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THYMIDINE KINASE, DNA SYNTHESIS AND CANCER*

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Summary

A resume has been presented of some recent investigations which show that DNA synthesis can be initiated in many types of quiescent animal cells by external stimuli, by introducing a quiescent nucleus into the cytoplasm of a proliferating cell, or by a virus infection. The components of the DNA replication apparatus are described. It is shown that deoxyribonucleoside triphosphate pools increase substantially in animal cells at the time DNA synthesis is initiated due to the enhanced activities of enzymes functioning in nucleotide synthesis. Especially striking is the increase of thymidine kinase activity, indicating that this enzyme may be a useful marker of the shift from the quiescent to the replicative state. The thymidine kinase isozymes of vertebrate cells have been characterized. Thymidine kinase F, which is found principally in the cytosol, is the isozyme that increases when G1(Go) phase cells are stimulated or infected with oncogenic viruses. Chick cytosol thymidine kinase F can also be reactivated by introducing differentiated chick erythrocyte nuclei into the cytoplasm of enzyme-deficient LM(TK$^-$) mouse cells. Furthermore, herpesviruses code for distinctive, virus-specific thymidine kinase isozymes, so that another way to transform thymidine kinase-deficient LM(TK$^-$) cells to kinase-positive cells is by infecting them with UV-irradiated herpes simplex viruses. The experiments on the activation of DNA synthesis and thymidine kinase F activity have been discussed in the context of the proliferative activity *in vivo* and the immortalization in culture of neoplastic cells. These experiments suggest that genes determining cell cycle proteins are readily accessible to transcription and translation in essentially all nucleated cells. The tendency of transformed cells to become multinucleated after cytochaliasin B treatment also suggests that one important difference between malignant cells and most normal cells may be the ability of malignant cells to 'stockpile' the proteins (and/or their messenger RNAs) of the DNA replicative apparatus and to maintain the 'stockpiles' in progeny cells.

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Introduction

A common thread, interwoven through much of the recent cancer research literature, is that cancer is a disorder of normal differentiation or gene expression. This basic concept has found favor with many virologists, immunologists, and biochemists[1–4]. There are numerous examples in the clinical literature of the ectopic production of polypeptide hormones by non-endocrine tumors. The immunological and biochemical literatures disclose that fetal antigens and isoenzymes, though absent from adult differentiated tissues, are detectable in tumors. The virology literature abounds with examples of the expression of endogenous oncornavirus functions in fetal and tumor tissues and in established cell lines. This recurring pattern has suggested that oncogeny results from block ontogeny or that cancer involves the re-expression in cancer cells of embryonic proteins which have been inactivated during embryonic development[2,3,5].

The retrogenesis and blocked ontogeny models describe the phenotypes of many cancer cells, but they have not as yet explained the proliferation *in vivo* and the 'immortalization' in culture of neoplastic cells. It is far from clear, for example, why the re-expression of fetal antigens and isoenzymes, or the formation of oncornavirus particles should enhance the growth and survival of re-differentiated cells. In the discussion that follows, a cancer hypothesis is presented which emphasizes the conditions permitting cancer cells to proliferate under environmental conditions in which normal cells cannot. I shall refer to this hypothesis as the 'stockpile' hypothesis. The 'stockpile' concept was suggested by experiments on DNA replication and enzyme induction in normal and virus-infected animal cells. Two ideas central to this hypothesis are that: (1) cancer cells and their progeny 'stockpile' proteins and/or their messenger RNAs which function in DNA replication; and (2) the DNA sequences determining these proteins are readily accessible to transcription in differentiating normal cells as well as malignant cells. The 'stockpile' hypothesis also strongly emphasizes nucleocytoplasmic interactions in controlling nuclear activity.

Results and Discussion

DNA Synthesis and differentiation

The theme that DNA synthesis and the cell cycle are essential for differentiation has been developed from numerous studies on myogenesis, erythrogenesis and chondrogenesis in chick embryos,[6] and from investigations of metanephrogenic mesenchyme differentiation[7] and erythropoietin-induced maturation of fetal liver cells in mice[8]. These experiments call attention to the fact that the cell cycle subserves 2 distinct functions: (1) it can yield 2 daughter cells with the same synthetic pathways as those of the mother cell or (2) it can yield 1 or 2 daughter cells with synthetic pathways very different from those active in the mother cell. Cell cycles leading to duplication of the mother cell's phenotype have been termed 'proliferative' cell cycles, whereas cell cycles leading to cells with new pathways have been termed 'quantal' cell cycles.[6] Poliferative cell cycles are responsible for increases in numbers of similar cells; quantal cell cycles are postulated as the means whereby genetic diversity is introduced into replicating systems. Although virtually nothing is known about the mechanisms by which the emergence of new synthetic programs is coupled to the prior or concurrent synthesis of DNA, it is thought that quantal cell cycles entail rearrangements in chromatin structure permitting the step-by-step readout of different mRNA's. These mRNAs correlate with different generations of covertly differentiated cells. DNA replication offers the opportunity for the chromatin rearrangements to take place. The thesis regarding quantal cell cycles has many interesting facets. One corollary worth noting is that cells not terminally differentiated are poised for DNA synthesis and cell division. The ability to initiate DNA synthesis is essential so that differentiation may take place.

Activation of DNA synthesis in vertebrate cells

The molecular events 'triggering' DNA synthesis and proliferative or quantal cycles in interphase vertebrate cells are poorly understood. Cells may remain in interphase for weeks, months, or even years. However, physiological stimuli such as cutting the skin, partial hepatectomy, anoxia or erythropoietin administration stimulate DNA synthesis and division in epidermal, liver, and blood cell precursors, respectively. Whether or not the ensuing cell cycles are proliferative or quantal and the particular line of differentiation taken by a pluripotent cell depends upon the 'microenvironment' (organ stroma and effector molecules) in which the stem cell finds itself[9].

The small lymphocyte which circulates in the peripheral blood has been regarded as a differentiated cell representing an end stage of lymphoid maturation. Certain data suggest that this cell may have a life span of several years, during which time it circulates without replicating DNA or undergoing mitosis. Yet, with appropriate stimuli, the resting lymphocyte enlarges, assumes the appearance of a less mature cell, undergoes chromatin decondensation, develops prominent nucleoli, replicates its DNA, and undergoes cell division. This process is referred to as lymphocyte transformation or blastogenesis. Various agents induce this lymphocyte transformation, including nonspecific stimuli (i.e., phytohemagglutinin), tissue antigens (i.e., homologous lymphocytes), and specific antigens and antisera.[10] Another way to activate DNA synthesis in mature lymphocytes

is by fusing them with actively growing cells. Cultures of rat lymphocytes do not normally synthesize nuclear DNA, but they do so after UV-Sendai virus induced fusion with HeLa cells[11].

Even more striking is the observation that mature hen erythrocyte nuclei can be activated to resume DNA synthesis when the nuclei are introduced by UV-Sendai virus fusion into the cytoplasm of mammalian cells. Within 2 hr of fusion, there is an influx of nuclear specific mammalian proteins into the chick erythrocyte nuclei. The erythrocyte nuclei gradually increase in mass and volume, undergo chromatin decondensation, and by 15 hr, they actively synthesize chick nuclear RNA. By about 16–24 hr, DNA synthesis can be detected in both erythrocyte and the mammalian cell nuclei. A day or 2 later, prominent nucleoli develop, RNA is transported from the erythrocyte nuclei into the cytoplasm of the heterokaryons, and chick-specific surface antigens and the chick enzyme, hypoxanthine-guanine phosphoribosyltransferase, are made[11–13]. Chick DNA synthesis can be activated by introducing erythrocyte nuclei into the cytoplasm of proliferating cells, such as HeLa, Ehrlich, L cells, or rat myoblasts, but not by introducing them into quiescent cells, such as rat myotubes or rabbit macrophages[11,14]. The phenotypic changes observed during erythrocyte activation are the reverse of those which take place when chick erythroblasts differentiate to polychromatic and mature erythrocytes[15]. The erythrocyte system is not a unique example of activation, for when the nuclei of terminally differentiated mouse neurons are introduced by fusion into the cytoplasm of undifferentiated monkey kidney fibroblasts, the neuronal nuclei re-initiate DNA replication[16]. Another demonstration that the capacity for DNA replication is not lost in neural cells comes from nuclear transplantation experiments. GURDON[17] transplanted nuclei of adult frog brains to unfertilized *Xenopus laevis* eggs and found that over 90% of the brain nuclei incorporated ^{3}H-TdR into DNA within 90 minutes. This did not happen when the brain nuclei were injected into the cytoplasm of oocytes, the cells which give rise to mature eggs. Further experiments have demonstrated that a cytoplasmic factor which induces DNA synthesis is absent from the nucleus and cytoplasm of growing oocytes, but appears a few hours after the administration of pituitary hormone. The inducing factor persists in the egg cytoplasm for at least an hour after fertilization or activation, but is effective in the absence of fertilization. The experiments clearly underline the importance of nucleocytoplasmic interactions in the induction of nuclear DNA synthesis.

