A Common Regulation of Genes Encoding Enzymes of the Deoxynucleotide Metabolism Is Lost after Neoplastic Transformation

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Abstract
We determined the cell cycle-dependent fluctuation of mRNAs that encode different enzymes of the deoxynucleotide metabolism in permanent cell lines of human and murine origin. In normal growing cells, dihydrofolate reductase, thymidine kinase, and both subunits of ribonucleotide reductase show exactly the same variation. The mRNAs rise near the G1-S boundary, peak in early S phase, and return in G2 to approximately the level of early G1. Deoxycytidine kinase mRNA does not follow this pattern, but remains essentially unchanged. Conversely, in DNA tumor virus-transformed cells, the levels of all these mRNAs remain relatively constant throughout all phases. These data provide evidence that DNA tumor viruses suppress a transcriptional down-regulation common to enzymes responsible for the DNA precursor pathway. The usefulness of analysis of mRNA levels of these genes for the detection of DNA tumor virus transformation is indicated.

Introduction
We have shown previously that the normal, cell cycle-dependent regulation of TK (EC 2.7.1.21) is altered in a large variety of virus-transformed or tumor-derived cell lines (1-3). Normal cells show a rise of TK enzyme activity near the G1-S boundary, which peaks in S phase, and in G2 returns approximately to the level of G1. In contrast to that, in DNA tumor virus-transformed or tumor-derived cells, TK activity remains high throughout S and G2 phases, although it is also stimulated at the onset of DNA replication. The pattern of TK mRNA variation during the cell cycle is similar to that of enzyme activity in normal cells but is higher and does not fluctuate throughout the cell cycle in transformed lines. Thus far, we demonstrated that DNA tumor virus transformation leads to a transcriptional deregulation of TK during the eukaryotic cell cycle. The question of whether transformation affects the regulation of related enzymes has not been defined.

TK catalyses the ATP-dependent phosphorylation of thymidine and deoxyuridine (Fig. 1). As this reaction is not part of the de novo synthesis for DNA precursors, TK is not essential for DNA replication (for a recent review, see Ref. 4). Why should malignant cells exclusively alter the regulation of this enzyme? This is of particular interest because we have shown previously that dCK (EC 2.7.1.74), which catalyses a similar reaction, is not similarly regulated; its mRNA is constitutive during the cell cycle of normal and transformed cells (5). We speculate that TK may be one representative of a family of enzymes with a common growth regulation, of which other members might be enzymes of more importance for DNA precursor metabolism. It is attractive to assume that, during malignant growth, the down-regulation of deoxynucleotide synthesis is switched off. TK may be a convenient indicator for such an effect, because the half-lives of the enzyme (6) and of its mRNA (7) are short enough to reflect variations during the cell cycle.

If this stands true, we should find this type of regulation in mRNA levels for other enzymes, but not necessarily in levels of enzyme activity, because these levels might be subject to additional regulation events. Plausible candidates are shown in Fig. 1, all enzymes which are part of the de novo synthesis of DNA precursors and which catalyse unidirectional reactions. To investigate if this deregulation is a more common phenomenon, we analyzed cell cycle regulation of mRNA levels of DHFR (EC 1.5.1.3) and both subunits of RR (EC 1.17.4.1) and compared it with TK and dCK.

Results
Separation of Cells in Different Phases of the Cell Cycle by Centrifugal Elutriation. Stimulated lymphocytes and five permanent cell lines of murine or human origin were separated according to their phase of the cell cycle by centrifugal elutriation. In some of the experiments, fewer and larger fractions were drawn in order to ensure a sufficient number of cells per fraction for the analysis of mRNAs. The DNA/cell distribution was routinely determined by flow cytometry, and the percentage of cells in G1, S, and G2 was calculated for each fraction. As indicated in Figs. 2 and 3, we were able to provide good and comparable separation of all analyzed lines. The first fraction of all elutriations contained constantly more than 80% G1 cells (mean 84%). The fractions most representative for S phase contained from 61 to 79% replicating cells (mean 68%). The amount of G2 cells (also including mitotic cells) in the end fractions varied from 62% (COP-8) to 82% (EBV lymph) (mean 76%). It is important to state that the separation quality of normal versus transformed cell types is comparable, which proves that the variations in mRNA expression patterns described below are not due to fractionation artifacts.

mRNA Levels during the Normal Cell Cycle. In the normal growing lines, PHA lymph, EBV lymph, and 3T6 (Fig. 2), we found a dramatic regulation of the mRNAs for TK,
DHFR, and for both subunits of RR during the cell cycle. A 6–10-fold stimulation from G1 to early S was followed by a similar decrease during late S and G2. Studying these enzymes separately, the induction of the expression has already been mapped near the G1–S boundary (reviewed in Ref. 8). We compared the mRNA regulation of these three enzymes by subsequent hybridization of the identical Northern blots. Within the detection limits of our method, the corresponding time courses were always in perfect agreement. These data show that the transcription of the studied enzymes is induced at the same point of the eukaryotic cell cycle. This consensus can hardly be explained on the basis of an accidental coincidence and is, therefore, most probably the effect of a common regulation mechanism.

