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# Complex Structure and Regulation of the *P16* (*MTS1*) Locus

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## Abstract

**The p16 gene (*P16, MTS1, CDKN2*) encodes a negative regulator of the cell cycle. Molecular genetic techniques have been used to explore the role of p16 in normal development and cancer. Two transcripts derived from the p16 gene with distinct protein coding potentials are described. The previously undescribed transcript form has the same exons 2 and 3 as the p16-encoding mRNA but contains a different exon 1. The human p16 transcripts are detected in various tissues, and the ratio of the transcripts is regulated in both a tissue-specific and cell cycle-specific manner. The *P16*-derived mRNAs are probably generated from separate promoters, and transcription from one of the promoters appears to be regulated, at least in part, by the retinoblastoma gene product.**

## Introduction

Many molecules have been identified that directly regulate the cell cycle (reviewed in Refs. 1–3). These include positive regulators, such as the cyclins and CDKs,[2] and an emerging set of negative regulators, such as p15, p16, p18, p21, and p27 (4–12). The negative regulators appear to act by inhibiting the kinase activity of the CDKs. In particular, the p16 protein may inhibit CDK4/6 and thereby prevent phosphorylation of Rb and cell cycle progression (7, 13, 14). Other recent reports provide evidence that p16 levels may be regulated by Rb protein (15, 16), suggesting that Rb in turn participates in a feedback loop to limit the levels of p16.

The p16 gene (*P16*; also called *MTS1, CDKN2,* and *INK4a*) is mutated or homozygously deleted in a high percentage of some types of tumors and tumor-derived cell lines (17–20). In addition, *P16* mutations segregate with predisposition to melanoma in several kindreds known to carry 9p21-linked melanoma susceptibility (21, 22). These results suggest that *P16* is a tumor suppressor gene. The biochemical behavior of p15, p18, p21, and p27 indicate that they too may be tumor suppressors, but detailed mutational analysis of their genes in tumors or cell lines has not been reported. Here we address issues pertinent to the structure and regulation of the *P16* locus and show that the gene is more complex then previously appreciated.

## Materials and Methods

**Genomic Clones.** P1 clones (Genome Systems), yeast artificial chromosomes, and cosmid subclones were isolated and characterized as described previously (23). *P16* clones were sequenced on an ABI model 373A DNA sequencing system using the PRISM Ready Reaction Dye-Deoxy Terminator Cycle sequencing kit (Applied Biosystems).

**cDNA Clones.** Human cDNA libraries were screened with a PCR-derived E2 fragment. Clones were purified and characterized by standard methods (24). *P16* cDNA clones were also isolated by hybrid selection procedures that have been described in detail.[3] Briefly, the entire p16 coding sequence was used to generate a biotinylated probe for the hybrid selection procedure. After hybridization capture, the selected cDNA clones were amplified and subjected to another round of capture and amplification. Human *P16* cDNA clones were isolated from lymphocyte, breast, ovary, pancreas, prostate, spleen, stomach, and thymus.

The 5′ ends of the α and β transcripts were analyzed by a modified hybrid selection procedure called hybrid capture RACE. Poly(A)$^+$-enriched RNA was isolated from the pancreas, spleen, stomach, and thymus tissues. First-strand cDNA synthesis reactions (24) used random 12-mers and Superscript II reverse transcriptase (Bethesda Research Laboratories). After second-strand synthesis, the cDNAs were "anchored" by ligation of a specific double-stranded oligo (dsRP.2) to their 5′ ends. The 5′ end of the second cDNA strand was the only phosphorylated DNA end in the ligation reaction. After the ligation, the anchored cDNA was purified by fractionation on Sepharose CL-4B columns. The anchored cDNA was captured with a biotinylated gene-specific oligo nucleotide, and the captured cDNA was amplified with a reverse primer (5′-GCTGCCCATCATCATGAC) derived from sequence common to both α and β transcripts in E2 and a nested version of RP.2 (RP.B). The amplified products (which crossed the E1/E2 splice junction) were gel purified and subjected to another round of capture and amplification. The resultant products were purified by gel electrophoresis, cloned, and sequenced.

**Cell Lines.** The cell lines used in this study were obtained from the Ludwig Institute for Cancer Research, from the American Type Culture Collection, or from sources described previously (25). UMSCC2 cells were provided by Thomas Carey, University of Michigan. Genomic DNA was isolated as described previously (26). RNA was isolated from cell lines using RNazol B (CINNA/BIOTECX Laboratories, Inc.) as described by the manufacturer.