Activation of DNA synthesis by virus infection of stationary phase cells in culture

DULBECCO and coworkers[18] were the first to show that nuclear DNA synthesis can be induced in stationary phase mouse kidney cultures by polyoma virus. Subsequent studies by these investigators and others extended the observation to SV40 and other oncogenic viruses[19,20]. Induction by SV40 of nuclear DNA synthesis was observed after productive infection of stationary phase monkey cells or abortive infection of mouse kidney cells; it was induced in human cells at the final step in their lifespan *in vitro*; and it could be restored in cells that had been blocked in a particular period of the cell cycle by elevated temperature, X-rays, or contact inhibition. Essentially the whole of chromosomal DNA participated in SV40- and polyoma virus-induced DNA synthesis[21,22]. The kinetics of the induction process suggested that virus infection did not induce an unscheduled replication cycle in host cells, but rather recruited more of the cells which would, in any case, undergo DNA synthesis under appropriate physiological conditions. Besides, nuclear DNA synthesis, mitochondrial DNA synthesis was turned-on after productive or abortive infections by SV40 and polyoma virus[23,24].

SV40 and polyoma virus induced nuclear DNA synthesis in differentiated cells which despite the capacity for division, were in postmitotic arrest. The peritoneal macrophage is naturally representative of this restriction. Derived from progenitors which have undergone mitosis, it maintains RNA and protein synthesis without entering the S period of DNA replication. However, mouse macrophages do synthesize cellular DNA after SV40 or polyoma virus infections[25,26]. Treatment with mouse interferon inhibited the virus-induced host DNA synthesis, suggesting that a virus-specific function was required for the induction process[26]. Proliferating transformants of the mouse macrophages have been observed at an average of 66 days after SV40 infection.

Established cell lines, positive for SV40 T antigen, from which virus could be rescued were also obtained. Their identity as transformed macrophages was substantiated by evaluation of cellular morphology, phagocytosis, acid phosphatase, β1c (complement) synthesis, and aminoacridine incorporation[25].

During the differentiation of skeletal muscle, mononucleated myoblasts fuse to form multinucleated myotubes. After fusion, neither DNA synthesis nor nuclear division is observed in multinucleated myotubes either *in vitro* or *in vivo*. Because DNA synthesis is completely suppressed upon the attainment of multinuclearity, the muscle system can be considered ideal to investigate the capacity of viruses to reactivate DNA synthesis. YAFFEE and GERSHON[27] infected cultures consisting of mixtures of rat myoblasts and myotubes with polyoma virus. They found that the virus induced cellular DNA synthesis and mitosis in the multinucleated cells. Also, LEE and coworkers[28] infected clones of chick embryonic leg muscle, consisting of both myoblasts and myotubes, with Rous sarcoma virus, and found that Rous sarcoma virus induced DNA synthesis in the nuclei of the myotubes within 24 hr, but not nuclear division. These studies provide additional evidence that the restriction of DNA synthesis in differentiated cells is not irreversible.

Human adenovirus type 12 activates cellular DNA synthesis in nuclei of non-growing hamster and human embryonic kidney cells[29]; human cytomegalovirus, a member of the herpesvirus group, does the same after permissive infections of human embryonic lung or nonpermissive infections of African green monkey kidney cells[30].

Marek's disease is a fatal lymphoproliferative disease of domestic chickens caused by a herpesvirus (MDV). Another herpesvirus, Epstein-Barr virus (EBV), causes infectious mononucleosis and probably Burkitt's lymphoma in man. An impressive biologic property of EBV is its capacity to change lymphoid elements from resting cells into cell lines that are 'immortal' [31]. Continuous cell lines from lymphocytes infected with MDV, but not from uninfected cells, have also been obtained[32]. Both EBV and MDV induce cellular DNA synthesis in mature lymphocytes[33,34].

To recapitulate, the studies cited above have shown that DNA replication can be initiated in quiescent animal cells by external stimuli, by introducing a quiescent nucleus into the cytoplasm of a proliferating cell, or by virus infection.

Proteins required for DNA synthesis

The components of the DNA replication apparatus are poorly understood in animal cells. However, rapid progress in understanding DNA replication in bacterial and viral systems has recently been made and will now be summarized.

Studies of temperature-sensitive *E. coli* mutants have disclosed that 8 genes (dna A-dna I) are required for bacterial DNA synthesis[35,36]. *E. coli* mutants in genes, dna A, dna C (D), dna H, and dna I are defective in initiating replication at the chromosome origin. They are unlike other mutants [dna B, dna E, dna G] whose DNA replication stops abruptly when the temperature is raised to a restrictive level; the origin defective mutants continue DNA synthesis until the chromosome duplication under way is completed. Among the abrupt stop mutants, dna G has been implicated in nascent chain starts and dna E shows a defect in DNA polymerase III. Possibly dna G serves as an RNA polymerase to generate oligoribonucleotide primers for DNA synthesis. Chain elongation from the RNA primers is achieved through the action of a multisubunit, holoenzyme form of DNA polymerase III. It has also been suggested that *E. coli* DNA polymerase I may function in filling gaps on replicating molecules and for excising RNA priming fragments. Polynucleotide ligase is needed to link the short DNA pieces formed during discontinuous replication. Additional factors include unwinding proteins, proteins that remove superhelical turns in the DNA, nucleases, and copolymerase III*. The replicating chromosome is attached to membrane sties, so that concurrent growth of new cell membrane may be necessary to separate the developing genomes. Finally, a balanced supply of nucleoside triphosphates is needed as substrates for the polymerases; they are generated by a number of enzymes which catalyze the *de novo* and *salvage* pathways of nucleotide synthesis. Gene dna F codes for a subunit, B1, of one of the *de novo* pathway enzymes, ribonucleoside diphosphate (RDP) reductase. Another gene, nrd B, mapping close to dna F, is the determinant for RDP reductase subunit B2. Bacterial mutants defective in RDP

reductase require exogenous UdR or TdR for growth[37].

The general requirements for DNA replication in mammalian cells appear to be similar to those in bacteria. DNA synthesis may be initiated in animal cells, as in bacteria, with RNA primers; replication is discontinuous and eukaryotic unwinding proteins function in replication. At least 4 DNA polymerase activities have been identified in animal cells: (1) a cytoplasmic DNA polymerase I (7S) that increases in replicating cells; (2) a small nuclear DNA polymerase II (3S) whose activity is relatively constant during changed cell replication; (3) a mitochondrial-specific DNA polymerase; (4) and a cytoplasmic polymerase that efficiently uses poly rA-oligo dT as a template. Possibly, DNA polymerase I functions in the formation of small 4S pieces during discontinuous DNA replication and DNA polymerase II functions in filling gaps in replicating molecules. The role of the mitochondrial DNA polymerase is self-evident, but that of the poly rA-oligo dT polymerase is unknown. Two molecular forms of polynucleotide ligase have been identified in animal cells, one having a molecular weight of 190,000 and the other 95,000. The larger molecular form is most likely a dimer of the smaller form. DNA synthesis in animal cells rapidly declines when protein synthesis is inhibited by cycloheximide or puromycin, suggesting that concurrent protein synthesis is essential for DNA replication. Studies by HAND and TAMM[38] indicate that these proteins function both in the initiation of DNA chains and in chain elongation.

Mouse epidermis, particularly the ear, has been the subject of intensive study on the control of DNA synthesis and mitosis[38a]. Mitotic activity is very low in the basal epidermis *in vivo* with most cells blocked in the G1 phase of the cell cycle. However, DNA synthesis and mitosis can be induced by heat or wounding in the ear. It is thought that epidermal cells produce epidermis-specific inhibitory proteins (chalones) which help limit the mitotic rate. Chalones specific for lymphocytes, liver, and fibroblast cells have also been identified[38b,c]. Another indication that inhibitory proteins control DNA synthesis has come from studies on chick erythrocyte-mammalian cell heterokaryons. It has been demonstrated that activation of chick erythrocyte nuclei in heterokaryons is suppressed by protease inhibitors, suggesting that inhibitory proteins must be removed to permit activation[38d].

Analysis of cells in culture have suggested that animal cells cease to increase in number under a diversity of suboptimal nutritional conditions, and that a uniformity of metabolic changes follow these nutritional shifts. Each of several diverse blocks to replication (high density, serum depletion, amino acid starvation, suboptimal glucose or phosphate ions, high levels of cAMP) arrest cells in the same quiescent state of the cell cycle, G1(Go), and the cells escape at the same point when nutrition is restored. The name, 'restriction point', has been proposed by PARDEE[39] for the specific time in the cell cycle at which the critical release from G1 arrest occurs. He has proposed that the restriction point control permits normal cells to retain viability by a shift to a minimal metabolism upon differentiation *in vivo* and *in vitro* when conditions are suboptimal for growth. There seems to be a fundamentally different response for virus-transformed cells to nutritional insufficiency as compared to normal cells. The restriction control may be lost by transformed cells, so that, under adverse conditions, they stop replicating at random points in the cell cycle rather than at G1(Go). For unknown reasons, they subsequently die.

In general, significantly lower serum concentrations are required for sustained growth of Rous sarcoma virus-transformed chick cells, murine sarcoma virus-transformed rat cells, SV40-transformed mouse, human, and monkey cells, or polyoma virus-transformed hamster cells than for uninfected host cells. At high serum concentrations (20%), virus-transformed cells generally attain higher population densities than uninfected cells, although both cell types grow at essentially the same rate[39–42]. Also, infection with SV40 induces mouse cells to synthesize DNA and divide in a medium lacking serum protein growth factors essential for division of uninfected cells[43].

