinct from those to induce G1 phase cells into S phase.

E1A protein of adenovirus can induce DNA synthesis and proliferation of quiescent primary baby rat kidney cells. Deletion analysis located the functional sites for these activities at the N-terminus and a segment of 20 amino acids further downstream of the protein (Moran and Zerler, 1988; Howe and Bayley, 1992). Deletion of the first region abolished the induction activity of DNA synthesis by E1A protein. Like the observation

in SV40-infected or -transformed cells, cells with deletion in the second region were induced into a second round DNA synthesis without entering mitosis and ended with a DNA content greater than that in G2. Furthermore, this mutant lost its binding ability to pRB and cyclin A. These findings suggest that pRB and cyclin A may involve in controlling of G2/M transition in the cell cycle.

1.4 The centromere-associated protein CENP-E and the cell cycle

Centromere associated proteins (CENP) were originally identified with autoimmune sera from patients with systemic sclerosis. Four centromere associated proteins were isolated called CENP-A, B, C and D (Earnshaw and Rothfield, 1985; Earnshaw et al., 1985; Kingwell and Rattner, 1987). CENP-A is a histone H3-like protein and CENP-B is localized to the alphoid rich region underlying the kinetochore of the centromere of chromosomes (Palmer et al., 1987; Cooke et al., 1990). Kinetochore provides spindle-microtubule binding site and locates on either side of the centromere. During mitosis both kinetochores capture spindle microtubules, which facilitate the alignment and separation of chromosomes, and at the onset of anaphase segregate with chromatid toward spindle poles. Chromosome segregation is driven by depolymerization of the microtubules or kinetochore associated motors (Pfarr et al., 1990). Since centromere associated proteins are closely related

to the kinetochore and the centromere, identification and characterization of molecular components of the kinetochore complex at centromere may help elucidate the biochemical processes occurring at the complex during chromosome segregation.

Yen et al. (1991) identified a fifth centromere associated protein, CENP-E, by developing monoclonal antibodies against chromosome scaffold proteins which are enriched for centromere/kinetochore proteins. This protein is about 300kD and has a markedly different localization through the cell cycle when compared with other centromere proteins CENP-A, B, C and D. The staining of CENP-E is not seen during interphase and appears at the centromere of chromosomes at prometaphase whereas staining of other CENPs can be observed throughout the cell cycle. Furthermore, CENP-E is redistributed from the centromere to the midplate at the onset of anaphase, and at telophase the protein is localized exclusively to the midbody of the cells. Cells can be blocked at metaphase after microinjection of the antibody against CENP-E. These findings demonstrate that CENP-E is a centromere associated protein but differs from other CENPs. Its differential localization suggests that its function is cell cycle regulated. Alteration of its function in mitosis could be responsible for different DNA content observed in SV40 transformed cells or in tumor cells.

1.5 Objectives:

Tumorigenesis involves multiple steps in which a number of cellular processes are altered to produce malignant tumor cells. To study the mechanism of tumorigenecity, transformation of in vitro cultured cells by DNA tumor viruses is widely used as a model system to mimic the changes occured in tumor cells. Although malignant tumor cells can be produced by introducing DNA tumor viruses into the cells, the biochemical functions of these viruses in the process is not clear. Among the viruses, SV40 has been used for almost four decades as an experimental system for investigation of tumorigenesis. However, the mechanism of transformation and immortalization by the virus is not well understood. In this laboratory, Small et al. (1982) demonstrated that enhanced transformation of human diploid fibroblasts (HDF) can be achieved by introduction of replication-defective (origin-defective) SV40 genome. Neufeld et al. (1987) investigated such SV40-transformed HDF and obtained immortalized derivatives.

To study the biochemical function of the large T antigen in the processes of transformation and immortalization, Dr. Ozer's laboratory generated temperature-sensitive transformants by introducing origin-defective SV40 genome encoding a heat-labile large T antigen (pSVtsA58) into human diploid fetal bone marrow fibroblasts. At permissive temperature 35°C, four of the transformants isolated from two separate experiments were studied for their growth property. These transformants designated SVtsA/HF-A, B, C and D grew well at 35°C and ceased to proliferate at the nonpermissive temperature 39°C. This finding

demonstrated that the growth of SVtsA/HF transformants is controlled by the heat-labile large T antigen which is encoded by SVtsA58 genome. After continued subculture of the transformant SVtsA/HF-A, immortalized transformants were isolated. In addition to mass-subcultured immortal SVtsA/HF-A, cloned immortal subclones AR-5 and HAL were obtained from it. The preceding work in the laboratory provides a useful experimental system in defining biochemical changes associated with transformation and immortalization.

It is well documented that large T antigen of SV40 can form complexes with antioncogenes pRB and p53. Complex formation between T antigen and antioncogenes may mimic the inactivation state of endogenous tumor suppresors in the malignant tumor cells. The growth property of tsA-transformants may be regulated by the binding of T antigen to these tumor suppressors. One might predict that complex formation would occur at 35°C and dissociation of the complexes when large T antigen becomes inactivated at 39°C. To study the mechanism of transformation and immortalization of HDF by SV40 and explore the biochemical changes in tumor cells, the following experiments will be performed:

1. Study the growth properties of the immortal transformants AR-5 and HAL by culturing them at permissive and nonpermissive temperatures.

2. Demonstrate the complex formation of large T antigen with antioncogenes pRB and p53. Compare the complexes formed at permissive and nonpermissive temperatures.

3. Use flow cytometry to analyze the DNA content of the ts transformants. Compare the results of flow cytometry for permissive and nonpermissive temperature cultured cells, or preimmortal and immortal transformants.

As I have mentioned in the introduction of the thesis, my project was extended to investigate the tetraploid phenomenon which was observed in preimmortal ts transformants after shift-up of the cells from permissive to nonpermissive temperature. The next two experiments were designed to answer the above question:

4. Construct an origin-defective SV40 genome (pSVtsAdl) which encodes a heat-labile large T antigen but no small t antigen. In temperature-dependent SV40-transformed human fibroblasts (SVtsA/HF) both large T and small t antigens are expressed. Inactivation of large T antigen at nonpermissive temperature abolishes its ability to autoregulate the expression of large T and small t antigens. Overexpressed small t antigen may act as promotor to induce the tetraploid DNA content in pre-immortalized transformants at nonpermissive. Removal of small t antigen from the transformed cells may shed light on the role of small t involved in tetraploid phenomenon.

5. Study the regulation of the CENP-E expression in the cell cycle. Since CENP-E is indispensable for the transition from metaphase to anaphase, it is important to determine CENP-E level in the SV40-transformed ts pre-immortals between permissive and nonpermissive temperature. Decreased production or inactivation of this centromere

associated protein may be responsible for the tetraploid DNA content in the pre-immortal SVtsA/HF-A transformants.

6. Study the phosphorylation states of CENP-E protein. From the primary sequence of CENP-E several consensus sites of $p34^{cdc2}$ kinase have been identified at the C-terminal. Phosphorylation of CENP-E by $p34^{cdc2}$ may regulate the function of the protein in mitosis. Different phosphorylation states of CENP-E between permissive and nonpermissive temperature may be responsible for tetraploid DNA content in the pre-immortal cells at the nonpermissive temperature.

Chapter 2

Materials and Methods

2.1 Cell Culture

All human fibroblasts cell lines were grown in DF10 or DF12 medium containing 10% Fetal Bovine Serum (Hyclone). Monolayer and suspension HeLa cells were cultured in DMEM and SMEM with 10% of FBS respectively. Antibiotics penicillin G phosphate and streptomycin sulfate (Hazelton 10,000 U/ml) were routinely added to a final concentration of 100 U/ml in the medium.

Monolayer cell lines were typically cultured in humidified incubator with 5 to 10% CO_2 at the desired temperature. Human diploid fibroblasts were cultured at 37°C. Preimmortal, immortal SV40tsA and SV40tsdl transformants were cultured and passaged at 35°C. Monolayer Hela cells were grown and passaged at 37°C while suspension HeLa cells were grown at 37°C in a roller bottle.

When cells became ~90% confluent, cells were passaged by brief treatment with trypsin-EDTA mixture (0.5 gm/L trypsin and 0.2 gm/L EDTA, Hazelton). Cells were resuspended in DF10 or DF12 medium containing 10% Fetal Bovine Serum (FBS) after removal of trypsin-EDTA mixture and then centrfuged for 5 minutes at 1000 rpm in an IEC clinical tabletop centrifuge. The supernatant was removed by aspiration and

fresh medium was used to resuspend cells followed by splitting cells to a desired number of petri dishes (usually cells were divided with a ratio of 1:3 to 1:5 at each passage). For determination of cell number, cells were diluted in phosphate buffered saline (PBS-150 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, pH7.4) and counted with Royco Tissue cell counter. For frozen storage, cells were suspended in DF10 or DF12 medium containing 10% FBS and 10% DMSO at a minimum concentration of $1x10^6$ cells/ml in Nunc Cryotubes. Cells were stored either in a -70°C freezer or liquid nitrogen.

2.2 Growth curves

AR-5 and HAL cells were seeded at 5 x 10^4 in 60 mm petri dishes at 35°C. The cells were grown at 35°C for several days before shift to 39°C. At each time point, three of the dishes from 35°C and 39°C were harvested respectively. Cells from each dish were counted separately and the average of all three dishes was used to determine the cell growth status.

2.3 Measurement of DNA synthesis by TCA-precipitation of [^{3}H]-thymidine

Cells were plated at 5 x 10^4 in 100-mm dishes and incubated at 35°C for 24 hours before shift to 39°C. At selected intervals, [^{3}H]-thymidine ([^{3}H]TdR; New England Nuclear; specific activity, 70 Ci/mmol) was added to a final concentration of 1-10 uCi/ml in 1.5uM thymidine (in DF10 medium) for 3 hours. The medium was removed and the cells were washed with PBS. The cells were lysed by the addition of 1 ml of lysis

buffer containing 0.1% sodium dodeceyl sulfate (SDS), 10 mM Tris [pH 7.5], 1mM EDTA. Samples were precipitated with 10% trichloroacetic acid (TCA), collected onto GF/A filter (Whatman, Inc., Clinfton, NJ) and dissolved with an NCS tissue solubilizer (Amershan Corp., Arlington Heights, Ill.). The filters were counted for the radioactivity in Liquifluor (New England Nuclear Corp.) by liquid scintillation spectroscopy.

2.4 Isolation of low molecular weight DNA by Hirt Extraction (Hirt, 1967)

Transfected cells in 60 mm dishes were washed three times with PBS at room temperature. 0.5 to 1 ml of Hirt lysis buffer containing 0.6% SDS, 10 mM EDTA, 10 mM Tris-HCl, pH 7.6 was added to the cells. After incubation at room temperature for 5 minutes the extracts were transferred to microcentrifuge tubes. 5 M NaCl was added to the extracts to bring the final concentration of the extracts to 1 M. The extracts were mixed gently by inverting the microfuge tubes, and incubated on ice for at least 4 hours or at 4°C for overnight. The extracts were then centrifuged at 4°C for 45 minutes in a microcentrifuge and the supernatants were transferred to polypropylene microfuge tubes. The supernatants were extracted once with TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) equilibrated phenol:chloroform:isoamyl alchohol with a ratio of 48:48:2 and followed once with chloroform:isoamyl alchohol of 24:1 ratio (Sambrook et al., 19889). After the extractions the aqueous phase which contains DNA molecules was transferred into a fresh microfuge tube. NaCl was added to a final concentration of 200 mM and the DNA was precipitated with 2 to3 volumes of ice cold 95% ethanol for 20-30 minutes at -20°C. The DNA

was recovered by centrifuging in a microcentrifuge at 4°C for 15 minutes. The supernatant was aspirated and the DNA pellet was dried in a speed vac for 5 minutes. The dried DNA pellet was resuspended in a desired volume of TE. For replication analysis of plasmid containing the origin of SV40 DNA, the plasmid DNA was digested with Dpn1 and EcoR1 to linearize the replicated plasmid before Southern blot analysis (Sambrook.et al., 1989).

2.5 DNA-$CaPO_4$ transfection

Recipient cells were seeded at a concentration of 0.5-1 x 10^6/100 m m dish 16-24 hours before transfection. Medium was replaced with 9 ml of fresh medium 4 hours prior to transfection. DNA-$CaPO_4$ transfection solution was made freshly for each experiment. 1 ml of DNA-$CaPO_4$ transfection solution was added to each 100 mm dish. Each milliliter of DNA-$CaPO_4$ transfection buffer consists of 0.5 ml of 2 X Hepes Buffered Saline (HBS) and 0.5 ml of 2X DNA-$CaCl_2$ solution. The solution for DNA-$CaPO_4$ transfection was prepared as described in Frederick et al. (1992).

For stable transfection, dishes were incubated at 35°C with 7.5% CO_2 for 4-16 hours, such as transfection of HDF with SVtsAdl DNA. For transient transfection, dishes were incubated at 37°C with 3% CO_2 for 8-12 hours, such as transfection of Hela cells with DNA encoding the C-terminal CENP-E fregment. The medium is replaced with fresh medium after the dishes are washed twice with sterile PBS.

2.6 Construction of pRNSVtsA58dl

SV40 dl888 (Jog et al., 1990) encodes wild type large T and mutant small t antigens. To substitute wild type small t in pMKtsA58 with mutant small t in SV40 dl888, the following work was performed:

1. CV-1 cells were infected with SV40 dl888. Viral DNA was extracted by the Hirt procedure. To obtain a large quantity of SV40 dl888 DNA, the DNA was digested with EcoR1 and cloned into the EcoR1 site of pUC 19 (2.7kb). After amplification of the SV40 dl888/pUC19 DNA (plasmid 308; 7.9kb), the plasmid 308 and pMKtsA58 were each digested with Nde1. The Nde1 fragment (0.9kb) containing mutation of small t (from p308) was ligated with the large Nde1 fragment (8.4kb) from pMKtsA58 (9.3kb). BamH1 and BstX1 were used to determine the right orientation of the ligation. The resulting plasmid encodes temperature sensitive large T and mutant small t antigens. This plasmid is called pMKtsA58dl.

2. Because of the difficulty in obtaining pMKtsA58dl transformants, pRNSVtsA58dl was constructed to select tsA58dl transformants in G418 after transfection. Plasmid IBI-30 (2.9kb from IBI, Cat. # 338830) and pMKtsA58dl were each digested with EcoR1; and tsA58dl was cloned into the EcoR1 site of IBI-30. The resulting plasmid IBI-30/tsA58dl (8.2kb) was digested with BamH1 and two fragments of 4.5kb and 3.6kb were generated. The fragment 4.5kb contained the sequence encoding temperature sensitive large T and mutant small t antigens. This fragment was cloned into the BamH1 site of plasmid RSVneo (5.5kb, Gorman et

al., 1983). The resulting plasmid contains G-418 resistant gene and tsAdl gene.

2.7 Isolation of pRNSVtsA58dl transformants

Early passage HDF cells were seeded at a density of 5 x 10^5 per 100mm-diameter dish. Two days later, the cells were transfected at 35°C with 10 microgram of closed circular origin-defective pRNSVtsA58dl DNA plus 10 microgram of salmon sperm DNA as carrier for each plate. Cells were subcultured at a 1:3 ratio two days later and colonies were selected in G-418 at 200 ug/ml for one week and 100 ug/ml for two more weeks. Colonies were identified and picked after 3 to 4 weeks at 35°C. Individual colonies were picked. Five transformants were grown to mass culture in 100-mm-diameter dishes for frozen storage and expanded for characterization of growth property or cell cycle study. It should be noted that the transformants had a flat colonial morphology unlike SVtsA transformed HDF.

2.8 Cell cycle analysis by flow cytometry

For analysis of human fibroblasts, the cells were harvested by trypsinization, centrifuged for 5 minutes at 1000 rpm in an IEC clinical tabletop centrifuge, and suspended in PBS at a concentration of 10^6 cells per ml. One milliliter was added to 9ml of 90% cold ethanol and stored at 4°C until analysis. The fixed cells were then sedimented, washed once with and resuspended in PBS (at 10^6 cells/ml), and stained with propidium iodide (50ug/ml) in the presence of Triton X-100 (1%, wt/vol) and 0.01%

ribonuclease A for 20 minutes at room temperature. Cells were analyzed for DNA content with a System 50H flow cytometer (Ortho Diagnostic Institutes, Westwood, Mass.) interfaced to an Ortho 2150 data analysis system in collaboration with Dr. Frank Traganos at Sloan Ketttering Memorial Institute (New York).

For analysis of HeLa cells, 10^6 cells were resuspended in 400 ul of citrate buffer (250 mM sucrose, 40mM trisodium citrate, pH 7.6, and 5 ml of DMSO in 100 ml citrate buffer). Cells can be stored at -70°C until analysis. 1.8 ml of solution A (3.4 mM trisodium citrate, 0.1% NP-40, 1.5 mM spermine, 0.5 mM Tris-HCl, pH 7.6, 0.003% trypsin) was added to 400 ul of above cell mixture and mixed by reverting the tube for several times. The mixture was incubated at room temperature for 10 minutes with inverting the tube every 2 minutes. After the incubation, 1.5 ml of solution B (3.4 mM trisodium citrate, 0.1% NP-40, 1.5 mM spermine, 0.5 mM Tris-HCl, pH 7.6, 0.05% soy bean, 0.01% ribonuclease A) was added to the tube and mixed followed by another 10 minutes incubation at room temperature. 1.5 ml of solution C (3.4 mM trisodium citrate, 0.1% NP-40, 1.5 mM spermine, 0.5 mM Tris-HCl, pH 7.6, 0.02% propidium iodide, 0.12% spermine) was added and mixed. The mixture was incubated on ice for at least 30 minutes (upto 4 hours) in dark followed by filtering the solution through nylon mesh (35 micron, Small Parts Inc.). Cells were centrifuged and resuspended with 1 ml of the same supernatant before flow cytometry analysis.

2.9 Immunoassays

Cell extracts of SV40-transformed cells were prepared from 100-mm dishes which had been seeded at 35°C. In a typical experiment, 1 x 10^6 to 2 x 10^6 cells were plated and either maintained at 35°C or shifted to 39°C for 3 to 4 days before harvest. Extracts were typically prepared directly on the dish at 4°C with 0.3 to 1ml of lysis solution containing 120 mM NaCl, 0.5% Nonidet P-40 (NP40), 50 mM Tris-HCl (pH 8.0) with a mixture of protease inhibitors which includes 0.15 mM of phenylmethylsulphonyl fluoride (PMSF), 0.1 mg/ml of alpha$_2$-macroglobulin, 0.3 mg/ml of leupeptin, and 0.1 ug/ml of aprotinin. The disrupted cells were harvested by scraping with a rubber policeman and centrifuged in a microfuge for 15 minutes at 4°C. The supernatant was used directly for further analsis.

The protein concentration of the extracts was determined by using the Bio-Rad protein assay. Extracts containing approximately 1 to 2 mg of protein were immunoprecipitated by the addition of the appropriate monoclonal antibody and Sepharose-conjugated protein A. In the case of pRb-1, an additional incubation with rabbit anti-mouse immunoglobulin G (IgG) was included because of the poor interaction of Sepharose-protein A with the antibody (IgG_1) to pRb-1. The immunoprecipitate was washed five times in buffer containing 100 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 20 mM Tris-HCl (pH 8.0), and the protease inhibitor mixture. The immunopreciptate was then dissolved in 50 ul of buffer containing 6% SDS, 250 mM NaCl, 2.5 M mercaptoethanol, 20% glycerol, and bromphenol blue followed by heating in a boiling-water bath for 5 minutes. Polyacrylamide gel electrophoresis (PAGE) and immunoblotting were performed as described in Harlow et al., 1988. Viral and cellular proteins were detected by appropriate monoclonal antibodies and affinity-

purified goat anti-mouse IgG conjugated with either horseradish peroxidase or alkaline phosphatase (Boehringer-Mannheim), used according to specifications provided by supplier. In some cases, the same nitrocellulose filter was assayed for individual proteins by sequential reactions. Polypeptides were determined by using the following monoclonal antibodies: SV40 T antigen was assayed by PAb 419 culture supernatant (1:20 dilution, Harlow et al., 1981), pRB was assayed by C36 ascites (1:100 dilution, Whyte et al., 1988), and p53 was assayed by PAb 421 culture supernatant (1:20 dilution, Harlow et al., 1981). PAb 210E8 culture supernatant against the *Escherichia coli* RNA polymerase B subunit was used as a negative control with 1:20 dilution (Rockwell et al, 1985).

