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Transcriptional Regulation of DNA Replication-related Genes in Cell Growth, Differentiation and Oncogenesis

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1. Introduction

Molecular mechanisms regulating eukaryotic cell growth and division are of central importance in biological science and oncology. Since the 1980s, numerous oncogenes and tumor suppressor genes have been identified. Activation of the former and/or defects in the latter, generated by mutation, have been identified in almost all cases of human cancers. These findings have provided a substantial basis for elucidating molecular mechanisms of oncogenic transformation of cells and clues for cancer diagnosis and therapy. Molecular mechanisms by which these genetic alterations cause oncogenic transformation of cells are less well understood. Biological research to examine the role of protooncogenes and suppressor oncogenes in normal cell growth and in differentiation, as well as studies on activated and viral oncogenes, are essential to achieve a better understanding.

Various lines of evidence indicate that most protooncogenes are structure genes encoding growth factors, growth factor receptors, or factors for signal transductions, including various protein kinases and transcription regulatory factors. Proteins encoded by tumor suppressor genes, such as p53 and retinoblastoma (Rb) genes, also seem to be components of transcription regulation.[1,2] These factors are members of various complex networks involved in growth signal transductions from the cell surface to the nucleus to regulate genes involved in cell proliferation and differentiation. One of the most important final targets of these signal networks is the DNA replication system.

In the next section, we discuss physiological and genetic factors involved in the regulation of DNA replication, then a short summary will be given of the cell cycle-dependent regulation mechanism of transcription of budding yeast DNA replication genes. The transcriptional regulatory mechanism of *Drosophila* DNA replication-related genes will be described in detail. This subject has not previously been reviewed. A brief discussion on recent findings in mammalian DNA replication-related genes is included. While detailed mechanisms related to protooncogenes or tumor-suppressor genes and DNA replication systems have not been elucidated, recent findings do shed some light on these events.

2. Physiological versus Genetic Mechanisms in Regulation of DNA Replication

When discussing the molecular mechanisms of eukaryotic cell proliferation, it is important to take account of related but separate biological processes.[3] One must consider molecular mechanisms determining whether cells are maintained in quiescence, or allowed to differentiate terminally, or proliferate actively. In actively proliferating cells, the mechanisms regulating the progression through phases of the cell cycle need to be analyzed in minute detail.

Replication of chromosomal DNA is carried out by complexes consisting of enzymes such as DNA polymerases and regulatory factors.[3,4] The onset of eukaryotic DNA replication requires activities of two levels of regulatory systems: one is the induction of coordinate expressions of many replication-related genes, and the other is assembly of the enzyme complex. The enzyme complex for DNA replication is formed at the G1/S boundary and is degraded during the mitotic phase of the cell cycle.

While the exact molecular mechanisms by which mammalian cells shift from G1 to S phase are unknown, the involvement of Rb protein, cyclin A and $p33^{cdk2}$ protein and p107 protein has been suggested.[2] A model in which the Rb protein is phosphorylated by $p34^{cdc2}$-related kinase at the late G1 phase so that phosphorylated Rb releases E2F transcription factor has been proposed. E2F then forms a complex with p107 and $p33^{cdk2}$/cyclin A to transcribe gene(s) essential for DNA replication. Mammalian genes required for DNA replication, such as those for dihydrofolate reductase, thymidine kinase, thymidylate synthase, DNA polymerase α and proliferating cell nuclear antigen (PCNA: an auxiliary protein for DNA polymerase δ), contain the E2F-binding consensus sequences, TTT(C/G)(C/G)CG(C/G), near their transcription initiation sites. However, the abundance of mRNA's and activities of DNA replication proteins, such as polymerase α and PCNA, was reported to increase only marginally at the G1/S boundary in proliferating mammalian cells.[5–10]