A viral gene function is responsible for inducing DNA synthesis and cell division in cells incubated in low serum. This has been shown in 2 ways. First, the inducing effects are abolished by UV-irradiation of SV40[44]. Second, confluent cultures of mouse (BALB-3T3) cells have been infected with wild-type and temperature-sensitive polyoma virus mutants and then incubated at permissive or nonpermissive temperatures in low serum media. Although wild-type virus induced

cellular DNA synthesis at both temperatures, a polyoma virus mutant, ts-3, was able to do so only at the permissive temperature[45].

The effects of serum on cell growth are complex. Some serum factors, such as insulin, act at the cell surface and increase the uptake of low molecular weight nutrients[41,46]. In sparse, quiescent mouse fibroblast cultures, a fibroblast growth factor (FGF) derived from bovine pituitary glands together with glucocorticoids and insulin can replace serum in stimulating DNA synthesis. FGF and the hormones increase intracellular cGMP and membrane-bound guanyl cyclase, but not cAMP or adenyl cyclase. Lectins also act at the cell surface when they stimulate lymphocytes to synthesize DNA and, concurrently, cGMP concentrations increase. Furthermore, exogenous cGMP and its derivatives stimulate resting mouse spleen lymphocytes to synthesize DNA with a time course similar to that caused by lectins[46a,b,c]. Other factors (multiplication stimulatory activity) appear to stimulate cells to pass from the G1 state to another condition, named Glc, where they have not yet begun DNA synthesis, but are committed to do so. Multiplication stimulatory activity is usually supplied as serum, but it may also be produced by cells[47].

Studies on temperature-sensitive Chinese hamster (K12) mutants have been employed to analyze the initiation of DNA synthesis in cells synchronized by serum depletion[48]. When serum was restored to G1 phase K12 cells synchronized by serum depletion at 33 °C, they initiated DNA synthesis about 9 hr later. However, if the K12 cells were shifted to 40 °C at the time of serum addition, they failed to initiate DNA synthesis. Thus, a temperature-sensitive block in this mutant affected a specific protein (or proteins) whose function was needed at a time between mitosis and the next initiation of DNA synthesis. In order to define more closely the execution point of the mutation, SMITH and WIGGLESWORTH[48] tried 2 kinds of experiments. First synchronized cells that had been shifted to 40 °C were shifted back to 33 °C after various periods of time. They found that being at 40 °C for the first 5 hr after shift-up did not affect subsequent initiation of DNA synthesis at 33 °C, whereas if the cells were left at 40 °C until after initiation of DNA synthesis, DNA synthesis was greatly reduced. Second, cells in serum-containing medium at 37 °C were synchronized at the G1/S transition with hydroxyurea, and then shifted to 40 °C 2 hr prior to the removal of hydroxyurea. DNA synthesis began immediately after the hydroxyurea was removed. The experiments demonstrate that the temperature-sensitive block was in the second half of G1, but before the step blocked by hydroxyurea. Temperature-shift experiments with somatic cell hybrids obtained by fusing the K12 cells with BHK(TK^-) or LM(TK^-) cells demonstrated that the mutation in K12 is recessive. It thus appears that this mutant has some points in common with the dna A mutants of *E. coli*.

To recapitulate, inhibitory proteins and suboptimal nutritional conditions restrict DNA synthesis at the G1(Go) phase of the cell cycle. The restriction block is overcome by the removal of suppressor proteins and by essential nutrients. The requirements for the initiation of DNA synthesis include: (1) multiplication stimulatory factors present in serum or made by cells after oncogenic virus infections; (2) a balanced supply of nucleoside triphosphates; (3) initiation factors defined by the temperature-sensitive mutation in K12 cells and by cycloheximide sensitivity; (4) RNA and DNA polymerases, polynucleotide ligase, and possibly nuclease activities; and (5) unwinding proteins.

Enzymes functioning in nucleotide synthesis

Deoxyribonucleoside triphosphate pools are small in animal cells, but increase substantially when DNA synthesis is initiated[49–51]. The addition of serum to the medium of G1 arrested mouse embryo cells, for example, results in a 3 to 5-fold expansion of dGTP pools, which are the smallest, and a 10 to 20-fold increase in the concentration of dCTP. Increases in dTTP and dATP are intermediate between those for dGTP and dCTP[52]. Intracellular dTTP concentrations increase by a factor of 3 during liver regeneration[53] and after polyoma virus infection of stationary phase mouse embryo cells[54]. The increased deoxyribonucleoside triphosphate pools result from a general enhancement of the activities of enzymes functioning in the *de novo* and *salvage* pathways of nucleotide synthesis. Table 1 summarizes a number of physiological conditions associated with increased DNA synthesis and growth. Enhanced activity of DNA polymerase and of the enzymes of nucleotide synthesis have been observed in these conditions.

Table 1

Enhanced enzyme activities associated with DNA synthesis and growth

Physiological condition	Enhanced enzyme activities	References
Phytohemagglutinin-stimulated lymphocytes	DNA polymerase, polynucleotide ligase, TdR kinase, CdR kinase, dTMP kinase, dCMP deaminase.	55–59, 59a
Regenerating rat liver	DNA polymerase, TdR kinase, dTMP kinase, dTMP synthetase, dCMP deaminase, RDP reductase.	60–61
Isoproterenol-stimulated mouse salivary gland	TdR kinase, CdR kinase.	65–66
Compensatory hypertrophy and hyperplasia of rat kidney after unilateral nephrectomy	DNA polymerase, TdR kinase, dTMP kinase, dCMP deaminase.	67
ACTH-stimulated DNA synthesis in guinea pig adrenal glands	DNA polymerase, TdR kinase.	68
Cell lines (HeLa, L, DON, BHK) in S phase vs. G1	DNA polymerase, TdR kinase, dCMP deaminase, RDP reductase.	62–64
SV40 and polyoma virus-infected stationary phase cells	DNA polymerase, polynucleotide ligase, TdR kinase, dCMP deaminase, dTMP kinase, dTMP synthetase, dihydrofolate reductase, CdR kinase, RDP reductase.	69

Enzyme increases have also been found when synchronized cells in culture entered the S phase of the cell cycle and after stationary phase cells were infected with oncogenic viruses[69–71]. Vaccinia virus, herpes simplex virus, and Marek's disease virus induce virus-specific DNA polymerases, and adeno- and papovaviruses enhance the activities of host DNA polymerases[69,74,74a]. Ribonucleotide diphosphate (RDP) reductase activities are also increased by unknown mechanisms in herpes simplex[72] and Yaba monkey poxvirus-infected cells[73].

One of the most consistent and striking enzyme increases that take place concomitant with the initiation of DNA synthesis is that of TdR kinase (thymidine kinase)[75]. High levels of this salvage pathway enzyme are found in various human and animal tumors, in cell cultures transformed by oncogenic viruses, and in fetal tissues (Table 1). Among normal adult tissues, TdR kinase activity is high in bone marrow, thymus, spleen, and small intestine, and low in kidney, brain, lung, pancreas, testes, and lactating mammary gland. In 9 transplanted rat tumors (nonhepatic) there is a strict correlation between TdR kinase activity and growth rate (doubling time ranging from 1–23 days)[76]. All minimal deviation hepatomas show elevated levels of TdR kinase activity compared with host liver. High levels of TdR kinase activity are found in hormone-stimulated tissues, phytohemagglutinin-stimulated lymphocytes, regenerating rat liver, and in kidney undergoing compensatory hypertrophy and hyperplasia following unilaterial nephrectomy. Urethane administration to partially hepatectomized rats inhibits liver regeneration, DNA synthesis and the induction of TdR kinase[77]. Fetal liver and spleen exhibit the highest TdR kinase activity at a time when they are most active in erythropoiesis. Proliferating intestinal epithelial cells have high levels of TdR kinase activity. However, when they mature and differentiate as they migrate towards the mucosal surface, there is a progressive reduction in the number of cells capable of further division and a sharp decline in TdR kinase activity. In human colonic mucosa, TdR kinase is primarily in the lower third, the region with the greatest proportion of proliferating cells[78]. At any time during the long period of diapause in silkworm pupae, injection of ecdysone or its secretion by the pupae's own thoracic gland provokes the initiation of adult development. Simultaneously, DNA synthesis and mitotic activity are resumed

after months of developmental standstill and TdR kinase activity increases 20-fold. In synchronized animal cell cultures, TdR kinase is low in G1 and rises sharply just prior to and during S phase. Periodic TdR kinase production has been observed in synchronous plasmodia of *Physarum polycephalum* and during synchronous division of fertilized sea urchin eggs. Very low TdR kinase activity is found in crude extracts of *Bacillus subtilis* spores as compared with extracts from vegetative cells. TdR kinase activity increases during spore germination simultaneously with the initiation of DNA synthesis.

TdR kinase activity is enhanced prior to the initiation of viral DNA synthesis in cells infected by numerous DNA viruses. Increased activities have been demonstrated in cells infected with vaccinia and other poxviruses, Shope fibroma virus, cytoplasmic icosahedral frog virus 3, bacteriophage T4, simian, human, and canine adenoviruses, papovaviruses SV40 and polyoma virus, and by 6 different herpesviruses[79].