Immunoprecipitation of CENP-E, $p34^{cdc2}$ and cyclin B was done with corresponding polyclonal antiserum from rabbit (1:500 dilution, Yen et al., 1992), polyclonal IgG from rabbit (1:100 dilution, UBI cat. # 06-181) and monoclonal IgG2b fron mouse (1:100 dilution, UBI cat. # 05-158) respectively. The immunoblotting technique was same as described in the above paragraph except for the secondary agent used. The secondary used for detecting CENP-E and $p34^{cdc2}$ was [^{125}I]-labeled protein A (1:2000 dilution, ICN) while [^{125}I]-conjugated rabbit anti-mouse IgG (1:2000 dilution, ICN) was used to detect cyclin B, pRb and T antigen in preimmortal transformants. Autoradiography was used in these immunoassays.

2.10 Immunoassays for CENP-E expression and its turnover

HeLa cells were grown in suspension at 37°C. The cells were synchronized twice at the G1/S boundary with thymidine followed by aphidocolin (Calbiochem) at a final concentration of 2.5 mM and 5 ug/ml respectively. Cells were first blocked with thymidine for 12-16 hours and then released from the block following centrifugation and subsequent wash with warm PBS. Released cells were resuspended in regular medium and incubated at 37°C for 10-12 hours. Cells were then blocked with aphidocolin for 12-16 hours and released as for the first block. Released cells were collected every 2 hours and cell synchrony was monitored by flow cytometry.

To determine the steady-state levels of CENP-E, synchronized cells (~4 x 10^6) were lysed in 1-2 ml of RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH7.5, 0.5% deoxycholate, 1.0% NP40, 0.1% SDS, 1 mM PMSF, 1 ug/ml each of leupeptin, aprotinin and pepstain) and centrifuged at 15,000*g* for 20 minutes. The pelleted nuclei and insoluble debris were boiled in SDS-sample buffer, separated by PAGE on a 4-10% gradient gel in SDS, and transferred onto Immobilin P (Millipore) for immunoblot analysis. Cytoplasmic extracts with the same concentration of protein were transferred to microfuge tubes and incubated with polyclonal antibody to CENP-E at 1:200 dilution for 2 hours at 4°C. A 1:1 slurry (40 ul) of washed Protein A Sepharose (Pharmacia) beads were added and incubated for 1 hour at 4°C. The immune complex was washed three times with 1 ml RIPA buffer, boiled in SDS sample buffer, and processed for immunoblot analysis as above. The same antibodies that were used for the immunoprecipitation were used for immunoblot analysis (used at 1:1000

dilution). [^{125}I]-labeled protein A (ICN) was used to detect bound primary antibodies.

To measure the turnover rate of CENP-E, pulse-chase labeling experiments with [^{35}S]-methionine were performed. Cells that were synchronized at late S or G2 were starved in methionine-free medium for 20 minutes and pulse-labeled for 1 hour by using Trans-label (ICN) at a final concentration of 100 uCi/ml in a methionine-free labeling medium of SMEM with 1% dialyzed FBS. Cells were washed three times with PBS after labeling and an aliquout of cells was immediately lysed as a zero time point (G2 cells). Three more aliquots of cells were taken at 1, 2 and 4 hours after the labeling. To block cells in mitosis, cells were chased in regular medium and 0.5 ug/ml of colcemid (Gibco). Cell lysate were processed for immunoprecipitation and SDS-PAGE. Labeled proteins were detected by flurography.

2.11 Expression and purification of wild type and mutants of CENP-E^{COOH368} in *Escherichia coli*

A 1.3 kb Dra 1 cDNA fragment that encodes the COOH-terminal 368 amino acids of CENP-E was cloned into pGEX-2T (Pharmacia) at the Nco1 site that had been filled in. Similarly, all four serine mutants were cloned into the same vector after site-directed mutagenesis. The resultant plasmids, pGEXDraB or the mutants, were transformed into BL21 host cells, and protein expression was induced with 50 uM isopropyl-B-D-thiogalactopy-ranoside (IPTG) for 4 hours at room temperature. Cells were resuspended in 1/10th volume of PBS, incubated with lysozyme (0.1

mg/ml) for 30 minutes on ice, and sonicated. After centrifugation at 25,000g for 20 minutes, the supernatants were passed through a 0.5 ml glutathione-Sepharose column (Sigma). After washing with PBS, the column was reequilibrated in thrombin digestion buffer. The protein was digested with thrombin (Boehinger) at concentration of 0.2 to 1% (w/w) of digested protein for 1 hour at 25 ^{o}C, and the COOH-terminus of CENP-E was eluted from the column, desalted, and concentrated in a microconcentrator (Amicon). Protein concentration was determined by Bio-Rad protein assay. Sample was stored at -80^{o}C for future use.

2.12 In vitro phosphorylation of bacterially expressed and purified CENP-E^{COOH368}

2-4 microgram of purified CENP-E^{COOH368} fragment was added to each labeling reaction which contained 10 mM $MgCl_2$, 0.05 mM ATP, 1 mM DTT and 5 uCi gamma ATP. 2 ul of p34^{cdc2} and cyclin B (same amount of cdk2 and cyclin A was used for different combinations of kinase reaction) were taken from their stocks, mixed and incubated at room temperature for 20 minutes. The stocks were crude non-detergent extracts (hypotonic lysate at ~5 mg/ml of total protein) of baculovirus-infected insect cells and contained human p34^{cdc2}, cdk2, cyclin A and cyclin B1 (gift from Dr. Morgan, Desai et al., 1990). p34^{cdc2}/cyclin B1 mixture (or other combinations) was added to the labeling reaction and incubated at room temperature for 5 minutes. The reaction was then stopped by boiling in the sample buffer for 5 minutes. The sample was then subjected to SDS-PAGE and autoradiography.

2.13 Mutagenesis of serine phosphorylation sites

A fragment that encoded CENP-E^{COOH368} was cloned into a single strand vector, M13 mp18. Four oligonucleotides were designed to contain the substitution mutation which converts serine amino acid to alanine at the predicted consensus sites for p34^{cdc2} kinase. The oligonucleotides of each mutant were annealed to M13/CENP-E^{COOH368} phage separately at equal molar concentration at 65°C in annealing buffer for 10 minutes. The mixtures were incubated at room temperature for 30-40 minutes to allow maximal annealing. The annealed oligonuleotides were extended to complete the synthesis of the complementary strand of M13/CENP-E^{COOH368} phage template. The double strand M13/CENP-E^{COOH368} phage was digested with appropriate restriction enzymes and the heteroduplex fragment was gel purified by electrophoresis in 0.8-1% of low melting agarose gel (FMC). The mutagenized fragment was subcloned into freshly digested M13mp18 vector and transformed into host strain JM101. Plaques expressing the mutants were identified by plaque hybridization that utilized the mutant [^{32}P]-oligonucleotides as probes and then confirmed by DNA sequencing. Positive mutants were then cloned back to pGEX-2T vector for expression in bacteria and purification as described above.

2.14 In vivo phosphorylation of CENP-E^{COOH368} fragment in transiently transfected HeLa cells

A vector encoding an HA epitope (hemagglutinin tag from influenza viruses) in the expression vector pSV2 was used. A 1.3 kb Dra1 cDNA

fragment that encodes COOH-terminal 368 amino acid of CENP-E was inserted downstream in the correct reading frame of the HA epitope-tag (Mulligan and Berg, 1981). HeLa cells were transiently transfected by calcium phosphate precipitation as described. Twenty hours after removing of the DNA precipitate, transfected Hela cells were labeled with [^{32}P]-orthophosphate (1 mCi/ml) for 3 to 4 hours in phosphorus free DMEM medium containing 1% of dialysed FBS and 0.5 mg/ml colcemid. After labeling, mitotic cells were separated from interphase cells by mechanical shake-off. Cells were washed three times with PBS and lysed in RIPA buffer containing protease and phosphatase inhibitors (50 mM NaF, 0.1 mM Na_3 VO_4, and 80 mM B-glycerophosphate). Extract was clarified by centrifugation at 15,000 *g* for 10 minutes at 4°C. Monoclonal antibody 12CA5 to HA (Babco) was added to the supernatant (1:500) and incubated for 2 hours at 4 °C. 40 ul of 50% agarose (vol/vol) coupled with antibody to mouse IgG was used to immunoprecipitate the immune-complex. After washing 5 times with RIPA buffer, the precipitate was boiled for 5-10 minutes in 0.2 ml of 1% SDS. The sample was diluted with RIPA buffer without SDS to a final concentration of 0.1% SDS. Antibody to DraB fragment was added at a final concentration of 1:200 and incubated at 4°C for one hour. The second immuno-complex was similarly processed, and analyzed on SDS-PAGE.

2.15 Tryptic digestion of in vitro and in vivo [^{32}P]-labeled CENP-E^{COOH368} fragment

Trypsin cleaves proteins on the C-terminal side of arginine or lysine residues. After SDS-PAGE and autoradiography of both in vitro and in

vivo labeled CENP-E^{COOH368} fragment, bands were sliced from the dried gel and digested with 20 microgram trypsin in 50 microliter of 50 mM ammonium bicarbonate(pH 8.05) at 37^{o}C for overnight. Supernatants of digestion reaction were separated from the gel slices and lyophilized after removal of the gel debris by centrifugation. Tryptic digested [^{32}P]-labeled phosphopeptides were dissolved in 3 ul of H_2O for thin-layer cellulose (TLC) analysis.

2.16 Two dimension TLC analysis of phosphopeptide

The number of phosphopeptides that were generated by tryptic digestion were determined by thin layer chromotography. Samples were separated on TLC plates in the first dimension by electrophoresis and then followed by chromatography in the second dimension. Samples were applied at the origin by using micropipettes in 0.2-0.5 ul steps. The sample was dried by blowing of air between applications. After concentrating the spotted sample, the plate was sprayed with electrophoresis buffer (100 ml glacial acedic acid, 10 ml pyridine, 1890 ml deionized water). The plate was air dried followed by electrophoresis at 1 kV for 1.5 hours.

After drying the TLC plate, separation in the second dimension was performed by ascending chromotography using chromotography buffer (v/v, 7.6% glacial acetic acid, 37.8% pyridine, 30.2% deionized water, 24.4% butanol) in an upright position in the tank. The chromotography was stopped when the buffer front reached to top of the plate (14-18 hours). The plate was dried with hot air for 10 minutes, marked with

radioactive ink and analyzed by autoradiography or Fujix Bio-imaging Analyzer (Fuji Photo).

2.17 Identification of the phosphoamino acid

After lyophilization of tryptic digested phosphopeptide, the sample was dissolved in 6 N HCl and hydrolyzed at 110 °C for 1 hour in a heat block. After hydrolysis, the sample was vacuum dried and resuspended in 3 ul of H_2O. Amino acids were separated by TLC in the 1st dimension as desribed for phosphopeptide. After electrophoresis, the TLC plate was dried and put in a tank for ascending chromatography in a buffer consisted of H_2O:isobutic acid with 1:1.7 (v/v). The plate was dried using a hot fan for 10 minutes, marked with radioactive ink and analyzed by autoradiography or Fujix Bio-imaging Analyzer (Fuji Photo).

Chapter 3

Results

SV40-transformatiom and immortalization of human diploid fibroblasts has been proposed as a cell culture (in vitro) model for human carcinogenesis in the intact organism (in vivo). In order to study the mechanisms involved in vitro, I utilized a virus-cell system developed in Dr. Ozer's laboratory in which function of the viral transforming agent, large T antigen, could be conditionally regulated. In particular, I investigated the nature of SVtsA/HF-A transformed by an origin-defective SV40 genome encoding a temperature-sensitive, heat-labile large T antigen at 35°C.

SVtsA/HF-A when first isolated as a transformed focus and grown to mass culture (P1) showed a rapid growth rate and high efficiency of colony formation (EOC). After passage 15, there was a gradual decrease in population growth and EOC. After passage 20 there was a progressive improvement in both parameters. Based on these and other data, it was concluded that cultures at early passage were composed of preimmortal cells, a modest form of crisis occured between passages 15-20, and populations subsequent to that time were composed of immortal cells. The minimal "crisis" reflected a mixed culture of preimmortal cells at the end of their extended lifespan and a significant number of cells which had become immortal at an early stage of its extended lifespan.

3.1 Characterization of the growth property of AR-5, HAL

Immortalized cell line AR5 and HAL are cloned derivatives of SVtsA/HF-A, AR5 was cloned at passage 2 of preimmortal SVtsA/HF-A. AR5 was studied by me when it was itself immortal. It had a population doubling time of 30 hours at 35°C. At nonpermissive temperature 39°C, AR-5 lost the ability to proliferate and showed a low efficiency of colony formation with a EOC ratio of 0.0006 (Ratio of the number of colonies at 39°C divided by that at 35°C). Furthermore, when logarithmically growing cultures were shifted to 39°C, there was a rapid cessation of growth, independent of cell density over a severalfold range (Figure 1 panel A). HAL was isolated from immortal SVtsA/HF-A at passage 40. The cells grew well at 35°C and doubled no more than once at 39°C (Figure 1 panel B). Finally, DNA synthesis is inhibited at 39°C as measured by incorporation of [^{3}H] thymidine (Figure 2).

3.2 Analysis of temperature sensitive immortals

To clarify the function of large T antigen in immortalized transformants, an essential step is to confirm the expression of large T antigen in the transformed cell line. These temperature sensitive (ts) transformants were characterized for their expression of large T antigen by using immunoprecipitation method followed by western blot analysis. Cells from both 35°C and 39°C were subjected to this procedure. As shown in the immunoblot in Figure 3, large T antigen is detected at both permissive and nonpermissive temperature, lane 1 and 2 respectively.

Cessation of growth despite continued expression of large T in the transformed immortals at 39°C suggests that heat-labile large T antigen is functionally inactivated at this temperature. A way to test this hypothesis is to transfect these cells with a plasmid containing a functional origin of SV40. Functional large T antigen will promote replication of the plasmid when introduced into a permissive or semi-permisive cells. pSV0 (Auborn et al., 1988) is a such plasmid and it was transfected into AR-5 to determine the function of large T antigen at 39°C. After transfection, cells were incubated at 35°C or 39°C respectively for 3 days. Viral DNA was isolated by Hirt extraction (Hirt 1967) and Southern blot procedure were performed to analyze for replicated plasmid. The purified DNA was incubated with the methylation specific endonuclease DPN1 prior to gel electrophoresis. The input DNA was methylated and sensitive to DPN1 digestion whereas becomes resistant to the restrictive enzyme if it were replicated in the AR5 cells, since the mammalian cells do not methylate the relevant GATC sequence. At 35°C replicated plasmid was detected as shown in lane 1 of Figure 4 while at 39°C no plasmid was seen in lane 2 of the figure. No plasmid was detected in the lanes 3 and 4 which are mock transfection for 35°C and 39°C respectively.

3.3 Complex formation between pRB and large T antigen in AR5

Above experiments demonstrate that the growth phenotype of ts transformants is correlated with the function of large T antigen. Furthermore, in Dr. Ozer's laboratory it was demonstrated that transfection of the temperature sensitive cells with wild-type SV40 genome can restore

cell growth at 39°C. These results indicate that the inability of these cell lines to proliferate at 39 °C is solely due to the inactivation of large T antigen function at the elevated temperature. One would suspect that the ability of T antigen to form complex with antioncogenes pRB or p53 may also be affected by inactivation of large T antigen at 39°C.

To test whether complex formation between large T antigen and cellular protein pRB correlated with T function, cell extracts from AR-5 and HAL were immunoprecipitated and analyzed by Western immunoblot. Extracts containing equal amounts of protein were immunoprecipitated in all cases. Initially, three fractions of HAL cell extract at 35°C were immunoprecipitated with antibodies to large T, pRB and *E.coli* RNA polymerase respectively. After SDS-PAGE, immunoblot was developed with antibodies to large T antigen and pRB. As shown in Figure 5 , antibody to T antigen brought down T antigen as well as some pRB (lane 1). This result indicated the complex formation between T antigen and pRB. The same result was obtained when antibody to pRB was used and both pRB and T antigen were detected (lane 2). In addition a doublet band of pRB is clearly shown in lane 2. The lower band represents hypophosphorylated form of pRB while the upper one is hyperphosphorylated pRB. Comparing lanes 1 and 2, it is estimated that about 5% of large T antigen binds to pRB. This fraction of T antigen seems to bind only to hypophosphorylated pRB. In the control lane, neither pRB nor T antigen was seen when antibody to *E.coli* RNA polymerase was used (lane 3).

To extend these findings to other ts immortals, AR-5 cell line was studied for the complex formation between T antigen and pRB at 35°C and 39°C. In parallel a temperature-insensitive transformant of wild-type SV40 (SV/HF-5/39) was included as a control. SV/HF-5/39 grew well at both 35°C and 39°C and demonstrated complexes at both temperatures Figure 6 (panel B). As other nonhuman SV40-transformants (Ludlow et al., 1989), T antigen preferentially bound to underphosphorylated (the faster-moving band) pRB (lanes 2 and 5). However, somewhat unexpectedly, when a fivefold greater amount of the immunoprecipitate was analyzed (lanes 3 and 6), a second (presumably phosphorylated) pRB was readily detected. Furthermore, most, if not all, of the underphosphorylated pRB was involved in complex formation when the same extracts precipitated with antibody to pRB (lane 7) or with antibody to T antigen (lane 3) were compared.

Figure 6 (panel A) shows the results of AR-5. Under all conditions, only a small proportion of the pRB was in the form of stable complexes with large T antigen. The doublet of pRB was only evident with the highest concentration of extract at 35°C (lane 3), while only multiple faint bands were detected at 39°C (lane 6) and no bands specifically reactive with antibody to pRB were observed. Antibody to pRB brought down both pRB and large T antigen (lane 7). These data confirm and extend the results with HAL cells. pRB-T antigen complexes were indeed demonstrable in a temperature-dependent manner in the cell lines expressing a heat-labile T function.

3.4 Complex formation between p53 and large T antigen in HAL cells

In addition to forming a complex with anti-oncogene protein pRB, large T antigen has been demonstrated to form complex with p53 in transformed cells. Mutations of p53 have been found to be associated with human carcinomas in many cases. To clarify the transformation function of large T antigen, formation of p53-T antigen complexes were studied and compared between permissive and nonpermissive temperature (Figure 7). Extracts from HAL cells were subjected to immunoprecipitation and immunoblot analysis as above. Temperature-independent cell line SV/HF-5/39 was used again as a control (panel B). Immunoprecipitation was performed with PAb 421 to p53 (lanes 1 and 2), PAb 419 to T antigen (lanes 3 and 4), control antibody to *E. coli* RNA polymerase (lanes 5 and 6). Immunoblot was developed with antibodies to p53 and large T antigen. As shown in panel B of Figure 7, p53-T antigen complex was observed at both 35°C and 39°C (lanes 1 and 2 respectively). Antibody to T antigen (lanes 3 and 4) also immunoprecipitateed p53-T complexes. There was no p53-T antigen complexes detected in control lanes (5 and 6).

Panel A of Figure 7 shows the results of HAL cells. Samples of lanes from 1 to 6 has the same order as in panel B. Comparing to lanes of 1 and 2 in panel B, the level of p53 was reduced and p53-T antigen complex was not seen at 39°C (lane 2 of panel A). The reduced level of p53 is consistent with a defect in intracellular complexes at 39 °C, since wild-type p53 has a short half-life and complex formation with T antigen has been reported to stabilize p53 (Reships et al., 1990). Restoration of growth at 39°C is concomitant with formation of p53-T antigen complexes after introducing wild-type large T antigen into HAL cells (lanes 1 and 2 of panel C). These

results demonstrate that the dissociation of p53-T antigen complexes occurred at nonpermissive temperature when the function of large T antigen is lost.

3.5 Cell cycle analysis of ts transformants

AR-5 and HAL have a temperature-dependent phenotype for growth. The cells grow well at 35°C and cease to proliferate at 39°C. At nonpermissive temperature, large T antigen becomes inactivated for SV40 origin-dependent DNA replication. Moreover, complexes between large T and antioncogenes pRB and p53 are becoming dissociated when function of T antigen is lost at 39°C. Release of hypophosphorylated pRB may cause cells to arrest in G1 at 39°C since hyperphosphorylation of pRB in G1 is thought to be important for cells entering S phase of the cell cycle. One would expect that temperature sensitive transformants undergo a G1 arrest in the cell cycle. However, the cell cycle analysis by using flow cytometry (FCM) demonstrated it was not the case.