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Biochemical studies revealed that cell cycle-dependent activations of DNA replication enzymes are regulated by physiological systems rather than by the genetic system. Using cell-free replication systems such as those for simian virus 40 DNA and extracts of *Xenopus* oocytes, it has been demonstrated that cell cycle-dependent organization and degradation of the DNA replication enzyme complex is mainly regulated through protein phosphorylation by the cdc2-protein kinase system.[11-13] The decay of DNA replication enzyme complex at the M phase depends on the *string* gene, a *Drosophila* gene encoding a protein phosphatase which activates cdc2 kinase (our unpublished data). The genetic and physiological regulatory systems critical for the onset of DNA replication at the G1/S boundary and decay of the DNA replication complex at the M phase require much further study.

3. Cell Cycle-dependent Expression of DNA Replication Genes in Budding Yeast[14]

In budding yeast (*Saccharomyces cerevisiae*), many DNA replication-related genes (Table I) are transcribed in the late G1 phase. This periodic transcription is mediated by a hexameric sequence, known as MCB (*Mlu*I cell cycle box) present in promoters of these genes. The specific binding factor DSC1 is required for expression at the G1-S boundary.[15, 16] It has been suggested that the DSC1 transcription factor complex contains the product of the *SWI6* gene and a 120 kDa polypeptide.[17-19]

In fission yeast (*Schizosaccharomyces pombe*), a similar complex containing products of *cdc10*$^+$ (homologue of *SWI6*) and *res1*$^+$ genes is involved in the activation of promoters containing the MCB sequence.[18, 20] However, expression of DNA replication-related genes of fission yeast does not change depending on the cell cycle,[21] a finding similar to events in mammalian cells rather than those with budding yeast.

Table I. Budding Yeast DNA Replication Genes Transcribed at G1/S under the Control of *Mlu*I Cell Cycle Boxes

Name of gene	Function
POL1	DNA polymerase I (α)
POL2	DNA polymerase II (ε)
POL3	DNA polymerase III (δ)
POL30	PCNA
DBP2	DNA polymerase II subunit B
DBP3	DNA polymerase II subunit C
PRI1	DNA primase I
PRI2	DNA primase II
RFA1	Replication factor A 70 kDa subunit
RFA2	Replication factor A 34 kDa subunit
RFA3	Replication factor A 14 kDa subunit
CDC9	DNA ligase
CDC21	Thymidylate synthase
CDC8	Thymidylate kinase
RNR1	Ribonucleotide reductase M2

4. Regulation of Drosophila DNA Replication Genes

Dramatic increases in animal cell DNA replication-related proteins occur when cells enter the proliferating state. Expression of genes for these proteins is reduced in quiescent cells reaching confluency or in association with cellular differentiation.[22-24] Thus, the expression of DNA replication-related genes is thought to be coordinately regulated by a common mechanism closely linked to regulatory mechanisms governing cell proliferation, as is the case for cell cycle-dependent transcription of budding yeast genes.

Little is known of *cis*-elements or *trans*-acting factors required for the appropriate expression of animal cell DNA replication-related genes, since only a few of these genes have been isolated. If a common molecular mechanism responsible for the coordinate induction and re-