**Lymphocytes.** Peripheral blood lymphocytes were isolated from whole blood by flotation on Ficoll-Hypaque gradients. The lymphocytes were further purified by counter current elutration as described previously (27). These authors estimated that a cell population, prepared in this manner, was 98% pure B and T cells. The purified cells were grown in RPMI (GIBCO) supplemented with 10% FBS. Quiescent cells were induced by 10 μg/ml phytohemaglutinin (Sigma Chemical Co.) and 10 units/ml IL-2 (Sigma). Cell cycle progression was monitored by flow cytometry. RNA was isolated from primary T cells using RNazol B (CINNA/BIOTECX Laboratories, Inc.) as described by the manufacturer.

**RT-PCR.** cDNA was synthesized from total RNA (24) isolated from T cells, cell lines, or human tissues (Clontech). The cDNA reactions used random 9-mers to prime DNA synthesis and Superscript II reverse transcriptase (Bethesda Research Laboratories). cDNA yields were calculated by including [$\alpha^{32}$P]dATP (Amersham) in the synthesis reaction (0.1 Ci/mmol) and determining the amount of radioactive nucleotide incorporated into the final product. *P16* α and β transcript levels were analyzed by PCR using α- or β-specific forward primers and nested reverse primers from E2 in two successive rounds of amplification. In the initial amplification, 2 ng of cDNA was amplified with the α-specific primer AS.1 (5′-CAACGCACCGAATAGTTACG) or the β-specific primer BS.1 (5′-TACTGAGGAGCCAGCGTCTA) and X2.R140′ (5′-AGCACCACCAGCGTGTC). The reactions were done on a Perkin-Elmer 9600 thermal cycler for 20 cycles under the following conditions: 97°C for 3 s; 65°C for 10 s; and 75°C for 20 s. These reactions were diluted 100-fold and

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[2] The abbreviations used are: CDK, cyclin-dependent kinase; Rb, retinoblastoma protein; RT, reverse transcription; ORF, open reading frame; IL, interleukin; RACE, rapid amplification of cDNA ends.

[3] T. Hattier, R. Bell, D. Shaffer, S. Stone, R. Phelps, S. Tavtigian, M. Skolnick, D. Shattuch-Eidens, and A. Kamb. A case study of hybrid selection: the search for BRCA1, submitted for publication.

reamplified with AS.1 or BS.1 and X2B (5′-CGTGTCCAGGAAGCCC). The X2B oligo was radiolabeled at its 5′ end (24) with [$\gamma^{32}$P]dATP (DuPont). PCR conditions were as above but for only 15 cycles. To eliminate problems due to genomic DNA contamination, the PCR products spanned the E1α or E1β/E2 splice junction. The products were resolved by electrophoresis through a denaturing 5% polyacrylamide gel. Dried gels were exposed to X-OMAT (Kodak) film overnight.

The quantitative behavior of the RT-PCR was confirmed by creating serial dilutions from the T-cell cDNA isolated after induction. The amount of target cDNA present in the undiluted sample was quantified by determining the dilution value at which the target was no longer amplifiable. These experiments were replicated with multiple primer pairs that generated predominately nonoverlapping PCR products. Although the results from the different PCR experiments were in agreement, the dilution experiments suggested that we could only detect changes in RNA levels if they were greater then 4-fold. The cDNA samples from the $Rb^+$ and $Rb^-$ cell lines were also analyzed in this manner and normalized against expression levels of cellular actin.

**E1β Mutation Screening.** Genetic characterization of the melanoma-prone pedigrees has been reported previously (28). Isolation of genomic DNA from melanoma-prone kindreds (22) and from cell lines (26) has been described previously.

PCR amplification for E1β was performed using the forward primer (5′-AGTCTGCAGTTAAGG) and the reverse primer (5′-GGCTAGAGGCGAATTATCTGT) for 30 cycles using the following conditions: 97°C for 3 s; 65°C for 10 s; and 75°C for 20 s. The amplification reactions were diluted 100-fold and amplified again under the same reaction conditions with the same forward primer and the reverse primer (5′-CACCAAACAAAACAAGTGCCG). PCR products were run on a 1% agarose gel and were extracted using Qiagen beads (Qiagen, Inc.). The products were sequenced using the Cyclist sequencing kit (Stratagene) with the forward primer mentioned above.

**ORF Statistical Analysis.** A function of the form $f(x) = (1/\lambda)e^{-x/\lambda}(\lambda = \langle L\rangle/\ln(2)$; ($\langle L\rangle$ = median size of ORFs representing the normalized distribution of ORF sizes in an arbitary sequence of defined base composition] was integrated to determine the probability ($P$) of observing an ORF of length 180 by chance to yield $P = e^{-180\ln(2)/\langle L\rangle}$. $P$s were computed for A = T = C = G = 0.25 and for the *P16* E1β composition: A = 0.15; T = 0.13; C = 0.35; and G = 0.36.