Uncloned immortal SVtsA/HF-A cells (passage 24) were used for the cell cycle analysis. Cells were seeded in 100mm dishes at 4×10^5 and incubated at 35°C until they reached log phase of growth. On day 0, one dish from 35°C was harvested for cell cycle analysis and four replicate dishes were shifted to 39°C. On following days, one of the 39°C dishes was harvested at daily intervals for analysis. On day four, one dish incubated for four days at 35°C was also analyzed to serve as a control of the experiment. As shown in panel A of Figure 8, cells in log phase of growth at 35°C showed normal cell distribution (50% in G1, 25% in S,

20% in G2/M). Cells which had been at 39°C for 24 hours (panel B) showed no changes in the distribution as compared to those at 35°C. However, the flow cytometry (panel C to E) showed a depletion in the number of cells in S phase (from 26.6 to less than 15.8%) and a increase in the number of cells in G2 (from 22 to 44%) by four days at 39°C. During the study, the dishes at 39°C showed no increase in cell number between 24 hours at 39°C and the end of the experiment while the number of cells increased four-fold in the control dish with an unchanged log phase distribution (panel F). This experiment demonstrate that the temperature sensitive transformant has experienced a G2 arrest when the large T antigen is inactivated.

Preimmortal SVtsA/HF-A was also analyzed by flow cytometry to characterize the distribution of the cells in the cell cycle at 39°C. It was found that a more complicated situation was occurring when the results of immortal transfromants were compared with preimmortals at 39°C. Cells at passage 6 were seeded at 1.9 x 10^6 in 100mm dish at 35°C and shifted to 39°C on the next day. Four days later, cells were subconfluent (3 x 10^6) with a more flattened appearance. FMC analysis (in panel B of the Fig 9) revealed that the 44% of the total population had a DNA content expected for cells in G2. In addition, 34% of the population had a DNA content higher than G2 cells (tetraploid population). When the subconfluent cells were subcultured at 1x10^6 in 100mm dishes at 39°C, populations of both G2 and tetraploid cells increased progressively in the following four days (panels C to F). Increases in these two compartments were concomitant with decreases in the populations of both G1 and S phase cells. The cell number only increased 20% over the course of the subculture at 39°C. The

same results could also be obtained if the cultures were seeded initially at lower cell density at 35°C and shifted to 39°C for an equivalent number of days. The results demonstrate that preimmortal transformants shows a different pattern of cell cycle arrest at 39°C in which a tetraploid DNA content is observed compared to G2 arrest of the immortal cells.

3.6 Temperature sensitive transformants which express heat-labile large T antigen but not small t antigen

The tetraploid DNA content seen in preimmortal SVtsA/HF-A at 39°C by cell cycle analysis may be due to either overexpressed mutant T or wild type t antigens since inactivation of large T antigen at 39°C abrogates its autoregulation function. In this circumstance, both large T and small t antigens are overexpressed. To clarify the situation, a SV40 genome which makes heat-labile large T antigen but not small t was constructed. A G418 selection marker was also put into the same construct to faciliate the isolation of transformants. This construct is called pRNSVtsA58dl.

HS74 was transfected with closed circular pRNSVtsA58dl DNA and colonies were isolated. After expansion of the picked colonies, one was chosen for detailed study. Western analysis showed that the cell line only had expression of large T antigen; no small t was detectable in the transformants (data not shown). These cells were temperature sensitive for growth as preimmortal transformants of SVtsA/HF-A. FCM analysis of SVtsAdl/HF at 39°C (Figure 10) showed that no tetraploid DNA content was detected in contrast to that of preimmortal SVtsA/HF-A. These data

suggest that small t is responsible at least in part for the tetraploid phenomenon observed in preimmortal cells at 39°C.

3.7 Expression of CENP-E is cell cycle regulated, its cDNA sequence suggests CENP-E as a kinesin-like motor protein

Centromere associated protein, CENP-E, binds to centromere region at prometaphase and relocates to the midbody by telophase (Yen et al., 1991). Microinjection of antibody to CENP-E into metaphase Hela cells blocks or significantly delays progression into anaphase of the cell cycle. This blocking effect may prevent cells going through mitosis and jeopardize chromosome segregation. Subsequently, blocked cells enter a second S phase without completion of mitosis and contain tetraploid DNA. Hence, it is reasonable to suspect that loss of CENP-E function in cells may result in a tetraploid DNA content. Since large T antigen has multiple functions it may directly or indirectly regulate CENP-E expression or function.

To clarified the function of CENP-E and the possible relationship with tetraploid phenomenon, it is useful to know the regulation of CENP-E expression. Unlike other centromere associated proteins, CENP-E is not seen in interphase cells by immunofluorescence staining. Its apparent absence during interphase and presence at mitosis suggest that expression of CENP-E is cell cycle regulated. To further clarify the expression of CENP-E during the cell cycle and as a prelude to its study in the SV40-transformed fibroblasts, Hela cells were synchronized and released at the G1/S boundary (Yen et al., 1992). Equal number of cells were harvested at

a 2-hour interval starting from 2 hours after release to examine the accumulated CENP-E. Cell extracts were immunoprecipitated with antibody to CENP-E followed by Western blot analysis. Progression of synchronized cells during cell cycle was monitored by flow cytometry. Cells in G1 and early S phases showed little detectable CENP-E in either cytoplasmic or nuclear fractions while the level of CENP-E in cytoplasm increased sharply during late S and G2/M phases (Figure 11, upper panel). At all times CENP-E is excluded from nuclei (Figure 11, lower panel). This result is consistent with the staining of interphase cells by immunofluorescence and the absence of a consensus nuclear targeting signal (Yen et al., 1992)

Pulse-chase experiment was performed to examine the degradation of CENP-E at the end of the mitosis (Yen et al., 1992). Late S phase cells were labeled with ^{35}S-methionine for 1 hour. One labeled dish was immediately lysed and used as a sample of G2 or early M phase cells. Other dishes were washed and chased in regular medium without labeling. At 1, 2 and 4 hours after release, chased dishes were harvested and analyzed for CENP-E degradation. At 1 hour chase (cells at G_2 or M phase), CENP-E synthesized earlier remained stable (Figure 12). When most cells had completed mitosis only about 20% of CENP-E remained (2 hours). At 4 hours, virtually all CENP-E was lost from previously labeled cells as the progenies of labeled cells entered following G1 phase. On the contrary, CENP-E remained stable up to 4 hours in the cells blocked in mitosis (Figure 12). These results indicate that CENP-E is quickly degraded after mitosis and the degradation is dependent on completion of mitosis.

cDNA of CENP-E was cloned by Yen et al. (1992). Comparison of the sequence of CENP-E with all sequences in current gene and protein data base suggested that CENP-E is a kinesin-like motor protein. Moreover, like other motor proteins, CENP-E showed microtubule-binding ability which is diminished in presence of ATP.

In summary, CENP-E is a putative kinetochore motor which is important for cells to progress from metaphase to anaphase in mitosis. The protein accumulates just before mitosis of the cell cycle and degrades after mitosis.

3.8 CENP-E is phosphorylated both in vivo and in vitro

Predicted amino-acid sequence shows that four consensus sites for $p34^{cdc2}$ kinase are located at the C-terminal of CENP-E. This finding suggested that the function of CENP-E might be regulated through phosphorylation by $p34^{cdc2}$ kinase. Maturation promoting factor (MPF, a complex including $p34^{cdc2}$ and cyclin B), is responsible for cells entering mitosis. It is activated by dephosphorylation of tyrosine-15. To initiate mitosis, activated $p34^{cdc2}$ phosphorylates histones, lamins and other cellular factors. Phosphorylation of these cellular proteins regulates their functions and results in chromosome condensation, nuclear envelope breakdown and so on. It is conceivable that the function of CENP-E may also be regulated by phosphorylation at the beginning of mitosis. The phosphorylation-consensus sites at C-terminal for $p34^{cdc2}$ make the kinase a good candidate.

To verify whether CENP-E is phosphorylated, an in vitro experiment was performed initially. A fragment containing 368 C-terminal amino acids of CENP-E (CENP-E^{COOH368}) was used for in vitro labeling since it contains all four consensus sites for phosphorylation. A GST-CENP-E^{COOH368} fusion protein, a bacteria protein expression system, was induced and purified from bacteria. The fusion protein was digested with thrombin to release the portion of CENP-E for labeling. This fragment (CENP-E^{COOH368}), unlike motor domain of kinesins, can bind to polymerized microtubules in a ATP-insensitive manner (Liao et al., 1994). Moreover, phosphorylation of CENP-E^{COOH368} inhibited its microtubule binding ability when MPF from clam embryos was used. These properties indicate that CENP-E^{COOH368} does not function as a motor, and binds to microtubules is its dephosphorylated form. To test whether CENP-E^{COOH368} can be phosphorylated by cdks from human source, p34^{cdc2} and cdk2 were used in different combinations with either cyclin B1 or A. Purified CENP-E^{COOH368} was labeled with gamma-ATP in the presence of either p34^{cdc2} and cyclin B1, p34^{cdc2} and cyclin A, cdk2 and cyclin B1, or cdk2 and cyclin A. The results with the p34^{cdc2} and cyclin B1 combination demonstrated the highest activity to the C-terminal fragment of CENP-E (Figure 13, lane 2). Combinations of other forms in p34^{cdc2} and cyclin A, cdk2 and B1, cdk2 and A gave orderly decreased labeling signal of CENP-E^{COOH368} (Figure 13, lanes 1, 3 and 4).

In vivo labeling of CENP-E was performed to confirm the in vitro finding. CENP-E^{COOH368} was inserted downstream of the HA epitope-tag in the expression vector pSV2. The plasmid was transfected into Hela

cells for C-terminal expression. One day after removal of DNA-$CaPO_4$ coprecipitates, one of the replicate dishes was harvested for western analysis and the other was labeled with [^{32}P]-orthophosphate for 4 hours. Cells in mitosis and interphase were collected separately by the mechanical shakeoff method. This procedure harvested mitotic cells in supernatant of the medium and left interphase cells on the dish. Separation of mitotic cells from interphase cells would help to determine whether phosphorylation occured in mitosis, or to detect changes in the degree of phosphorylation if the protein was phosphorylated before entering mitosis. After collection, all the samples were immmunoprecipitated. To reduce the background of the labeling, labeled cell extracts were immunoprecipitated first with antibody to HA epitope-tag followed by antibody to C-terminal of CENP-E. Then, labeled samples were analyzed by autoradiography after SDS-PAGE. Unlabeled samples were processed by Western blot.

Western blot showed that the C-terminal of CENP-E was expressed in both mitosis and interphase cells (Figure 14, lanes 1 and 2 of panel B). In addition, the band of mitotic cells (lane 1) migrated slower than that of interphase cells (lane 2). The upward shift of mitotic CENP-$E^{COOH368}$ indicated that extra phosphate(s) have been added to the protein as the cells enter mitosis. This prediction was confirmed by in vivo labeling. As expected, in vivo labeled cells showed corresponding bands in autoradiography (Figure, lanes 1 and 2 of panel A). Above all, phosphorylation of endogenous CENP-E was also demonstrated by immunoprecipitation and autoradiogaphy (Figure 15). However, the signals were weak due to its low copy number in Hela cells. On the other

hand, a transfected construct which overexpresses CENP-E could provide workable signals for further phosphopeptide analysis.

3.9 Phosphopeptide map of C-terminal CENP-E showed similar pattern in vitro and in vivo

It has been demonstrated that C-terminal of CENP-E can be phosphorylated both in vivo and in vitro (preferentially by $p34^{cdc2}$ and B1 complex). Inspection of amino acid sequence of CENP-E revealed that four consensus sites of phosphorylation reside in this part of CENP-E. To estimate the number of amino acid residues being phosphorylated, two dimensional thin layer chromatography (TLC) was performed. In addition, this method would provide information of additional phosphorylation sites which were not expected by comparison of phosphopeptide mapping between in vitro and in vivo or interphase and mitosis.

After confirmation by autoradiography, in vitro or in vivo labeled protein bands were excised from SDS-PAGE gel. The gel slice was digested with trypsin and subjected to 2D TLC analysis. 10 spots were generated in the in vitro map (Figure 16) and 7 spots were generated in the in vivo map (Figure 17A). Spots 1 to 4 of in vitro and in vivo map showed a similar pattern. The number of the common phosphopeptides (spots 1 to 4) generated matched the number of putative sites for MPF. The extra phosphopeptides of in vitro and in vivo labeling could be due to either partially digested phosphopeptides of the spots 1 to 4 or different labeling condition between in vitro and in vivo. These observation

suggested that $p34^{cdc2}$ might be responsible for the phosphorylation of spots 1-4.

To study the changes occured at G2/M transition, the phosphopeptide map of interphase (Figure 17) and mitosis (Figure 17B) were compared. Spot 3 became heavily phosphorylated as the cells entering mitosis. The change in the degree of phosphorylation of spot 3 indicated the possible function regulation of CENP-E at mitosis.

3.10 Serine is the only amino acid being phosphorylated; confirmation of the consensus sites by mutagenesis

Phosphopeptide mapping demonstrated that four major spots were generated by tryptic digestion. Analysis of amino acid sequence revealed that only the serine residue was involved in phosphorylation consensus sites of $p34^{cdc2}$. To verify the phosphorylation sites individually, oligonucleotide mutagenesis method was used to direct substitution of serine with alanine. Before start of mutagenesis, phosphoamino acid analysis was done to determine whether serine was the only amino acid being phosphorylated. In vitro labeled C-terminal was digested with trypsin, followed by hydrolysis with 6N HCl. The hydrolysate was separated in the presence of unlabeled phosphoamino acid standards by 2D TLC. The standards were visualized by spraying the plates with ninhydrin. The TLC plate was then analyzed by autoradiograph. The autoradiograph of phosphoamino acid analysis showed that only one kind of phosphoamino acid was detected and co-migrated with unlabeled

phospho-serine standard (Figure 18). This result was consistent with the composition of amino acid in the consensus sites.

Four mutant oligonucleotides containing the substitution of serine for alanine were designed to abolish putative phosphorylation of serine within the consensus sites. The oligonucleotides were annealed to the corresponding wild-type sequences of the CENP-E fragment in M13 phage. After synthesis of the minus strand, the sequence containing mutation was cloned and analyzed by sequencing. Finally, the desired mutants were cloned into pGEX-2T to produce fusion proteins with corresponding mutations. The GST-mutant fusions were digested and purified as described in section 3.9. The mutants were labeled with gamma-ATP in the presence of $p34^{cdc2}$ kinase and cyclin B1 complex. All the mutations were the substitution of serine for alanine. Figure 19A showed the result of oligonucleotide mutagensis of serine-2617, and spots 1, 9, 10 were missing in the phosphopeptide map. Mutation of serine-2571, 2602 abolished spots 2 and 3 in the map respectively (Figures 19B and 19C). Spots 4, 5 and 6 were not seen in the mutation of serine-2568 (Figure 19D). Spots 7 and 8 were consistantly seen in all mutation maps. The concomitant disappearance of phosphopeptides in mutagenesis study confirmed the partial digestion of phosphopeptides. Those which did not disappear (spots 7 and 8) with either mutant represented the additional sites of phosphorylation by MPF or contamination of comigrating phosphopeptides.

3.11 Verification that in vitro phosphorylation sites are the phosphorylation sites in vivo

Although the phosphopeptide mapping of in vitro and in vivo labeling revealed a similar pattern and number of spots, a quadruple mutant of all four serine was required to determine the relationship between in vitro and in vivo phosphorylation. The quadruple mutant of all four serines was made by oligonucleotide mutagenesis method with sequentially adding mutation of serine to a existed serine mutant. Finally, the C-terminal fragment containing all four serine mutations was cloned into pGEX-2T and pSV2 with a HA epitpope-tag (pSV2/HA-tag) for fusion protein and transient expression in cells respectively.

In vitro, the results showed that no phosphorylation of the quadruple mutant could be detected with gamma-ATP labeling in presence of $p34^{cdc2}$ and cyclin B1 (Figure 20-upper panel, lane 6) while labeling of wild-type and other mutants was seen easily (Figure 20-upper panel, lanes 1 to 5). Absence of any labeled band was not due to low level of the quadruple-mutant protein since the protein was easily visualized by staining (Figure 20-lower panel, lane 6) at an intensity seen for that of wild-type and other mutants (Figure 20-lower panel, lanes 1 to 5). The quadruple mutant prohibited the phosphorylation of all four serines. To verify the same result was true for in vivo phosphorylation, Hela cells were transfected with pSV2/HA-tag containing the quadruple mutant followed by [^{32}P]-orthophosphate labeling. As shown in Figure 21, the 4 major phosphopeptides were not observed in vivo. However, the quadruple mutant did not change the phosphorylation pattern and number of the expected minor spots.

3.12 Comparison of the complex formation between large T antigen and pRB in preimmortal SVtsA/HF-A at permissive and nonpermissive temperature

pRB can form complex with E2F, a transcription factor. The complex is believed to inhibit transcription of some cellular genes (Nevins, 1992). One such cellular target is $p34^{cdc2}$ kinase (Dalton, 1992). The transcription of $p34^{cdc2}$ is repressed by pRB/E2F complex. This result could be extended to the tetraploid phenomenon observed in preimmortal SVtsA/HF-A. If pRB/T antigen complex become dissociated at 39°C, free pRB would form complex with E2F transcription factor to inhibit the expression of $p34^{cdc2}$. Decreased $p34^{cdc2}$ may hinder its ability to phosphorylate cellular proteins involved in mitosis. Phosphorylation of CENP-E, one such protein which may be involved in chromosome segregation (Yen et al., 1991), could be prohibited. Thus, one would speculate that decreased level of $p34^{cdc2}$ cannot fully activate CENP-E to induce chromosome segregation resulting in metaphase block. The blocked cells may start a second round DNA synthesis leading to a tetraploid DNA content. To study the dissociation of pRB and T antigen in preimmortal SVtsA/HF-A at 39°C, extracts of these preimmortal transformants were immunoprecipitated and analyzed by immunoblot.

The same amount of cell extracts from 35°C and 39°C was immunoprecipitated with antibody to pRB. Following SDS-PAGE, immunoblot was reacted with antibodies to pRB and large T antigen, [^{125}I] conjugated anti-mouse IgG followed by autoradiography. Lanes 1, 2 and 3 of Figure 22 represented cell extracts of 35°C, 39°C and control

respectively. There was less T antigen detected at 39°C than that at 35°C. The level of pRB at 39°C showed a slight increase as compared with the level at 35°C. Neither T antigen nor pRB was seen in control lane 3. Together, these results demonstrated pRB and T antigen formed complex at 35°C and the complex became dissociated when the cells shifted to 39°C.

3.13 Level of $p34^{cdc2}$ and cyclin B1 in ts transformants

CENP-$E^{COOH368}$ was preferentially phosphorylated by $p34^{cdc2}$ and cyclin B1 complex in the in vitro labeling experiment. The study of phosphopeptide mapping and mutagenesis confirmed that the number and the sites of phosphorylation in CENP-$E^{COOH368}$ were consistent with the consensus sites deduced from amino acid sequence. Moreover, phosphorylation of serine-2602 (spot 3) increased 3 fold when cells entered mitosis. These results suggested that the fragment of CENP-E was phosphorylated in the late S or G2 at all four major phosphorylation sites. Increased phosphorylation at serine-2602 occured at G2/M transition. The increased phosphorylation might help to regulate the function of CENP-E in mitosis and $p34^{cdc2}$/cyclin B1 might be responsible for the phosphorylation. To study the correlation of tetraploid phenomenon with the function of CENP-E, the level of $p34^{cdc2}$ and cyclin B1 was determined in preimmortal SVtsA/HF-A since the free pRB might form complex with E2F to inhibit the expression of $p34^{cdc2}$ or cyclin B at 39°C.

Cells were seeded at 35°C and incubated for 24 hours before shift to 39°C. Three days after incubation at 35°C and 39°C, cells were harvested

and extracted with EBC buffer. Equal amount of cell extracts were immunoprecipitated with antibodies to $p34^{cdc2}$. The immunoblot of $p34^{cdc2}$ immunoprecipitation was reacted with antibody to $p34^{cdc2}$. Extracts from different temperatures were compared to see whether the protein levels were changed. As shown in figure 23 (panel A), the protein level of $p34^{cdc2}$ did not change after cells were cultured at 39 °C for three days (lanes 1 for 35 °C and 2 for 39 °C). Lane 3 was a control sample where antibody to HA-epitope was used. The protein level of cyclin B was also determined in a similar experiment except that the antibody used was anti-cyclin B. Cells contained same amount of cyclin B at 39°C as that at 35°C (Figure 23 panel B, lanes 1 for 35 °C and 2 for 39 °C, lane 3 for control).