Bromodeoxyuridine-resistant, TdR kinase-deficient cell mutants

The observations cited strongly suggest that TdR kinase has an important role related to DNA synthesis. The nature of this role will be discussed later. It should be noted, however, that TdR kinase is not absolutely indispensable for cell growth. In 1963, KIT and coworkers[80] isolated mouse fibroblast [LM(TK$^-$)] mutants highly deficient in TdR kinase activity. The mutant cells were resistant to BrUdR, IUdR, FUdR and to inhibitory levels of TdR, failed to incorporate either TdR or BrUdR into nuclear DNA, and they were killed when cultivated in medium containing aminopterin, a drug which inhibits *de novo* dTMP synthesis. Nevertheless, they grew as rapidly as parental cells in standard tissue culture growth medium. Additional TdR-deficient mouse [mKS(BU100)], human [HeLa(BU25)], and Syrian and Chinese hamster, rat, and bacterial mutants have been obtained by us and by others[81–83]. LITTLEFIELD[83] and DAVIDSON and EPHRUSSI[84] recognized that the BrUdR-resistant cell lines could be very useful for selecting somatic cell hybrids. This could be accomplished by co-cultivating BrUdR-resistant and azaguanine-resistant lines in HATG (hypoxanthine, aminopterin, thymidine, glycine) medium. Since parental BrUdR-resistant and azaguanine-resistant lines lack TdR kinase and hypoxanthine phosphoribosyltransferase activities, respectively, they are killed when the *de novo* pathways of dTMP and IMP synthesis are inhibited by aminopterin. However, somatic cell hybrids can obtain the TdR kinase and hypoxanthine phosphoribosyltransferase genes from each of their respective parental lines, and thus, can utilize the TdR and hypoxanthine in the HATG medium. They therefore grow, despite the aminopterin inhibition of *de novo* biosynthetic pathways.

Numerous somatic cell hybrids have now been obtained using drug-resistant cell lines and selective medium[85]. They have been employed to study genetic complementation and linkage, the role of genes in the regulation of cellular synthetic activities, and for numerous experiments in virology and oncology. HARRIS and coworkers[86], for example, have studied the suppression of malignancy in somatic cell hybrids formed by fusing high and low malignancy mouse lines.

One of the important observations emanating from studies on human-mouse somatic cell hybrids was the finding that human chromosomes are often selectively lost from the hybrids. The hybrid cell lines must retain the determinant for human TdR kinase[87], but when they are back selected in BrUdR medium, they lose the human determinant for TdR kinase. By applying these principles, MILLER and coworkers[88] and MCDOUGALL *et al.*[89] were able to show that the human TdR kinase gene is on chromosome E17 and is located on the long arm in the proximal region of 17q22. Also, BIRON and coworkers[90] demonstrated that E17 is one of three human chromosomes essential for the repication of human adenovirus type 12 in human-mouse somatic cell hybrids. The other 2 chromosomes are A3 and D14.

In our laboratory, the LM(TK$^-$) cells have been used to show that herpesviruses and poxviruses induce virus-specific dTR kinases in enzyme-deficient cells[69]. In addition, pox- and herpesvirus mutants deficient or temperature-sensitive in TdR kinase inducing activity were obtained. These studies provided the first genetic evidence that pox- and herpesviruses code for TdR kinase[69,79]. Similarly, using TdR-kinase deficient *E. coli* cells, bacteriophage T4 mutants deficient in enzyme-inducing activity were

obtained and the TdR kinase gene (tk) was mapped[91].

Genetically distinct mitochondrial TdR and CdR kinase activities

One of the unexpected discoveries resulting from the use of TdR kinase-deficient cell mutants was the finding that a genetically distinct isozyme of TdR kinase exists in mitochondria. While attempting to demonstrate the activation of SV40 DNA synthesis in transformed monkey cells (permissive for SV40 replication), KIT and MINEKAWA[92] carried out centrifugation experiments in cesium chloride-ethidium bromide (CsCl-EtBr) density gradients and noticed that rather large amounts of ^{3}H-TdR were incorporated into heavy density (superhelical) DNA molecules. It was recognized that mitochondrial DNA as well as SV40 DNA consists of circular superhelical DNA molecules. Since the DNA molecules obtained from the permissive SV40-transformed monkey cells were not infectious, it was suspected that they were of mitochondrial rather than viral origin. The DNA was further characterized and the experiments were extended to the study of DNA from normal monkey (CV-1) and human (HeLa) cells, and from TdR-kinase deficient human [HeLa(BU25)] and mouse [mKS(BU100)] cells. The results of these experiments demonstrated that the superhelical DNA synthesized in SV40-transformed monkey cells was, in fact, mitochondrial DNA. However, they also showed that the incorporation of ^{3}H-TdR into the mitochondrial DNA of HeLa(BU25) and mKS(BU100) cell mutants occurred at rates comparable to those in wild-type cells, despite the fact that the mutant cells incorporated ^{3}H-TdR poorly into nuclear DNA. These results suggested that: (1) TdR kinase-deficient cells still contained a TdR phosphorylating activity even though the major TdR kinase activity of the cells had been lost by mutation; (2) the mitochondrial enzyme could not substitute for the parent cell enzyme in the utilization of ^{3}H-TdR for nuclear DNA synthesis; and (3) the mitochondrial enzyme had selective value, since it was retained in mitochondria despite prolonged growth of TdR-deficient cells in high concentrations of BrUdR. Subcellular fractionation experiments were then carried out and proved that the mitochondria of HeLa(BU25) cells contained a TdR phosphorylating enzyme. At about the same time, similar experiments were carried out by CLAYTON and coworkers[93,94] and by ATTARDI and ATTARDI[95], who also found that a TdR phosphorylating enzyme was present in LM(TK$^-$) cell mitochondria.

Further studies on the TdR phosphorylating enzymes of parental and BrUdR-resistant mammalian cells have shown that: (1) the principle enzyme of parental cells, accounting for 90% or more of the total TdR phosphorylating activity, is in the cytosol (post-microsomal supernatant); (2) the cytosol TdR phosphorylating enzyme is also present in small amounts in the mitochondrial fraction of wild-type cells; (3) the cytosol enzyme of parental cells differs biochemically from the mitochondrial enzyme of mutant HeLa(BU25), mKS(BU100) and LM(TK$^-$) cells; (4) the mitochondrial isozyme is found in the mitochondria of wild-type cells, but the cytosol isozyme is absent from both mitochondrial and cytosol fractions of mutant cells; and (5) the specific activity of the mitochondrial isozyme is considerably greater in human and monkey cells than in mouse and Chinese hamster cells[96–99].

Deoxycytidine kinase, another salvage pathway enzyme, was investigated by de SAINT-VINCENT and BUTTIN[100] in wild-type and 1-β-D-arabinofuranosylcytosine (ara-C)-resistant Chinese hamster cells. Their observations paralleled the findings on BrUdR-resistant cells. Chinese hamster cells contained 2 biochemically distinct isozymes of CdR kinase. One, present primarily in the cytosol, functioned in the utilization of CdR for nuclear DNA synthesis, and a second isozyme, located in mitochondria, permitted the utilization of CdR for mitochondrial DNA synthesis. Ara-C resistant cells contained the mitochondrial CdR kinase, but not the cytosol isozyme, while parental cells contained both CdR kinase isozymes. More recent studies strongly suggest that the mitochondrial TdR and CdR kinases are one and the same enzyme, although cytosol TdR and CdR kinase are 2 distinctly different enzymes (W.-C. LEUNG and S. KIT, unpublished experiments).

It is interesting that yeast cells contain neither a cytoplasmic nor a mitochondrial TdR kinase[101]. However, yeast cells are permeable to nucleotides. Algae lack a cytoplasmic TdR kinase, but do contain a chloroplast-specific TdR kinase[102].

Characteristics of the TdR phosphorylating enzymes of vertebrate cells

The cytosol and mitochondrial TdR phosphorylating enzymes are TdR kinases or ATP; thymidine 5′-phosphotransferases (EC2.7.1.2.1). They catalyze the transfer of the γ-phosphate of ATP to TdR, and analogs of UdR. It is the cytosol TdR kinase that increases markedly when cells begin DNA synthesis, and it is the cytosol isozyme that decreases markedly when cells enter the stationary phase of the growth cycle. In contrast, the mitochondrial isozyme remains relatively constant in activity under a variety of conditions that induce changes in the cytosol form by a hundred-fold. In non-growing tissues of adult animals and in G1 phase cells in culture, the mitochondrial TdR kinase is the major cellular isozyme[103–105].

Submitochondrial fractionation experiments indicate that the mitochondrial TdR kinase isozyme is located mainly in the mitochondrial matrix[106]. Drug inhibition studies suggest that the enzyme is determined by nuclear rather than than by mitochondrial DNA, synthesized on cytoplasmic polyribosomes, and translocated to mitochondria. Consistent with this conclusion is the finding that small amounts of the mitochondrial TdR kinase isozyme can be detected in cytosol and microsomal fractions of HeLa(Bu25) cells. The determinant for human mitochondrial TdR kinase is on a different chromosome than E17, the determinant of human cytosol TdR kinase[107].