Separation by elutriation centrifugation is based on sedimentation behavior of cells and can, therefore, not absolutely discriminate G1 from S. Hence, the actual induction of mRNAs is more pronounced than the slopes presented in Fig. 2 because late G1 fractions contain a considerable proportion of S phase cells. Accordingly, we plotted the percentage of S phases per fraction of these three sets of elutriations (data not shown). The increasing part of the so-obtained curves neatly matched the corresponding mRNA fluctuations. These data allow us to conclude that, both in mice and humans, transcription of these genes is switched on at a defined point immediately before the onset of S phase.

Furthermore, the half-lives of the different mRNAs must all be very short to explain the homogeneous declines during the second half of the cell cycle. Interestingly, the steady state mRNA levels decrease earlier as one would expect, assuming ongoing transcription until the end of S phase.

As also presented in Fig. 2, the levels of dCK mRNA never displayed any significant cell cycle-dependent changes. Obviously, this gene is not a target for the regulation observed with the already described enzymes. (Note that all mRNA values are normalized to β2-microglobulin, which is constitutively expressed. Therefore, the normal increase by factor two from one mitosis until the next one was compensated.)

mRNA Levels during the Cell Cycle of Transformed Cells. Although transformed by different types of virus (papilloma, SV40, and polyoma virus), the tested lines (HeLa, SVMK, and COP-8) all showed the same pattern; mRNA levels of all tested genes were constant during the entire cell cycle, with only minor fluctuations (Fig. 3). Obviously, during viral transformation, the common regulation of TK, RR, and DHFR observed in normal cells is lost; all patterns appear like in the manner that dCK behaves in normal and transformed cells.
These observed relative distributions during the cycle of the transformed cell may be caused either by a loss of induction of expression at the G1-S boundary or by a cell cycle-independent high promoter activity. In the first case, transformed cells would express a constant and lower mRNA level, compared with normal cells. Considering the second case, the level of mRNA in transformed cells should be much higher.

One would favor the second possibility, as it is attractive to assume that during viral transformation, the synthesis of deoxynucleotides is up-regulated. One possible reason could be the advantage for the viruses of a prolonged period of optimal conditions for their own replication by securing a high supply with phosphorylated DNA precursors.

Relation of mRNA Amounts in Normal and Transformed Cells. In order to test if transformed cells exhibit a higher or lower mRNA expression of the studied enzymes, we further quantitated the Northern blot experiments presented in Figs. 2 and 3. Each hybridization had originally been exposed for different time periods, and we chose the autoradiographs of comparable intensity, which still represented the linear range. The highest points of each curve (set to 100) have been scanned by densitometry, and the obtained values were each divided by the according exposure time and normalized to the appropriate amount of β₂-microglobulin. This allows comparison of mRNA quantities of each particular gene in all studied cell lines. (Because the cDNA probes specific for the studied genes were different in respect to length and radioactivity, these relative numbers do not allow the comparison of mRNA levels from different genes within one cell line.)

For a negative control, it is most important to notice that the mRNA of dCK is of about the same level in all studied cell lines. This proves that the elevated mRNA levels in the other cases are not caused by unspecific effects like higher gene dosage, general overexpression, dramatic stabilization of mRNAs, etc.

As mentioned above, the mRNAs of the regulated genes seem to be transcribed in a relatively short period (approximately one-half of S phase) during the normal cell cycle. This means that, in this short time period, a real equilibrium between formation and degradation of mRNA is not reached, because transcription is switched off again before reaching steady-state level. On the other hand, our data conclusively show that transformed cells constitutively transcribe these genes all the time. Considering this, the level of mRNAs in transformed cells must be higher by a factor of two or more than the peak level in normal cells. The data presented in Fig. 4 perfectly support this conclusion.