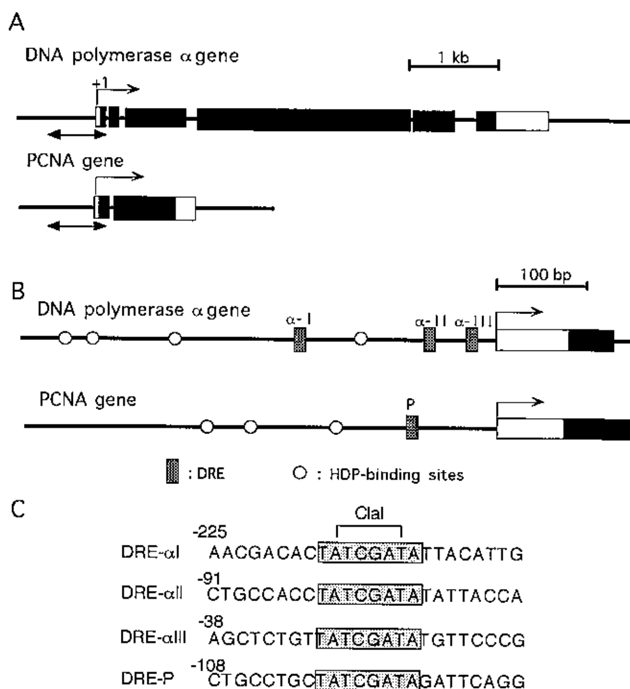

Fig. 1. *Drosophila* genes of DNA polymerase α and PCNA. A. Structures of genes, in which boxes indicate exons. Closed parts represent open reading frames. B. Regions with two-headed arrows in A represent the transcription regulatory elements. Localization of DRE and homeodomain binding sites is indicated by shaded boxes and open circles, respectively. C. Nucleotide sequences of DREs in which common 8 bp palindromic sequences center. The six base-pair sequence in DRE is the site for restriction endonuclease *Cla*I.

pression of animal cell DNA replication genes does exist, it may be activated by growth signals, including those from oncogenes. In attempts to clarify the genetic basis for regulation of DNA replication genes, we isolated *Drosophila* genes for PCNA[23] and DNA polymerase α.[25]

(i) *Drosophila* genes for DNA polymerase α and PCNA (Fig. 1)

The PCNA gene has a coding region for a 260 amino acid residue polypeptide and is interrupted by a single intron of 60 bp. The amino acid sequence of PCNA is highly conserved among various organisms, including plants.[23,25] Neither a TATA box nor a CAAT box was found within the 600 bp region upstream from the major transcription initiation site. Clusters of a 10 bp sequence similar to the binding sites for *Drosophila* homeodomain proteins were found in the regions from −127 to −413. DNase I-footprint analysis revealed that the recombinant *Drosophila* homeodomain proteins coded by *even-skipped* and *zerknüllt* genes can specifically bind to these sites. Therefore, expression of the PCNA gene seems to be under the control of homeodomain proteins which are transcription factors involved in gene regulation for morphogenesis.

The gene and cDNA for DNA polymerase α were also cloned.[24] This gene consists of 6 exons separated by 5 short introns. The nucleotide sequence of the open reading frame revealed a polypeptide of 1,504 amino acid residues with a molecular weight of 170,796. The amino acid sequence of the polypeptide was 37% identical with that of the catalytic subunit of human DNA polymerase α. This sequence contains six regions with order and amino acid sequences highly conserved among a number of viral and eukaryotic DNA polymerases.[3] We identified 7 amino acid residues between the 639th and 758th positions, identical to those essential for the active site of *Escherichia coli* DNA polymerase I-associated 3′→5′ exonuclease. Thus, the exonuclease activity may be associated with *Drosophila* DNA polymerase α, and with functions in "proofreading" to maintain fidelity during DNA replication.[26] The upstream region of this gene, like the PCNA gene, lacks TATA and CAAT boxes and has a number of sites for homeodomain proteins.

Levels of mRNAs for PCNA and the DNA polymerase α are very high in unfertilized eggs and early embryos, moderately high in adult female flies and second-instar larva, and low at other stages of development. These features of expression seem to coincide with the proportions of proliferating cells in various stages of development.