Although the major TdR kinase isozyme of animal cells is mostly in the cytosol, small amounts are detectable in mitochondria and nuclei. The genetically distinct mitochondrial isozyme is also detectable in cytosol and microsomal fractions. It, therefore, seems inappropriate to refer to these 2 isozymes as cytosol and mitochondrial dT kinases. STAFFORD and JONES[105] have called the TdR kinase isozymes fetal and

Table 2

Summary of the biochemical properties of TdR phosphorylating enzymes of vertebrate cells

Enzyme and source						Phosphate donor			
Enzyme	Cell origin	Subcellular localization	Disc PAGE (Rm)	Isoelectric focusing (pI)	Sedimentation coefficient (S)	ATP	CTP	UMP dCMP	dCTP inhibition
TdR kinase F	human, monkey (owl and AGMK) rabbit	cytosol (mitochondria and nuclei)	0.25	9.7	5.2	+	–	–	–
	mouse	cytosol	0.31	9.0	5.2	+	–	–	–
	chick, duck	cytosol (mitochondria and nuclei)	0.20	9.7	5.0	+	–	–	–
TdR kinase A	human, monkey (owl and AGMK)	mitochondria (small amounts in cytosol)	0.6–0.7	5.7	4.5	+	+	–	+
	mouse	mitochondria	0.8	5.0	4.5	+	+	–	+
	chick, duck	mitochondria (small amounts in cytosol)	0.65–0.70	~6.0	4.5	+	+	–	+
TdR kinase B	human [KB, HeLa(BU25), HeLa S3]	mitochondria	0.4	8.4	5.2	+	–	–	–
Nucleoside phosphotransferase C	chick, duck	cytosol (mitochondria)	0.4–0.5	5.0	8.6	–	+	+	–
Nucleoside phosphotransferase D	chick, duck	cytosol (mitochondria)	0.8	4.1	3.4	–	+	+	–

adult forms, respectively, but the latter terminology fails to emphasize that the 'fetal' form is very active in growing adult tissues and is very low in G1 tissue culture cells. We propose that the major TdR kinase isozyme of replicative cells be designated, TdR kinase F, and the genetically distinct mitochondrial form be called TdR kinase A.

Some of the biochemical properties of the vertebrate TdR kinase F and A isozymes are summarized in Table 2. Disc PAGE, isoelectric focusing, and glycerol gradient centrifugation experiments have revealed that mitochondrial TdR kinase A has a larger electrophoretic mobility, and a smaller isoelectric point and sedimentation coefficient than cytosol TdR kinase F. Both isozymes are inhibited by dTTP, but only TdR kinase A is significantly inhibited by dCTP. Unlike cytosol TdR kinase F, mitochondrial TdR kinase A efficiently utilizes GTP, CTP, or UTP in place of ATP as a phosphate donor, but neither isozyme efficiently utilizes UMP or dCMP as phosphate donors[79,96–99]. TdR kinase A differs from TdR kinase F in pH optimum and Km value (TdR).

A third molecular form, TdR kinase B, is prominent in the matrix and inner membrane submitochondrial fractions of HeLa S3, HeLa-(BU25), and KB cells. TdR kinase B has not been detected in other established human lines, but may be present in mitochondrial extracts of mouse cells. TdR kinase B decreases significantly when HeLa(BU25) cells are cultivated in medium containing chloramphenicol. The origin and biological significant of TdR kinase B are unknown. It is unclear whether this molecular form represents a genetically distinct isozyme or a modified form of TdR kinase F or A[75].

Properties of the TdR phosphorylating enzymes induced by animal viruses

As indicated previously, pox-, herpes-, adeno-, and papovaviruses induce TdR phosphorylating enzymes. In all cases, the primary site of enzyme induction is the cytosol. All of the viral induced enzymes utilize ATP as the preferred phosphate donor (TdR kinase). The viruses enhance the TdR kinase activities of infected cells in different ways. Biochemical, genetic, and immunological experiments strongly suggest that pox- and herpesviruses code for virus-specific TdR kinases. In contrast, the papovaviruses and adenoviruses derepress cytosol TdR kinase F. The evidence for the latter conclusion is that papovaviruses and adenoviruses enhance the TdR kinase activities of stationary phase, TdR kinase-positive cell lines, but not those of mutant cell lines deficient in cytosol TdR kinase F. The cytosol enzyme that is enhanced by papova- and adenoviruses in stationary phase cultures of parental cells is indistinguishable from the host cell TdR kinase F with respect to electrophoretic mobility, isoelectric point, sedimentation coefficient, and phosphate donor specificity[108,109].

The mechanisms by which adenoviruses derepress the host cell cytosol TdR kinase F are unknown. However, recent studies by MCDOUGALL and coworkers[89,110,111] may be pertinent. They observed that adenovirus types 12 and 31 induced non-random breaks and gaps at a specific site on human chromosome E17. The human genes for cytosol TdR kinase F and galactokinase are closely associated with the sites of virus-induced gaps and breaks. They suggested that a virus-coded protein made early after infection may bind to host DNA and promote the uncoiling of chromosome E17 in the region coding for TdR kinase F. Transcription of the TdR kinase F gene would be facilitated in the uncoiled region, which would also be susceptible to chromosome breakage.

Some of the properties of the TdR kinases induced by vaccinia virus and by herpesviruses are summarized in Table 3. The following points may be noted: (1) TdR kinase activities are induced by vaccinia virus, herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), marmoset herpesvirus (MarHV), and pseudorabies virus (PRV) in cell lines deficient in cytosol TdR kinase F, providing genetic evidence that they are virus-coded proteins; (2) the TdR kinases induced by the 6 different mammalian and avian herpesviruses have remarkably similar properties; (3) the properties of the enzymes induced by HSV-1, HSV-2, and MarHV are the same whether the host cells are human, monkey, mouse, or rabbit; (4) the herpesvirus-induced TdR kinases are distinctly different from cytosol TdR kinase F and the vaccinia virus-induced TdR kinase; and (5) the herpesvirus-induced TdR kinases differ from mouse mitochondrial TdR kinase A, but they resemble human, monkey, and chick mitochondrial TdR kinase A in electrophoretic mobility, isoelectric point, and

Table 3

Summary of the properties of the cytosol dT phosphorylating enzymes induced by vaccinia virus and by avian and mammalian herpesviruses

Enzyme source		Disc PAGE	Isoelectric focusing	Sedimentation coefficient	Phosphate donor			
Infected by	Host cells	(Rm)	(pI)	(S)	ATP	CTP	UMP dCMP	dCTP inhibition
Vaccinia (IHD)	Mouse [LM(TK$^-$)] or Human [HeLa(BU25)]	0.9	4.0	5.4	+	–	ND[b]	–
HSV-1 (C1 101 and HF)	Mouse [LM(TK$^-$)], Human [HeLa(BU25)] or Rabbit	0.6–0.7	6.0–6.5[a]	5.2	+	+	–	–
HSV-2 (333)	Mouse [LM(TK$^-$)] or Human [HeLa(BU25)]	0.6–0.7	6.3	4.9	+	+	–	–
Marmoset herpesvirus (Falk)	African green monkey kidney (Vero) Owl monkey kidney Mouse [LM(TK$^-$)] or Rabbit	0.6–0.7	6.0	5.1	+	+	–	–
Pseudorabies virus (Aujesky)	Mouse [LM(TK$^-$)]	0.57–0.61	5.5–6.5[a]	5.1	+	+	–	–
ILTV (Lederle)	Chick CAM	0.6–0.7	6.1	4.9	+	+	ND	–
HVT (Nazerian)	Chick embryo fibroblasts	0.6–0.7	6.0	5.3	+	+	ND	–

[a] Considerable microheterogeneity is observed after isoelectric focusing
[b] Not done

phosphate donor specificity. Nevertheless, the herpesvirus-induced TdR kinases are distinctly different from the latter TdR kinase A isozymes. The herpesvirus-induced TdR kinases have larger sedimentation coefficients, they are less sensitive to dCTP inhibition, and the HSV-1- and HSV-2-induced enzymes are more heat-labile than mitochondrial TdR kinase A. Also, the HSV-1 and HSV-2 TdR kinases differ from both TdR kinases F and A in antigenic determinants. Likewise, the HSV-1 and HSV-2 TdR kinases differ serologically from each other and from the other 4 herpesvirus-induced TdR kinases, indicating that they are similar, but not identical polypeptides[112–114].

The data presented in Table 2 show that besides TdR kinases F and A, avian cells contain at least 2 additional TdR phosphorylating activities, nucleoside phosphotransferases C and D. In contrast to TdR kinases, the nucleoside phosphotransferases utilize dCMP, UMP, OMP, dTMP and other low energy phosphate donors for the phosphorylation of TdR. Furthermore, several nucleoside diphosphates and triphosphates are effective donors with crude cell extracts and UdR, CdR, cytidine, and uridine are alternative nucleoside acceptors. The nucleoside phosphotransferase catalyzed reactions are not inhibited by dTTP, an end product inhibitor of the TdR kinase reaction, but the reactions are inhibited by ATP, the preferred phosphate donor of the TdR kinase reaction[79]. The existence of nucleoside phosphotransferase activities in avian cells complicates the identification of avian herpesvirus-induced TdR kinases. Comparison of Tables 2 and 3, however, show that the avian herpesvirus (ILTV and HVT)-induced TdR kinases are distinguishable from nucleoside phosphotransferases C and D. Nucleoside phosphotransferase C has a smaller electrophoretic mobility, a lower isoelectric point, and a larger sedimentation coefficient than the ILTV

and HVT-induced enzymes, while avian nucleoside phosphotransferase D has a larger electrophoretic mobility, lower isoelectric point, and a smaller sedimentation coefficient than the viral induced enzymes.