These experiments did not show a decreased protein level for both $p34^{cdc2}$ and cyclin B at 39°C as proposed for an effect of Rb on transcription. However, it was possible that there was an effect on the kinase at the protein level. To become functionally active, $p34^{cdc2}$ must be complexed with cyclin B. If complex between $p34^{cdc2}$ and cyclin B was dissociated at 39°C the inactivated kinase would not be able to phosphorylate its substrates including CENP-E. To investigate the complex formation between $p34^{cdc2}$ and cyclin B, the techniques used were same as used in studying T antigen and pRB complex. Cells were seeded at 35°C and cultured for additional 24 hours at 35 °C for dishes shifted to 39°C. Three days after shifted to 39°C, cells were collected along with those incubated at 35°C. Equal amounts of total protein were immunoprecipitated with anti-cyclin B1 antibody and the immuno blot was reacted with antibodies to $p34^{cdc2}$ and cyclin B1. After incubation with

[^{125}I] conjugated anti-mouse IgG, the filter was autoradiographed. Figure 24 showed the results of this experiment. At both temperature cyclin B1 was easily detected with a increased level at 39°C (upper bands of lanes 1 for 35°C and 2 for 39°C). The p34^{cdc2} was seen at both 35°C (lower band of lane 1) and 39°C(lower band of lane 2). The complex between p34^{cdc2} and cyclin B1 was not dissociated since the level of p34^{cdc2} at 39°C increased concomitantly as that of cyclin B1 at 39°C (Figure 24).

3.14 Level of CENP-E in ts transformants

When T antigen becomes inactivated at 39°C in ts transformants, nonfunctional T antigen may decrease both expression and function of CENP-E, and result in tetraploid DNA content. The level of CENP-E was determined at both 35°C and 39°C. Preimmortal cells (SVtsA/HF-A) were seeded at 5 x 10^5 and incubated at 35°C for 24 hours before shifted to 39°C. The dishes at 39°C were harvested daily for four days. To compare the expression of CENP-E at 35°C and 39°C, the extracts containing same amount of protein were analyzed by immunoprecipitation and Western blot procedure. As shown in Figure 25, the level of CENP-E at 35°C (lane 1) was higher than that of 39°C extracts (lanes 2, 3 and 4). The longer the cells were incubated at 39°C, the lower the level of CENP-E was. The results indicated that the decreased level of CENP-E might be responsible for the tetraploid DNA content at 39°C.

Chapter 4

Discussion

Tumorigenesis is a multistep process involving both inherited and somatic genetic alterations. These alterations result in activation of oncogenes or inactivation of tumor suppressor genes to induce cells to enter a tumorigenic state. Many experimental systems were established to study the mechanism of tumorigenesis. Among them, fibroblasts transformed by DNA tumor viruses provide an excellent system for identifying the cellular processes which are responsible for malignant transformation. In order to promote their own multiplication, viruses have to alter the cellular processes of their host. The perturbance of biochemical processes and genetic structure may result in uncontrolled cell proliferation and transformation. Studies of the interactions between viral protein products and their targeted cellular proteins therefore may elucidate the mechanism of tumorigenesis.

Transformation of human fibrobalsts by simian virus 40 has been utilized for many years as a model system for tumorigenesis. Human diploid firoblasts can be transformed and their limited lifespan may be extended indefinitely following introduction of SV40. It is well established that large T antigen is responsible for cellular transformation and immortalization. However, the mechanism of these changes is not well understood. It has been demonstrated that large T antigen can form complexes with tumor suppressors pRB and p53. Formation of the

complexes phenotypically mimics inactivation of these tumor suppressors. The hypothesis is that large T antigen of SV40 interacts with growth-suppression factors of the cells and disrupts their normal function. Restoration of the function of the tumor suppressors would inhibit cellular growth.

Human fibroblasts immortalized by a SV40 genome encoding a temperature-sensitive T antigen were generated to be able to manipulate viral gene function. Such SVtsA/HF-A were characterized to determine their growth property. The transformants grew well at 35°C and ceased to proliferate after being shifted to 39°C. At nonpermissive temperature DNA synthesis was inhibited as demonstrated by incorporation of [^{3}H]-thymidine. This inhibition was due to loss of large T antigen function since T antigen was detected at a similar level at 39°C. The temperature-dependent cell growth demonstrated that functional large T antigen was indispensable for both initiation and maintenance of immortalization of human fibroblast cells.

Immunoprecipitation and Western blot analysis were used to determine complex formation between large T antigen and pRB and compare the complexes between 35°C and 39°C. SV/HF-5/39 which encodes a heat-stable T antigen was used as a control. The cell extracts from both 35°C and 39°C showed stable complexes between T antigen and pRB. However, a noticeable decrease of T antigen/pRB complexes was observed with the fibroblasts transformed by an SV40 genome encoding a heat-labile large T antigen when samples of SV/HF-5/39 and SVtsA/HF-A from permissive temperature were compared. The result was not due to

dissociation of the complexes during washing of immunoprecipitates since unwashed samples displayed the same reduction in the complex formation (data not shown). On the other hand, it is possible that the extraction process may disrupt some of preformed complexes. It should be noted that heat-labile T antigen of tsA58 has been observed to form more labile complexes with cellular proteins, such as DNA polymerase alpha, at the permissive temperature in transformed rodent cells (Gannon, 1990).

Although the complexes between T antigen of tsA58 and pRB were seen in a reduced level at 35°C, detection of the complexes was greatly reduced when extracts were prepared from cells incubated at 39°C for three days. At 39°C underphosphorylated or active pRB was released from the T antigen/pRB complexes. The free active pRB could now interact with its cellular component or components in a normal fashion to curtail the unlimited cell proliferation. This speculation is supported by the fact that dissociation of T antigen/pRB complex was concomitant with growth arrest and cessation of DNA synthesis at nonpermissive temperature. Moreover, the region (amino acid 102-115) which binds to pRB has been demonstrated involved with cell immortalization and transformation in rodent cells (Srinivasan et al., 1989). Together, these result strongly suggested that interactions between large T antigen and pRB had functional importance in maintaining transformed and immortalized phenotype of SVtsA/HF-A cells

Suppression of cell growth by pRB at 39°C is closely related to its biochemical activities. First, the carboxy-terminal of pRB can bind to DNA, although no specific sequence has been identified (Lee et al.,1987;

Wand et al., 1990). This interaction between DNA and pRB might interfere with transcription of cellular genes which are important for promoting cell growth. Inhibition of these transcripts would cause growth cessation of immmortalized human fibroblasts. Second, underphosphorylated pRB can form complex with E2F and other cellular proteins. E2F-1 is a transcription factor that is reported to activate genes required for G1 and S phase transition. This transcription factor can be replaced or dissociated from the E2F/pRB complex by either viral oncogenes in transformed cells or hyperphosphorylation of pRB before cells entering S phase. At 35°C large T antigen of SVtsA58 displaced E2F from the pRB/E2F complexes. As a free molecule, E2F transactively stimulated the expressions of genes involved in cell-growth control and promoted unlimited cellular proliferation. When the temperature sensitive transformants were shifted to 39°C, underphosphorylated pRB was released from heat-labile T antigen. Underphosphorylated pRB would be able to form the complexes with E2F and other cellular proteins to regain the cell growth control.

Experimental observations by others indicate the significance of the interaction between pRb and E2F-1 in controlling cell growth: First, little E2F-pRb complex is detected in cells which produce a nonfunctional pRb protein which is present in an uncomplexed form (Chellappan et al., 1991). Secondly, viral proteins of T antigen, E1A and E7 can dissociate the E2F-pRb complex and the dissociation is dependent on the domains which are essential for their oncogenic activity (Chellappan et al., 1992). Third, the sequences on pRb for interaction with E2F is also required to supress cell growth (Goodrich, 1991). Fourth, co-transfection of pRb and E2F inhibits

E2F-dependent cellular transcription and the inhibition is correlated with the pRb and E2F interaction (Hiebert et al., 1992). Fifth, promoters of genes which contain E2F sites include proteins necessary for DNA synthesis and cell growth control, such as DHFR (Blake, et al., 1989), thymidine kinase (Kim et al., 1991), DNA polymerase alpha (Pearson et al., 1991) and cdc2 kinase (Dalton, 1992). Many of these genes are activated in late G1 phase. These observations are in good agreement with the findings that thymidine kinase and DHFR are activated in both SV40- and adenovirus-infected cells (Yoder et al., 1983). One would suspect that the interaction between E2F and underphosphorylated pRB may play important roles in G1 arrested and senescent cells (Stein, 1990). However, the involvement of other cellular proteins in cell growth control cannot be neglected as discussed later.

p53 is another tumor suppressor which can form a complex with large T antigen of SV40. The p53-binding domain is localized to the carboxy-terminal of large T antigen and lies between amino acid 272-517. The complex between p53 and T antigen could also inactivate the function of p53 resulting in uncontrolled cell proliferation. To determine the additional factors of cell growth control, the complexes between heat-labile T antigen and p53 were studied in immortalized SVtsA/HF-A cell line. The p53/T antigen complexes were detected in the extract of cells culture at 35°C. After immortalized transformants were cultured at 39°C for three days, the complexes of T antigen/p53 were not observed. These results indicated that p53 can be inactivated by forming complex with large T antigen and lost its function as a cell proliferating inhibitor at 35°C after introduction of SV40 into HDF. The inactivation of p53 at permissive temperature may

partially contribute to uncontrolled cell growth. At nonpermissive temterature, the complexes between T antigen and p53 are disrupted. Free p53 resumes its natural ability to suppress uncontrolled cell growth.

The cellular processes in which p53 functions to control cell growth is not fully understood. p53 can interact with TATA-binding protein or other transcription factors to suppress promotors containing TATA elements (Kley et al., 1992; Mack et al., 1993). One such example is that c-fos promotor is repressed by wild-type p53. In immortalized SVtsA/HF-A cells p53 might modulate the expression of c-fos cellular oncogene to induce cells to arrest at 39°C after being freed from p53/T antigen complex. On the other hand, a body of experimental evidences indicate that p53 can transactivate expression of a cellular protein which is a inhibitor of cyclin-dependent kinase named p21$^{CIP1/WAF1}$ (El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993; Dulic et al., 1994). The promotor of the protein contains p53-binding sequence and its transcription can be induced by wild-type p53 (El-Deiry et al., 1993). Introduction of p21$^{CIP1/WAF1}$ into tumor cells, such as brain tumor cell lines and lung adenocarcinoma, can suppress their cell growth (El-Deiry et al., 1993). Moreover, p21$^{CIP1/WAF1}$ can inhibit the function of cyclin-dependent kinase by forming complex with Cdks and associated cyclins A, D, and E (Harper et al., 1993). Subsequently, the phosphorylation of their substrates, such as histone H1 and pRb, is inhibited by p21$^{CIP1/WAF1}$. These Cdk-cyclin complexes include cdc2 complexed with cyclins A and B, Cdk2 complexed with cyclins A and E, and Cdk4 complexed with cyclins D1 and D2. Furthermore, increasing the expression of p21$^{CIP1/WAF1}$ results in a reduction in the number of the cells in S-phase and the reduction can be

reversed by introduction of SV40 T antigen. This result indicates that T antigen can counteract the p21$^{CIP1/WAF1}$ function or dissociates the complexes between the inhibitor and Cdk-cyclin complex. The finding may explain the phenomenon in which the complex between the inhibitor and Cdks cannot be demonstrated in SV40 transformed HDF as shown in normal diploid fibroblasts (Xiong et al., 1993). Finally, Dulic et al (1994) demonstrated that Cdk inhibitor, p21$^{CIP1/WAF1}$ which is induced by elevated p53 subsequent to the irradiation of HDF in the G1 interval, is responsible for the cell G1 arrest. This cell cycle block is correlated with inactivation of cyclin E, and cyclin A-dependent kinases and inhibition of pRb phosphorylation. Together, these observations suggested that p53 may function as a checkpoint controller. By regulating the expression of cyclin-dependent kinase inhibitor, p21$^{CIP1/WAF1}$, p53 prevents the phosphorylation of proteins which are involved in cell growth control, such as pRb, to curtail the cell cycle progression.

Putting this information together, one could propose a model to elucidate the possible pathway by which T antigen transforms HDF. First, inactivation of pRb or p53 through gene mutations can generate tumor and cause infinite cell growth in normal cells. SV40 large T antigen introduced into HDF can inactivate both pRb and p53 by forming complexes with them to extend the their lifespan or immortalized them. The protein interactions between T antigen and the cellular components have the same effect as their gene mutations. Second, the infinite continuation of the cell cycle more than likely is due to the abnormality at the checkpoint controls, from G1 to S phase and from G2 to M phase. Experimental results indicate that these primary cell cycle transitions are controlled by Cdks.

Cdks regulate cell cycle transitions by phosphorylating proteins which are necessary for the events (Meyerson et al., 1992; Reed, 1992). Third, pRb, one of the Cdks substrates, needs to be phosphorylated to become inacitvated for cell cycle progression through checkpoints (Dulic et al., 1994; Karantza et al., 1993). Fourth, phosphorylation of pRb is potentiated by inactivation of p53 by forming T/p53 complex since complexed p53 cannot induce the Cdk inhibitor at G1/S transition; it therefore does not suppress Cdks-dependent phosphorylation of pRb. pRb is known for its antiproliferation as underphosphorylated form and becomes inactivated after being phosphorylated. Finally, pRb does not form a complex with transcription factor E2F due to its phosphorylation. Free E2F activates genes which are important for DNA synthesis and cell cycle control. To activate E2F for transcription and keep continuous cell cycle progression, T antigen not only sequesters underphosphorylated pRb but also indirectly induces its hyperphosphorylation. Apparently, pRb plays a pivotal role in relaying the signal of cell growth control and T antigen cleverly uses this system for its own reproduction or "immortalization" by living in infinitely growing cells.

Altogether, the immortal cell line of SVtsA/HF-A, by virtue of its temperature-dependent growth, has served as a useful model to study the immortalized phenomenon of viral transformed cells. The temperature-dependent proliferation of immortalized transformants is in a large T-dependent manner. The cessation of cell growth at 39^{o}C is concomitant with the inactivation of T antigen function, T/pRB and T/p53 complex dissociation. The inability of incorporation of [^{3}H]-thymidine into DNA

suggests that the cells might be blocked from entering S phase by function of either pRB or p53 at 39°C.

Although these experiments suggest that large T antigen immortalizes human cells through inactivation of pRB and p53, it cannot rule out its other roles for cellular processes. Besides formation of complex with pRB and p53, large T antigen forms complex with pRB related proteins, such as p107 and p130. These pRB-related proteins have a similar structure which is substantially homologous to the pocket regions of pRB. Like pRB, these pRB-related proteins are able to interact with viral oncoproteins in a pocket-dependent fashion. It has been shown that p107 negatively regulates the progression of cells entering S phase of the cell cycle (Zamanian et al., 1993). Moreover, p107 can also repress cellular transcriptions through interacting with E2F transcription factor (Schwarz et al., 1993). Though the function of p130 is still under investigation it would not be surprising to find that p130 may function like pRB and p107. Therefore, pRB-related proteins could well be other cellular proteins which are responsible for the immortalization of human fibroblasts in SVtsA/HF-A model system.

Genetic mutations of pRB and p53 are found in many human tumors but these mutations are not universally identified in all different tumors. Nonrandom alternations of the long arm chromosome 6 (6q) have been observed in ovarian carcinoma (Ehlen and Dubeau, 1990), non-Hodgkin's lymphoma (Schouten et al., 1990), malignant melanoma (Trent et al., 1989) and many others. These observations indicate that genes other than pRB and p53 are involved in transformation and immortalization of cells.

Pereira-Smith and Smith (1988) reported that a limited number of genes might be responsible for immortalization. In that study, complementation results showed that many SV40-transformed cells result from loss of function of a particular gene product. In Dr. Ozer's laboratory, Hubbard-Smith et al. (1992) have shown that nonrandom deletions in chromosome 6q have been identified in immortalized SVtsA/HF-A cells. These changes in the chromosome are absent in their preimmortal parental cells. At same time Ray and Kraemer (1992) also studied the deletions of chromosome sequences in SV40 immortalized human fibroblast cells. Although nine frequent chromosomal deletions were observed, abnormal chromosomes with 6q21 were identified with highest frequency while chromosomes 13 and 17 in which pRb and p53 reside were not detected in immortalized cell lines. Recently, Sandhu et al. (1994) demonstrated that SV40-immortalized cells became senescent when normal human chromosome 6 was introduced into these cells. The results suggested that the immortalization of DHF caused by SV40 needs additional cellular changes. At the begining of immortalization process, T antigen inactivates pRb and p53 to initiate this complicated event in absence of deleted tumor-suppressor genes as in preimmortal SV40 transformed cells. Since p53 is suggested to play a role in genomic stability (Bischoff et al., 1990) inactivation of this protein most likely increases the possibility of deletion of chromosome 6 in immortalized SV40 transformants.

To characterize the population distribution of preimmortal and immortal tsA-transformants, flow cytometry (FCM) was performed for cells cultured at both permissive and nonpermissive temperatures. The results showed that both preimmortal and immortal transformants had a

normal population distribution at 35°C. However, at nonpermissive temperature immortal SVtsA/HF-A showed a depletion of S phase cells with concomitant accumulation of G2 cells. There was no increase in G1 cells. This population transition was consistent with the absence of an increase in cell number and the inhibition in DNA synthesis as measured by incorporation of ^{3}H-thymidine. Experiments of prelabelling cellular DNA with ^{14}C-thymidine demonstrated that the stable cell number was not due to the balance between cell division and concominant cell loss from monolayer but growth arrest of cells following up-shift to nonpermissive temperature. FCM study of preimmortal SVtsA/HF-A also showed a similar accumulation of cells with G2 content of DNA. However, a intriguing finding in preimmortal SVtsA/HF-A was the predominance of hyperdiploid cell at 39°C.

It is expected that removal of T antigen in both preimmortal and immortal SVtsA/HF-A would revert the cells to the normal (T^{-}) phenotype with G1 phase arrest. Wright et al. (1989) transfected HDF with SV40 whose large T and small t antigens were driven by a steroid inducible mouse mammary tumor virus. Both preimmortal and immortal transformants were isolated in the presence of the inducer (dexamethasone) and their continuous growth was dependent on the inducer. Both preimmortal and immortal cells experienced G1 arrest when the expression of T and t antigens were stopped by removal of dexamethasone in their experimental system. There are obvious differences in experimental design between Dr. Ozer's laboratory and their's including the cell line used, Wright et al. did their studies in IMR-90, a fetal lung fibroblasts while we used fetal bone marrow fiboblasts.

Another difference is that the function of small t would not be impaired in SVtsA/HF-A at 39°C but both large T and small t were turned off by removal of dexamethasone. Indeed, one might even expect, the level of small t polypeptide to become elevated due to the inactivation of the autoregulatory function of large T antigen on the transcription of mRNAs of both proteins (Tegtmeyer et al., 1975), although there is no evidence to demonstrate this to be the case in these cells.

To study G2 arrest and the hyperdiploid phenomena, SVtsAdl/HF transformants were isolated by introduction of a SVtsA genome with deleted small t into HDF. FCM results showed that the preimmortal SVtsAdl/HF did not have the same profile of population distribution as preimmortal SVtsA/HF-A (Figures 9 and 10). The results demonstrated the accumulation of cells with G2 but cells with a hyperdiploid DNA content were not observed. This result suggests that small t antigen might be responsible at least in part for induction of tetraploid population at non-permissive temperature in preimmortal transformants.

Hyperploid DNA contents were also reported in SV40-infected permissive, semipermissive and nonpermissive cells. Acquisition of >G2 DNA content was shown to coincide with the accumulation of hypophosphoyrlated pRb in SV40-infected CV-1 cells (Friedrich et al., 1993). However, hypophosphorylated pRb was only restricted to the G1 phase of uninfected CV-1 cells and the cells passed M phase. These observations indicate that reappearance of hypophosphorylated pRb before the infected cells pass M phase may be relevant to the hyperploidy in infected cells. Friedrich et al. (1992, 1994) demonstrated that cells infected

with SV40 encoding temperature sensitive large T antigen (SVtsA30) accumulated tetraploid DNA content at 37°C but not at 40.5°C. At non-permissive temperature, infected cells were induced to reach G2 but not undergo a second round of DNA synthesis. These findings suggest that large T antigen could influence reappearance of hypophosphorylated pRb and induce hyperploidy in SV40 infected CV-1 cells.

To induce successive rounds of cellular DNA sythesis without mitosis, T antigen needs to uncouple the normal pathway linking the completion of S phase to mitosis. $p34^{cdc2}$ plays a important role in the pathway. Therefore, disruption of $p34^{cdc2}$ function may prohibit G2/M transition. Recently, Scarano et al. (1994) studied the function of $p34^{cdc2}$ in SV40-infected and uninfected CV-1 cells. The results showed that the activity of $p34^{cdc2}$ was inhibited in the SV40-infected cells as evidenced by a reduced H1 kinase activity compared to that of uninfected cells passing through mitosis. Moreover, electrophoresis of cyclin B-associated $p34^{cdc2}$ showed slowly migrating form of $p34^{cdc2}$ (due to hyperphosphorylation), and this hyperphosphorylated form contained phosphotyrosine. It is known that $p34^{cdc2}$ will become activated after removal of phosphate from tyrosine-15. Therefore, retaining the phosphate at tyrosine-15 may block activation of $p34^{cdc2}$ in SV40-infected cells (protein levels of $p34^{cdc2}$ kinase and cyclin B and p34cdc2/cyclin B were normal). The results indicate that either large T or small t antigen may prevents the activation of MPF through inhibition of dephosphorylation of the kinase.