(ii) Mechanisms regulating transcription[27,28]

The 5′-flanking regions of the PCNA gene and DNA polymerase α gene were placed upstream of the chloramphenicol acetyltransferase (CAT) gene of the CAT vector, then transient expression assays of CAT activity

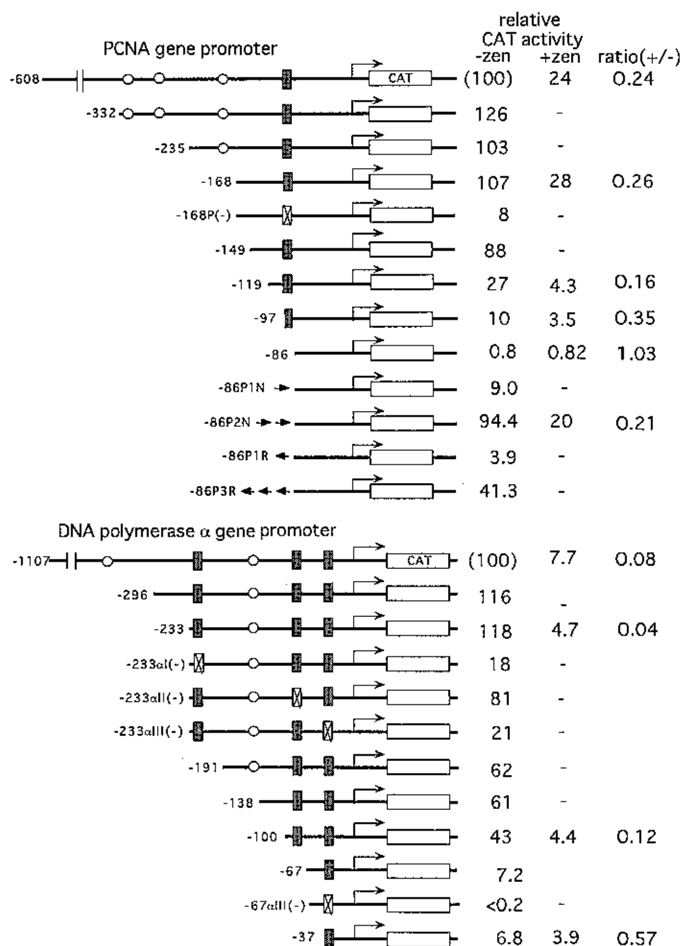

Fig. 2. Involvement of DREs in activities of promoters from PCNA and DNA polymerase α genes. Results obtained in several independent experiments were normalized and represented in terms of relative CAT expression activity. Upstream regions indicated in Fig. 1B were ligated to the CAT gene and various derivatives were constructed to determine CAT expression. Open boxes with X indicate DREs with 2 bp (GC)-insertional mutations at the center. Arrowheads indicate numbers and orientations of the chemically synthesized DRE-containing sequences (24 bp), which are identical with DRE-P and were ligated with the minimal promoter of the PCNA gene. CAT expression values obtained in cells transfected with *zen*-expression plasmid are also represented to indicate that *zen* repressed the promoter activity in the presence of DRE.

in *Drosophila* Kc cells transfected with either of these plasmids or their 5′-deletion derivatives were carried out (Fig. 2). The promoter function was located within a 192 bp region (−168 to +24 with respect to the transcription initiation site) of the PCNA gene. In the case of the

DNA polymerase α gene, the region within 233 bp upstream from the transcription initiation site is required for the high promoter activity in Kc cells. Since these promoter regions of both genes lack homeodomain protein-binding sites, except one in the DNA polymerase α gene, these sites do not seem necessary for promoter functions, at least in this particular culture cell system.

In regions required for the promoter functions of both genes, we detected sites containing a novel common 8 bp-palindromic sequence, 5′-TATCGATA, termed DRE (DNA replication-related element). Three DREs and one DRE are present in the DNA polymerase α gene (starting at the −217, −83 and −30 nucleotide positions with respect to the transcription initiation site) and the PCNA gene (−100), respectively, Deletions or 2-bp insertional mutations of DRE sequences led to extensive reduction of promoter activities of both genes (Fig. 2). Chemically synthesized oligonucleotides containing DRE sequences greatly stimulated the activity of the heterologous promoter of the *Drosophila* metallothionein gene, in addition to the promoter of the PCNA gene, when placed upstream of these promoters in a normal or a reverse orientation.

