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A Binding Site for Transcription Factor E2F Is a Target for *trans* Activation of Murine Thymidine Kinase by Polyomavirus Large T Antigen and Plays an Important Role in Growth Regulation of the Gene

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The promoter of the murine thymidine kinase gene contains a binding site for transcription factor E2F. Using cell lines (3T3-LT) conditionally expressing polyomavirus large T antigen from a hormone-responsive promoter and reporter gene constructs carrying the thymidine kinase promoter with intact or mutated E2F sites, we show that this E2F site is the target for *trans* activation by the viral protein. Transcription of the growth-regulated endogenous thymidine kinase gene can be activated in serum-starved, quiescent 3T3-LT cells by induction of T antigen. Activation of transcription from the thymidine kinase promoter requires an intact binding site for the retinoblastoma protein in the T antigen. The same promoter region was furthermore shown to play a major role in growth regulation of the gene. As several other DNA synthesis enzymes also carry E2F binding sites in their promoters, our observations suggest a common mechanism of growth regulation of these genes and that they all might be targets for *trans* activation by DNA tumor virus proteins.

Enzymes involved in DNA synthesis and precursor production are coordinately regulated with growth in mammalian cells; they are induced in late G_1 to early S phase of the cell cycle. The molecular basis of this regulation has yet to be established. Promoter sequences known for some of the enzymes differ markedly. For instance, while the promoters for cytoplasmic thymidine kinase (TK) of human and hamster cells have TATA and CCAAT boxes, the mouse promoter lacks both of these elements (reviewed in reference 27). Common to all of these promoters are GC boxes containing binding sites for transcription factor SP1. In addition, experiments on the promoter of mouse dihydrofolate reductase have shown that this promoter carries a binding site for transcription factor E2F (17) which is recognized by a protein (HIP1) whose relation to E2F is not clear at present (24). Similar motifs are known to be present in promoters of other DNA synthesis enzymes, namely, those of DNA polymerase α (31), TK (35), and possibly thymidylate synthase (21). We show here that the E2F binding site present in the murine TK promoter is the target for *trans* activation of the TK gene by polyomavirus large T (LT) antigen and is likely to play a major role in growth regulation of this gene. As the LT antigens of polyomavirus and simian virus 40 share with the E1A protein of adenovirus and the E7 gene product of human papillomaviruses the capacity to interact with the retinoblastoma protein (pRb), a negative regulator of E2F during the G_1 phase of the cell cycle (reviewed in references 7 and 16), these observations indicate that promoters for DNA synthesis enzymes may be regulated by E2F and pRb.

MATERIALS AND METHODS

Plasmids. Plasmids used for transfection are schematically shown in Fig. 1. They were constructed by standard procedures (34), using parts of the genomic clones of mouse TK (33, 35) and a commercially available chloramphenicol acetyltransferase (CAT) plasmid. Mutations in the E2F site or in MT3 were introduced by using oligonucleotides carrying the mutations (synthesized in a Pharmacia Gene Assembler) and the in vitro mutagenesis system of Amersham as recommended by the supplier. The presence of the mutations in the plasmids was verified by determination of the DNA sequence.

Cell culture, transfection, and CAT assays. Swiss 3T3 or 3T3-LT cells (28) were grown in medium containing 10% fetal bovine serum. For growth arrest by serum deprivation, the serum concentration was reduced to 0.2% and cells were used 3 days thereafter. For transfection of plasmids, cells growing logarithmically on 10-cm-diameter dishes were treated with 20 μg of the CAT plasmids, using the calcium phosphate coprecipitation technique (15). After 8 h, cells received fresh medium. They were grown overnight and then trypsinized, and equal numbers were seeded onto two dishes, one of which received dexamethasone to a final concentration of 10^{-6} mol/liter for full induction of LT antigen; the other dish served as a control not expressing the viral protein. CAT assays (15) were carried out 28 h later, using equal amounts of cellular extracts.

