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Coordinated *trans* Activation of DNA Synthesis- and Precursor-Producing Enzymes by Polyomavirus Large T Antigen through Interaction with the Retinoblastoma Protein

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Previously constructed Swiss mouse 3T3 fibroblasts producing polyomavirus large T antigen after addition of dexamethasone were used to study the transcriptional activation by the viral protein of five genes coding for enzymes involved in DNA synthesis and precursor production, namely, dihydrofolate reductase, thymidine kinase, thymidylate synthase, DNA polymerase α, and proliferating-cell nuclear antigen. It was found that all these genes, whose expression is stimulated at the G_1/S boundary of the cell cycle after growth stimulation by serum addition, are coordinately *trans* activated when T antigen is induced in cells previously growth arrested by serum withdrawal. Cell lines carrying the information for a mutant form of large T antigen, in which a glutamic acid residue in the binding site for the retinoblastoma protein was changed into aspartic acid, were constructed to test the involvement of an interaction of T antigen with the retinoblastoma protein in this reaction. It was found that the mutated T protein is incapable of stimulating transcription of any one of the genes. The promoter of three of the genes (dihydrofolate reductase, thymidine kinase, and DNA polymerase α) unequivocally carries binding sites for transcription factor E2F, suggesting that complexes forming with this growth- and cell cycle-regulating transcription factor are the targets for T antigen. Although there is so far no evidence that thymidylate synthase and proliferating cell nuclear antigen are regulated via E2F, our data indicate that the retinoblastoma protein still is involved in the control of these genes. mRNA for E2F itself increases in amount at the G_1/S border in serum-stimulated cells but not during polyomavirus T antigen-induced transcriptional activation of DNA synthesis enzymes in arrested cells.

Enzymes involved in DNA synthesis and precursor production are regulated during growth stimulation and during the cell cycle. Their synthesis is induced at the beginning of the S phase, and in many cases controls at different levels, transcriptional as well as posttranscriptional, are crucial (for reviews, see references 32 and 42). Transcriptional regulation plays the major role when cells are stimulated to grow from an arrested state. Members of a family of transcription factors called E2F were recently found to play a key role in transcriptional regulation of many important genes in the G_1 phase and at the G_1/S boundary of the cell cycle (reviewed in references 4, 15, and 27). E2F was originally identified as a cellular protein binding to the adenovirus E2 promoter, but binding sites for E2F were later also found in cellular promoters and were shown to be important for the transcription of cell cycle-regulated genes such as c-*myc*, dihydrofolate reductase, and thymidine kinase (2, 17, 27, 30, 38). E2F is a target for the retinoblastoma tumor suppressor protein (pRB); a related protein, p107 (reviewed in references 4, 15, and 27); and possibly other so-called pocket proteins; complexes formed on binding sites for E2F were also found to contain a member of the cyclin family and a cyclin-dependent kinase (cdk), which appear to contribute to the regulation of the transcriptional activity of E2F (7, 9, 24, 26, 31, 37).

A most significant advance in relation to the function of E2F and pRB was the demonstration that pRB and p107 are focal points of the action of oncogenic proteins of DNA tumor viruses (see, e.g., references 3, 8, 17, 27, and 30 and references therein). Thus, the adenovirus E1A protein, the large tumor antigens (LT) of simian virus 40 and polyomavirus, and the E7 protein of human papillomaviruses were found to bind to these proteins, causing an activation from E2F-regulated promoters apparently by removing pRB or p107 and thereby inducing the transcriptional activity of E2F. Complex formation with pRB or p107 requires a binding site carrying the motif Asp/Asn-Leu-X-Cys-X-Glu, which is present in all of these tumor virus proteins (see reference 3 and references therein). DNA tumor viruses require host cells in S phase for their own replication (40). Therefore, an important cellular function of one or more proteins encoded by these viruses is to comply with this demand. One important step in this direction could be the *trans* activation of genes coding for proteins and enzymes important for DNA synthesis.

To contribute to the understanding of the functions of the pleiotropic LT of polyomavirus, we have previously constructed cell lines which carry the information for the protein in inducible form under the direction of the mouse mammary tumor virus promoter (28). These cell lines (3T3-LT) produce T antigen after addition of 0.01 to 1 μmol of dexamethasone per liter (29). Using these cell lines, we have recently shown that the endogenous mouse thymidine kinase gene is transcriptionally activated by polyomavirus LT protein at the E2F site of the promoter (30). In this work we have extended these studies to several other genes coding for DNA synthesis enzymes and precursor-producing enzymes. We show here that dihydrofolate reductase, thymidylate synthase, thymidine kinase, DNA polymerase α, and proliferating-cell nuclear antigen, a protein cooperating with DNA polymerase δ in DNA replication, are all coordinately *trans* activated in cells growth arrested by serum starvation, when polyomavirus LT is induced by hormone addition. This *trans* activation requires the intact binding

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site for pRB or p107 within the T antigen. The LT-induced increase in levels of mRNA encoding DNA synthesis enzymes in the absence of serum parallels that observed upon serum stimulation.