## Results

***P16* Transcripts.** During efforts to clone the melanoma susceptibility gene *MLM*, we isolated genomic sequences that corresponded to the previously identified cDNA sequence of the p16 gene (18). *P16* cDNA clones were isolated by hybrid selection procedures[3] and by conventional cDNA library screening. These clones were assembled into composite full-length cDNAs that revealed the nature of the processed transcripts from *P16* (Fig. 1*A*). Two different forms of *P16* transcripts were identified: a form (the α form) identical to the revised p16 cDNA (7, 29); and a form (the β form) containing a different exon in place of the first p16 coding exon. These alternative first exons were named E1α (which encodes the first 43 amino acids of the p16 protein) and E1β.

Translation of E1β in the p16 reading frame (ORF 1; extrapolated from the reading frame used in p16 coding exons 2 and 3) revealed an in-frame stop codon positioned only nine codons upstream of the splice junction between E1β and exon 2 (E2; Fig. 1*B*). The position of the stop codon was confirmed by analysis of the genomic sequence. The first potential initiation codon downstream of this stop is in the p16 reading frame, immediately 3′ of the E1/E2 splice junction. This potential start codon is flanked by sequences that do not closely resemble the consensus Kozak sequence (30). If translated in the p16 reading frame, the E1β transcript of the p16 gene would encode a protein of 105 amino acids.

Additional analysis of the β cDNA revealed that it possessed a large ORF in a different frame than the one used to encode p16 (Fig. 1). The ORF (referred to as ORF 2) extended through E1β and continued for 67 amino acids into E2. The entire ORF could encode a protein of 180 amino acids. However, the reading frame remained open at the 5′ end of E1β, and, therefore, may be incomplete. Statistical analysis suggested that an ORF of this size was unlikely to occur by chance in DNA composed of random sequence ($P = 0.003$). However, given the base composition of the β transcript, the probability was higher ($P = 0.16$). The predicted polypeptide was not similar to any previously described protein.

Mouse P16 cDNAs were isolated and compared to their human homologues to identify the conserved portions of the *P16* transcripts.[4] The β transcript was present in the mouse, and E1β contained stop codons in the reading frame used to encode p16. Also, the mouse β transcript contained a large ORF (ORF 2) in a different reading frame then the one used to encode p16 (ORF 1). Although the nucleotide sequence of E1β was conserved in the mouse (61% identical to human), the protein coding capacities of the mouse and human E1βs were very different. The E1β-encoded portion of the ORF 2 polypeptides were only 28% identical. In contrast, the mouse and human p16 protein sequences were 60% identical. In addition, the E2-encoded ORF 2 polypeptides were as similar (42%) as the polypeptides encoded by the third reading frame in E2 (ORF 3). These results suggest that ORF 2 has not been selectively maintained and probably does not encode a protein. The secondary structure of the human and mouse β RNAs were also compared (data not shown). No striking similarities were identified. Collectively, these results suggest that the β transcript is functional, by virtue of its presence in both mice and humans; and if translated, the encoded protein probably initiates at the first methionine in exon 2 (Fig. 1*B*). Despite these arguments, it remains possible that ORF 2 encodes a novel protein.

The exon structure of the p16 gene was determined by comparing the sequence of the composite cDNA clones to the genomic regions from which they were derived. A combination of genomic Southern blots, sequence analysis of the genomic region containing *P16*, and long PCR was used to map the positions of the *P16* exons (Fig. 2 and data not shown). The p16 gene spans approximately 30 kb of genomic DNA. E1β is the most 5′ of the *P16* exons.

**Two *P16* Promoters.** The different *P16* mRNA forms could be generated in two possible ways. Transcription could initiate from different promoters, or the mRNA could be derived from a single promoter and then alternatively spliced to generate the different forms of the transcript. Evidence for separate α transcript and β transcript promoters was obtained by demonstrating that the α form was transcribed in cell lines, even when the upstream E1β sequences were deleted. Cell lines A375 and SK-mel 93 contain a deletion with one breakpoint between E1α and E1β (Fig. 2). The centromeric breakpoint was not precisely mapped in either cell line but is at least 85 kb upstream of the 5′ end of E1β. Using RT-PCR with α-specific primers, both of these cell lines were shown to express the α transcript (Fig. 3). This suggests that the α transcript initiates from a promoter independent of sequences 5′ of E1β. An alternative explanation is that the deletions fused ectopic promoter sequences to E1α. However, this seems unlikely, given that A375 and SK-mel 93 are independently isolated cell lines. Therefore, it seems most likely that the α and β transcripts are transcribed from separate promoters, $P_\alpha$ and $P_\beta$, and that the alternative first exons are spliced to a common E2 (Fig. 2).