Inactivation of MPF may block G2/M transition to allow cells to have a second round DNA synthesis. Both large T and small t antigens may be

involved in the second round DNA synthesis in SV40-infected or transformed cells. Large T can induce DNA synthesis by at least three functions which include binding to p53, pRb and a novel function found in the DNA-binding domain (Dobbelstein et al., 1992). The idea of influence of small t on DNA synthesis was supported by result of Cicala et al. (1994). Microinjection of small t antigen along with SV40 DNA increased viral DNA replication. Moreover, CV-1 cells (G1) infected with wild-type SV40 demonstrated that 46% of the cells with S or G2 DNA content, compared with 26% of the cells infected with SV40/dl888. The results suggests that small t antigen probably can induce cells to enter S phase and stimulate cellular DNA synthersis.

With these findings, one may build a model to explain the hyperploid phenomenon in both our experimental and infected CV-1 systems. Assumption needs to be made that hyperploid DNA content is produced by a combination of two separate events: 1) cells were blocked at G2/M transition (G2 arrest) by accumulation of hypophosphorylated pRb, 2) after the transition blockage, DNA synthesis was induced by large T antigen or large T and small t antigens. It is reasonable to suspect that hypophosphorylated pRb might block G2/M transition because hypophosphorylated pRb can interact with E2F-1 and may suppress the expression of $p34^{cdc2}$ (Dalton, S., 1992). In addition, hypophosphorylated pRb can interact with $p34^{cdc2}$ and the interaction may reduce the kinase activity. However, our experimental results showed that both $p34^{cdc2}$ and cyclin B1 protein levels were not reduced and the $p34^{cdc2}$/cyclin B1 complex was intact at at 35°C and 39°C. Thus, I would suspect that the

kinase activity was inhibited by tyrosine phosphorylation on $p34^{cdc2}$ in SVtsA transformants (like in SV40-infected CV-1 cells).

How does this model explain the tetraploid phenomenon in SV40-infected CV-1 cells and SVtsA transformants ? In SVtsA30-infected CV-1 cells, functional large T antigen produces G2 blockage by inducing hypophosphorylated pRb at 37 °C. At permissive temperature, large T antigen continuously stimulates DNA sythesis while the infected cells were blocked at G2. Thus, the tetraploid DNA content was produced at 37°C. At non-permissive temperature (40.5°C), non-functional large T antigen is less effective in DNA synthesis in the infected cells. In this situation cells would experience a G2 arrest if first round DNA synthesis were accomplished in presence of hypophosphorylated pRb at 40.5°C (the hypophosphorylated pRb likely being produced from dissociation of T-pRb complexes). If first round DNA synthesis were not accomplished, cells would remain in the G1 phase of the cell cycle.

In applying the above information to interpret the hyperdiploid phenomenon in preimmortal SVtsA/HF-A transformants, differences between the two systems cannot be neglected. First, semipermissive human diploid cells were used in the transformation experiments versus permisive monkey cells used in the infection. Second, the temperature sensitive mutants used in these experiments were not same, with tsA58 for HDF transformation and tsA30 for CV-1 infection. Although both of the ts mutants are temperature sensitive for DNA synthesis, the phenotype of different mutants may not be identical (Loeber, 1989). Both mutants show reduced ability to replicate viral DNA at nonpermissive temperature but

with different efficiency. For example, at 39°C tsA30 had a 12% replication efficiency compared with wild type virus while replication efficiency of tsA58 was below 0.1%. Topologically, tsA58 mutant has a amino acid substitution of Ala with Val at amino acid position of 438 which involves in ATP-binding. tsA30 has a substitution of Arg with Lys at position 357 which may facilitate dimerization of T antigen.

In my experiments, the tetraploid phenomenon was not observed in preimmortal SVtsA58/HF-A transformants at 35°C althrough a graduate appearance of tetraploid cells does occur over many generations (i. e., repeated passage) at the the permissive temperature or with wild-type SV40 transformants. I speculate that a failure of large T antigen to stimulate the accumulation of hypophosphorylated pRb is a more likely explanation rather than the loss of its ability to induce DNA synthesis since large T can induce DNA replication at 35°C. At 39°C, hypophosphorylated pRb was freed from T-pRb complex to block both preimmortal and immortal transformants in G2 phase. At 39°C, heat-labile large T antigen loses its function to stimulate DNA synthesis. In this situation cells will remain in G2 phase without further DNA accumulation to tetraploidy unless DNA synthesis is resumed by some other mechanism. At nonpermissive temperature, small t antigen is not affected and could function as a positive factor for DNA synthesis (Cicala et al., 1994). Tetraploid DNA content was observed in preimmortal but immortal transformants. The discrepancy can be due to their different growth properties. After shifted to 39°C, preimmortal cells can still proliferate to make confluent dish for suculture before cease growth while immortal cells stop proliferation and never make a confluent dish. The

continuous proliferation may allow small t to have an oppertunity to induce second round DNA synthesis in presence of free hypophosphorylated pRb. However, in preimmrtal SVtsA58dl transformants, cells will be blocked in G2 phase even if the transformants were able to proliferate at 39°C since positive simulator-small t was not present any more.

The following discussion will focus on the characterization of a human centromere-associated protein called CENP-E. The studies on CENP-E susgest that it plays a role in chromosomal segregation. In addition, some of its function is regulated by $p34^{cdc2}$ kinase at the onset of mitosis, and phosphatase during exit from mitosis. I characterized the expression of CENP-E during the cell cycle and also determined whether the protein is a substrate of $p34^{cdc2}$ kinase in vitro and in vivo. These investigations would result in elucidating the mechanism of cell cycle control at G2/M transition. Inactivation of CENP-E may produce mitotic arrest and lead to a second round of DNA synthesis in preimmortal cells. The study was done in Dr. Yen's laboratory at Fox Chase Cancer Center.

CENP-E is a kinesin-like motor protein which associates with kinetochores during chromosomal condensation and relocates to the spindle midzone at anaphase (Yen et al., 1992). Immunofluorescence localization of CENP-E showed that the protein was not detectable in about 90% of interphase cells. This observation suggests that the abundance of the protein might be dependent on the phase of the cell cycle. Synchronized HeLa cells showed little detectable CENP-E in cytoplasm opf G1 cells, but CENP-E levels rose sharply during late S and G2/M, ultimately reaching the level found in cells arrested in prometaphase by

microtubule inhibitors such as colcemid. The increase in steady-state levels of CENP-E correlated with the increase in synthetic rate as determined by pulse-labeling experiments (data not shown). Examination of CENP-E turnover rate by pulse-chase experiments showed that virtually all CENP-E was lost from cells after completion of mitosis. These results suggest that the expression of CENP-E is dependent on the phase of the cell cycle and the peak of the protein level coincides with the onset of mitosis. The correlation of CENP-E expression with mitosis imply that CENP-E might play an important role in mitosis

Yen et al. (1992) showed that N-terminal 335 amino acids of CENP-E share extensive homology with the motor domain of all known kinesin-like proteins. Within this region, a sequence of 120 residues is virtually identical to the highly conserved nucleotide- and microtubule-binding sites which are characteristic of the kinesin family of proteins (Endow, 1991). CENP-E, like other kinesin motor proteins, demonstrates ATP-dependent microtubule interaction. Depletion of ATP or addition of inactive ATP analogue (AMP-PNP) will stimulate co-sedimentation of CENP-E with microtubule while addition of ATP dissociates the interaction between CENP-E and microtubule. Moreover, Liao et al (1994) demonstrated that the N-terminal 540 amino acids contain the ATP-sensitive microtubule binding property. These informations strongly suggest that the N-terminal region of CENP-E is the motor domain of the protein.

A second microtubule-binding site was identified in CENP-E by deletion analysis and was confined to the COOH-terminal 99 amino acids (Liao et al., 1994). The interaction of C-terminal domain with microtubules

is different than its motor domain as it is insensitive to ATP. This microtubule-binding domain contains high proline content (13%) and has a basic isoelectric point (pI=9.4). All these properties are also observed in a separate class of microtubule-associated proteins (MAPs). But unlike MAPs, the interaction of this domain with microtubule resists salt extraction up to 2 M NaCl and is not sensitive to subtilisin-digested microtubules. Thus, the binding of the C-terminus of CENP-E to microtubules is different than conventional MAPs. Moreover, CENP-$E^{COOH368}$ extracted from mitotic cells or phosphorylated by p34cdc2/cyclin B complex in vitro cannot interact with microtubules while the unphosphorylated form that is extracted from interphase cells can. Finally, immunoflurescence staining of transfected CENP-$E^{COOH368}$ showed that the C-terminal fregment is diffusely distributed throughout the cytoplasm in metaphase cells but is colocalized with microtubules in anaphase cells.

These results demonstrate that phosphorylation of CENP-E by p34cdc2/cyclin B can regulate the interaction between C-terminal domain of CENP-E and microtubules in vivo. At the onset of mitosis, phosphorylation of CENP-E carboxyl terminus prevents it from binding microtubules. As the cells enter anaphase, the p34cdc2/cyclin B complex becomes inactivated and subsequent the dephosphorylation of C-terminal domain activates microtubule binding. Along with the microtubule binding by the N-terminus, CENP-E may function as a microtubule cross-linker during anaphase. The function of cross-linking may help to stabilize the overall structure of the anaphase spindle or serve to push the overlapping

antiparallel microtubules past each other and elongate the spindle poles during anaphase B.

Inspection of the amino acid sequence of CENP-E revealed consensus phosphorylation sites for MPF at the carboxyl terminus. Together, these findings suggest that not only might CENP-E be involved in the mitotic process but also its function may be regulated by MPF which triggers cell entry into mitosis. To confirm this hypothesis, the C-terminal fragment of CENP-E was labeled both in vivo and in vitro followed by amino acid content analysis. Purified CENP-$E^{COOH368}$ fragment was labeled by combination of recombinant Cdks and cyclin in which $p34^{cdc2}$ and cyclin B1 showed highest activity in the in vitro labeling experiment (Figure 13). Combinations of cdk/cyclin A and cdk/cyclin B showed little activity while $p34^{cdc2}$/cyclin A exhibited moderate activity. To compare the phosphorylation pattern of this domain in vivo, the same fragment of CENP-$E^{COOH368}$ was introduced into Hela cells by transfection. Cells were labeled with [^{32}P]-orthophosphate and interphase and mitotic cells were separated after mechanical shakeoff. CENP-$E^{COOH368}$ fragment from both interphase and mitotic cells was phosphorylated. A slower migrating form of CENP-$E^{COOH368}$ fregment was observed in mitotic cells (Figure 14, panels A and B). Phosphorylation study of endogenous CENP-E showed that the protein was preferentially phosphorylated in mitosis while phosphorylation of CENP-E in interphase cells was in a much reduced level (Figure 15). These results demonstrate that CENP-E becomes phosphorylated after its expression and extra phosphoric group or groups are added to the protein as cells enter mitosis.

Comparison of the phosphorylation intensity between endogenous CENP-E and the exogenous C-terminal fregment, it is obvious that full length CENP-E became more heavily phosphorylated in mitosis than in interphase while labeling of the C-terminal fragment showed the opposite result. Immunoprecipitations of labeled endogenous CENP-E from either interphase cells or mitotic cells were successively performed by using the supernatant after clearing the exogenous CENP-E with anti-HA antibody. Since same cell extracts were used, factors such as cell growing status or technical handling between differ experiments were unlikely causes. It is possible that expression of exogenous CENP-E under the control of viral promotor used in the DNA construct is not regulated by the cell cycle. Therefore, the transfected C-terminus may be overexpressed in the interphase cells while the expression of endogenous CENP-E is not.

Experiments in vitro and in vivo demonstrate that CENP-E can be phosphorylated at least at the C-terminus of the protein with the combination of $p34^{cdc2}$/cyclin B1 showed highest activity. This is consistant with observations that this is the predominant complex in mitosis in HeLa cells. This finding suggests that $p34^{cdc2}$/cyclin B1 could be the kinase that phosphorylates CENP-E in vivo. This was confirmed by two methods:

1), Comparison of in vitro and in vivo phosphopeptide map showed overlapping patterns that indicate four of the same residues are phosphorylated in vivo and in vitro. Although phosphopeptide mapping generated from both in vitro and in vivo labeling experiments revealed similar pattern and intensity (Figure 16 and Figure 17), phosphopeptide map derived from kinase reaction in vitro demonstrates six additional

minor spots (Figure 16, spots 5-10) which cannot be seen in in vivo mapping while in vivo mapping contains three spots (Figure 17) which cannot be detected in in vitro. These phenomena suggest that spots 5, 6 and 7 of in vivo mapping could be phosphorylated by kinase other than $p34^{cdc2}$/cyclin B1 or preferentially phosphorylated under in vivo condition. Failure to show the in vitro spots 5 to 10 could be due to the weak signal in in vivo labeling experiment. Spots 5 to 10 in the in vitro phosphopeptides could also be preferentially phosphorylated under in vitro condtions or some contaminating phosphorylated protein in the kinase reaction.

2), Direct mapping of the phosphorylation sites by mutagenesis showed that they lie in consensus phosphorylation sites for p34cdc2/cyclin B kinase complex. Mutagenesis of the putative phosphorylation sites for $p34^{cdc2}$/cyclin B1 not only confirmed the sites involved in phosphorylation but also helped to verify these extra spots (Figure 19). Since spots 1 to 4 were seen in both in vitro and in vivo it could be that these four represented the complete digestion of the C-terminal fragment. If this were the case, spots 5 and 6 of in vitro labeling fregment were the partial digestion of spot 4 since these two cannot be seen when spot 4 cannot be phosphorylated. Spots 9 and 10 were the partial digestion of spot 1 and cannot be detected in spot 1 site mutation. Phosphopeptides 7 and 8 were consistantly seen in all four mutations of their 2-D mapping. These two phosphopeptides could be either due to extra serine sites in in vitro labeling or a protein co-migating with CENP-$E^{COOH368}$ fragment. Quadruple mutation for in vitro mapping failed to show spots 7 and 8 (data not shown). This result suggests that phosphorylation of spots 1-4 could be important for phosphorylation of spots 7 and 8 since spots 7 and

8 can be seen consistantly in all four mutations. As expected, quadruple mutation of all serine sites in in vivo mapping also failed to show those 10 spots seen in in vitro phosphopeptide mapping (Figure 21) but those three additional spots seen in in vivo. Together, these results indicates MPF phosphorylates the same serine in in vivo as does in vitro. Moreover, other kinase may also be involved in CENP-E phosphorylation in vivo.

The finding of four major phosphopeptides is in good agreement with amino acid sequence analysis which revealed four consensus phosphorylation sites for MPF. Further more, phosphoamino acid analysis demonstrated that serine is the only amino acid that was phosphorylated (Figure 18). These results along with the mutagenesis strongly suggest that MPF is the kinase which regulates the phosphorylation state of the C-terminus of CENP-E. Phosphopeptide mapping of in vivo labeled C terminal fregment revealed that spots 1 to 7 were phosphorylated in interphase cells. Among these, only spot 3 got heavily phosphorylated in mitotic cells (Figure 17 A and B). This change indicates that some aspect of CENP-E's function may be affected by phosphorylation during the transition of G2 phase to mitosis in the cell cycle.

Sequence of CENP-E indicates that the protein is a kinesin-like microtubule-based motor protein. In general, motor proteins can be classified as microtubule-based and microfilment-based motors (Sawin and Scholey, 1991). These motors hydrolyze ATP to generate energy to move along microtubules (kinesin and dynein) or microfilment such as myosin. The movement depends on at least two domains of a motor protein, motor domain and anchorage domain. The motor domain

hydrolyzes ATP to generate force while the anchorage domain binds to tubulin or actin. Combination of the two factors results in the movement of motor which transports specific cargo such as vesicles or chromosomes along its track.

Based on these informaions, a model of chromosomal segregation involving CENP-E could be proposed. During each cell cycle, CENP-E accumulates to high levels at G2/M boundary. Since the C-terminus of CENP-E can be phosphorylated as cells mitosis, these phosphorylations prevent this domain from binding microtubules. In addition to regulating microtubule binding by MPF, the hyperphosphorylation of serine residue 2602 (spot 3) at the C-terminal CENP-E may regulate binding of this protein to kinetochores of the chromosomes during prometaphase. Thus, CENP-E acts to push chromosomes toward metaphase-plate as its carboxyl terminus is fixed the kinetochore with amino terminus "walking" along the microtubule in plus end direction.

During anaphase, $p34^{cdc2}$/cyclin B becomes inactivated, and the activation of unidentified phosphatase removes the phosphates from the C-terminal CENP-E. Dephosphorylation may disrupte the interaction between the C-terminal and kinetochores. This interruption probably not only dissociates the CENP-E from kinetochores but also initiates the interaction of C-terminus with microtubules at midzone of the spindle at anaphase. With C-terminus anchored on a microtubule, the N-terminal motor domain reaches to an adjacent antiparallel microtubule and walks toward the plus end of the microtubule to push the spindle poles apart.

Finally, CENP-E concentrates in the developing midbody and becomes degraded as cells complete mitosis (Brown et al, 1994).

According to the above hypothesis, inactivation of both CENP-E and its function regulator-MPF could contribute to the sessation of mitosis and hyperploid DNA content in the tsA transformants at nonpermissive temperature or SV40-infected monkey cells. The protein level of CENP-E, $p34^{cdc2}$ and cyclin B, and MPF complex was compared between permissive and non-permissive temperature in preimmortal SVtsA/HF-A. Results showed that both protein level of $p34^{cdc2}$ and cyclin B did not decrease but increased to some extent (Figure 23). The complex between $p34^{cdc2}$ and cyclin B also remained complexed at nonpermissive temperature (Figure 24). These results did not exclude the possibility of retaining of phosphate(s) group at tyrosine-15 of $p34^{cdc2}$. At non-permissive temperature, although the proteins level sustained and complex remained intact retaining of phosphate(s) might prevent MPF to be activated.

Comparison of CENP-E level between 35°C and 39°C revealed decreased expression of the protein at 39°C (Figure 25). Since CENP-E is involved in chromosomal motion during mitosis, inhibition of the protein expression could impede or delay the cell cycle progression to allow a second round DNA synthesis or arrest cells in G2/M phase of the cell cycle. Thus, hyperploidy and G2/M arrest in tsA preimmortal and immortal transformants may be caused by combination of two factors which are inactivation of $p34^{cdc2}$/cyclin B kinase complex and inhibition of the expression of kinesin-like motor protein, CENP-E.

Figure 1. Growth curves of immortal AR5 and HAL cells

5 x 10^4 cells were seeded in 60 mm diameter dishes at 35°C. In panel A, replicate sets of dishes of AR5 were shifted to 39°C at the times indicated by arrows. In panel B, HAL cells were propagated for several days at 35°C and then shifted to 39°C (at day 0) or maintained at 35°C. Cell number was determined as described in chapter 2. Symbols: solid circles represent cell number at 35°C, open circle represent cell number at 39°C. The two growth curves were performed separately.

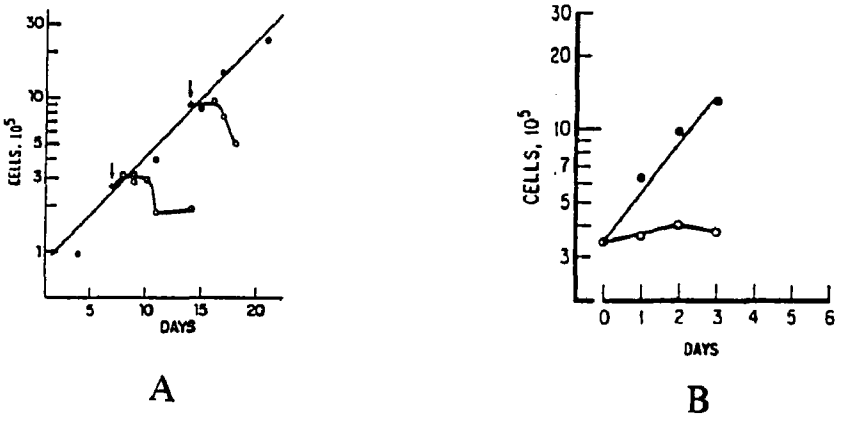


Figure 1. Growth curves of immortal AR5 and HAL cells

Figure 2. DNA synthesis of AR5 by [^{3}H] thymidine incorperation

Cells (4 x 10^5) were plated into 100-mm dishes and incubated at 35 ^{o}C for 24 hours. Replicate sets of dishes were shifted to 39^{o}C at day 0. At days 0, 1, 2 and 3, cultures from 35^{o}C and 39^{o}C were incubated with [^{3}H] thymidine for 3 hours and TCA-precipitable counts were determined. Symbols: solid circles represent cell number at 35^{o}C, open circle represent cell number at 39^{o}C.