Polyoma Virus Large T Antigen Alone Is Sufficient to Alter RR mRNA Regulation. It has already been shown how a constitutively high expression of TK (3) and DHFR (9) can be caused by the permanent presence of tumor antigen. To prove if such a mechanism also effects the two reductase subunits, we analyzed their mRNA regulation after specific expression of polyoma virus large T antigen in otherwise normal cells (Fig. 5). In these cells, we were able to switch from an S phase-dependent up-regulation to a constitutive pattern of steady-state mRNA by expression of this antigen. Strikingly, this effect perfectly matches the behavior of TK. (It is important to mention that dexamethasone treatment does not influence the regulation of these genes in 3T3 control cells (Ref. 3 and data not shown). Therefore, we
suggest that RR is regulated during the cell cycle by a mechanism analogous to that of TK.

**Discussion**

In earlier studies, we demonstrated that DNA tumor virus transformation changes the cell cycle-dependent expression of TK (1, 2). Normal cells exhibit a transient stimulation of enzyme activity during S phase, paralleled by TK mRNA content. In transformed cells, we observed a higher TK activity caused by an elevated and unregulated expression of this gene. In addition, activation of one viral oncogene alone was sufficient to induce this transformed pattern. In 3T3 mouse fibroblasts containing the gene for polyoma virus large T under the control of a hormone-inducible promoter, we were able to switch from one status to the other by activating the promoter (3).

It is most unlikely that transformed cells exclusively alter the regulation of TK. Speculating that TK is one of a family of enzymes regulated simultaneously during onset of DNA synthesis, we were interested if other related enzymes also express this behavior. In this study, we show that TK, RR, and DHFR, but not dCK, share this regulation, which is dependent on the transformation status. In the normal eukaryotic cell cycle, TK, both subunits of RR, and DHFR are transcribed during the same short and very distinct time period, ranging from very late G1 to mid S. In contrast, transformed cells express these genes permanently. From such an expression, one must expect a permanently elevated level of these four mRNAs, which is at least 2-fold higher than the peak value of the normal cell cycle. Accordingly, we demonstrated a 2–6-fold excess of these messages in DNA tumor virus-transformed cell lines, comparing peak values. As normal cells contain low amounts of these mRNAs during most of their cell cycle, we expect an even higher difference (4–12-fold) comparing randomly growing cultures. These data point out that analysis of mRNAs of the mentioned genes can be a helpful tool to distinguish transformed from normal cells.

What could be the mechanism causing this deregulation, and what could be its biological significance? De novo synthesis of deoxynucleotides is strictly correlated with the time period of DNA synthesis: a period of extremely high metabolism during S phase alternates with very low or undetectable activities during the rest of the cell cycle or in resting cells (reviewed in Ref. 10). Very effective processes for activation and/or deactivation must be necessary to control this regulation. Recently, a group of transcription factors called E2F were found to play a key role in transcriptional regulation of many genes encoding enzymes of the deoxynucleotide metabolism (reviewed in Ref. 11). DHFR and DNA polymerase α contain sequences in their promoter region that serve as a binding site for E2F (12–14; reviewed in Ref. 15). Furthermore, it was demonstrated that the E2F binding site in the TK gene promoter is the target for trans activation by the large T antigen of polyoma virus (16) and that enzymes like DHFR, TK, DNA polymerase α, and thymidylate synthase are all coordinately trans activated in cells growth-arrested by serum starvation, when polyoma virus large T antigen is induced by hormone addition (17; reviewed in Refs. 15, 18, and 19). Other factors besides viral early proteins may also interfere with the regulation of E2F. For instance, we previously found deregulation of TK during the cell cycle also in many tumor cell lines, which were not in obvious connex with the viruses mentioned before (1–3), one example being cells derived from a retinoblastoma, which exhibited a loss of functional pRb (20).

Obviously, this system of regulation can explain the expression patterns of TK and DHFR described in this report; but can it also be responsible for our observed data with RR? The S phase-specific expression of the genes R1 and R2 has already been shown (21), and mechanisms for the regulation of both genes independent from the transcription factor E2F have been postulated (22, 23). Were the promoter sequences of these two genes for possible E2F binding sites (data not shown) and could not detect any definitive site. Using only the so-far described consensus sequences, one cannot rule out binding of this factor at a not-yet-defined site. Nevertheless, our data shown in Fig. 5 prove a direct connection between polyoma T antigen and the regulation of RR mRNAs.

Additional evidence that analysis of primary structure of DNA alone is not sufficient to define a possible role of this transcription factor for the studied promoter come from our data with dCK. The promoter region of dCK has a sequence which can be considered as a binding site for E2F (24), but this gene never displays any cell cycle-dependent fluctuation (this paper and Ref. 5).