Preparation of nuclear extracts and band shift assays. 3T3 or 3T3-LT cells growing logarithmically in medium containing 10% serum or arrested by serum starvation were used. Dexamethasone was added to the cultures to a final concentration of 10^{-6} mol/liter for the time indicated in individual experiments. Nuclear extracts were then prepared as described previously (1), and band shift assays were carried out (30) by using a labeled synthetic double-stranded oligonucleotide corresponding to that part of the TK promoter which contains the E2F site (see the E2F oligonucleotide in Fig. 1a). The nuclear extract was treated with deoxycholate (DOC) as described previously (5). Anti-pRb monoclonal antibody XZ142 (19) or anti-p107 monoclonal antibody SD9

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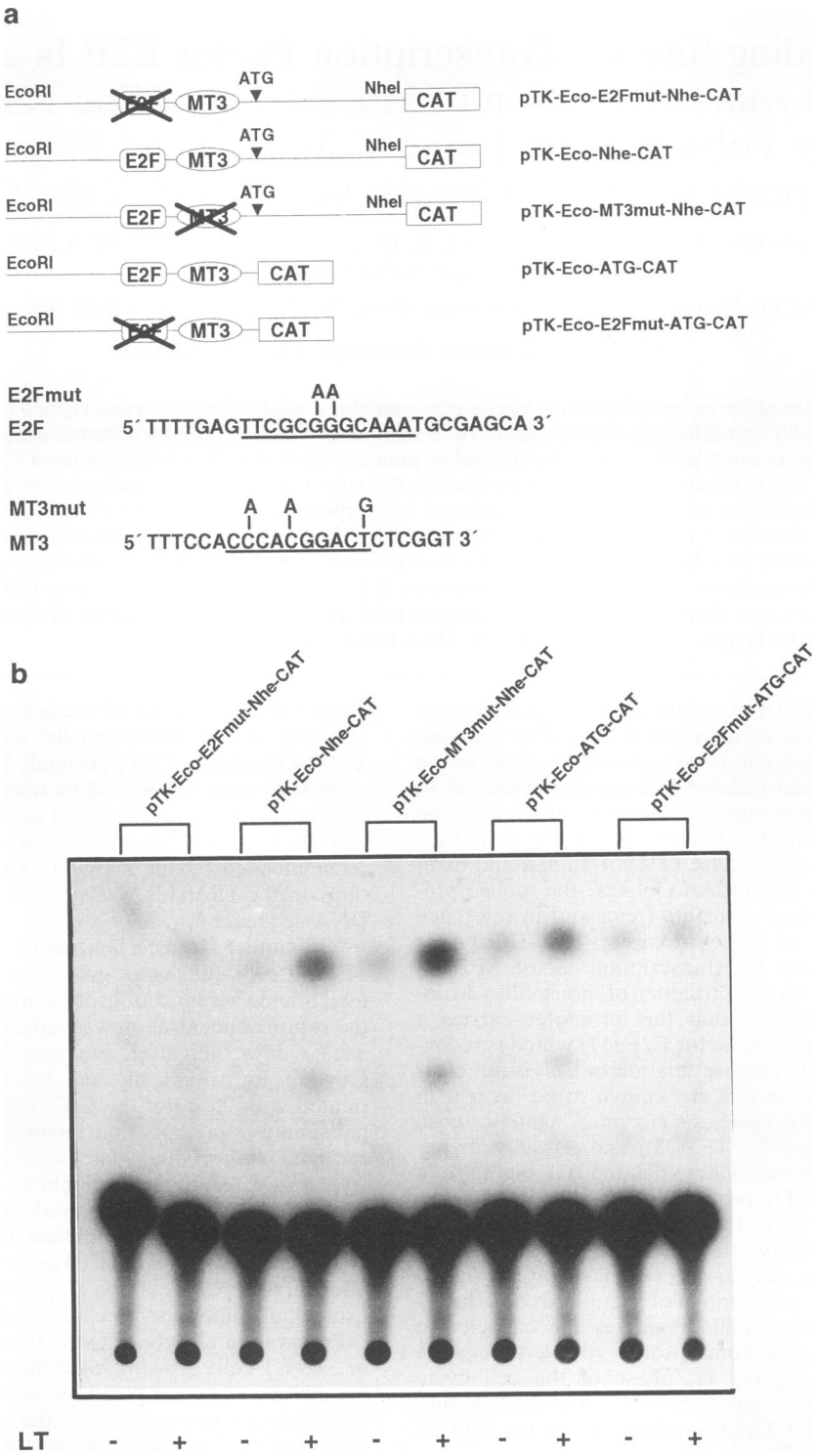