MATERIALS AND METHODS

Cell lines. The 3T3-LT cell line expressing polyomavirus LT after addition of dexamethasone was described previously (28). Cell lines producing LT mutated within the Asp/Asn-Leu-X-Cys-X-Glu motif involved in pRB binding were prepared as follows. The glutamic acid residue (amino acid 146 of the LT sequence) was mutated into an aspartic acid residue by oligonucleotide-directed in vitro mutagenesis, using plasmid pMMTV-PyLT (28) and the mutagenesis system of Amersham as recommended by the manufacturer. The presence of the correct mutation was verified by sequencing. This yielded plasmid pMMTV-PyLTmut. Then 18 μg of pMMTV-PyLTmut and 2 μg of pSV2-*neo* were cotransfected into Swiss 3T3 cells as described previously (28). Neomycin-resistant cells were selected by growth in the presence of geneticin (600 μg/ml of medium), and individual clones were expanded and grown into mass cultures. These were then tested by Western immunoblotting for the production of LTmut protein after addition of 1 μmol of dexamethasone per liter. The antibodies used were the rat monoclonal anti-polyomavirus T antibody αPyC4 (6) followed by rabbit anti-rat immunoglobulin and finally by peroxidase-conjugated goat anti-rabbit immunoglobulin (Accurate). In a modification of the method used previously (28), we used the ECL Western blotting analysis system (Amersham), as recommended by the vendor, to visualize the protein.

Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. For growth arrest, semiconfluent cultures were fed with medium containing 0.2% serum and used 3 days later either for induction of LT protein by addition of dexamethasone (keeping the serum concentration low) or for serum stimulation by addition of fetal calf serum to 20%.

For transient chloramphenicol acetyltransferase (CAT) assays 3T3-LT or 3T3-LTmut cells were transfected as described previously (30) with a plasmid carrying the CAT gene under the control of the murine thymidine kinase promoter. Preparation of cell extracts and determination of CAT activity were done as described by Gorman (13).

Preparation and analysis of RNA. Cytoplasmic RNA was extracted by the method of Favaloro et al. (10), except that Macaloid was omitted from the lysis buffer. RNA (30 μg per lane) was separated in formaldehyde-agarose gels and transferred to GeneScreen nylon membranes. After UV fixation, membranes were hybridized with ^{32}P-labeled probes generated by random-primer labeling (34). After exposure of an X-ray film, filters were washed to remove the probe and then rehybridized with a different one. The bands on the films were scanned, and the values obtained for each time point were corrected for RNA loading by equalizing the signal obtained with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

Band shift assays. Cellular (31) or nuclear (1) extracts were prepared from Swiss 3T3, 3T3-LT, or 3T3-LTmut cells previously growth arrested by serum withdrawal followed by addition of dexamethasone (final concentration, 1 μmol/liter) for the induction of LT protein. The oligonucleotide used carried the E2F site of the murine thymidine kinase promoter (30), and band shift assays were done as described previously (31).

Replication assay. The function of polyomavirus LT or LTmut in allowing replication of plasmids carrying a polyomavirus origin of replication was analyzed essentially as described previously (28). In short, the plasmid pGEMPyA3ori, which contains the polyomavirus origin and enhancer region of strain A3 (from nucleotides 5021 to 84), was transfected into 3T3-LT or 3T3-LTmut cells by the DEAE dextran method. Dexamethasone (final concentration, 1 μmol/liter) was then added to half of the cultures, while the other half remained in medium without the hormone. Plasmid DNA was extracted about 24 h later, digested with *Dpn*I to remove unreplicated input plasmid, linearized with *Eco*RI, and analyzed by Southern blotting as described previously (28).