Hybrid capture RACE (see "Materials and Methods") was used to isolate cDNA clones containing sequence extending toward the 5′ end of the β transcript. Although the exact location of the β promoter is not clear, the longest β-derived hybrid capture clone extended 289 nucleotides 5′ of the E1β/E2 splice junction. Despite numerous at-

[4] P. Jiang, S. Stone, R. Wagner, P. Dayananth, B. Wold, and A. Kamb. Comparative analysis of human and mouse CDK inhibitor genes p15 and p16, manuscript in preparation.

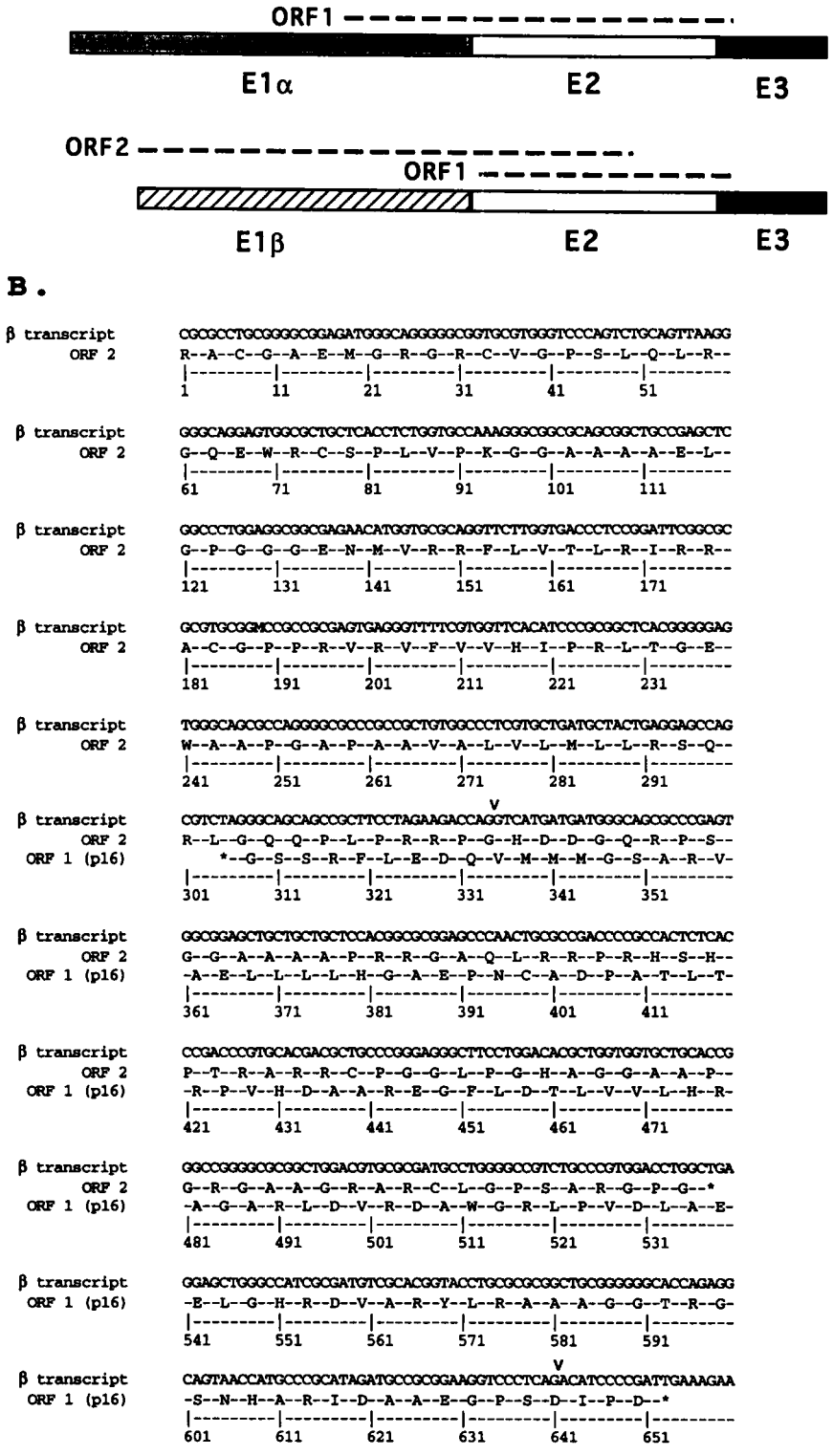


Fig. 1. *A*, schematic of *P16* transcripts. The α transcript (containing E1α) and the β transcript (containing E1β) are shown. The *dashed lines* above each transcript form indicate the position and length of potential ORFs. The complete ORF 1 (α transcript) encodes p16. It is unknown if ORF 2 encodes a protein. *B*, nucleotide sequence and protein coding capacity of the β transcript. E1β extends 5′ at least to position 45. The sequence upstream of position 45 was derived from genomic clones.*, stop codons; *v*, splice junctions.

tempts, we were unable to isolate cDNA clones that extended beyond this position. However, since the 5′ end of the β transcript has not been mapped directly, it is possible that the full-length transcript contains additional upstream sequence.