FIG. 1. (a) Constructs used. The CAT gene was placed under the control of the promoter of the murine TK gene either ranging from an *Eco*RI site at nucleotide −555 upstream of the initiation codon and ending at an *Nhe*I site in exon 3, thus including enhancer sequences within the expressed part of the gene (33) (upper three constructs), or ending at the initiation codon of TK (lower two constructs). The E2F and MT3 sequences are indicated; mutated forms are crossed out. The drawing is schematic and not to scale. The sequences of the E2F binding site and of MT3 are shown, including the mutations introduced for inactivation. (b) CAT assay. Induction of LT antigen by addition of 10^{-6} mol of dexamethasone per liter (indicated as + LT) to 3T3-LT cells (28) previously transfected with CAT constructs shown in panel a causes an increase in CAT expression which is eliminated by mutation of the E2F binding site but not by mutation of the MT3 sequence.

was added to the nuclear extract prior to complex formation with labeled oligonucleotide.

Isolation of RNA and Northern (RNA) analysis. The isolation of cytoplasmic RNA and the Northern analysis were carried out as described previously (34). Equal amounts (30 μg) of RNA were applied to individual lanes. The murine TK cDNA was used as the hybridization probe.

RESULTS AND DISCUSSION

When the upstream region of the murine TK gene was placed in front of the CAT gene and the resulting construct was transfected into 3T3-LT cells which conditionally express polyomavirus LT protein (28), transient CAT expression was significantly higher in cells expressing the polyomavirus protein than in the controls (Fig. 1). As LT antigen is likely to exert its *trans*-activating ability, at least in part, via an interaction with pRb in an E2F-pRb complex and as the TK promoter carries a binding site for transcription factor E2F (TTCGCGGGCAAA; nucleotides −68 to −80 upstream of the ATG [35]), we considered it likely that this binding site is involved in the upregulation of the promoter by LT protein. To test this presumption, the E2F site was mutated by replacing the two central G residues by A residues. This change eliminated the binding capacity of the sequence, as shown by mobility shift experiments (Fig. 2a) and the *trans* activation of the promoter by LT antigen (Fig. 1). The murine TK promoter is weak but is upregulated by sequences within the expressed part of the gene. In particular, we have observed the presence of enhancer sequences within intron 2 of the TK gene (33). Addition of these sequences to the CAT constructs led to an increase in CAT activity but did not cause any change in the *trans* activation by LT protein and the specificity of this process as regards the requirement for an intact E2F binding site (Fig. 1). Dou et al. (12) reported recently that another region of the murine TK promoter (called MT3; nucleotides −29 to −42 [11]) is involved in growth regulation of the gene. These authors suggested that pRb may be involved in the regulation through this site. As our CAT constructs included this sequence, we tested whether this region contributes to the observed *trans* activation. The sequence was mutated in vitro at three positions within a stretch important for its function (11). We found that this alteration of the MT3 site did not influence the *trans* activation by LT protein (Fig. 1), which, together with results described below, suggests that the E2F site is the sole or at least the major target for this effect.

So far we have tested for *trans* activation by transient transfection of reporter gene constructs. To extend this study to the role of the E2F site within the TK promoter in regulation of the endogenous TK gene, the following experiments were carried out. Serum-starved Swiss 3T3 or 3T3-LT cells were treated with 10^{-6} mol of dexamethasone per liter, which causes full induction of LT protein in the 3T3-LT line (28). Nuclear extracts were prepared prior to and after addition of the hormone and tested in DNA mobility shift experiments for the presence of complexes forming at a synthetic E2F binding site of the TK promoter (Fig. 2). As shown in a number of other studies, mobility shift experiments using E2F sites reveal the formation of several complexes, the simplest one containing free E2F. This complex can also be obtained by dissociation of higher-order complexes with DOC (5). We assume that part of the major slowly migrating complex contains pRb in addition to E2F, and we tentatively call this complex E2F-pRb. This