RESULTS

Coordinated *trans* activation of five DNA synthesis and precursor-producing enzymes by polyomavirus LT. In growth-stimulated cells, DNA synthesis enzymes and several precursor-producing enzymes are induced during the traversion from the G_1 to the S phase of the cell cycle (reviewed in references 32 and 42). This was shown previously for individual enzymes. For the purpose of our present study, we have analyzed the time course of induction of stable mRNA for five DNA synthesis enzymes after serum addition to Swiss 3T3 cells previously arrested by serum withdrawal (Fig. 1). All of the G_1/S-regulated enzymes and proteins probed in this work were induced at approximately the same time after serum stimulation. In fact, when the hybridization signals were quantitated by scanning and the increase in the amount of mRNA was plotted as a function of the time after serum addition, no significant difference could be observed in the timing of induction of mRNAs for the DNA synthesis enzymes within the time points chosen in this work, although it is quite evident that the relative increase in the steady-state levels of different mRNA varied. The amount of mRNA for GAPDH was found to be fairly independent of the growth status of these cells and served as a control for RNA loading. DNA synthesis in 3T3 cells started with our experimental protocol about 12 h after serum addition (results not shown). Several of the genes coding for DNA synthesis enzymes were already found to carry binding sites for transcription factor E2F in the promoter. This is specifically true for dihydrofolate reductase (2, 17) and thymidine kinase (30), for which there is strong evidence for a role of E2F in growth regulation of gene expression. In addition, the human gene coding for DNA polymerase α carries E2F-binding motifs within the promoter region (33), although their involvement in the control of gene expression has yet to be shown. We have therefore chosen these three enzymes for our study and have complemented them with two further proteins for which there is so far no evidence for a participation of E2F in the regulation, namely thymidylate synthase (19) and proliferating-cell nuclear antigen (43). By using experiments involving retardation in gel electrophoresis, several laboratories have indicated a shift in complexes formed on the E2F-binding site at the boundary between the G_1 and S phases of the cell cycle from those containing several proteins to those supposedly containing only free E2F (see, e.g., references 3, 30, and 35). Because the amount of free E2F increases much more than could be accounted for by its production from higher-molecular-weight complexes, we also examined the level of E2F1 mRNA during serum stimulation, and, in agreement with a recent independent report (38), we found this to increase with kinetics similar to that of mRNAs for DNA synthesis enzymes (Fig. 1), indicative of synthesis of new E2F at the G_1/S boundary of the cell cycle.

Having shown coordinated expression of the chosen DNA synthesis enzymes during serum stimulation, we next asked