JAMIESON and coworkers[115,116] have recently shown that HSV-1- and HSV-2-induced TdR kinases catalyze the phosphorylation of CdR as well as TdR. In this property, they resemble mitochondrial TdR kinase A and they differ from cytosol TdR kinase F of baby hamster kidney and LM cells. Experiments on partially purified enzymes from the author's laboratory have confirmed that the HSV-1 TdR kinase phosphorylates CdR, and have demonstrated that the MarHV- induced enzyme also does so[117]. It should be emphasized, however, that the Km values of TdR are considerably lower than those for CdR.

Unlike the HSV-induced enzymes, the TdR kinases induced by PRV and vaccinia virus phosphorylate TdR, but not CdR[116,117]. Thus, the various cellular and viral-induced TdR kinases exhibit interesting individual differences in nucleoside substrate specificity. The differences between the HSV TdR-CdR kinases and cytosol TdR kinase F may have a practical application, permitting selective chemotherapy of HSV infections by 5-bromo- or 5-iododeoxycytidine[118].

Herpesvirus mutants deficient in TdR kinase-inducing activity

Mutants strains of HSV-1, MarHV, and PRV, defective or temperature sensitive in TdR kinase inducing activity have been isolated[69,79]. One of the HSV-1 mutants produced a TdR kinase at the permissive temperature that was more heat-labile than the enzyme induced by wild-type virus[119]. These observations provide additional genetic evidence that the herpesvirus-induced enzymes are virus-specific.

In contrast to the other herpesviruses, JAMIESON *et al.*[115] found that equine herpesviruses types 1 and 2 do not induce TdR kinase activity in baby hamster kidney cells. Similar results were obtained in our laboratory after infection of owl monkey kidney (OMK) cells by equine herpesviruses types 1 and 2. These findings indicate that common isolates of equine herpesviruses resemble mutant HSV-1, MarHV, and PRV strains in that they lack the ability to induce TdR kinase[79].

Control of viral-induced TdR kinase activity

Regulation of the levels of 'early' enzyme activities has been shown to occur in both bacteriophage and animal virus systems[69,120]. This regulation of enzyme activities can be altered by blocking viral RNA or DNA synthesis in infected cells. GARFINKLE and MCAUSLAN[121] demonstrated that the messenger RNA for the HSV-1 TdR kinase is an 'early' viral transcript, but not an 'immediate-early' transcript. The HSV-1 specific TdR kinase was not made when cells were treated at the time of infection with cycloheximide. Also, termination of TdR kinase formation was dependent on viral DNA synthesis and the formation of a 'late' messenger RNA. If infected cells were treated with drugs which block viral DNA and 'late' viral RNA synthesis, the switch-off of TdR kinase synthesis did not occur and superinduction of TdR kinase was observed. Similarly, UV-irradiation of HSV-1 or vaccinia virus prior to infection inactivated viral infectivity and viral DNA synthesis in infected cells without preventing the induction of virus-specific TdR kinases[69]. Indeed, superinduction of enzyme has been observed when cells were infected with HSV-1 and vaccinia virus that had been subjected to an appropriate dose of UV-irradiation. These experiments focus attention on 2 control points in viral-specific TdR kinase formation: (1) an immediate-early viral gene product is required for the formation of TdR kinase; and (2) a late viral protein is needed to shut-off synthesis of the enzyme.

Experiments with bacteriophage T4 *'far'* mutants are also instructive[122]. *'Far'* mutants are folate-analog-resistant phage. There are 2 classes of *'far'* mutants. One class overproduces TdR kinase, dCMP deaminase, dihydrofolate reductase, and deoxycytidine triphosphatase. The overproduction of dihydrofolate reductase by these *'far'* mutants is not affected by the absence of DNA synthesis. The second class of *'far'* mutants that affect the synthesis of early enzymes causes overproduction in the absence of DNA synthesis of some of the above enzymes, but not of dihydrofolate reductase. All of the mutants show delays in DNA synthesis, phage production and lysis. Thus, overproducing *'far'* mutants have mutations in phages T4 genes controlling the expression of the T4 genome. The mutants map near phage genes 56 and 52, distant from either

the TdR kinase or the dCMP deaminase and dihydrofolate reductase genes. The *'far'* mutants illustrate that abrogation of normal control mechanisms at loci removed from the promoter sites of the enzyme genetic determinants can lead to enhanced formation of enzymes functioning in deoxyribonucleotide synthesis.

Conversion to TdR kinase-deficient cells to the TdR kinase-positive phenotype by HSV infection

The observation that UV-irradiation of herpesviruses inactivates infectivity, but not TdR kinase inducing activity, enabled MUNYON and coworkers[123,124] to transfer the HSV TdR kinase gene to TdR-kinase deficient cells. They showed that LM(TK$^-$) or HeLa(BU25) cells lacking TdR kinase activity stably acquired the ability to synthesize the enzyme after they were infected with HSV-1 or HSV-2 and cultivated in selective (methotrexate, thymidine, adenosine, guanosine, glycine) medium. The medium does not permit growth of TdR kinase-deficient cells but allows the growth of TdR kinase-positive cells. The TdR kinase acquired by the converted cells is identical to the enzyme made after productive virus infection in the following properties: electrophoretic mobility, isoelectric point, sedimentation coefficient, phosphate donor specificity, thermal stability, and antigenic determinants[113,114,124]. LIN and MUNYON[125] have further shown that the HSV-specific TdR kinase in converted cells is controlled differently than the cellular TdR kinase of wild-type mouse cells. In asynchronously dividing L cell cultures, TdR kinase F activity rose and declined rapidly and coordinately with DNA synthesis. When net cell increase stopped, TdR kinase F activity was at a minimum. In contrast, the TdR kinase activity of HSV-converted cells remained at a minimum during rapid DNA synthesis and gradually increased as the rate of DNA synthesis decreased. When net cell increase stopped, TdR kinase activity was at a maximum. In synchronous L cell cultures, TdR kinase F activity rose and fell coordinately with the rate of DNA synthesis. In synchronous cultures of HSV-converted cells, no increase in TdR kinase activity was observed during the rapid period of DNA synthesis (S phase). Infection of mouse cells with HSV mutants lacking TdR kinase inducing activity decreased the activity of the host TdR kinase by 80%, but superinfection of converted mouse cells with the same HSV mutants enhanced the activity of the HSV-specific TdR kinase by five-fold. Stable transformation of TdR kinase-deficient rat cells to the TdR kinase positive phenotype by HSV infection has also been described[126].

The HSV-converted cells are of interest in connection with the oncogenic properties of certain herpesviruses. They illustrate that viral genes determining enzymes functioning in DNA replication can be brought into cells by transforming viruses. It was therefore of interest to investigate whether the oncogenic herpesviruses, Epstein-Barr virus (EBV) and Marek's disease virus (MDV), also exhibited TdR kinase-inducing activity.

Radioautographic experiments by HAMPAR and associates have suggested that EBV does have this capability[127]. They investigated a TdR kinase-deficient Burkitt lymphoma line, P3HR-1(BU). In this Burkitt line, as in most other Burkitt lines, EBV production is repressed in most cells. EBV formation can, however, be activated by BrUdR treatment. Cells activated to produce virus can be recognized by the formation of early antigens (EA) and viral capsid antigens (VCA). In contrast, in Burkitt lymphoma cells not producing virus, the only EBV antigen detected so far is a nuclear antigen, named EBNA[128]. The EBNA antigen, superficially at least, resembles the T antigens of SV40, polyoma virus, and adenovirus-transformed cells.

HAMPAR and coworkers[127] found that activation of EBV formation in P3HR-1(BU) cells resulted in the uptake of ^{3}H-TdR into nuclei of only those cells which were positive for EBV EA and VCA antigens. Their experiments suggest that a TdR phosphorylating enzyme was activated at the time that EBV EA and VCA antigens were produced. However, the experiments provide no direct evidence that the enzyme was virus-specific rather than a derepressed or hitherto 'silent' cellular enzyme.

In contrast to the radioautographic experiments, enzyme analyses on extracts of P3HR-1(BU) cells have failed to demonstrate a virus-specific TdR kinase[79]. Since few P3HR-1(BU) cells were actively producing virus, it is possible that the activity of an EBV-specific TdR phosphorylating enzyme was too low to be detected by the enzyme assay, although readily detected by radioautography. A number of

TdR-kinase-positive Burkitt lymphoma lines have also been investigated. They contained very high levels of cytosol TdR kinase F activity, but did not exhibit detectable levels of a herpesvirus-specific TdR kinase. Similarly, primary chick and duck embryo cells infected by MDV and a chicken lymphoblastoid line transformed by MDV exhibited enhanced activities of cytosol TdR kinase F, but did not show detectable amounts of a herpesvires-type enzyme. The enzyme experiments therefore suggest that oncogenic herpesviruses may resemble papova- and adenoviruses in that they derepress the cellular TdR kinase of transformed cells. The experiments leave open the possibility that, in addition, a herpesvirus-specific TdR kinase is induced when the virus genetic information is more fully expressed.