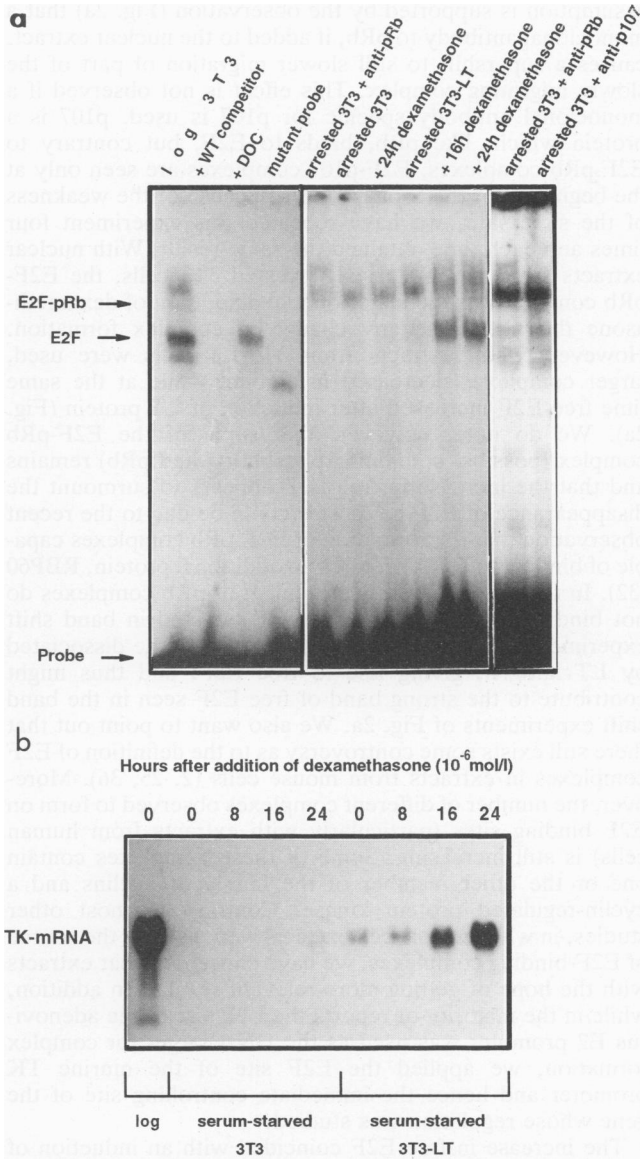

FIG. 2. (a) Mobility shift assays. Band shifts in gel electrophoresis indicate the formation of complexes on the E2F binding site from the TK promoter with proteins present in nuclear extracts from 3T3 or 3T3-LT cells. Addition of cold oligonucleotide (100-fold molar excess) effectively competes for complex formation with the labeled probe, proving the specificity of the reaction. Mutation of the E2F site (indicated in Fig. 1a) eliminates the formation of proper complexes (the faster-moving band in the lane with the mutant probe is an artifact). Addition of DOC dissociates higher-order complexes; the band arising is due to free E2F (5). Growth arrest of cells caused by serum starvation gives rise to complexes containing E2F and pRb but very little free E2F. The presence of pRb in these slowly moving complexes is indicated by a supershift following addition of a monoclonal antibody against pRb. Addition of dexamethasone to arrested 3T3 cells does not change the pattern of complexes; in contrast, addition of the hormone to arrested 3T3-LT cells, which results in the synthesis of LT antigen (accumulation of significant quantities of LT requires about 6 h after dexamethasone addition [28]) causes an increase in the amount of free E2F and a reduction of E2F-pRb complexes. WT, wild-type. (b) Northern blot. Concomitant with the shift in E2F complexes toward those containing free E2F, there is a dramatic increase in the steady-state level of TK mRNA in 3T3-LT cells. 3T3 cells, serving as a control, exhibit neither the change in complexes on the E2F site nor an induction of TK mRNA upon hormone addition.