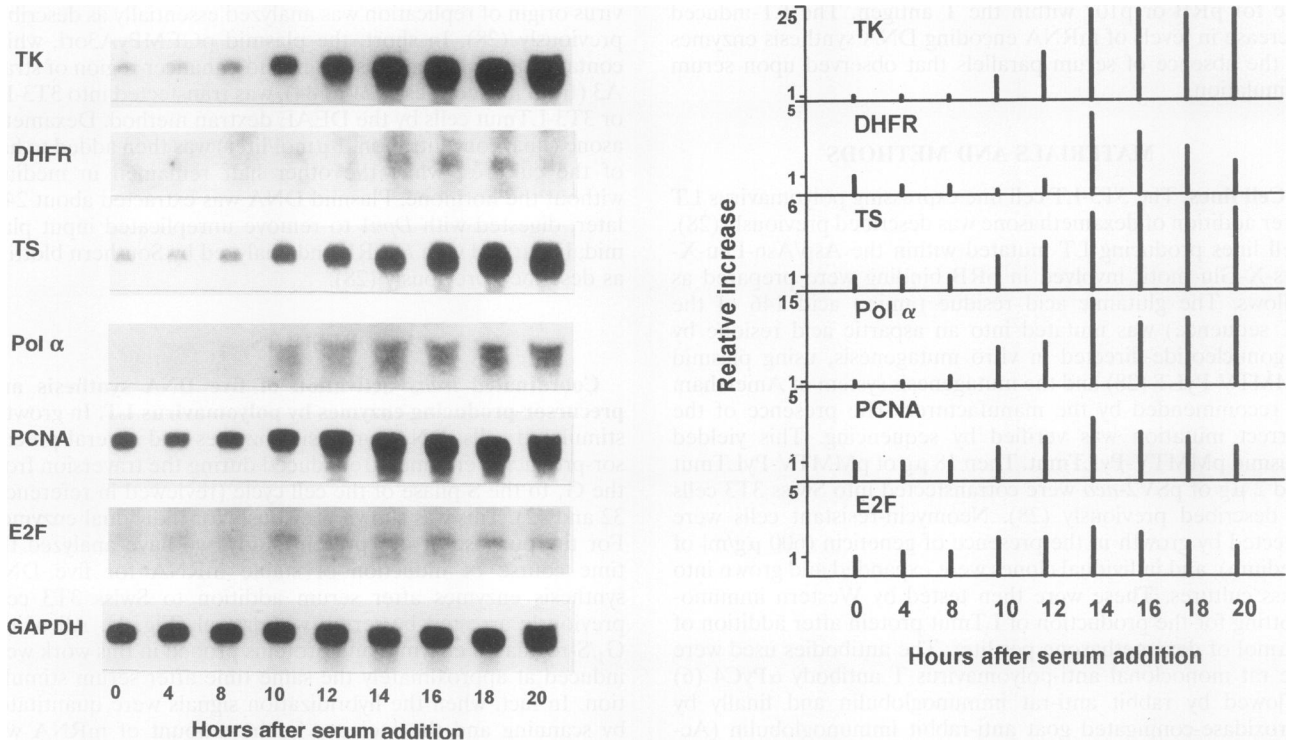

FIG. 1. Coordinated induction of mRNAs for DNA synthesis enzymes during growth stimulation of arrested cells by addition of serum. 3T3-LT cells were arrested by serum withdrawal for 3 days and then stimulated by addition of medium containing 20% fetal calf serum. RNA was isolated at the times indicated and separated by electrophoresis for Northern analyses. Blots were consecutively hybridized to labeled cDNA probes for thymidine kinase (TK), dihydrofolate reductase (DHFR), thymidylate synthase (TS), DNA polymerase α (Pol α), proliferating-cell nuclear antigen (PCNA) transcription factor E2F, and GAPDH. The last probe was used as an example of a gene whose expression is only weakly dependent on the growth status of our cells. It therefore served as a control for RNA loading. The right panel of the figure shows the quantitation of the data as obtained by scanning of the autoradiograms and correcting for RNA loading by using the signal for GAPDH. These data are presented as relative increase of RNA, setting the signal obtained at the 0-h time point as 1. Because the fold increase varies between different mRNAs, the unit of 1 in the ordinate was adjusted for each mRNA, taking into account the maximal stimulation found for the respective mRNA.

whether all of these proteins are *trans* activated by polyomavirus LT protein. With our experimental setup we are able to measure transcriptional activation simply by adding dexamethasone to serum-starved arrested cells and then determining steady-state levels of mRNA by Northern blotting as an indicator of an expression of the respective genes from their normal location within the mouse genome. The results of this experiment are shown in Fig. 2. It is apparent that all of the genes coding for DNA synthesis proteins were *trans* activated by polyomavirus LT. As a control we used several genes not involved in S-phase activities, such as those coding for β-microglobulin, for α-actin, and for GAPDH. No gene of the control group was *trans* activated; the analysis of GAPDH is included in Fig. 2 as a negative control. Most significantly, transcriptional activation in all cases depended on an intact binding site for pRB within the LT protein. This is shown here for one 3T3-LTmut line producing fair amounts of the mutated LT protein (see below and Fig. 3); a similar negative result was obtained with two other cell lines carrying identically mutated LT antigen. It is furthermore noteworthy that hybridization to the cDNA for E2F1 did not reveal any increase in the expression of the E2F1 gene as the result of LT production. This result was obtained in three completely independent sets of experiments. It indicates that, in contrast to the situation in serum-stimulated cells, E2F1 expression is not turned on by T antigen in parallel to that of DNA synthesis enzymes, although, as shown below, band shift experiments with an oligonucleotide carrying the E2F site present in the murine thymidine kinase promoter revealed a significant increase in free E2F in cells producing polyomavirus LT.

Wild-type and mutant T antigens are produced in comparable amounts and show no significant difference in stability. In this study we used a 3T3-LT cell line employed before, which carries the information for wild-type polyomavirus LT under the control of the mouse mammary tumor virus promoter and which, upon addition of very small amounts of dexamethasone, gives rise to significant levels of LT protein (28, 29). In addition to a previously used cell line carrying a mutated T antigen (30), which has a lysine residue in place of a glutamic acid residue in position 146 within the pRB-binding sequence, we have for the present work constructed several cell lines (called 3T3-LTmut) carrying a mutant form of LT in which the glutamic acid residue was changed to aspartic acid. This mutation was shown previously to preclude the pRB-binding and immortalizing ability of polyomavirus LT (23); we considered it superior to the lysine mutant because we expected less potential influence of this amino acid change on properties of the pleiotropic protein other than pRB binding. To exclude the possibility that the failure of mutant LT protein to *trans* activate any one of the DNA synthesis enzymes studied in this work was due to reasons unrelated to the absence of the pRB-binding capacity, we carried out several control experiments. In particular, Western blotting analyses (Fig. 3) showed that wild-type and mutant T antigens were induced to compa-