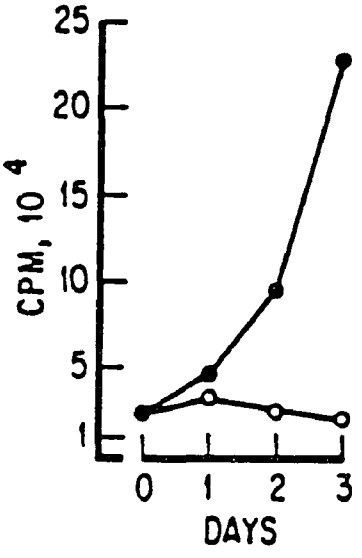


Figure 2. DNA synthesis of AR5 by [^{3}H] thymidine incorporation

Figure 3. Immunoblot for T antigen

AR5 cells were seeded at 35°C and incubated for 3 days at 35 °C (lane 1) or 39°C (lane 2). Cells were harvested and extracted, and equivalent amounts of protein were immunoprecipitated with the monoclonal antibody PAb419 as described in chapter 2. The amount of large T antigen in the immunoprecipitates was quantified by immunoblot, using monoclonal antibody PAb419 and peroxidase-conjugated goat antimouse immunoglobulin G. Prestained markers of 200, 97, 68, 45, and 25 kilodaltons are in lane M.

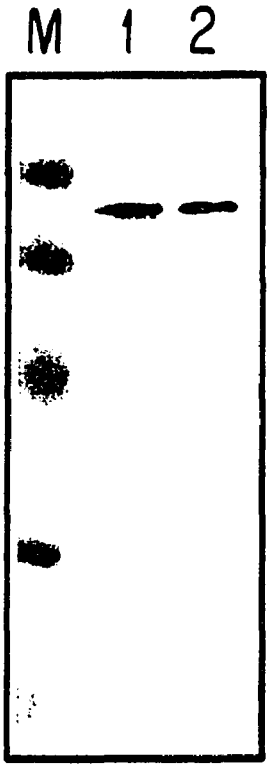


Figure 3. Immunoblot for T antigen

Figure 4. Replication of plasmid DNA containing SV40 origin in AR5 cells

Cells were seeded at 35°C and transfected for 4 hours on the following day with 1 ug of pSVO with carrier DNA (lanes 1 and 2) or carrier DNA alone (lanes 3 and 4) as described in chapter 2. Cultures were maintained at 35°C (lanes 1 and 3) or 39°C (lanes 2 and 4) for three days. DNA was extracted by the Hirt procedure and analyzed for replicated SV40 sequences by the Southern blot procedure. DNA was digested with Dpn1 and EcoR1 (to linearized all forms) before electrophoresis. pSV0 labeled with [^{32}P]dCTP by the random-primer method was used as a probe.

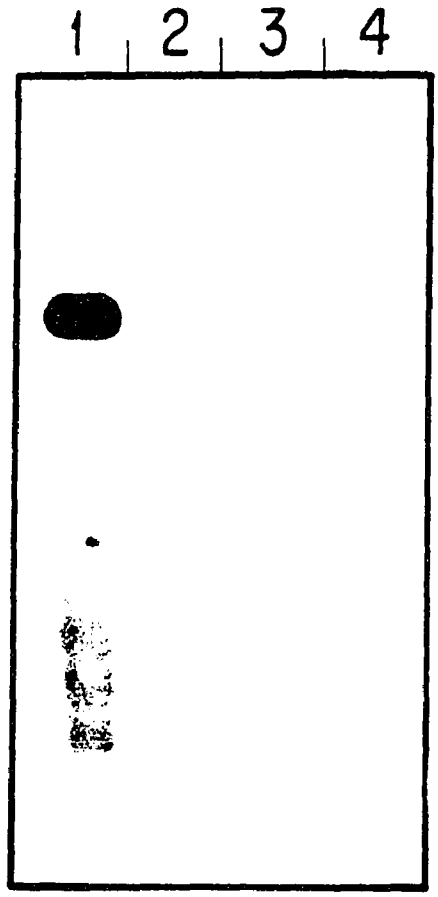


Figure 4. Replication of plasmid DNA containing SV40 origin in AR5 cells

Figure 5. pRb-T complexes in SVtsA58-transformed human cells

Extracts were prepared from SVtsA58-transformed HAL cells cultured at 35°C and analyzed for Rb-T complexes by immunoblot as described in chapter 2. Immunoprecipitation was performed with pAb 419 to T antigen (lane 1), PAb C36 to pRb (lane 2), and control antibody to *E. coli* RNA polymerase (lane 3). The immunoblot was initially reacted with antibody to T antigen and horseradish peroxidase-conjugated antiglobulin and subsequently with antibody to pRb and alkaline phosphatase-conjugated antiglobulin. Markers are indicated for Rb and T antigen (T).

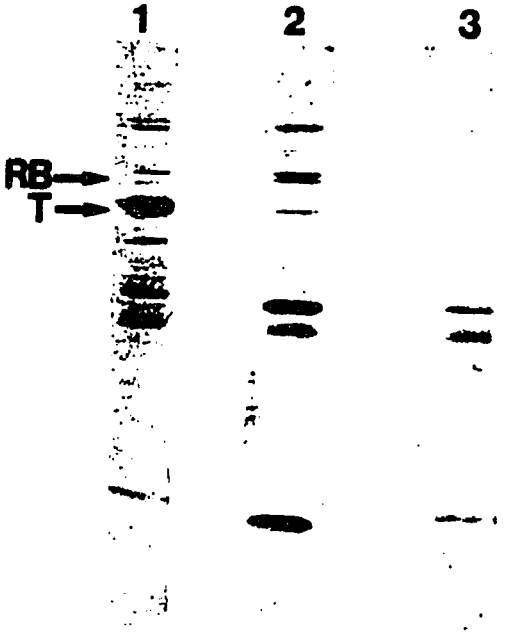


Figure 5. pRb-T complexes in SVtsA58-transformed human cells

Figure 6. pRb-T complexes in temperature-sensitive and temperature-independent human cell lines

Cells were seeded at 1 x 10^6 in 100 mm dishes at 35°C, and cultured at 35°C and 39°C for three days. SVtsA58-transformed AR5 cells (panel A) and SV40-transformed SV/HF-5/39, a temperature-independent transformants which showed continuous cell growth at 39°C (panel B), were harvested for immunoprecipitation. Extracts containing equal amount of protein were immunoprecopitated in all cases, but different amounts of the dissolved precipitate (in 50 ul) were analyzed. Lanes 1, 2, 3, 7, and 4, 5, 6, were the samples from 35°C and 39°C respectively for both panel A and B. Immunoprecipitation was performed with PAb419 to T antigen (lanes 1 to 6) and PAb C36 to pRb (lane 7). The immunoblot was reacted with both PAb 419 and PAb C36 and developed with alkaline phosphatase-conjugated antiglobulin. Lane 1, 2 ul; lane 2, 8 ul; lane 3, 40 ul; lane 4, 2 ul; lane 5, 8 ul; lane 6, 40 ul; lane 7, 50 ul. Markers are indicated for pRb, T antigen (T), and IgG heavy chains (H).

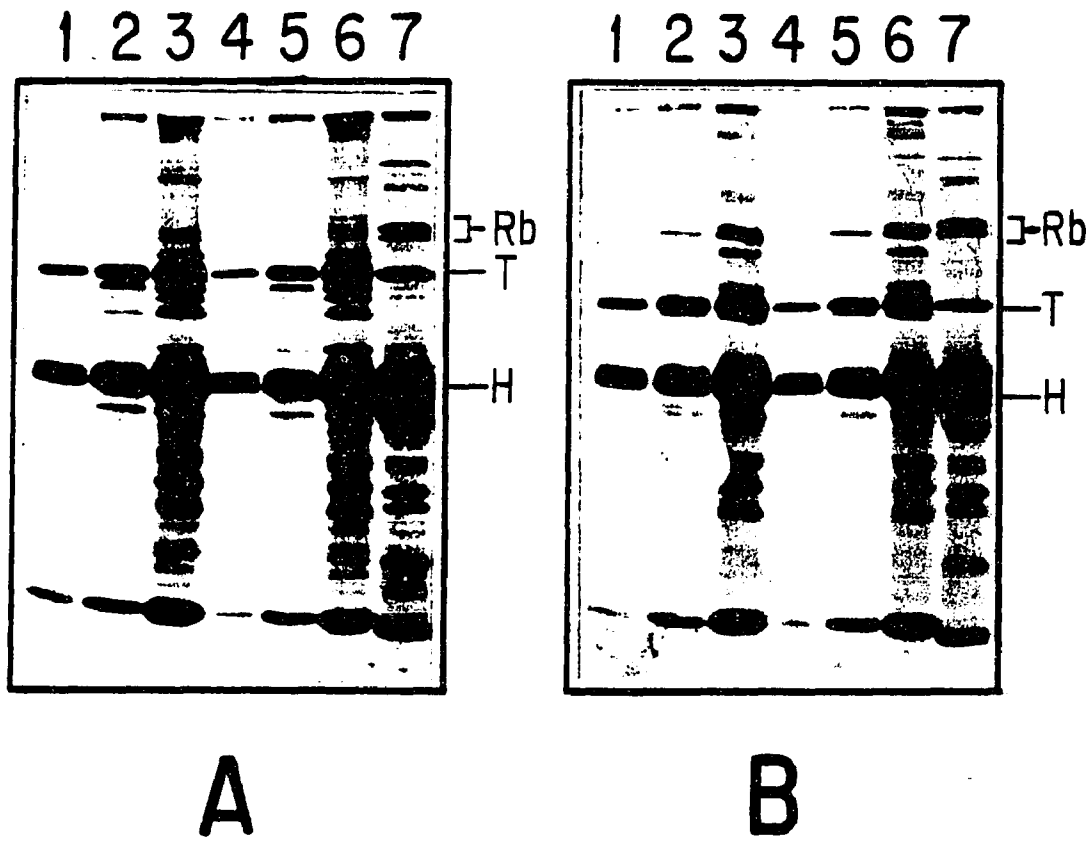


Figure 6. pRb-T complexes in temperature-sensitive and temperature-independent human cell lines

Figure 7. p53-T complexes in human cell lines

HAL cells (panel A), SV/HF-5/39 (panel B) and SV40-transformed HAL cells (HAL/SV-WT) (panel C) cultured for 3 days at 35°C (lane 1, 3, and 5) or 39°C (lanes 2, 4, and 6) were analyzed after immunoprecipitation with different monoclonal antibodies as described in the legend to Fig. 5. Immunoprecipitation was performed with PAb 421 to p53 (lanes 1 and 2), PAb 419 to T antigen (lanes 3 and 4), and control antibody to *E. coli* RNA polymerase (lanes 5 and 6). The immunoblots were reacted with PAb 421 and PAb 419 and developed with alkaline phosphatase-conjugated antiglobulin. Immunoblots of panel A and B were performed together and separately from that of panel C. Markers are indicated for T antigen (T), p53, and IgG heavy chain (H).

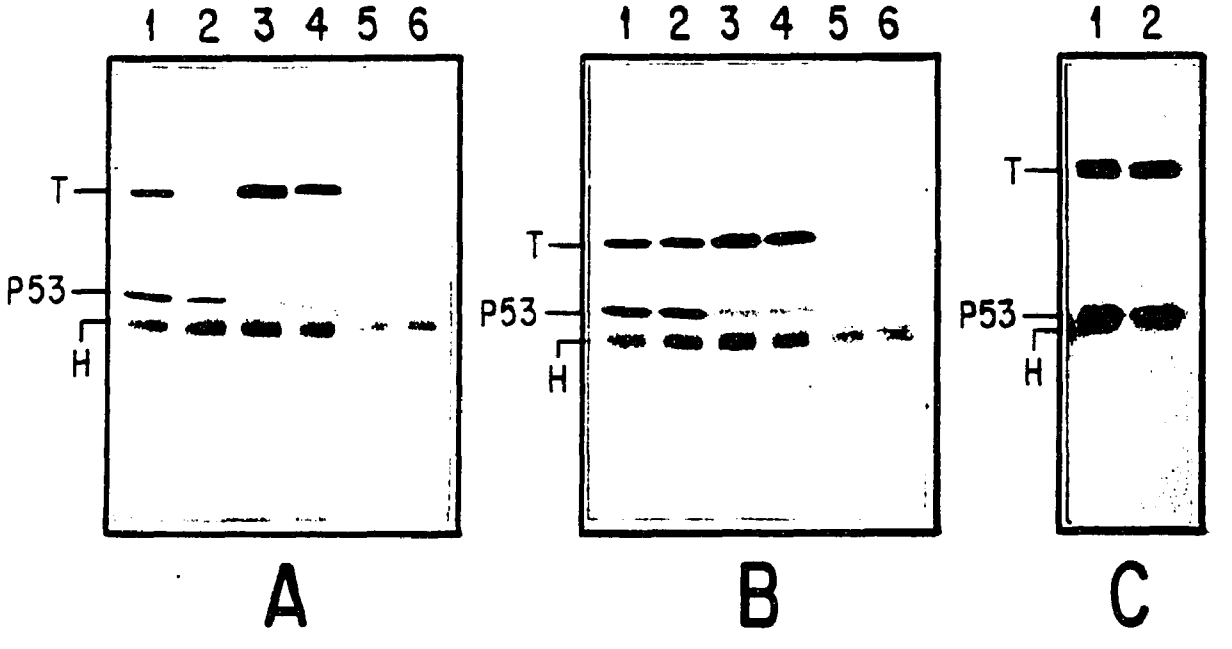


Figure 7. p53-T complexes in human cell lines

Figure 8. Cell cycle analysis of immortal SVtsA/HF-A

Dishes were seeded with immortal SVtsA/HF-A cells (passage 24) and incubated at 35°C until cells were verified to be in logrithmic growth. On day 0, replicate cultures were shifted to 39°C. Cells were harvested at daily intervals and analyzed by flow cytometry as described in chapter 2. (A) day 0, 35°C; (B) day 1, 39°C; (C) day 2, 39°C; (D) day 3, 39°C; (E) day 4, 39°C; (F) day 4, 35°C. Frequency on the ordinate represents number of cells, and channel number on the abscissa represents relative DNA content per cell.

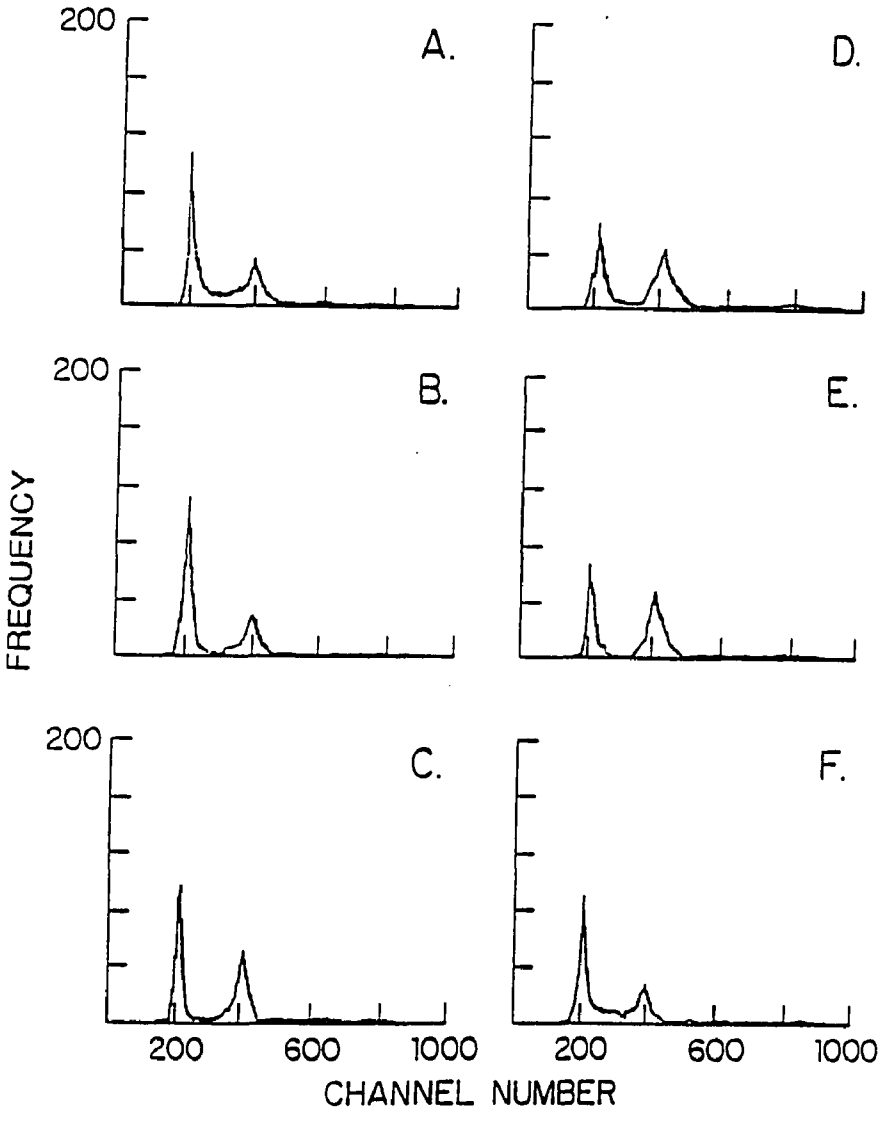


Figure 8. Cell cycle analysis of immortal SVtsA/HF-A

Figure 9. Cell cycle analysis of preimmortal SVtsA/HF-A

Cells were harvested and analysed by FCM as described in figure 8. Dishes were seeded at 35°C and maintained at 35°C (panel A) or shifted to 39°C for 4 days (panel B) followed by subculture at 39°C for 1 day (panel C), 2 days (panel D), 3 days (panel E) and 4 days (panel F) as described in the text. Frequency on the ordinate represents number of cells, and channel number on the abscissa represents relative DNA content per cells.

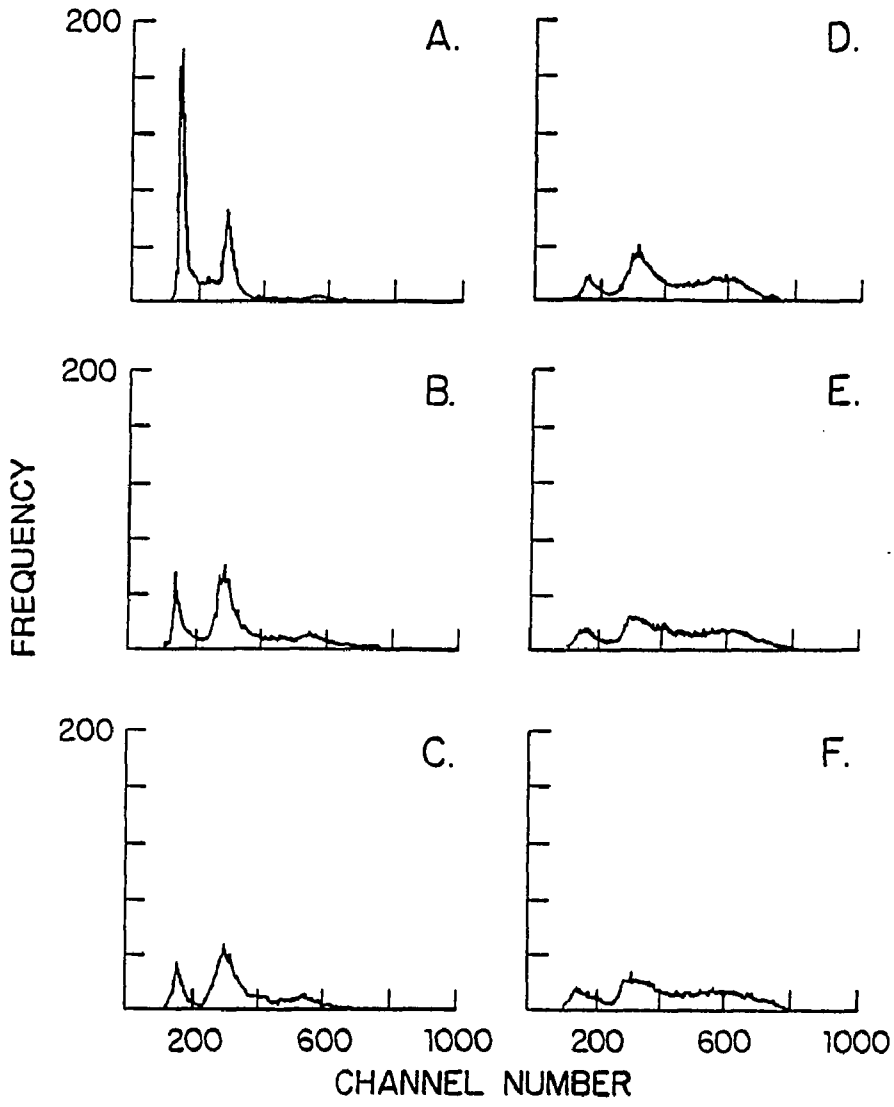


Figure 9. Cell cycle analysis of preimmortal SVtsA/HF-A

Figure 10. Cell cycle analysis of preimmortal pRNSVtsAdl

Cells were harvested and analysed by FCM as described in figure 8. Dishes were seeded at 35°C and maintained at 35°C or 39°C for 4 days and then subcultured for continuous incubation. Panels A and F represented subcultures at 35°C for 1 and 4 days respectively. Panels B, C, D and E were subcultures at 39°C for 1, 2, 3 and 4 days respectively. Frequency on the ordinate represents numer of cells, and channel number on the abscissa represents relative DNA content per cells.