assumption is supported by the observation (Fig. 2a) that a monoclonal antibody to pRb, if added to the nuclear extract, causes a supershift to still slower migration of part of the slowly migrating complex. This effect is not observed if a monoclonal antibody specific for p107 is used. p107 is a protein which, like pRb, binds to E2F, but contrary to E2F-pRb complexes, E2F-p107 complexes are seen only at the beginning of the S phase (36). Because of the weakness of the supershift, we have repeated this experiment four times and each time obtained the same result. With nuclear extracts from serum-starved, arrested 3T3 cells, the E2F-pRb complex was the major one, and addition of dexamethasone did not cause any change in complex formation. However, when extracts from 3T3-LT cells were used, larger complexes decreased in amount while at the same time free E2F increased after induction of LT protein (Fig. 2a). We do note, however, that some of the E2F-pRb complex (possibly containing phosphorylated pRb) remains and that the increase in free E2F appears to surmount the disappearance of E2F-pRb. This could be due to the recent observation that the formation of E2F-pRb complexes capable of binding to DNA requires an additional protein, RBP60 (32). In the absence of this protein, E2F-pRb complexes do not bind to DNA and thus are not detected in band shift experiments. Nonetheless, they are likely to be dissociated by LT antigen, giving rise to free E2F, and thus might contribute to the strong band of free E2F seen in the band shift experiments of Fig. 2a. We also want to point out that there still exists some controversy as to the definition of E2F complexes in extracts from mouse cells (2, 25, 36). Moreover, the number of different complexes observed to form on E2F binding sites (particularly with extracts from human cells) is still increasing. Some of these complexes contain one or the other member of the family of cyclins and a cyclin-regulated protein kinase. Contrary to most other studies, in which whole cell extracts were used as the source of E2F-binding complexes, we have chosen nuclear extracts with the hope of getting more relevant results. In addition, while in the majority of reports the E2F site of the adenovirus E2 promoter was used as the DNA target for complex formation, we applied the E2F site of the murine TK promoter and hence the immediate controlling site of the gene whose regulation was studied.

The increase in free E2F coincided with an induction of TK mRNA in serum-starved cells, as shown by Northern blotting (Fig. 2b). This result indicates that the endogenous TK gene is *trans* activated by LT antigen via the E2F site. This conclusion agrees well with results of a study on the effect of the E1A protein on the expression of dihydrofolate reductase (17). We want to point out one important advantage of our experimental system over those commonly used to measure *trans* activation: after having proven the target sequence for *trans* activation by transient transfection of TK promoter reporter gene constructs, one can measure transcriptional activation in this experiment without any need for transfection, as both the target gene and the *trans* activator are already present in the cell if the latter is induced by hormone addition. Thus, one can avoid problems that may arise when the target or the *trans* activator, or both, have to be introduced into the cells by transfection.

Polyomavirus LT antigen and proteins of other DNA tumor viruses are thought to activate transcription by forming a complex with the hypophosphorylated form of pRb in transcriptionally inactive E2F-pRb complexes, thereby releasing transcriptionally active free E2F (3, 4, 5, 13; reviewed in references 7, 16, and 38). The LT proteins from