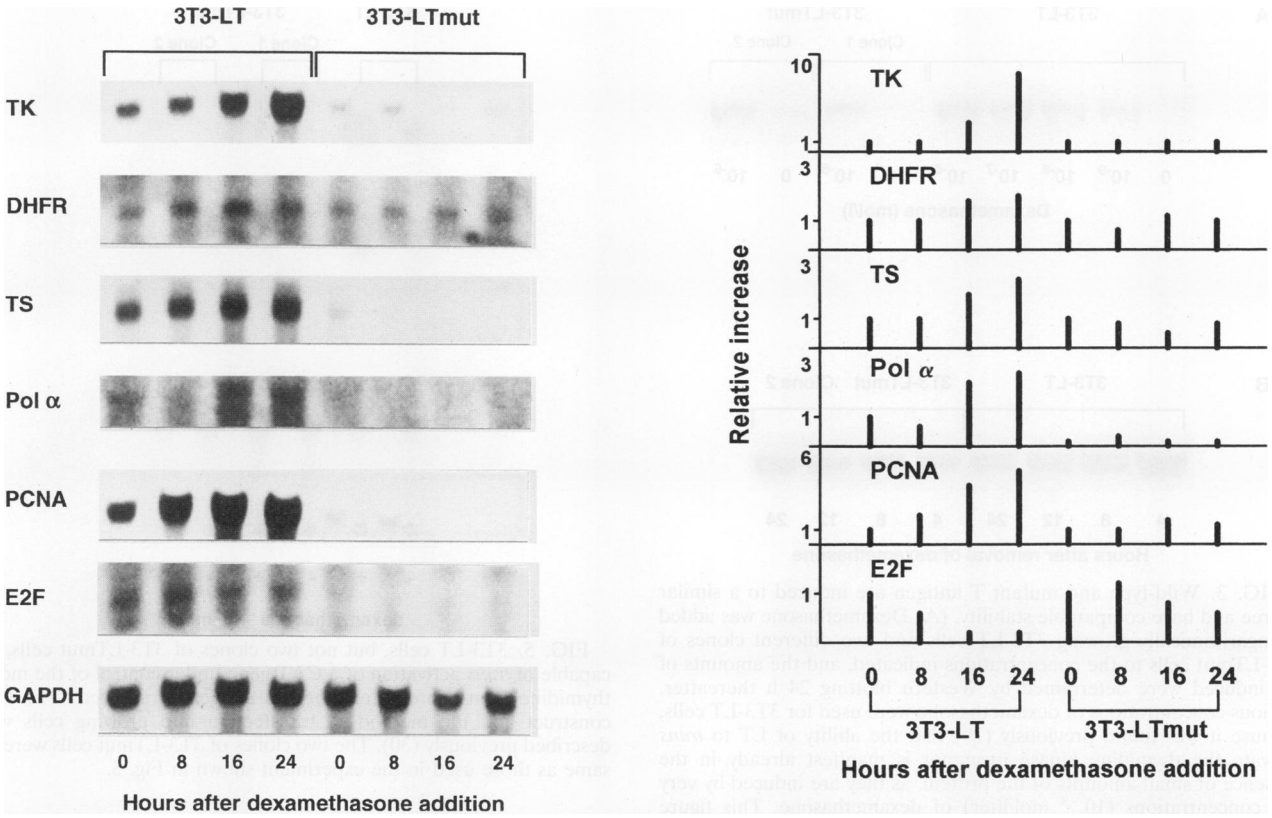

FIG. 2. DNA synthesis enzymes are coordinately transactivated by polyomavirus LT but not by a mutant T antigen which is defective in pRB binding. Cells carrying the information for wild-type (3T3-LT) or mutated (3T3-LTmut) T antigen were arrested by serum starvation for 3 days. Dexamethasone was then added to a final concentration of 1 μmol/liter, keeping the serum concentration at 0.2%. RNA was isolated at the times indicated and analyzed by Northern blotting as described in the legend to Fig. 1. Because of the small amounts of E2F1 mRNA produced in serum-starved cells, exposure times for analysis of this mRNA were considerably longer than in the other cases. The right panel of the figure again shows a quantitation of the bands in the autoradiograms obtained as described in the legend to Fig. 1.

rable levels in the cell lines and that the proteins had similar stability. In affirmation of this result, we found that the mutant T antigen was still capable of supporting the replication, in logarithmically growing 3T3-LTmut cells, of a plasmid carrying a polyomavirus origin of replication (Fig. 4), although previous experiments indicated that the plasmid replication function required much larger amounts of LT than did the activation of transcription (29).