Using a gel mobility shift assay method, we detected a protein factor, named DREF (DRE-binding factor), in the nuclear extract of Kc cells, and this factor specifically binds to DREs of both genes. The DREF binds to DRE 24 bp region of the DNA polymerase α gene in which 8 bp palindromic sequences are centered. A UV-cross-linking experiment revealed that a polypeptide of about 90 kDa in the nuclear extract interacts directly with the DRE sequence. DREF was affinity-purified from the Kc cell nuclear extract using DRE-oligonucleotide-conjugated lutex particles. By comparing results obtained by SDS-polyacrylamide gel electrophoresis and gel mobility shift experiments, we concluded that the DREF is associated with the Mr 86,000 polypeptide (Fig. 3). In the gel filtration chromatography, a single peak of DREF activity was recovered in fractions corresponding to a molecular mass of 170 kDa, and the 86 kDa polypeptide was detected only in the corresponding fractions, so the active DREF is possibly associated with a form of homodimer of the 86 kDa polypeptide. DREF may play important roles in coordinating expressions of *Drosophila* DNA replication-related genes. Whether or not the DREF has a structure and function similar to yeast DSC1 or mammalian E2F remains to be determined.

(iii) Repression of promoter activities by the *zerknüllt* homeodomain protein

To examine the molecular mechanism involved in the differentiation-associated repression of DNA replication-related genes, we first directed our attention to a homeobox gene, *zerknüllt(zen)*. During cellularization and gastrulation of *Drosophila* embryos, the expression of *zen*

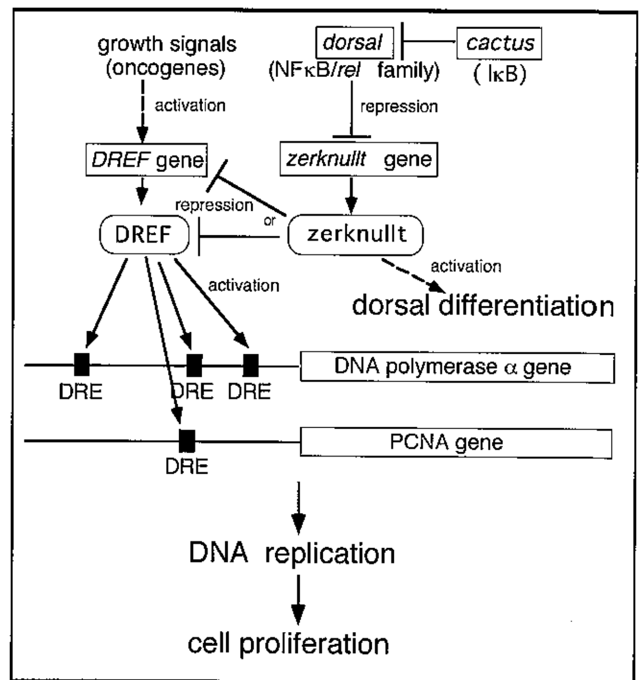

Fig. 3. DRE/DREF system in the regulation of *Drosophila* DNA replication genes. A hypothetical regulatory mechanism of DNA replication genes in the dorsal region of *Drosophila* early embryo is presented, based on the observations described in the text.

is restricted to the dorsal-most cells of the middle body region,[29, 30] an area where the presumptive optic lobe and amnioserosa are located, according to the embryo fate map.[31, 32] Cells in this region do not divide after the 14th cleavage cycle, perhaps because DNA replication is repressed by *zen*.

Cotransfection with *zen*-producing plasmid specifically repressed the CAT-expression controlled by either PCNA or DNA polymerase α gene promoters (refs. 27 and 48). The repression by *zen* was at the step of transcription. The target sequence of the repression by *zen* was mapped within the 34 bp region (−119 to −86) of the PCNA promoter located downstream of the previously determined *zen* protein-binding sites (−357 to −165), indicating that the homeodomain protein-binding sequences of the PCNA gene are not the target of repression by *zen* (Fig. 2).