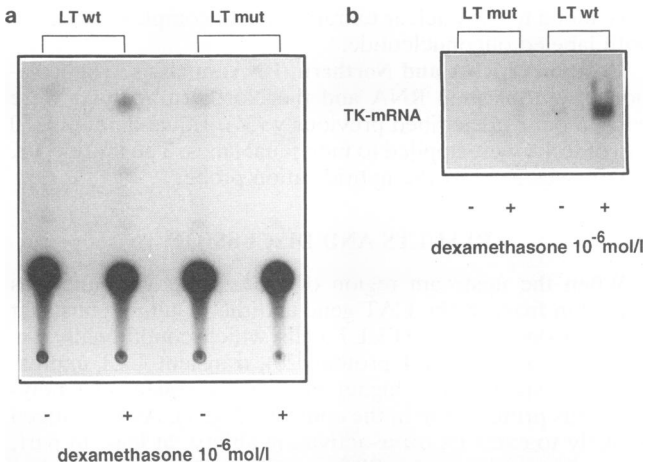

FIG. 3. Evidence that *trans* activation of the TK gene requires an intact pRb binding site in the LT antigen. Mutations within the pRb (and p107) binding region of LT (creating LTmut) were introduced by using the in vitro mutagenesis system of Amersham and synthetic oligonucleotides carrying the mutations. (a) Wild-type plasmid or mutated plasmid (15 μg) was cotransfected together with 15 μg of the CAT construct pTK-Eco-Nhe-CAT (Fig. 1a) into 3T3 cells, and LT antigen was transiently induced by addition of dexamethasone. CAT assays were carried out 48 h after trypsinization and reseeding of the cells (see Materials and Methods). It is shown that the capacity of the mutated LT protein (the Glu-146-to-Lys mutation was used here) to *trans* activate the TK promoter in transient CAT assays is eliminated. (b) A mouse mammary tumor virus-LT plasmid carrying the mutated gene for LT (LTmut; the Glu-146-to-Asp mutant is shown, but identical results were obtained with a Glu-146-to-Lys mutant) was used to create a 3T3 cell line carrying the LT mutant information stably integrated into the 3T3 genome, using previously published methods (28). These cells were then made quiescent by serum starvation, and T antigen was induced by addition of 10^{-6} mol of dexamethasone per ml. RNA was isolated 24 h thereafter, and TK mRNA was analyzed by Northern blotting. For comparison, TK mRNA produced in 3T3-LT cells (carrying wild-type T antigen [LT wt]) was analyzed in parallel.

polyomavirus or simian virus 40, the E1A protein of adenovirus, and the human papillomavirus E7 protein all contain a stretch with the consensus sequence Asp/Asn-Leu-X-Cys-X-Glu which is required for binding of pRb and p107. Mutating the Glu residue within this sequence in simian virus 40 LT protein to Lys eliminates pRb binding (9); the same was observed when the Glu residue within the pRb binding sequence of polyomavirus LT antigen (amino acid 146) was changed into Asp (22). We have introduced these mutations (Glu-146 into Lys and Glu-146 into Asp) into the polyomavirus LT information in a plasmid described earlier (28) which carries the LT gene under the mouse mammary tumor virus promoter. That an interaction between the LT protein and pRb is important for *trans* activation of the TK gene was proven by showing that the mutated protein had a strongly reduced ability to *trans* activate the TK promoter in transient cotransfection assays (Fig. 3a). Even more convincing is the result of the experiment shown in Fig. 3b. In this experiment, 3T3-LTmut cell lines carrying the information for the mutated LT antigen stably integrated in the genome of 3T3 cells, produced as described previously (28), were used. Steady-state levels of TK mRNA were measured in such cells after serum starvation and induction of the mutated LT antigen by hormone addition. Compared with 3T3-LT cells (see also Fig. 2b), 3T3-LTmut cells have almost

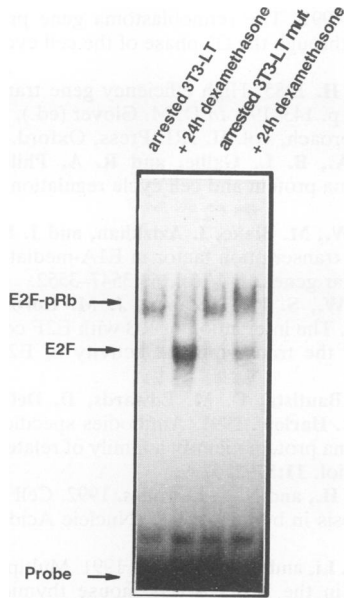

FIG. 4. Evidence that LT antigen with a mutated pRb binding site is very inefficient in the induction of free E2F. Band shift experiments were carried out as described for Fig. 2 and in Materials and Methods. 3T3-LTmut cells, carrying the Glu-146-to-Asp mutation, were used for the preparation of nuclear extracts, and 3T3-LT cells (carrying the wild-type LT antigen) were used as a control.