The 3T3-LTmut lines used in the present work were, furthermore, found to be incapable of *trans* activating a transfected gene coding for chloramphenicol acetyltransferase (CAT) when this gene was attached to the murine thymidine kinase promoter which carries a binding site for transcription factor E2F. As shown in Fig. 5, transcriptional activation was observed only in 3T3-LT cells synthesizing wild-type LT after hormone addition but not in two different cell lines carrying the mutation from glutamic acid to aspartic acid at position 146 of the LT protein.

Transcriptional activation by LT was accompanied by changes in protein complexes at the binding site for E2F; a shift from higher molecular complexes to free E2F was observed (Fig. 6). This shift occurred on hormone addition to serum-starved 3T3-LT cells but not when either 3T3 cells (carrying no information for polyomavirus LT) or 3T3-LTmut cells (possessing information for mutated LT) were used. It is worth noting that the shift in E2F complexes obtained with extracts of 3T3-LT cells can already be seen 8 h after hormone addition, whereas levels of mRNAs for DNA synthesis enzymes increased significantly only after 16 h (Fig. 2). This is probably because sufficient levels of mRNA have to build up to be seen in Northern analyses of steady-state mRNA. A more precise correlation between the timing of the interaction of LT protein with E2F complexes and an increase in transcriptional activity at the promoters would require run-on experiments. Irrespective of this, however, results of all the control experiments support the conclusion that, in contrast to wild-type LT, the mutant LT protein, which is unable to interact with pRB, fails to stimulate transcription from promoters of G_1/S-regulated genes; they exclude the possibility that this failure is due to insufficient amounts or to inferior stability of the protein induced by dexamethasone in 3T3-LTmut cells.

DISCUSSION

The expression of five enzymes involved in DNA synthesis or precursor production (dihydrofolate reductase, thymidine kinase, thymidylate synthase, DNA polymerase α, and proliferating-cell nuclear antigen) chosen for the present study is uniformly regulated at the boundary of the G_1 and S phases of the cell cycle. According to current evidence, the control of some of these enzymes, namely dihydrofolate reductase (2, 38), thymidine kinase (30), and probably also DNA polymerase α (33), is mediated by transcription factor E2F, whose activity is restrained in early G_1 by the tumor suppressor protein pRB

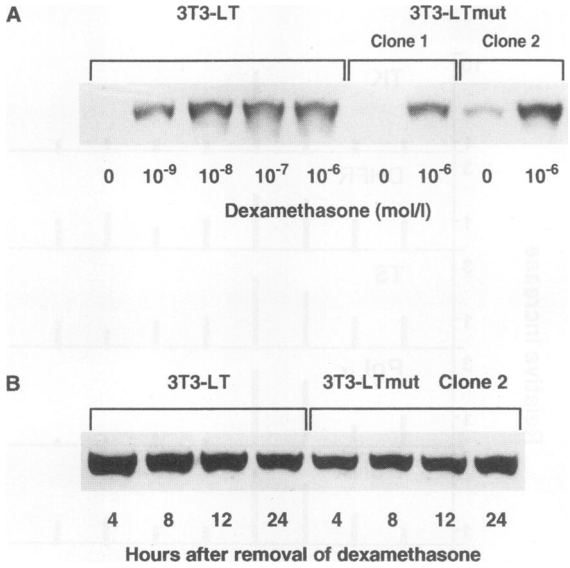

FIG. 3. Wild-type and mutant T antigen are induced to a similar degree and have comparable stability. (A) Dexamethasone was added to logarithmically growing 3T3-LT cells and two different clones of 3T3-LTmut cells to the concentrations indicated, and the amounts of LT induced were determined by Western blotting 24 h thereafter. Various concentrations of dexamethasone were used for 3T3-LT cells, because it was found previously (29) that the ability of LT to *trans* activate the thymidine kinase promoter is manifest already in the presence of small amounts of the protein, as they are induced by very low concentrations (10^{-8} mol/liter) of dexamethasone. This figure shows that the quantities of LTmut protein induced by 1 μmol of dexamethasone per liter (the concentration routinely used) exceed those minimally required for *trans* activation by wild-type LT. (B) The stability of wild-type and mutated LT was tested by inducing the protein in the respective cells by addition of 1 μmol of dexamethasone per liter for 24 h. The hormone was then removed, and LT protein was analyzed by Western blotting at various times thereafter. There is no difference in the stability of wild-type and mutant proteins.