In transgenic flies carrying the PCNA gene regulatory region (−607 to +137 or −168 to +137) fused with *lacZ*, zygotic expression of *lacZ* was not observed in the dorsal region where the *zen* gene is expressed. When the transgenic flies were crossed with the *zen*$^-$ mutant to form homozygous *zen*$^-$ mutation, we observed ectopic

expression of *lacZ* in the abnormally expanded dorsal region of gastrulating embryos carrying the transgene with either construct.[27] These results indicate that *zen* indirectly represses PCNA gene expression, probably by regulating the expression of transcription factor(s) that binds to the PCNA gene promoter.

Results obtained using various deletion derivatives of the promoter regions of both genes and chemically synthesized oligonucleotides of DRE sequence revealed that the DRE sequences are responsible for repression by *zen* protein (Fig. 2). Furthermore, the nuclear extract of Kc cells transfected by the *zen*-expressing plasmid contained lesser amounts of DREF than did that of untransfected or mutant *zen*-expressed cells (ref. 48). These results suggest that *zen* protein represses DNA replication by reducing DREF, which is required for expression of DNA replication-related genes.

During the preceding period of cellularization, the maternally deposited product of the *dorsal* gene, a *Drosophila* member of the NFκB/*rel* gene family, was present in nuclei in the ventral region of the embryo, but it remained in cytoplasm in dorsal region.[33, 34] The transport of the *dorsal* protein into the nucleus is inhibited in the dorsal region by the *cactus* protein, like the IκB (an inhibitor of NFκB in mammalian cells). The *dorsal* protein is a transcription factor repressing *zen* gene expression, and thus, *zen* expression does occur in the dorsal region. The regulatory mechanism of *Drosophila* DNA replication genes, in which DRE and DREF play central roles, is summarized in Fig. 3.

The repression of *zen* by mechanisms such as ectopic nuclear accumulation of *dorsal* protein in the dorsal area, may allow for expression of DREF and consequently, DNA replication gene expression and cell proliferation. At the same time, differentiation of the dorsal structure may be halted. Since arrest of cell proliferation is evident during differentiation in various tissues, homeobox genes other than *zen* are likely to be involved. We propose that both the induction and repression of DNA replication-related genes occur through physiological and (or) genetic alterations of DRE/DREF. From this standpoint, a most interesting feature is that the expression of two DNA replication-related genes is controlled by regulatory elements with identical sequences and a common *trans*-acting factor. Further studies on the function of DRE/DREF and the regulatory mechanisms for the gene coding for DREF are expected to reveal important molecular mechanisms of cell proliferation and/or cell differentiation. Recently, we succeded in isolating cDNA for DREF. The fluctuation of DREF mRNA during *Drosophila* developmental stages is similar to those of PCNA and DNA polymerase α, indicating the importance of DREF in the transcription regulation of DNA replication-related genes (our unpublished data).

5. Transcriptional Regulation of DNA Replication-related Genes in Mammalian Cells

The gene for PCNA has been studied most extensively among mammalian genes of DNA replication-related proteins. Although the mammalian PCNA protein and mRNA levels change relatively little during the cell cycle,[35, 36] they can be induced upon growth stimulation of quiescent cells by serum, growth factors and infection with tumor virus.[37–41] Viral oncogenes such as the adenovirus E1A gene are responsible for induction of the PCNA gene. Since the induction of PCNA mRNA levels by these agents requires protein synthesis and occurs after a delay of several hours,[37, 40] the PCNA gene is a so-called late growth-regulated gene, like the thymidine kinase gene and other genes coding for proteins of the DNA-synthesizing machinery.[41]