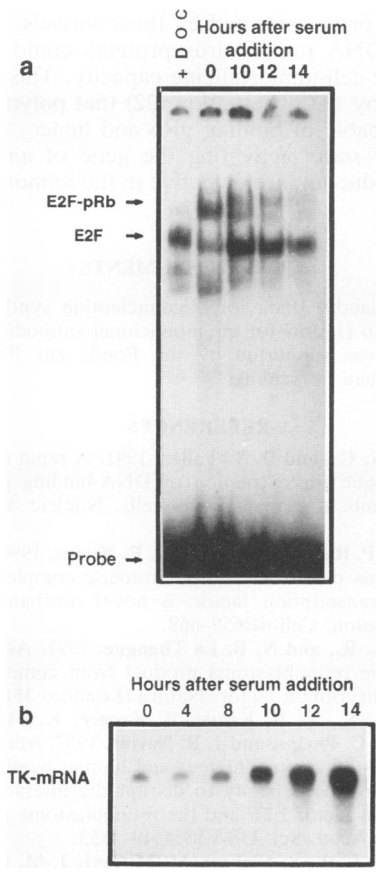

FIG. 5. Mobility shift assays. A shift in complexes toward free E2F (a) coincides in time with the appearance of TK mRNA (b) in 3T3 cells that had been growth stimulated by addition of serum. Swiss 3T3 cells were growth arrested by reducing the serum concentration to 0.2% for 3 days. For stimulation of growth, serum was added to 20%. Band shift assays and Northern analyses were carried out as described in Materials and Methods. DNA synthesis (not shown) starts between 10 and 12 h after serum addition.

totally lost the ability to induce the synthesis of TK mRNA. Figure 4 shows that in agreement with these results, LT antigen mutated at the pRb binding site is inefficient in its capacity to induce the appearance of free E2F. That a mutation at the pRb binding site of LT antigen does not completely eliminate a release of free E2F may be due to the likely possibility that introduction of a point mutation at the binding site greatly reduces the interaction with pRb but does not repress it totally. Western immunoblot analyses (not shown) were carried out to prove that the mutated protein is produced in the cells in amounts comparable to those synthesized in 3T3-LT cells and that the protein is stable for the time chosen for the experiment. Using immunofluorescence staining, we proved as a further control experiment that the mutated LT protein localizes to nuclei just as does the wild-type protein. Moreover, independent experiments (29) have shown that *trans* activation requires only very small quantities of LT antigen. It is thus highly unlikely that the negative result obtained with 3T3-LTmut cells is due to any other reason but the inability of the mutated protein to bind to pRb.

In a normal cell cycle, pRb is phosphorylated during G_1 and S phase (8, 23), which again apparently results in a rearrangement of E2F complexes and a potential activation of E2F (5, 7, 14, 16, 18, 36). To investigate the role of the E2F site in growth regulation of TK, addition of serum to serum-starved 3T3 cells was studied with relation to complexes formed at the E2F site (Fig. 5). Gel electrophoresis showed a significant shift of complexes toward free E2F between 10 and 14 h after serum addition, while at the same time, slowly migrating complexes (possibly containing cyclin A and cdk2 [10, 26, 30, 36]) appeared (Fig. 5a). We again notice (i) that some of the putative E2F-Rb complex remains into S phase, which is in agreement with published data (36), and (ii) that the quantities of free E2F seem to be larger than could be expected from their sole production from higher-order DNA binding complexes. The shift toward free E2F corresponds in time with the induction of TK mRNA at the G_1/S transition (Fig. 5b), providing strong evidence for an important role of the E2F site in growth control of the murine TK gene.

The presence of E2F binding sites in promoters of several other genes coding for DNA synthesis enzymes suggests that growth regulation of these enzymes may take place by a common mechanism. This is similar to the regulation of DNA synthesis enzymes in the yeast *Saccharomyces cerevisiae*, in which a regulatory element, identical in sequence with the recognition site for restriction enzyme *Mlu*I (ACGCGT), plays a central role (20). The *trans* activation by DNA tumor virus proteins of genes coding for DNA precursor-producing enzymes is probably part of the S-phase-inducing capacity of these viruses, a prerequisite for their replication which requires cells in S phase. In addition, it may, however, cause an imbalance in the pool of DNA synthesis precursors. This imbalance, in turn, might be followed by reduced fidelity of DNA replication and thus could account for the higher mutation rate and the chromosome instability (see, e.g., reference 37) observed in cells

immortalized or transformed by these viruses. In fact, this property of DNA tumor virus proteins could be a major cause of their cell-immortalizing capacity. This assumption is supported by the observation (22) that polyomavirus LT mutants incapable of binding pRb and hence, as shown in this work, of *trans* activating the gene of an enzyme of precursor production are defective in the immortalization of rat cells.

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