**Expression Pattern of *P16*.** Clues to the function of genes may emerge from analysis of their expression pattern in different tissues. To determine the expression pattern of *P16*, a set of cDNA samples prepared from 11 tissues were screened by PCR with α and β specific

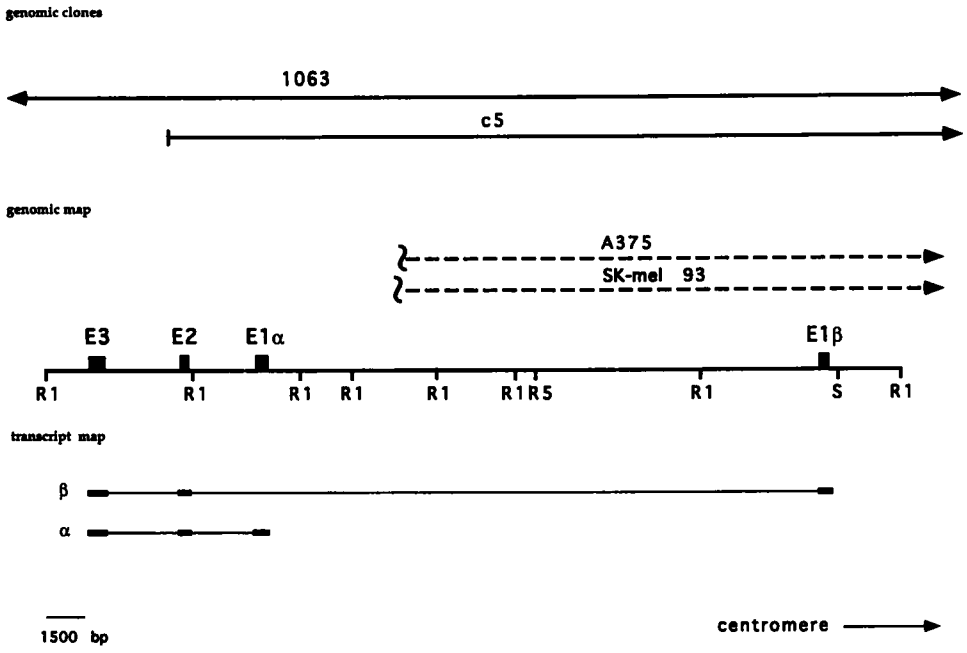


Fig. 2. Map of the *P16* gene. Above the restriction map are the positions of genomic clones (cosmid c5 and P1 1063) and the positions of the deletions in cell lines A375 and SK-mel 93. *Dashed line*, deleted DNA. The exact location of the telomeric breakpoint in either A375 or SK-mel 93 is not known. However, they have been mapped to the interval between E1α and the sequence-tagged site c5.3 (18).[5] On the genomic map are E1α, E1β, E2, and exon 3 (E3). The positions of the exons are indicated by the *filled boxes*. Also indicated are the positions of restriction sites *Eco*RI (*R1*), *Eco*RV (*RV*), and *Sal*I (*S*). The transcript map indicates the splicing patterns that generate the α and β mRNAs. Transcription proceeds from *right* to *left*.

primers (Fig. 4*A*). Both forms of *P16* transcript were detected in all tissues examined, although there were some differences. For example, in spleen, the ratio between the α and β forms was skewed toward β. In contrast, the ratio in breast favored α. These expression data suggest roles for p16 in multiple tissues, consistent with studies that found deletions and point mutations of *P16* in cell lines derived from many different tissue types (18, 26).

Recent reports suggest that some mitogenic and antimitogenic signals affect cell cycle progression, at least in part, by regulating the activity of CDK inhibitors (9, 31–34). For example, p27 may be negatively regulated during IL-2-induced mitogenic activation of quiescent T lymphocytes. Given the biochemical function of p16, demonstrated *in vitro* to be an inhibitor of CDK4 and CDK6 (7, 13, 15), the expression of *P16* was analyzed during T-cell activation. Human peripheral blood lymphocytes were stimulated by phytohemaglutinin plus IL-2, and cells were harvested at different times after stimulation. These cells were analyzed by flow cytometry to determine their cell cycle stage, by RT-PCR to determine the relative levels of *P16* gene expression, and by Western blot to determine the levels of p16 protein.