In the upstream region of the mouse PCNA gene, we identified consensus sequences for transcription factors AP-1, ATF/CREB and E2F,[42] all of which are required for full promoter activity. Both ATF/CREB and E2F sites are responsible for the stimulation by adenovirus E1A protein.[43] E1A protein interacts with Rb protein to activate the E2F transcription factor. Thus, one of the regulatory mechanisms of the PCNA gene is the Rb/E2F system. This system may be responsible for some of the increase of PCNA gene expression at the late G1 phase.[10] However, regulatory events other than the E2F system are also involved. This situation is similar to that of other genes for DNA replication proteins, including DNA polymerase α.[44]

Mutation, including deletion, of the p53 gene is one of the most frequently observed genetic alterations in human cancers.[45] It was proposed that function of the product of the p53 gene is to act as "guardian of the genome."[46] When DNA is damaged by mutagenic or carcinogenic agents such as ultraviolet irradiation, the level of the wild-type p53 is elevated to arrest cells at G1 phase so that the damaged DNA is repaired during this arrest. Mutant-type p53, however, fails to arrest cells, and therefore permits replication of damaged DNA, which causes various genetic aberrations such as gene amplification.[47] Thus, genetic instability resulting from mutation in the p53 gene is possibly one cause of transformation of cells. The molecular mechanism by which the wild-type p53 induces G1 arrest has not been clarified. p53 may function as a transcription regulatory factor by interacting with the general transcription factor, the TATA-binding protein (TBP or TFIID), to repress transcription.[1]

In the light of all these observations, the assumption seems justified that the wild-type p53, as a transcription factor, represses DNA replication genes but does not repress DNA repair-related genes. We examined the

effect of p53 on the promoters of the PCNA gene, a replication-related gene, and of DNA polymerase β gene, a repair gene. As expected, the wild-type p53 repressed the former but not the latter in a mouse cell system (our unpublished data). Furthermore, we found in the promoter region of the mouse DNA polymerase β gene the presence of an unique 10 bp sequence, in the presence of which p53 did not repress promoter activity. When this sequence was introduced into the PCNA gene promoter, p53 was no longer repressive to this promoter. It is tempting to postulate that the differential effect of p53 on replication and repair genes is a mechanism by which this tumor-suppressor gene product functions as "guardian of the genome."

6. Conclusions and Perspectives

Regulatory systems of DNA replication-related genes can be described as follows. (1) Genes for many DNA replication enzymes and factors are coordinately induced under the control of a common transcription factor, the expression of which is regulated as an important target of growth-signaling systems. (2) Some transcription factors governing differentiation-related genes may also function as negative regulatory factors for DNA replication genes, by repressing the above-mentioned common regulatory mechanism in various differentiating tissues. This mechanism might well be vital to induce local differentiation-coupled arrest of cell growth under general proliferating states of cells during embryogenesis.

Our tentative hypothesis is that the DNA replication genes in cells in the resting stage can be activated directly through activation of common transcription factors of DNA replication genes such as DREF and E2F, depending on the excess supply of various growth signals, or, in the presence of usual growth signals, on an inactivation of differentiation-specific regulatory systems which function to repress DNA replication genes. This inactivation, in turn, results in deregulation of DNA replication genes, in addition to loss of the differentiated phenotypes. Oncogenic transformation of cells may be related to either of these mechanisms. It should be pointed out that this hypothesis, while remaining to be proved, does provide mechanisms for generating tumor cells with differentiation phenotypes and those with degenerated phenotypes, depending on which activation occurs with mutations.

The physiological and genetic growth-signaling systems affecting common transcription factors for DNA replication genes such as DSC1 in budding yeast, DREF in *Drosophila*, and E2F in mammalians require urgent attention. Whether or not these three factors are functional homologues to each other is unknown. It is also important to study the involvement of various differentiation regulatory mechanisms, including homeodomain proteins in the regulation of DNA replication genes. Such studies should result in an important break-through in our understanding of the mechanisms of cell growth regulation in differentiation and oncogenesis.

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