or by the related protein p107 (reviewed in references 4, 15, and 27). Both of these proteins are subject to cell cycle-dependent phosphorylation by cyclin-dependent protein kinases, which is thought to result in a release of the suppressor from E2F and an activation of the transcription factor (5, 12, 35). It is so far uncertain whether E2F is also involved in the regulation of thymidylate synthase and proliferating-cell nuclear antigen. pRB and p107 are both targets for proteins

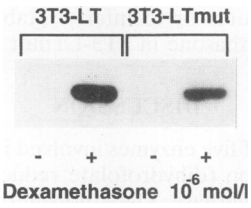

FIG. 4. T antigen mutated in the pRB-binding motif is capable of supporting the replication of a plasmid carrying a polyomavirus origin of replication. Plasmid pGEMPyA3ori was transfected into logarithmically growing 3T3-LT or 3T3-LTmut cells. T antigen was induced by addition of dexamethasone to half of the cultures, and replication of the plasmid was measured as described in Materials and Methods (for details, see reference 28). Low-molecular-weight *Dpn*I digestion products of unreplicated DNA are omitted.

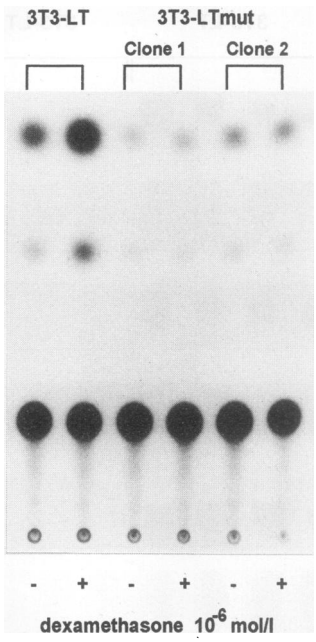

FIG. 5. 3T3-LT cells, but not two clones of 3T3-LTmut cells, are capable of *trans* activation of a CAT gene under control of the mouse thymidine kinase promoter when T antigen is induced. The CAT construct and the method of transfection into growing cells were described previously (30). The two clones of 3T3-LTmut cells were the same as those used in the experiment shown in Fig. 3.

encoded by DNA tumor viruses (3, 8, 17, 27, 30). Thus, adenovirus E1A protein, simian virus 40 or polyomavirus large T antigen, and the E7 protein of human papillomaviruses all bind to the underphosphorylated form of pRB or p107 via a sequence motif present in all of these proteins. It was shown previously that E1A can *trans* activate dihydrofolate reductase (17), and we recently reported the *trans* activation of murine thymidine kinase by polyomavirus LT (30). In this paper we show that polyomavirus LT coordinately activates the transcription of all five DNA synthesis enzymes described above and that this *trans* activation requires an intact pRB-binding site in the viral protein (Fig. 2). The control experiment described in Fig. 3 and our previous observation that the transcriptional activation by polyomavirus LT requires only very small amounts of the protein (29), supported by the finding that the mutant protein is still sustaining the replication of plasmids carrying a polyomavirus origin of replication (Fig. 4), rule out the possibility that the failure of mutant protein to *trans* activate is due to the production of insufficient amounts of LTmut protein in hormone-treated 3T3-LTmut cells. We could, furthermore, show that wild-type and mutant proteins display comparable stability. Hence, our data support a model according to which polyomavirus LT and other DNA tumor virus proteins stimulate the transcription of DNA synthesis enzymes by interaction with pRB, p107, and other potential "pocket" proteins which are capable of forming complexes with the viral proteins. This could satisfactorily explain the *trans* activation of genes having a binding site for E2F within their control region. No clear evidence has so far been obtained for the presence of E2F motifs in the promoters of the genes coding for thymidylate synthase (19) or for proliferating-cell nuclear antigen (43). Hence, our data can be explained by assuming that within these promoters there are, in fact, also sequences which function as recognition sites for