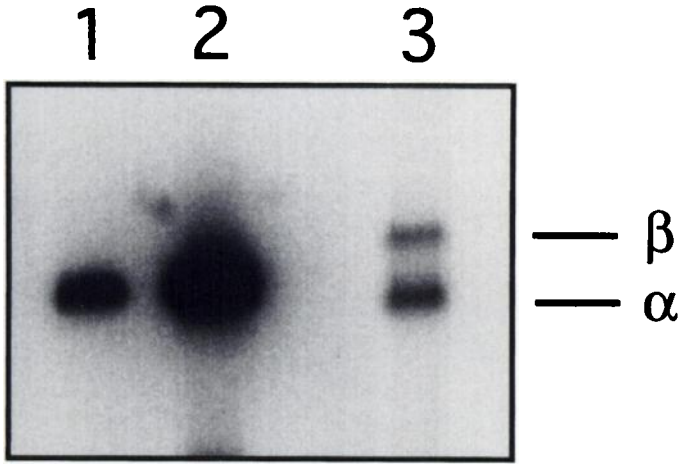


Fig. 3. Expression of the α transcript in cell lines that contain deletions of E1β. cDNA was derived from total RNA isolated from the indicated samples. A radiolabeled primer was included in the reactions to amplify the *P16* transcripts. Equal volumes of the α and β amplifications were mixed, and the products were resolved on a denaturing 5% polyacrylamide gel: *Lane 1*, cell line SK-mel 93; *Lane 2*, cell line A375; *Lane 3*, quiescent human T cells.

The ratio of the two forms of *P16* transcript changed dramatically through the cell cycle (Fig. 4, *B* and *C*). Initially, the β form was low, but by 30 to 40 h after stimulation, the level began to rise. During this time, the expression level of the α form remained relatively constant, perhaps increasing slightly. By flow cytometry, the ratio change was correlated with cells exiting $G_0$ and entering S phase (data not shown). The quantitative behavior of the RT-PCR was examined by template dilution experiments (data not shown; see "Materials and Methods"). Based on those experiments, RT-PCR was sensitive to 4-fold or greater changes in transcript level. The β induction was estimated to be at least 10-fold. Therefore, as T cells entered the cell cycle, they altered the relative amounts of the two forms of the *P16* transcript so that the β:α ratio increased.

We also examined the level of p16 protein expression as the T cells traversed the cell cycle. Protein was isolated from the cells at various times after mitogenic induction, and the isolated protein was subjected to Western analysis. The levels of p16 protein were determined using a p16 antibody raised against the 20 COOH-terminal amino acids of the complete polypeptide. As the cells exited $G_0$, the level of p16 protein remained relatively constant (data not shown). Thus, both the p16-encoding RNA (the α transcript) and p16 protein remained relatively constant during the cell cycle. Others have reported a moderate increase in p16 levels during S phase (16). We did not see an accumulation of p16, which might reflect differences in p16 regulation in different cell types or reflect problems in detecting a 2- to 3-fold increase in protein (or mRNA) levels. Interestingly, a $M_r$ 10,000 protein was not detected by the anti-p16 antiserum in these experiments.

**Expression of *P16* in Tumor Cell Lines.** Previous studies have suggested that Rb influences the expression of p16 (7, 13, 15, 16). We tested the effect of the Rb status of cells on the expression of the β mRNA (Fig. 4*D*). cDNA was prepared from a set of cell lines, five of which contained wild-type Rb protein, and six of which contained nonfunctional Rb protein (13, 25).[6] As expected, α transcript was only

[5] S. Stone *et al.*, unpublished observations.

[6] D. Parry and G. Peters, unpublished observations.