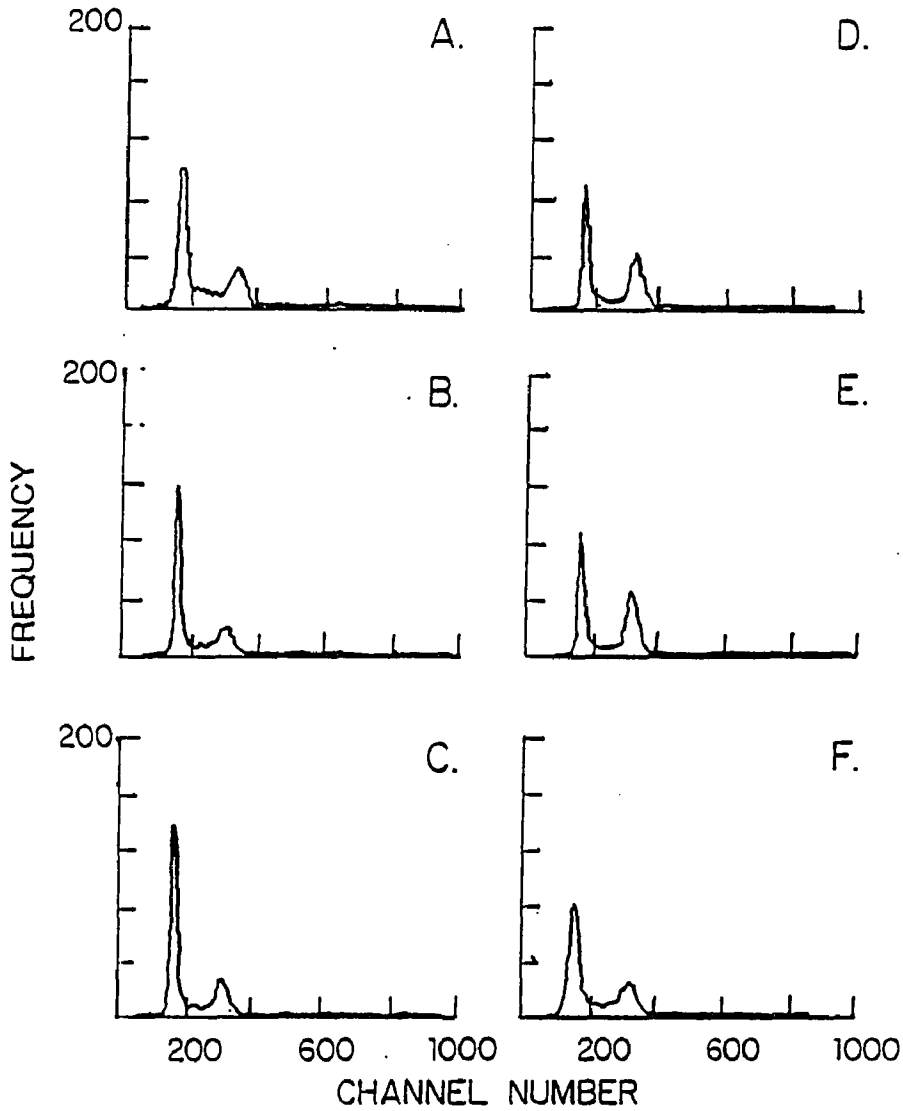


Figure 10. Cell cycle analysis of preimmortal pRNSVtsAdl

Figure 11. Cell cycle-dependent accumulation of CENP-E

Steady-state levels of CENP-E in the cytoplasm (panel A) and nuclei (panel B) of cells at different stages of the cell cycle (marked above the panel). Suspension HeLa cells were synchronized at G1/S by successive thymidine and aphidocolin blocks. Cells were released from the G1/S boundary and samples were collected at 2 hours intervals (marked below the panel) and extracts with the same concentration of protein were used for immunoassays as described in chapter 2. The pelleted nuclei and insoluble debris were boiled in SDS-sample and applied directly to the gel. Polyclonal antibody to CENP-E was used for both immunoprecipitation and immunoblot analysis. [^{125}I]-labeled protein A was used to detect bound primary antibodies. Col, cells blocked in mitosis by addition of colcemid. Markers are indicated for CENP-E and protein markers 202kD, 116,kD 96kD. Cell synchrony was monitored by flow cytometry.

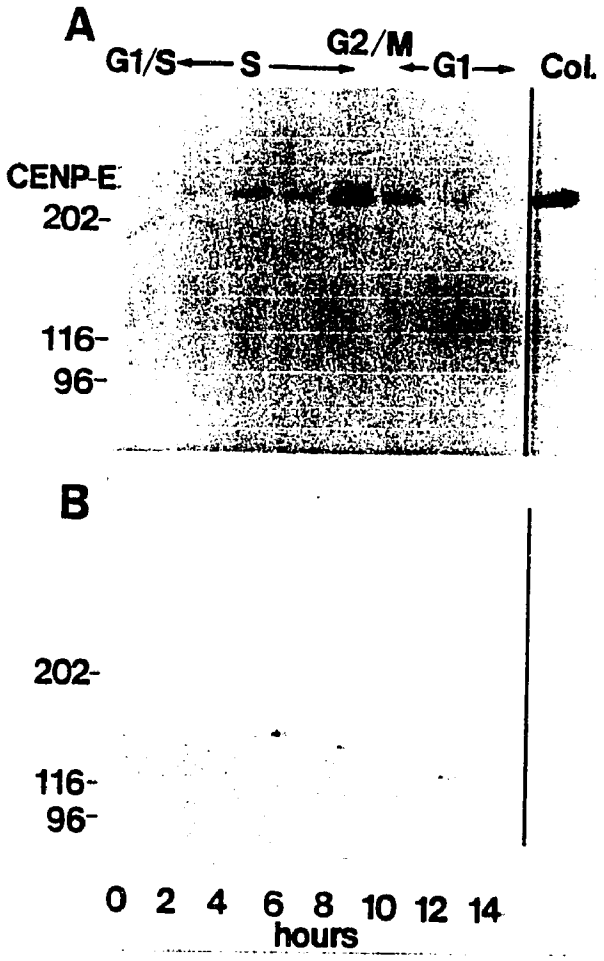


Figure 11. Cell cycle-dependent accumulation of CENP-E

Figure 12. CENP-E degradation by pulse-chase analysis

The turnover rate of CENP-E was examined by pulse-chase experiments. Synchronized late S or G2 phase cells were pulse-labeled with [^{35}S]-methionine for 1 hour. Cells were washed after labeling and an aliquot of cells was immediately lysed as a zero time point (marked below the panel). The remaining cells were chased in unlabeled complete medium and samples were taken at 1, 2, 4 hours that correspond to G2/M, G1 and G1, repsectively (marked above the panel). Cell lysate were processed for immunoprecipitation, SDS-PAGE and autoradiography as described in chapter 2. Markers are indicated for CENP-E and protein markers of 202kD, 116kD and 96kD. Col, colcemid.

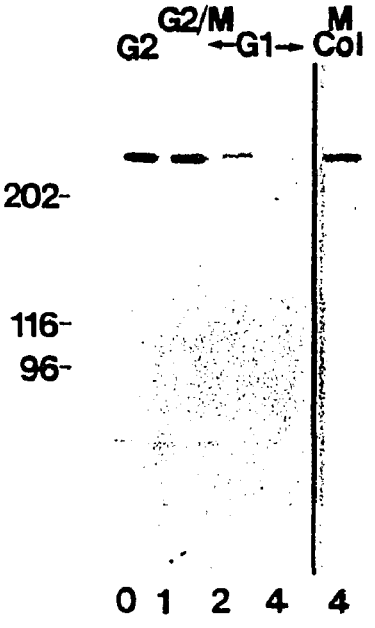


Figure 12. CENP-E degradation by pulse-chase analysis

Figure 13. In vitro phosphorylation of C-terminal fragment of CENP-E with recombinant cdks

The activity of different cdks toward the C-terminal fragment of CENP-E was tested. Bacterially expressed and purified CENP-E^{COOH368} fragment was incubated with either recombinant human p34^{cdc2} and cdk2 that were complexed with cyclin A or cyclin B1 in equal amount of a hypotonic baculovirus infected sf-9 cell lysate. Lanes 1, 2, 3 and 4 represent combinations of p34^{cdc2}/cyclin A, p34^{cdc2}/cyclin B1, cdk2/cyclin A and cdk2/cyclin B1 respectively. After the labeling reaction the samples were boiled in the sample buffer and subjected to SDS-PAGE followed by autoradiography. Markers are indicated for the C-terminus of CENP-E (C) and protein markers of 66kD, 57kD, 40kD.

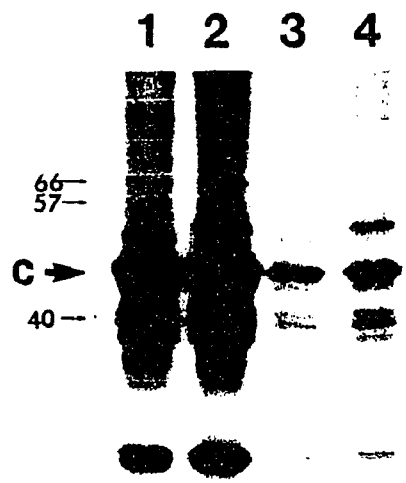


Figure 13. In vitro phosphorylation of C-terminal fragment of CENP-E with recombinant cdks

Figure 14. In vivo phosphorylation of transfected CENP-E^{COOH368} fragment in mitotic HeLa cells

An expression vector encoding the C-terminal fragment of CENP-E fused to the HA epitope-tag was transfected into HeLa cells. Twenty hours after removing of the DNA precipitate, transfected Hela cells were labeled with [^{32}P]-orthophosphate (1 mCi/ml) for 4 hours. After labeling, mitotic cells were separated from interphase cells by mechanical shake-off. Cell extracts were immunoprecipitated successively with antibodies to HA followed by C-terminus-specific antibody to reduce the background. After the second immunoprecipitation, the samples were boiled in the sample buffer and subjected to SDS-PAGE followed by autoradiography. Lanes 1 and 2 of panel A represents mitotic and interphase cells respectively. Panel B was the immunoblot of unlabeled, transfected samples with lanes 1 and 2 for mitotic and interphase cells respectively. Markers on the side are indicated for the C-terminus of CENP-E (C)and protein marker of 40kD.

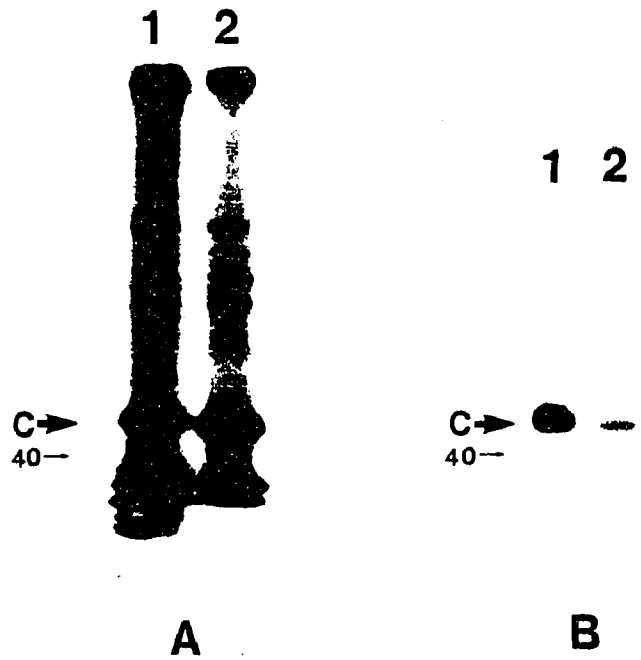


Figure 14. In vivo phosphorylation of transfected CENP-E^{COOH368} fragment in mitotic HeLa cells

Figure 15. Phosphorylation of endogenous CENP-E at mitosis

Supernatants of mitotic and interphase HeLa cells were immunoprecipitated with polyclonal antibodies to CENP-E and processed the same way as for the transfected C-terminus. Lanes 1 and 2 are samples from mitotic and interphase cells respectively. Markers on the side are indicated for CENP-E (E) and protein marker of 202kD.

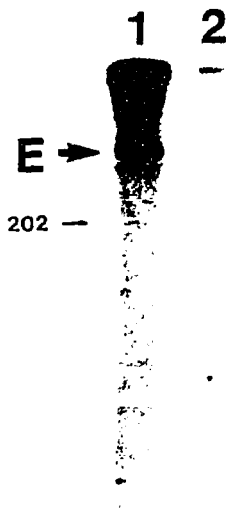


Figure 15. Phosphorylation of endogenous CENP-E at mitosis

mitosis

Figure 16. Phosphopeptide map of in vitro phosphorylated C-terminus of CENP-E

Phosphopeptides generated by tryptic digestion of in vitro phosphorylated C-terminus were resolved in two dimensions on TLC plates. In vitro phosphorylated C-terminal fragment was excised from the gel after SDS-PAGE. The gel slice was digested with trypsin and samples were separated by electrophoresis in the first dimension (arrow) followed by ascending chromatography in the second dimension (arrow). The TLC plate was analyzed by autoradiography or Fujix Bio-imaging Analyzer (Fuji Photo) for phosphopeptides. 10 spots (1 to 10) were detected in the map as indicated by arrows.

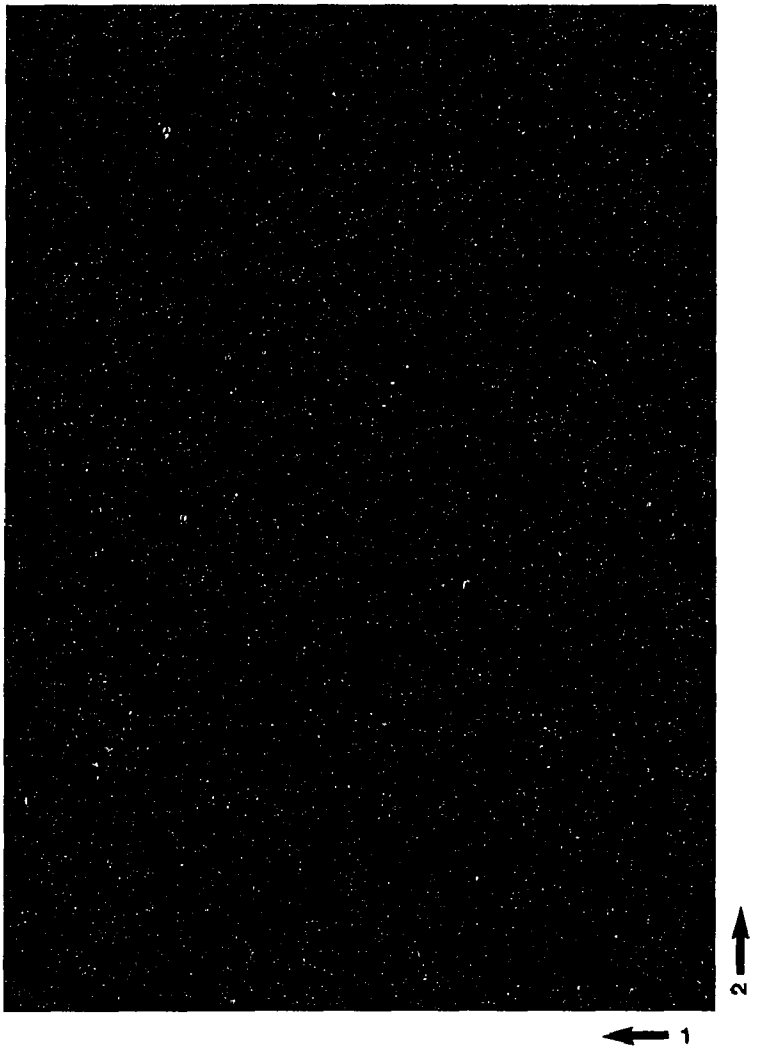


Figure 16. Phosphopeptide map of in vitro phosphorylated C-terminus of CENP-E

Figure 17. Phosphopeptide map of in vivo phosphorylated C-terminus of CENP-E

The number of phosphopeptides generated by tryptic digestion of in vivo labeled C-terminal CENP-E were determined in two dimensions on TLC plates. HeLa cells were transfected for the expression of C-terminal CENP-E and phosphorylation in vivo. After the labeling, mitotic cells were separated from the interphase cells by mechanical shake-off. Interphase cells were extracted and immunoprecipitated for the C-terminus. After SDS-PAGE, the correspondent gel slice (containing labeled protein) was excised from the gel and digested with trypsin. Digested samples were applied at the origin (ori) followed by two dimenssions TLC analysis and analyzed by Fujix Bio-imaging Analyzer (Fuji Photo) for phosphopeptides. 7 spots (1 to 7) were detected in the phosphopeptide map as indicated. 17A shows the phosphopeptide map of interphase cells. 17B shows the phosphopeptide map of mitotic cells.

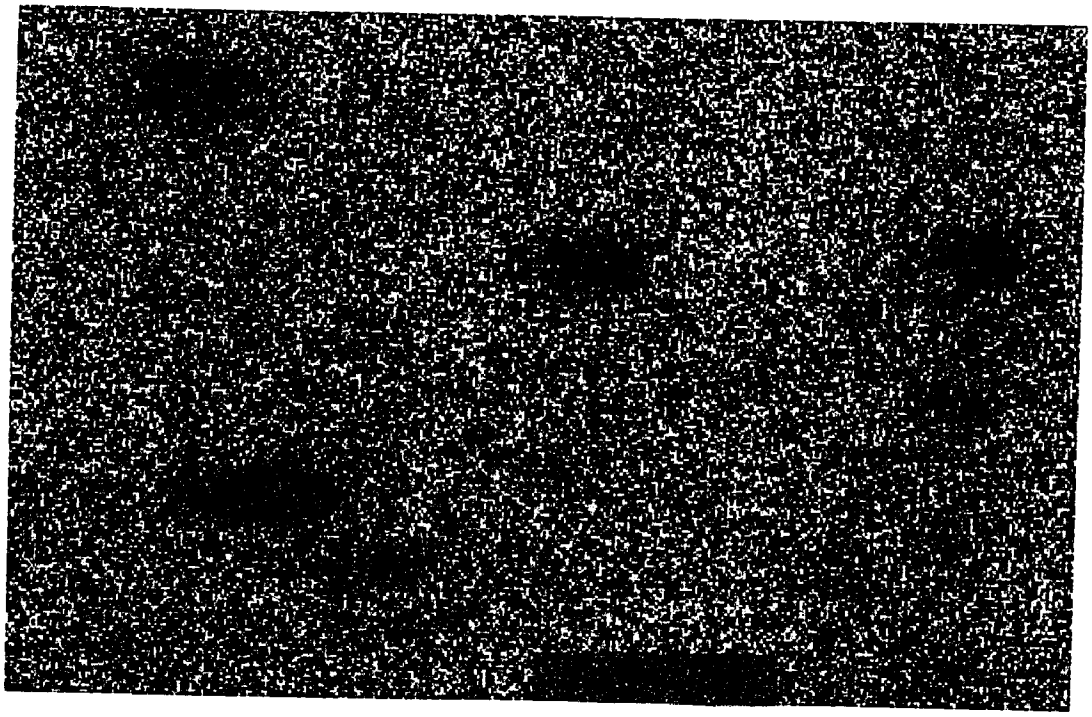


Figure 17A Phosphopeptide map of in vivo phosphorylated C-terminus of CENP-E

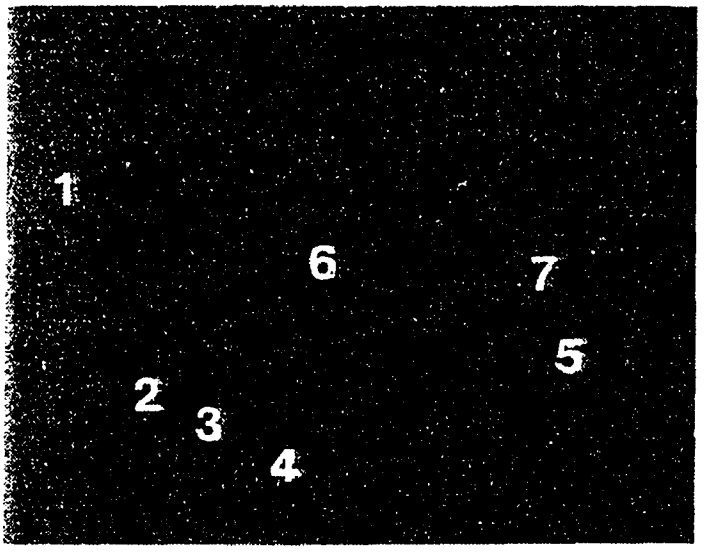


Figure 17B. Phosphopeptide map of in vivo phosphorylated C-terminus of CENP-E (mitotic cells)

Figure 18. Phosphoamino acid analysis of in vitro-phosphorylated CENP-$E^{COOH368}$

In vitro-phosphorylated CENP-$E^{COOH368}$ fragment was subjected to SDS-PAGE for separation. The correspondent gel slice was excised from the gel and digested with trypsin. The sample was then hydrolyzed with 6N HCl to obtain individual amino acids. After the acid hydrolysis, the sample was applied to TLC for two-dimenssion analysis as described in chapter 2. Autoradiography demonstrated that only one kind of phosphoamino acid comigating with serine control was detected. Dotted circle were the controls of phosphoserine (Y), phosphothreonine (T) and phosphoserine (S).