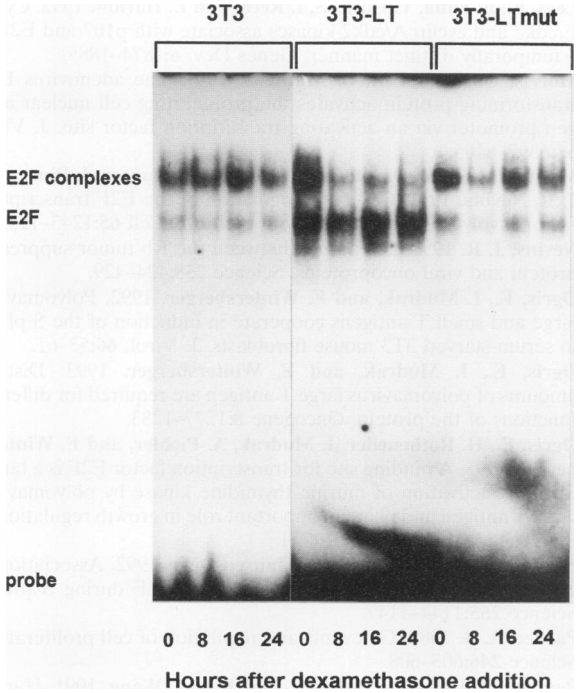

FIG. 6. Wild-type LT, but not the mutant protein incapable of binding pRB, causes a shift of higher molecular complexes formed on the binding site for transcription factor E2F toward free E2F. Cell extracts were prepared from growth-arrested cells at various times after addition of dexamethasone to 1 μmol/liter, and band shift experiments were carried out with an oligonucleotide containing the E2F site of the thymidine kinase promoter (30) as the probe.

E2F. Alternatively, the possibility must be considered that pRB, p107, or another pocket protein intervenes with transcriptional factors other than E2F but having a similar effect on gene expression. Indeed, pRB has already been shown to be capable of interaction with a variety of transcriptional factors, negatively or positively regulating their activity (21, 22, 41). On the other hand, it is conceivable that T antigen also cooperates with different proteins in the cell, and, although unlikely, it cannot be entirely ruled out that the mutation in the pRB-binding region caused a change in the three-dimensional structure of the protein which inactivated, apart from the interaction with pRB and p107, other functions of the protein, including the *trans* activation of the promoters of thymidylate synthase and proliferating-cell nuclear antigen. In this regard it is worth recalling that adenovirus E1A protein was found to *trans* activate the promoter for proliferating-cell nuclear antigen via a binding site for activating transcription factor (25). It is not known whether this activity is affected by a mutation in the pRB-binding motif of E1A. Interestingly, activating transcription factor type 2 was shown to be a target for pRB in the control of transforming growth factor β2 (TGF-β2) gene expression (22).

Band shift experiments carried out in a variety of laboratories provided evidence for a change of complexes formed between cellular proteins and the E2F-binding site when cells move from the G_1 into the S phase. In all cases an increase in the amount of free E2F was observed while higher molecular complexes partially disappeared. As shown in Fig. 1 and confirming a previous report (38), this increase could be due to new synthesis of the protein during G_1/S transition. Polyomavirus LT causes a similar shift in complexes at the E2F site, but in this case the increase in the level of E2F cannot be explained by increased synthesis of the transcription factor because there does not seem to be any induction of E2F transcription by polyomavirus LT (Fig. 2). Recent evidence implies that there are several proteins binding to the E2F site, two of which, E2F1 (16, 20, 36) and DP-1 (11), have so far been cloned. E2F, furthermore, appears to bind as a heterodimer (18). Since in band shifts either one of these proteins probably will react, while the probe used in the Northern blots was E2F1 cDNA, clarification of the situation will require the use of cDNA probes for the various members of the E2F family in Northern (RNA) blots as well as specific antibodies against the purified transcription factors in band shift experiments, once all of these reagents are available.

Finally, we would like to mention that it is highly unlikely that the coordinated *trans* activation of the five DNA synthesis enzymes studied in this work is due to an LT-elicited shift of serum-starved cells into the S phase rather than to a direct interference of the viral protein with the transcription apparatus in the promoters of the genes. First, we showed previously that LT protein alone causes only a very small fraction of arrested 3T3 cells to move into the S phase (28); the increase we observed in mRNA levels by far exceeded that expected from a low percentage of cells moving into the S phase. Second, as shown in Fig. 1, cells driven into the S phase by serum stimulation exhibit increased synthesis of E2F1 mRNA. This was not observed in the experiments involving LT as *trans* activator (Fig. 2).

Induction of DNA synthesis enzymes by proteins encoded by DNA tumor viruses is probably part of the program by which these viruses cause infected cells to move into the S phase, thereby satisfying a requirement for the replication of viral DNA. It is inspiring to speculate that this ability of the viruses is also responsible for their ability to immortalize cells. The observed connection between pRB binding and immortalization in the case of polyomavirus LT (14, 23) suggests that deregulation of the expression of DNA synthesis enzymes might play an important role in the immortalization of cells by polyomavirus. In contrast to precrisis cells, immortalized ones are commonly aneuploid and display general characteristics of genome mobilization such as DNA amplification and recombination. This phenotype, which is also characteristic for cells transformed by simian virus 40 (39), could be the consequence of altered pools of DNA precursors resulting from the *trans* activation of precursor-producing and DNA synthesis enzymes by proteins of DNA tumor viruses.

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