But why is TK a member of this family of regulated enzymes? Or in other words, why is dCK not a member when TK is? Both enzymes are postulated to be members of the salvage pathway (25). This metabolism has the purpose to provide the cell with a low supply of deoxynucleotides (e.g., for DNA repair) during the time interval when the de novo synthesis is not active. (This should not be confused with the salvage of free purine bases via phosphoribosyltransferases.) But from the metabolic reactions sketched in Fig. 1, it is obvious that dCK alone is sufficient for this purpose because all four deoxynucleotide triphosphates can be synthesized from the products of dCK. The observation that dCK is constitutively expressed in resting cells and during the whole cell cycle fits in that picture.

On the other hand, TK would be a very poor salvage enzyme if its activity is down regulated just during that time period, where salvage is necessary. The only plausible conclusion, therefore, is that dCK is the salvage enzyme and
that TK has another function, which is so far unknown, but which should be connected with de novo synthesis or with DNA replication.

Materials and Methods

Cells. 3T6 cells (permanent line of contact-inhibited fibroblasts; American Type Culture Collection CCL 96), COP-8 cells (mouse fibroblasts transformed with polyoma virus), and SVMK (primary mouse kidney cells transformed with SV40) were grown on plastic Petri dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and antibiotics (30 mg/ml penicillin and 50 mg/ml streptomycin sulfate). HeLa cervix carcinoma cells (papillomavirus transformed; American Type Culture Collection CCL 2) and EBV lymph (EBV-transformed human lymphocytes) were cultivated in 75-cm² flasks containing RPMI 1640 supplemented with 10% calf serum and antibiotics. For specific expression of polyomavirus LT, we used 373LT, which are stable transfected mouse fibroblasts; the genetic information for polyomavirus LT was placed under control of the dexamethasone-inducible mammary tumor virus promoter, and mouse 3T3 fibroblasts were transfected with this construct, together with a gene conferring neomycin resistance (26). All cultures were maintained at 37°C and 5% CO₂ and routinely screened for the absence of Mycoplasma.

Human lymphocytes (PHA lymph) were isolated from the heparinized peripheral blood of a male normal subject using the Ficoll-Hypaque gradient method and washed three times in phosphate-buffered saline. Lymphocytes were stimulated with phytohemagglutinin and analyzed after reaching logarithmic growth.

Separation of Cell Cycle Phases. Centrifugal elutriation of randomly growing cells was performed as described (27). The elutriation system consisted of a Beckman J2-21M centrifuge and a J-6B rotor equipped with a standard separation chamber (Beckman Instruments, Inc., Palo Alto, CA). The rotor was kept at a speed of 2000 rpm, temperature was 20°C, and medium flow was controlled with a Cole-Parmer Masterflex pump. Elutriation medium was phosphate-buffered saline supplemented with 0.9 mmol/l CaCl₂, 0.5 mmol/l MgCl₂, and 2% calf serum. Consecutive fractions of 150–200 ml were collected at increasing flow rates. Cell cycle distribution was determined using a PAS-II flow cytometer (Partec, Münster, Germany) after staining of DNA with 6 μmol/l 4,6-diamidino-2-phenylindol-dihydrochloride (Merck, Darmstadt, Germany).

RNA Extractions and Northern Blotting. Total cytoplasmatic RNA was prepared from the elutriated fractions according to a method described by Favoro et al. (28), except that Macaloid was omitted from the lysis buffer. RNA was isolated after cell lysis in an isotonic buffer containing the nonionic detergent Nonidet P-40 and after removal of nuclei by centrifugation. RNA (20–40 μg) of each sample was separated in a 1% formaldehyde agarose gel and transferred to Biotrans nylon membranes (ICN, Costa Mesa, CA). After UV fixation, filters were hybridized sequentially with 32P-radiolabeled probes specific for the different enzymes and for β₂-microglobulin sequences (either mouse or human, in accordance with the cellular origin). Autoradiographs were scanned and quantitated with a Quick Scan densitometer (Helena Laboratories, Beaumont, TX).

Enzyme-specific cDNA Probes. The radioactive probes were prepared from TK cDNAs of either human or murine origin (29), from a 710-bp cDNA fragment specific for human dCK (5), from a 1600-bp cDNA fragment specific for mouse DHFR (30), from a 1373-bp fragment specific for the subunit R1, and from a 1487-bp fragment specific for the subunit R2 of murine ribonucleotide reductase (courtesy of L. Thelander; Ref. 31).

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References

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