Activation of chick cytosol TdR kinase F activity after fusion of chick erythrocytes with TdR kinase-deficient mouse cells

When chick erythrocyte nuclei are introduced into the cytoplasm of tissue culture cells of the same or a different species, they are activated to resume DNA synthesis[11]. Since numerous studies have shown that initiation of DNA synthesis is associated with enhanced activity of TdR kinase, it was of interest to learn whether activation of chick erythrocyte TdR kinase occurred in conjunction with the initiation of chick DNA synthesis. This question cannot be answered when the erythrocyte nuclei are introduced into TdR kinase-positive cells. Chick erythrocytes were therefore fused with TdR kinase-deficient LM(TK$^-$) cells and the heterokaryons were incubated with ^{3}H-TdR or ^{3}H-CdR. The rationale of the experiment was as follows: neither chick erythrocytes nor LM(TK$^-$) cells contain significant cytosol TdR kinase F activity. Hence, H^3-TdR would be incorporated into chick nuclear DNA only if a chick TdR phosphorylating enzyme were active in the heterokaryons. The LM(TK$^-$) cells do contain CdR kinase activity. Thus, the number of LM(TK$^-$) cells in S phase and the activation of chick DNA synthesis could be assessed by the incorporation of ^{3}H-CdR into chick and LM(TK$^-$) nuclei.

Radioautographic experiments revealed that about half of the LM(TK$^-$) cells were in S phase after fusion with chick erythrocytes and the erythrocyte nuclei in the heterokaryons did incorporate ^{3}H-CdR into their DNA[129]. Hence, the erythrocyte nuclei in heterokaryons were activated to synthesize DNA, as expected. About half of the erythrocyte nuclei in heterokaryons also incorporated ^{3}H-TdR into their DNA, showing that a TdR phosphorylating enzyme was present.

Enzyme analyses of heterokaryons were carried out but failed to reveal significant TdR kinase activity. This result suggested that the TdR phosphorylating activity in the heterokaryons might be avian nucleoside phosphotransferase rather than TdR kinase (Table 2). Assay of extracts from chick erythrocytes demonstrated that nucleoside phosphotransferase activity was present in cytosol fractions, though at much lower levels than in chick embryo cells. A small but detectable nucleoside phosphotransferase activity was also present in chick erythrocyte nuclei, suggesting that small amounts of this enzyme might be introduced into heterokaryons during fusion. Nevertheless, enzyme assays of heterokaryons failed to reveal significant nucleoside phosphotransferase activity. It is therefore uncertain to what extent pre-existing chick erythrocyte nucleoside phosphotransferase or re-activated chick TdR kinase F contributed to the phosphorylation of ^{3}H-TdR and the incorporation of ^{3}H-dTTP into chick nuclear DNA 24 hr after fusion. Possibly the combined action of low levels of both enzymes enabled the heterokaryons to generate the ^{3}H-dTTP used for the first round of ^{3}H-TdR incorporation into DNA detected by autoradiography[130].

One problem associated with the analysis of enzyme activation in chick mouse heterokaryons is that many of the heterokaryons undergo mitosis 24 to 48 hr after erythrocyte nuclei are introduced. To suppress mitosis so that the erythrocyte nuclei in the heterokaryons would remain discrete long enough to develop nucleoli, HARRIS[11] irradiated the mouse cell nuclei with a microbeam. Under these conditions, chick erythrocyte nuclei in heterokaryons incorporated radioactive uridine into heterogenous nuclear RNA, but significant transport of the labelled RNA to the cytoplasm did not occur until after the chick erythrocyte nuclei developed nucleoli (usually 2–3 days after fusion). In heterokaryons of irradiated mouse cells and chick erythrocytes, chick-specific proteins (e.g., surface antigens, diptheria toxin receptor, and hypoxanthine

phosphoribosyltransferase) were synthesized only after nucleoli developed. If this result were applicable to chick TdR kinase F, it would be doubtful that the reactivation of chick TdR kinase F could occur early enough to permit incorporation of ^{3}H-TdR into DNA at 24 hr after fusion. The experiments by HARRIS[11] rule out massive transfer of newly synthesized RNA from the chick erythrocyte nuclei to the cytoplasm of the heterokaryons, but they do not preclude the transfer of the messenger RNA for chick cytosol TdR kinase F. It seems unlikely that the amount of TdR kinase F messenger RNA which might be transferred to the cytoplasm would be detected by autoradiography. Therefore, the experiments of HARRIS[11] are not inconsistent with the possibility that chick cytosol TdR kinase F was reactivated within 24 hr after fusion. Additional methods of preserving heterokaryons so as to permit a greater early reactivation of this enzyme may reveal whether it is synthesized in the heterokaryons.

Because of the failure to detect chick TdR kinase F activity by assay of heterokaryon extracts, an alternative approach was taken[124]. Chick-mouse somatic cell hybrids were readily selected by cultivating heterokaryons in HATG medium. Fifteen somatic cell hybrids formed from embryonic chick erythrocytes and LM(TK$^-$) cells and 5 somatic cell hybrids derived from adult hen erythrocytes and LM(TK$^-$) cells were isolated and analyzed. Since chick TdR kinase F differs from mouse TdR kinase F in electrophoretic mobility and isoelectric point, it was possible to ascertain whether the chick-mouse somatic cell hybrids contained chick or mouse cytosol TdR kinase F (Table 2). The results showed conclusively that all of the chick-mouse somatic cell hybrids contained chick and not mouse cytosol TdR kinase F[129]. These findings indicate that: (1) chick TdR kinase F had been reactivated in the somatic cell hybrids; (2) mouse cytosol TdR kinase F had not been generated by back mutation of LM(TK$^-$) cells; and (3) the TdR kinase F mutation in LM(TK$^-$) cells is a mutation in structural and not in a regulatory gene.

The somatic cell hybrids contained mouse but not chick mitochondrial TdR kinase A activity. This signifies that chick mitochondrial TdR kinase A was not reactivated or that the determinant for the chick mitochondrial enzyme was lost during selection of the somatic cell hybrids. Surprisingly, chick nucleoside phosphotransferase activity was absent from the somatic cell hybrids[130]. Nucleoside phosphotransferase activity greatly exceeds cytosol TdR kinase F activity in chick embryo fibroblasts. Yet, it was the chick cytosol TdR kinase F and not the nucleoside phosphotransferase that was reactivated and preserved in the chick-mouse somatic cell hybrids. The close association between cytosol TdR kinase F and DNA replication in numerous cell systems suggests that cytosol TdR kinase F may be preferred when selective conditions necessitating the salvage of TdR are applied. The physiological function of nucleoside phosphotransferase activity is not known, but its primary role may be other than that of TdR salvage during DNA synthesis.

The enzyme experiments strongly suggested, but did not prove, that a chick determinant for TdR kinase was present in the somatic cell hybrids. Therefore, karyotypes were analyzed by by the method which sequentially reveals Q- and C-bands[131]. Four hybrid clones contained the full complement of mouse chromosomes and 1–3 chick micro-chromosomes. Counterselection of the hybrids in BrUdR medium resulted in the loss of chick cytosol TdR kinase F activity and the acrocentric chick chromosome, which stained weakly with quinacrine mustard, but mouse mitochondrial TdR kinase A was unaffected. These BrUdR-resistant clones could not grow in HATG medium. Consequently, the results demonstrated that the chick cytosol TdR kinase F gene is on a member of the micro-chromosomes and selection in HATG and BrUdR-containing medium involves only cytosol TdR kinase F.

Biological significance of TdR salvage pathway

TdR kinase functions in the salvage pathway of dTTP synthesis. It reduces the loss of dTMP(dUMP) which would normally result from the combined activities of phosphatase and nucleoside phosphorylase. When thymidine-3′-phosphate is generated through the successive actions of endo- and exonucleases, it cannot be used for DNA synthesis until it is dephosphorylated by nucleotidases. TdR kinase then rephosphorylates the TdR moiety to thymidine-5′-phosphate, salvaging it for DNA synthesis.

The term salvage is unfortunate as there is a

connotation of nonessentiality. There are indications, however, that in addition to the functions already mentioned, TdR kinase and the TdR salvage pathway have a more positive role in DNA synthesis and may be essential under certain conditions. As indicated earlier, cell and viral mutants deficient in TdR kinase activity have been isolated. TdR kinase-deficient cell mutants grow as rapidly as wild-type cells in enriched growth media. Both wild-type and mutant HSV strains, defective in the determinant for TdR kinase, exhibit similar growth patterns in exponentially growing TdR kinase-positive or mutant TdR kinase-deficient cells. However, only wild-type HSV, but not HSV mutants lacking TdR kinase inducing activity, grows to any appreciable extent in serum-starved cells where there is a low level of *de novo* pyrimidine synthesis. Thus, the HSV-specified TdR kinase is indispensable for virus growth in serum-starved cells[115,116].

LAWRENCE[132] has shown that equine herpesvirus, which lacks TdR kinase inducing activity, replicates only in S phase cells. Similarly, PAGES *et al.*[133] found that cells have to pass through a critical stage in late G1 or early S before papovavirus DNA replication can be initiated. Thus, there is a correlation between the lack of viral determinants for TdR kinase induction and the dependence of viral DNA synthesis on the cellular environment found in S phase.

We would like to suggest that a major function of TdR kinase is to act in conjunction with RDP reductase to provide a balanced pool of deoxyribonucleoside triphosphates for DNA synthesis. RDP reductase is the key enzyme functioning in the *de novo* synthesis of deoxyribonucleoside triphosphates. When RDP reductase is inhibited by hydroxyurea, deoxyribonucleoside triphosphate pools are rapidly depleted. Short DNA fragments with a sedimentation coefficient of about 4S accumulate and they are only slowly converted to longer DNA strands[133]. RDP reductase is dispensable for bacteriophage T4 DNA replication provided that phage nucleases and salvage pathway enzymes are functional. In contrast, T4 mutants deficient in nuclease-inducing activities are sensitive to hydroxyurea (hus mutants).