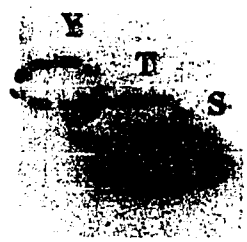
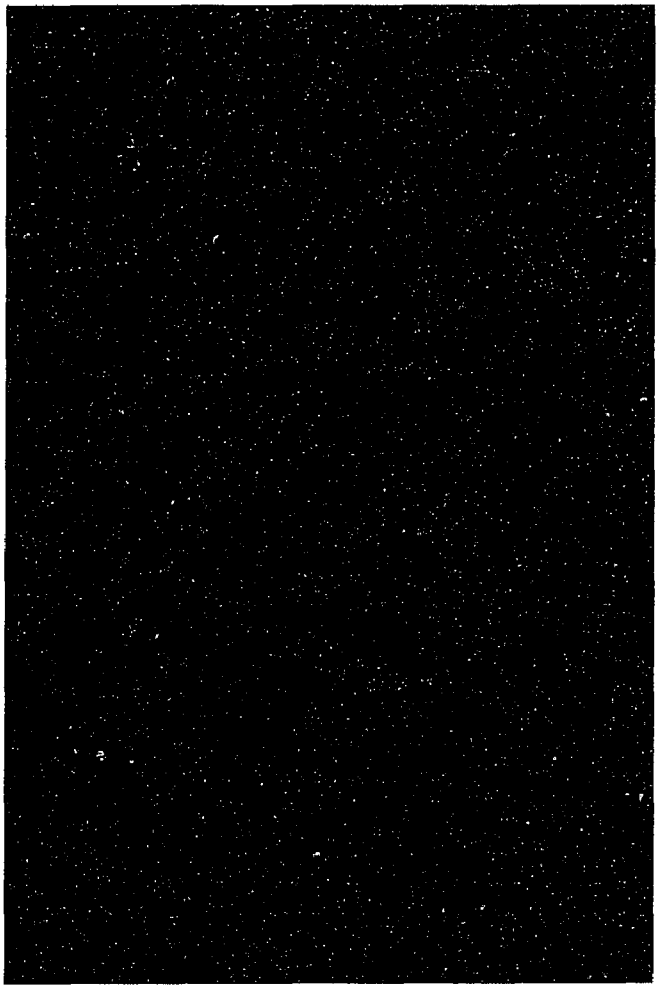


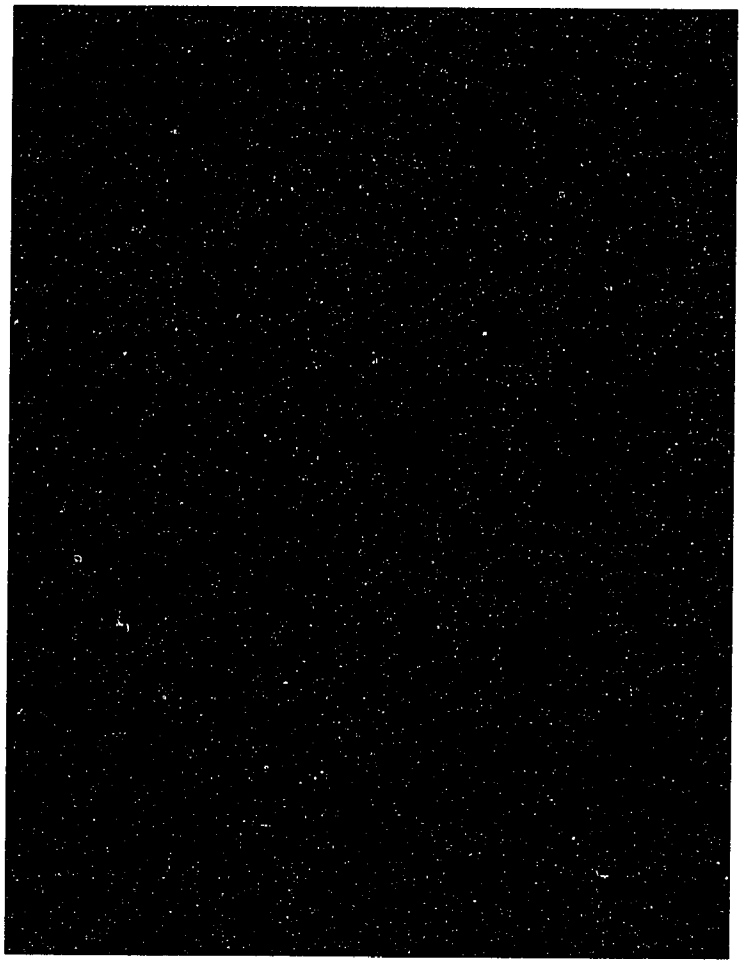
Figure 18. Phosphoamino acid analysis of in vitro-phosphorylated CENP-E^{COOH368}

Figure 19. Phosphopeptide map of serine-2617, 2571, 2602 and 2568 mutants

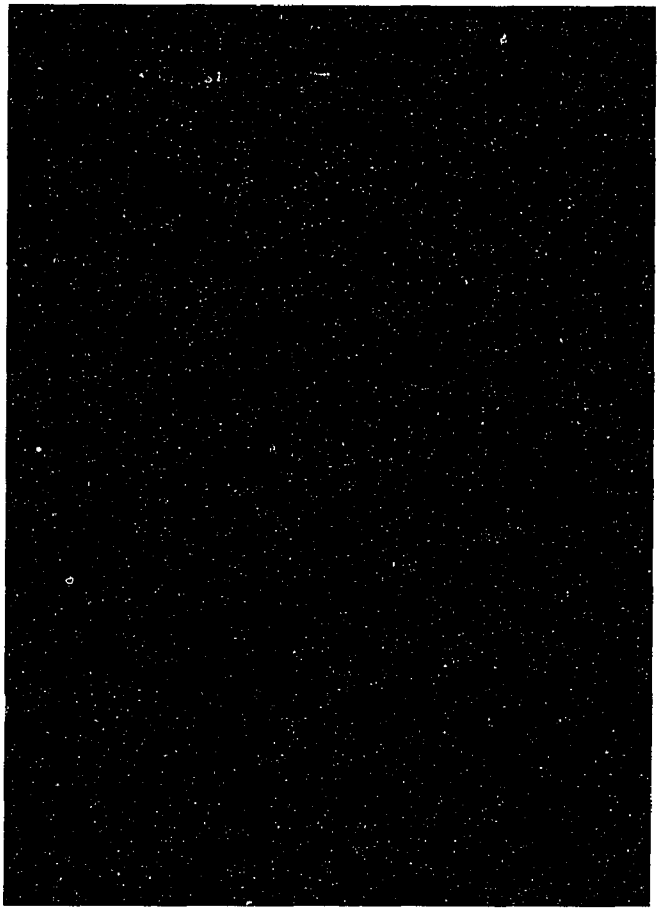
In vitro labeled C-terminal fragments containing mutation of serine-2617 (spot 1), serine-2571 (spot 2), serine-2602 (spot 3) and serine-2568 (spot 4) were subjected to SDS-PAGE followed by trypsin digestion individually. The two dimensions TLC plate was analyzed by autoradiography for phosphopeptide map. (A). Phosphopeptide map of serine-2617 mutant: Spots 1, 9, 10 were not detected in the two dimensional map (circles) while spots 2 to 8 remained unchanged. (B). Phosphopeptide map of serine-2571 mutant: autoradiography of serine-2571 mutant shows that spots 2 was not detected in the two dimensional map (circle) while others remained unchanged. (C). Phosphopeptide map of serine-2602 mutant: autoradiography shows that spots 3 was not detected in the two dimensional map (circle) while others remained unchanged. (D). Phosphopeptide map of serine-2568 mutant: autoradiography shows spots 4, 5, 6 were not detected in the two dimensional map (circles) while spots 1 to 3 and 7 to 10 remained unchaged.



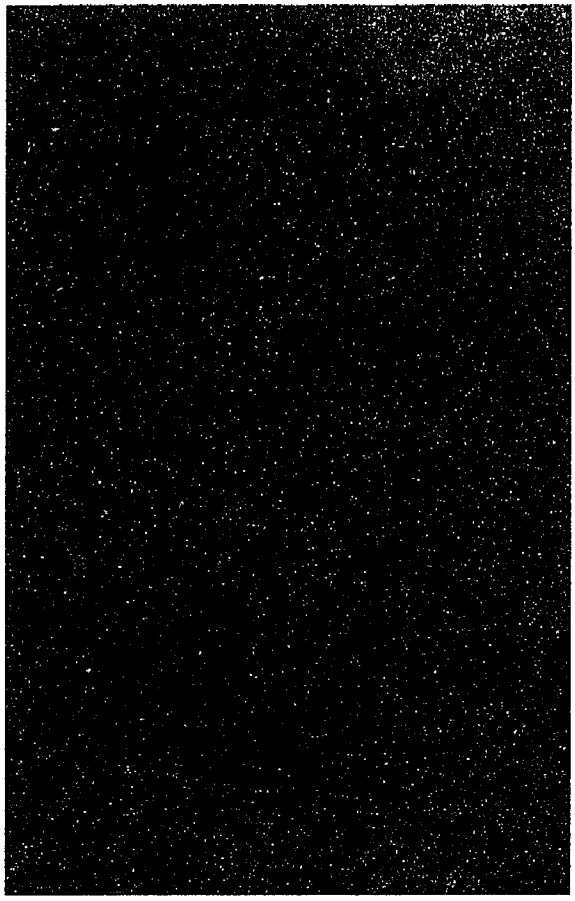
19A. Phosphopeptide map of serine-2617 mutant



19B. Phosphopeptide map of serine-2571 mutant



19C. Phosphopeptide map of serine-2602 mutant



19D. Phosphopeptide map of serine-2586 mutant

Figure 20. Quadruple mutation (serine-2617, 2571, 2602 and 2568) of C-terminal CENP-E

C-terminal CENP-E containing single or quadruple mutations was labeled with [^{32}P] orthophosphate under the same condition as in figure 19. After electrophoresis, the gel was stained with Coomassi blue followed by dessication and autoradiography. Lanes 1 to 6 of the panel A (autoradiography) represents wild type, mutants of serine-2568, 2571, 2602, 2617 and quadruple respectively. Phosphorylated C-terminus were seen for all mutants but quadruple mutant. Panel B is the Coomassi stained gel. C-terminal CENP-E were seen in lanes 1 through 6. C, C-terminal CENP-E.

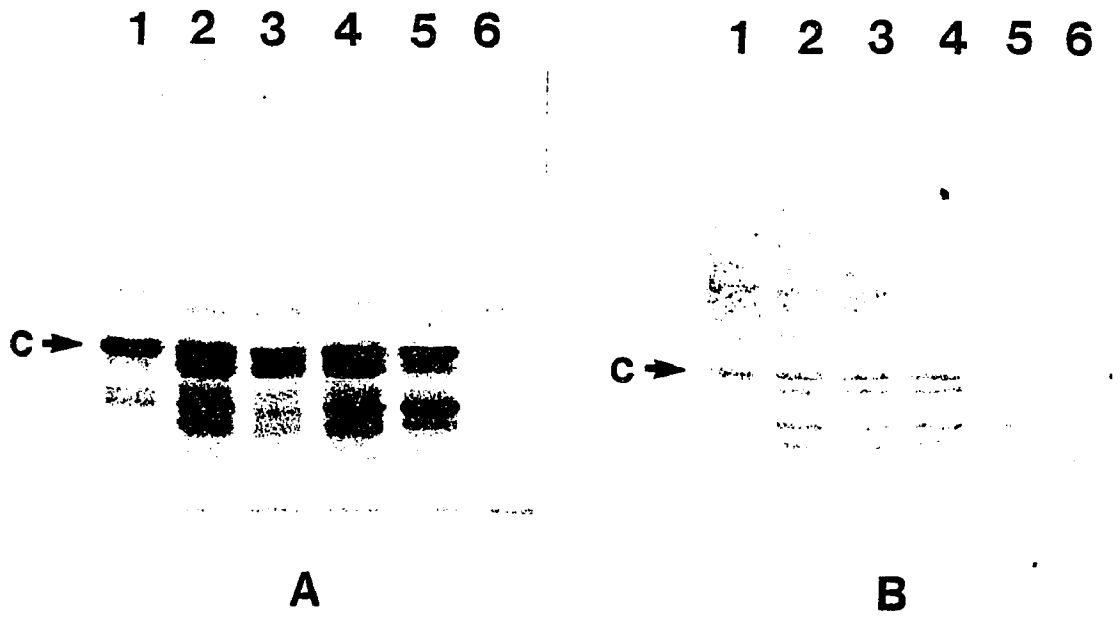


Figure 20. Quadruple mutation (serine-2617, 2571, 2602 and 2568) of C-terminal CENP-E

Figure 21. Analysis of transfected quadruple mutant in vivo

HeLa cells were transfected for expression of the quadruple mutant and phosphorylation in vivo. Phosphopeptide map of two-dimension did not show spots 1 to 4 while spots 5 to 7 were still intact. The high background was due to prolonged exposure time since the signals were weak.

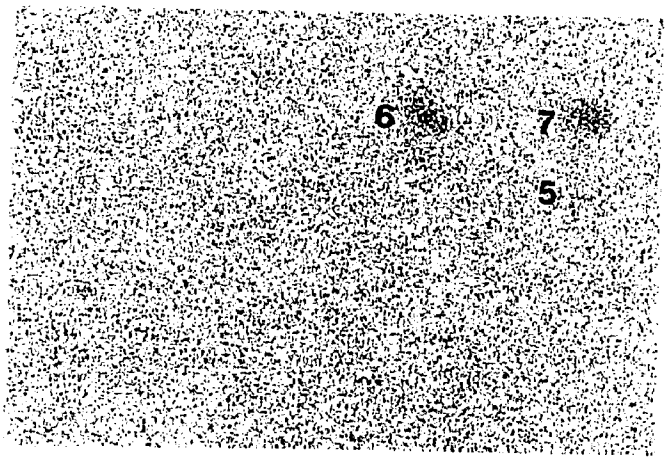


Figure 21. Analysis of transfected quadruple mutant in vivo

Figure 22. pRb-T complexes in preimmortal SVtsA/HF-A at permissive and nonpermissive temperature

The same amount of cell extracts from cells incubated at 35°C and 39°C for 3 days was immunoprecipitated with antibody to pRB. Following SDS-PAGE, immunoblot was reacted with antibodies to pRB and large T antigen sequentially. [^{125}I] conjugated anti-mouse IgG was used for detecting bound primary antibodies. Lanes 1, 2, 3 represented cell extracts of 35°C, 39°C and control respectively. pRb and T for T antigen were marked on the side of the panel.

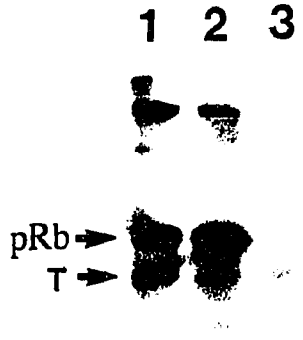


Figure 22. pRb-T complexes in preimmortal SVtsA/HF-A at permissive and nonpermissive temperature

Figure 23. Protein level of $p34^{cdc2}$ and cyclin B1 in the preimmortal ts transformants

Equal amounts of total protein of extracts from cells incubated at 35°C and 39°C for 3 days were immunoprecipitated with antibodies to either human $p34^{cdc2}$ or cyclin B1. The immunoblot of $p34^{cdc2}$ immunoprecipitation was reacted with antibody to $p34^{cdc2}$ (panel A). [^{125}I]-labeled protein A was used to detected bound primary antibodies. The protein level of cyclin B was deteced with antibody to cyclin B1 followed by [^{125}I]-conjugated sheep anti-mouse antibody (panel B). Lanes 1, 2 and 3 of panel A and B represent samples of 35°C, 39°C and control respectively. cyc, cyclin B1. cdc, $p34^{cdc2}$.

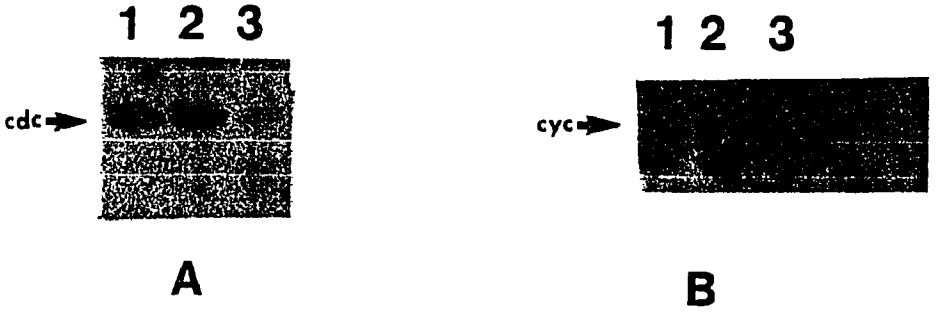


Figure 23. Protein level of $p34^{cdc2}$ and cyclin B1 in the preimmortal ts transformants

Figure 24. $p34^{cdc2}$-cyclin B1 complex in the preimmoratl ts transformants

Equal amounts of total protein as in figure 23 were immunoprecipitated with anti-cyclin B1 antibody and the immuno blot was reacted with antibodies to p34cdc2 and cyclin B1. After incubation with [^{125}I] conjugated anti-mouse IgG and [^{125}I]-labeled protein A, the filter was autoradiographed. Lanes 1 and 2 were samples of 35°C and 39°C respectively. cyc, cyclin B1. cdc, $p34^{cdc2}$.

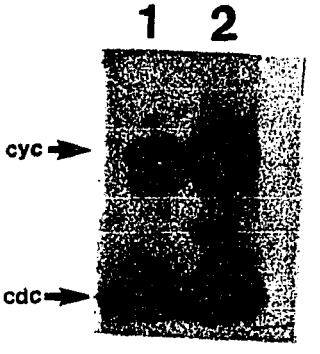


Figure 24. p34^{cdc2}-cyclin B1 complex in the preimmoratl ts transformants

Figure 25. Protein level of CENP-E in preimmortal ts transformants

Preimmortal cells (SVtsA/HF-A) were seeded at 5 x 10^5 and incubated at 35°C for 24 hours before shifted to 39°C. The dishes at 39°C were harvested daily for two days. To compare the expression of CENP-E at 35°C and 39°C, the extracts containing same amount of protein were analyzed by immunoprecipitation and Western blot procedure. Lanes 1, 2, 3, were samples of two days at 35°C, one day at 39°C and two days at 39°C respectively. E, CENP-E.

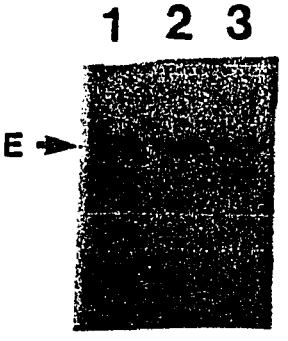


Figure 25. Protein level of CENP-E in preimmortal ts transformants

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