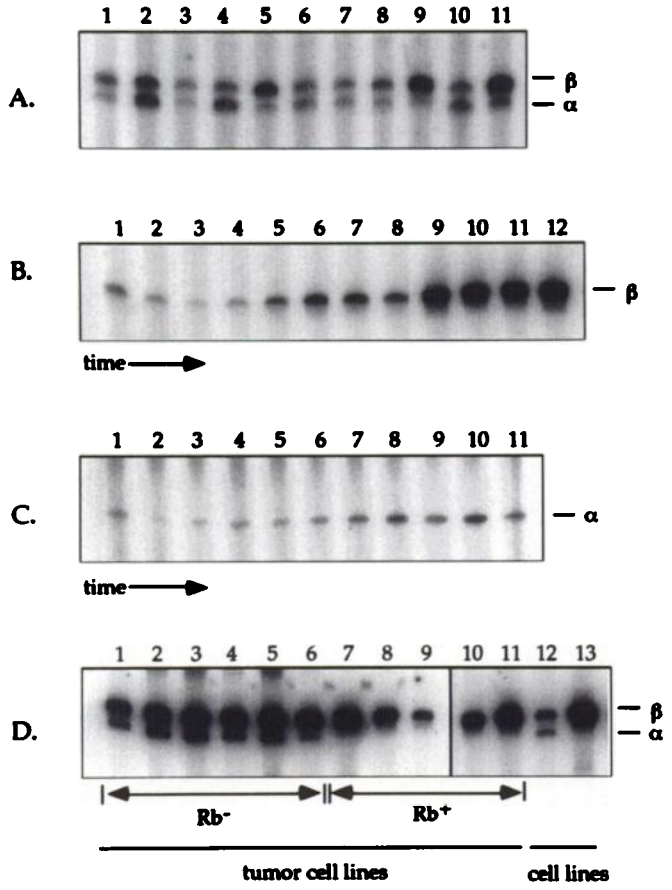


Fig. 4. Expression of *P16* transcripts. A radiolabeled primer was included in the reactions to amplify the *P16* transcripts, and the products were resolved on a denaturing 5% polyacrylamide gel. In *A* and *D*, the α and β reactions from a common sample were mixed prior to electrophoresis. *A*, the relative levels of *P16* transcripts in RNA derived from various human tissues: *Lane 1*, brain; *Lane 2*, breast; *Lane 3*, kidney; *Lane 4*, lung; *Lane 5*, lymphocyte; *Lane 6*, ovary; *Lane 7*, pancreas; *Lane 8*, prostate; *Lane 9*, spleen; *Lane 10*, stomach; *Lane 11*, thymus. *B*, the relative amount of the β transcript in human lymphocytes as a function of time after mitogenic induction: *Lane 1*, 0 h; *Lane 2*, 1 h; *Lane 3*, 2 h; *Lane 4*, 4 h; *Lane 5*, 8 h; *Lane 6*, 16 h; *Lane 7*, 24 h; *Lane 8*, 32 h; *Lane 9*, 40 h; *Lane 10*, 48 h; *Lane 11*, 56 h; *Lane 12*, 64 h. *C*, the relative amount of the α transcript in human lymphocytes as a function of time after mitogenic induction: *lanes*, same as in *B*, but the 1-h time point was omitted. The expression of other molecules that are either suspected to influence cell cycle progression or that are regulated at the transcriptional level during the cell cycle was also analyzed (data not shown). In agreement with previous results, levels of CDK4 and GoS 2 (a molecule of unknown function, but the transcription of which is induced when quiescent T cells entered the cell cycle) increased upon T-cell induction (41, 42). In contrast, the RNA levels of p27 appeared unchanged during the course of the experiment (11, 32). *D*, *P16* transcripts as a function of Rb status. $Rb^-$ cell lines: *Lane 1*, WERI; *Lane 2*, CaSki; *Lane 3*, SiHa; *Lane 4*, C33A; *Lane 5*, 5637; *Lane 6*, MDA MB 468. $Rb^+$ cell lines: *Lane 7*, T24; *Lane 8*, HaCaT; *Lane 9*, ZR75; *Lane 10*, Bristol 8; *Lane 11*, UMSCC2; *Lane 12*, diploid human fibroblast MRC5, passage 28; *Lane 13*, KIT (43). The quantitative behavior of the RT-PCR was verified by template dilution experiments (see "Materials and Methods").

detected in Rb-negative lines. However, the β transcript was present in both Rb-positive and Rb-negative cell lines. Although there were apparent differences in the amount of β transcript present in the cell lines, those differences did not correlate with the status of Rb. Therefore, in contrast to α, expression of the β RNA was independent of the mutation state of Rb in tumor-derived cell lines (within the resolution limits of RT-PCR).

**Mutation Analysis of E1β.** Both the preponderance of homozygous deletions, which inactivate *P16* in tumor derived cell lines, and the 9p21-linked melanoma-prone kindreds that do not reveal mutations in *P16*, have led others to propose the presence of another gene(s) near *P16* that is also involved in cancer formation (35, 36). If E1β encoded a protein that was involved in regulating cell growth, then the exon could contain mutations in either sporadic and/or familial cancer that would have been missed in earlier studies. Therefore, E1β was screened for mutations in cell lines derived from various tumors and in some melanoma-prone kindreds.

No sequence variants of E1β were detected in a set of 24 cell lines derived from 4 tumor types (Table 1) or in 6 melanoma kindreds with significant haplotype sharing among affected family members (28), but which did not reveal *P16* mutations in a previous study (22). These experiments suggest that mutations in E1β are not a common event during tumor progression, nor are they responsible for 9p21-linked melanoma susceptibility in these kindreds.

## Discussion

We have investigated the genomic structure and transcriptional regulation of the p16 gene in humans. Two types of *P16*-derived mRNAs were identified. One type was identical to the cDNA reported to encode the p16 protein (7, 29). We refer to this cDNA type as the α form. The other type, the β form, was identical to the α form in E2 and exon three (E3) but contained a different first exon, which we termed E1β (Fig. 1*A*). The β form appears to be the more abundant transcript in at least some tissues, based on the frequency of occurrence in cDNA libraries. The two RNAs do not arise from alternative splicing but instead arise from separate promoters. The α and β transcripts are similar in size, which may have complicated previous efforts to measure *P16* RNA levels by Northern blot. Thus, the human p16 gene is complex, with two partially overlapping transcripts with distinct coding potential, produced from separate promoters, $P_\alpha$ and $P_\beta$.