RDP reductase catalyzes the reduction of all 4 purine and pyrimidine ribonucleoside diphosphates (ADP, GDP, UDP, CDP) to deoxyribonucleoside diphosphages. The enzyme is under complex allosteric control. ATP is an allosteric effector promoting the reduction of CDP and UDP. Reduction of CDP and UDP is pre-requisite for the formation of dTMP from dUMP by way of the dTMP synthetase catalyzed *de novo* pathway. It is highly significant that the reduction of purine ribonucleotides requires dTTP as an allosteric effector[52,135,136]. dTTP is specifically required for the reduction of GDP to dGDP, and the dGTP generated from dGDP is the allosteric effector for the reduction of ADP to dADP. It is to be emphasized that another salvage pathway enzyme, GdR kinase, is of minor importance in animal cells. The major pathway for the incorporation of GdR into DNA is via the formation of hypoxanthine and guanine, the conversion of the latter to IMP and GMP, and the subsequent reduction of GDP by RDP reductase[137]. Hence, animal cells are notably depending on RDP reductase for the formation of dGDP and dGTP, and the latter reactions require dTTP.

It is suggested that the observed increases in TdR kinase activities associated with rapid DNA synthesis can be understood in terms of the critical requirement for purine deoxyribonucleoside triphosphates[79]. The concentration of dGTP in cells has been estimated as sufficient for only about 30 seconds of DNA synthesis, emphasizing the need for this DNA precursor[52,54]. The metabolic reactions initiated by TdR kinase provide a shunt leading to the rapid increase in the concentration of dTTP to a level which permits the conversion of RDP reductase to the conformational state favorable for the reduction of GDP to dGDP. Contrariwise, a deficiency of TdR kinase activity might impede this process, particularly when conditions demand rapid DNA replication. The latter conditions prevail during viral replication and cell growth in enriched medium. Unbalanced growth, induced experimentally by deoxyribonucleoside triphosphate deficiency, is lethal to cells.

The stockpile hypothesis of the cancer cell

To return to the cancer problem, it may be worth emphasizing that there are probably almost as many types of malignant cells as there are differentiated cells. Among the lymphomas and leukemias, malignant T-cells, B-cells, plasma

cells, erythroleukemic, and chloroleukemic cells are known, suggesting that many types of cells may be the targets of carcinogens. In tissue culture, differentiated cells from embryonic, newborn or adult tissues are readily transformed by oncogenic herpesviruses, macrophages by papovaviruses, and myoblasts, differentiated iris epithelium, and neuroretinal cells by Rous sarcoma virus[138].

The presence of differentiated cell products in malignant cells shows that either pluripotent stem cells are not the only targets of carcinogens or that they undergo further differentiation after they become malignant. In the latter case, the block in differentiation cannot be absolute. A striking example where differentiation can occur in a malignant cell population is that of the proerythroblastoid cell line, T3-C1 2. This cloned line was originally derived from a splenic focus that arose in a mouse infected with Friend leukemia virus. When cultured under standard growth conditions, T3-Cl 2 cell were stable, and like authentic proerythroblasts, contained no detectable hemoglobin or hemoglobin messenger RNA. However, when grown in the presence of low concentrations (0.5 to 2.0%) of dimethyl-sulfoxide (DMSO), cells of this and other similar lines became smaller in size and synthesized hemoglobin, erythrocyte-specific membrane antigens, and globin messenger RNA[139,140]. A rise in hemoglobin synthesis was accompanied by a decrease in the malignancy of the cells[141]. Although the mechanism of action of DMSO is far from clear, the inducing effects of DMSO illustrate that the microenvironment and the responsiveness of cells to the microenvironment may alter the transcriptional pattern of malignant cells.

The presence of fetal antigens and isozymes may signify that in adult animals, precursor cells of a given developmental series are more frequently transformed by carcinogens than are more highly differentiated cells of the same series. Another possibility is that some fetal antigens and isozymes may be characteristic of replicating cells rather than of fetal or malignant cells, *per se*. The multiplicity of differentiated products in many tumor cells also suggests that the presence or absence of 'luxury' molecules is less significant than the capacity of the cells to initiate new rounds of DNA synthesis and to undergo proliferative cell cycles.

Most impressive is the fact that peripheral lymphocytes and the nuclei of avian erythrocytes can initiate DNA synthesis within 24 hr, when appropriately stimulated. Mouse peritoneal macrophages, which do not synthesize DNA *in vitro*, can do so 3–7 hr after fusion with melanocytes[142]. Adult frog brain nuclei synthesize DNA within 90 minutes of the time they are introduced into the cytoplasm of egg cells[17].

The experiments on chick erythrocyte-LM(TK$^-$) heterokaryons and somatic cell hybrids strongly suggest that formation of chick cytosol TdR kinase F was activated by factors present in the cytoplasm of LM(TK$^-$) cells[129,130]. TdR kinase F was taken as an example of one of the enzymes associated with DNA synthesis. Further studies may reveal that synthesis of other essential proteins of the proliferative cell cycle can be reactivated in erythrocyte nuclei. The observations further suggest that synthesis of TdR kinase F and related proteins is not subject to the same class of epigenetic controls as that regulating immunoglobulin or hemoglobin formation. Unlike the genes for immunoglobulin and hemoglobin, the genes determining cell cycle proteins seem to be readily accessible to transcription and translation in essentially all nucleated cells. Thus, nuclei of terminally differentiated cells appear to be poised to respond to cytoplasmic signals for the resumption of DNA synthesis.

If terminally differentiated cells are prepared to duplicate their DNA, what distinguishes them from malignant cells? One important difference between malignant cells and most normal cells may be the ability of malignant cells to 'stockpile' the proteins (and/or their messenger RNAs) of the DNA replicative apparatus and to maintain the stockpiles in progeny cells. Presumably, the proteins that are stockpiled are enzymes functioning in DNA replication and other proteins analogous to those determined by *E. coli* genes, dna A-dna I.

The following figure of speech illustrates the 'stockpile' concept. Cancer cells may be better 'chess players' than normal cells. They are 5 or 6 moves ahead in preparing for multiple rounds of DNA replication, whereas normal cells are only 1 or 2 moves ahead.

Two examples from work on *Drosophila* eggs and rodent trophoblasts may further illustrate the 'stockpile' concept. The *Drosophila* egg is a

remarkably differentiated cell. As with most insects, the *Drosophila* egg does not undergo cleavage in the usual sense. Instead, after fertilization, the nuclei undergo 8 rapid and synchronous divisions over the course of about 100 minutes and migrate to the peripheral cytoplasm. The embryo is now called a preblastoderm. The nuclei in the preblastoderm embryo divide synchronously 4 more times to form a syncytium containing about 4,000 nuclei, of which about 3,500 are on the surface. At this time, 160 minutes after egg deposition, the plasma membranes form simultaneously over the entire egg surface and 3,500 cells are formed all at once to produce a blastoderm[143]. In rodents, trophoblast differentiation results in the production of cells with 256 to 2,048 times the normal amount of diploid cell DNA, and, thereafter, the differentiated trophoblast rarely divides. The polyploidy results from replication of the trophoblast genome without cell division, rather than from fusion of cells to form syncytia, as in muscle development, or from gene amplification, as with amphibian ribosomal cistrons[144]. The *Drosophila* egg and the rodent trophoblast are prime examples of cells 'stockpiled' for DNA replication.

Recent studies involving the drug, cytochalasin B, suggest that cells transformed by oncogenic viruses, like egg and trophoblast cells, have 'stockpiled' for DNA synthesis. Cytochalasin B at low concentrations is an inhibitor of cell division, but not of nuclear division. Cultured human lymphocytes and several normal mouse, human, and hamster cell lines were treated with cytochalasin B. Most of the normal drug-treated cells became bi- or trinucleate. In contrast, several virus-transformed lines became much more highly nucleated than the normal cells. Some SV40-transformed mouse tumor cells exhibited as many as 27 nuclei after cytochalasin B treatment[145–149]. The differences in multinucleation of normal and transformed cells after cytochalasin B treatment probably reflects a fundamental difference between them. Comparison of additional cell lines is obviously necessary to assess whether or not the differential response of normal and malignant cells to cytochalasin B is an inevitable consequence of transformation. A plausible explanation for the tendency towards polynucleation in transformed cells is that they have 'stockpiled' the factors needed to continue nuclear division in the absence of cytokinesis, whereas most normal cells have not.

In this essay, we have not focused on the mechanisms which might lead to persistent stockpiling by progeny of malignant cells. Suffice it to say that there are many models which might explain this result. For example, the bacteriophage T4 *far* mutants illustrate that mutation in regulatory genes can result in overproduction of enzymes functioning in deoxyribonucleotide synthesis. The experiments on HSV-converted LM(TK$^-$) and HeLa(BU25) cells show that virus infection can introduce determinants for TdR kinase into cells which lack them. The papovavirus T antigen may examplify the acquisition through virus infection of a protein necessary for DNA synthesis[150]. Perhaps this is also true of the EBV EBNA antigen[128].

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