Although we and others (13, 15) have shown that the levels of p16 RNA are affected by Rb, it is interesting that this regulation is not evident in cycling human T cells. The level of the α transcript remains relatively constant, although the phosphorylated state of Rb, and hence its activity, changes as the cells traverse the cell cycle (37). Tam *et al.* (16) have reported recently that the level of p16 peaks during S phase in human fibroblasts, consistent with the proposed regulation by Rb. They also reported that p16 protein was undetectable in human T cells. There is evidence that p16 is a member of a multigene family (12). By analogy with other multigene families, the members of this family might carry out redundant functions, different functions, or function in different temporal or tissue-specific patterns. Therefore, given the low level of p16 protein and apparent lack of *P16* regulation by Rb, it is possible that *P16* does not regulate the cell cycle in T lymphocytes. However, because the β transcript is dramatically induced upon T cell induction and because *P16* is deleted in a high percentage of T cell-derived tumors (38), it seems likely that p16 carrys out an important function in human T cells. A dramatic affect of Rb on p16 has been observed only in virally transformed or tumor-derived cell lines. Perhaps *P16* is regulated in some other manner in normal tissue.

Although the role of the β transcript is unclear, our results suggest that it is important for the function of the p16 locus because: (*a*) E1β

Table 1 *Cell lines screened for E1β mutations*

| Type | Number |
|---|---|
| Lung | 3 |
| Bladder | 7 |
| Glioma | 9 |
| Melanoma | 5 |
| Total[a] | 24 |

[a] These cell lines were shown previously not to contain homozygous deletions in the *P16* region or harbor *P16* coding sequence mutations (Q. Liu, personal communication). Based on previous results (26), a similar number and type of cell lines would have contained four point mutations in the p16 coding sequence, confined to the bladder and melanoma groups.

is conserved in mouse; (*b*) the relative amount of the β transcript is regulated in both a tissue-specific and cell cycle-dependent manner; and, (*c*) two cell lines harbor homozygous deletions that remove E1β but not E1α. These results suggest that E1β is required for wild-type *P16* function. Similar results are also described by Mao *et al.* (39) in this issue of *Cancer Research*.

Comparison of the mouse β transcript to the human suggests that E1β is not a protein coding exon. Only the sequence comprising the p16 reading frame in E2 was rigorously conserved. Therefore, if the β transcript were translated, it seems likely that the protein would initiate in E2 and be translated in the same frame used to encode p16. The deduced polypeptide would have a calculated molecular weight of 10,000 and retain 2¾ of the 4 ankyrin repeats present in p16. p15 contains only 3½ ankyrin repeats (9), and other proteins fold and function with only two repeats (40). Preliminary experiments using a commercially available p16 antibody have failed to identify a p16-related $M_r$ 10,000 protein. These results are equivocal. The putative protein could be present, but at levels too low to detect by Western blot (indeed, the p16 signal from T-cell lysates is extremely weak). Therefore, whether a p10 molecule exists *in vivo*, and if it exists, whether it inhibits CDK4/6 remains to be tested.

If the role of the β transcript were to inhibit cell growth, we might find mutations that disrupt E1β in tumor-derived cell lines. Consistent with this view are two melanoma cell lines with deletions that remove E1β but that do not abolish the α transcript. The p16 coding sequence is normal in these cell lines (data not shown). No small genetic lesions in E1β (*e.g.*, base substitutions) were found in a set of 24 tumor cell lines. Therefore, it is difficult to conclude that E1β was the target of the homozygous deletions. If the E1β exon does not encode a protein, small genetic lesions may be insufficient to disrupt its function. Alternatively, the target of the deletions mentioned above might have been some other gene. For example, it is possible that the p15 gene was the relevant target of the deletions in these melanoma cell lines. In that view, E1β was deleted simply because it is closer to *P15* then E1α. However, since we were unable to detect *P15* point mutations in a variety of cell lines,[7] and because there were no cell lines that contained deletions which specifically removed *P15* (18), this explanation seems unlikely.

Genetic evidence suggests that p16 and Rb are members of a growth-regulatory pathway often inactivated during tumor progression. If the role of the β transcript is to regulate cell growth negatively, perhaps it is part of another pathway that must be mutated independently from p16 and Rb. This would explain why deletions that specifically disrupt E1β have only been seen in Rb− cell lines. Based on its expression pattern, it seems likely that E1β plays a role in actively cycling cells. A definitive conclusion on the role of E1β awaits analysis of its expression *in vivo*.

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