BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **247,** 146–153 (1998)
ARTICLE NO. RC988497

Structure of the Gene Encoding the Human Cyclin-Dependent Kinase Inhibitor p18 and Mutational Analysis in Breast Cancer

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Received February 26, 1998

The cyclin-dependent kinase (CDK) inhibitor p18 blocks progression of the cell cycle by associating with the cyclin D-dependent kinases CDK6 and CDK4. To better understand the regulation of p18 gene expression, we isolated full-length cDNA clones from a human BT-20 breast cancer cell cDNA library. These clones were then used to isolate the human gene from a human genomic DNA library. The human p18 gene spans at least 7.5 kb and is composed of three exons, two of which encode the p18 protein. The genomic clone we isolated contained 5 kb of putative promotor sequence which directed expression of the luciferase reporter gene in transient transfection experiments. The longest cDNA that we isolated from BT-20 cells contained 2103 nucleotides which corresponds to the size of the major RNA transcript detected by Northern analysis in these cells. Transcription start sites mapping to the 5′ end of the putative full-length cDNA were identified by ribonuclease protection assays. A novel polymorphism was identified in the 3′ untranslated region of BT-20 cell cDNA clones that contained the previously described codon 72 mutation. The codon 72 mutation was also detected in 3 of 35 breast tumors analyzed using a mismatch PCR/RFLP strategy.

Key Words: **cell cycle; cyclin-dependent kinase inhibitor; p18; gene; breast cancer.**

The execution of the S phase of the cell cycle requires the activity of gene products whose expression is controlled by the retinoblastoma protein (pRb), a cell cycle transcriptional regulator (1). pRb restricts gene expression to the appropriate phases of the cell cycle by binding to and sequestering transcription factors such as E2F that regulate S-phase genes. This activity of pRb is regulated by phosphorylation events in a cell cycle-dependent manner. Throughout the early part of G1, pRb is found in an underphosphorylated form which is capable of associating with E2F. Phosphorylation of pRb by cyclin-CDK complexes late in G1 results in the release of E2F and the activation of S-phase genes. Passage through the pRb-controlled G1-S checkpoint is required for the mitogenic effect of growth factors (2, 3). The importance of this growth control checkpoint is further underscored by the fact that it is frequently disrupted in cancer cells, either through deletions of the pRb gene or the overexpression of cyclins (4).

Cyclin-dependent kinase inhibitors (CKIs) are a group of low molecular weight proteins that associate with cyclin-CDK complexes or CDKs alone and inhibit their activity (5). The INK4 family of CKIs is comprised of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. The INK proteins specifically bind and inhibit CDK4 and CDK6, thereby preventing cyclin D-dependent phosphorylation of pRb (6). Each INK4 protein consists of repeated ankyrin domains which presumably play a role in protein-protein interactions (6). Inactivating mutations in the ankyrin domains of p16 have been identified in several human tumors and cancer cell lines (7, 8). The p16 and p15 genes are also inactivated by chromosomal deletions or transcriptional silencing, alterations which perturb the pRb-controlled checkpoint (9, 10). On the other hand, no mutations were found in the closely related p19 gene (11).

The human INK4 p18 cDNA was first isolated in 1994 (12). Alterations of the p18 gene, though less frequent than those affecting p16, have been observed in human cancer. We have isolated a mutant form of p18 from human BT-20 breast cancer cells that is defective in CDK6 binding whereas Zariwala *et al.* (13) isolated a p18 variant of unknown functional properties from T47D breast cells (13, 14). Homozygous deletions of the p18 gene were observed in three of 21 acute lympho-

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0006-291X/98 $25.00

blastic leukemias examined and in one of 17 cases of multiple myeloma examined (15, 16). Loss of heterozygosity in the p18 gene was found in one case of non-small-cell lung cancer (17). Moreover, the p18 gene resides on chromosome 1p32, a region that is disrupted in a variety of human cancers (12 and references therein).

To better understand the role of p18 in the control of cell proliferation and its involvement in human cancer, we proceeded to clone the human p18 gene. We report here the isolation of full-length p18 cDNA clones as well as the structure and sequence of the human p18 gene and a mutational analysis of p18 in breast cancer cell lines and tumors.

MATERIALS AND METHODS

DNA and RNA isolation. The cell lines used for RNA/DNA analyses and cDNA library preparation were obtained from the American Type Culture Collection. BT-20 and MCF-7 human breast cancer cells were maintained as described (14). Myelogenous leukemia K562 cells were maintained in RPMI 1640 medium with 10% fetal bovine serum and antibiotics. Genomic DNA and total RNA was isolated from cells using Tri-Reagent according to the manufacturer's instructions. Polyadenylated RNA was isolated by two cycles of chromatography on oligo-(dT)-cellulose columns (18).

Characterization of full-length p18 cDNA clones. A UNI-ZAP cDNA library was prepared using human BT-20 breast cancer cell poly(A)$^+$ RNA according to the supplier's protocol (Stratagene Cloning Systems). A partial cDNA fragment comprising the coding sequence of the human p18 cDNA (GenBank accession number U17074) was amplified from BT-20 RNA by RT-PCR and cloned into pcDNA3 vector as described (14). To isolate full-length p18 cDNAs, this fragment was labelled with [α-^{32}P]-dCTP and used as a probe to screen approximately 1.2×10^6 clones from the BT-20 cDNA library. Prehybridization, hybridization, and washes were performed as previously described (19).

Double-stranded DNA fragments were subcloned into pBluescript II. The longest p18 cDNA was sequenced in both orientations using T7 and T3 vector primers and additional oligonucleotide primers synthesized in our laboratory.

Isolation of genomic p18 clones. To isolate the human p18 gene, we screened a genomic human leucocyte λEMBL-3 DNA library (Clontech, Palo Alto, CA) using standard procedures (18, 19). The purified full-length p18 cDNA fragment was labeled to high specific activity ($\geq 1 \times 10^9$ cpm/μg DNA) and used to screen approximately 9×10^5 clones. Genomic subclones were generated by digestion with *Sac* I and cloned into *Sac* I-digested pBluescript II KS(+) plasmid vector. To confirm the structure of the human p18 gene, DNA fragments overlapping each *Sac* I site were amplified from genomic DNA by PCR using oligonucleotide primers flanking each site, subcloned and sequenced. Genomic restriction fragments subcloned in pBluescript II KS(+) were sequenced in both orientations. Sequence analyses were conducted using the Wisconsin GCG Package Version 9.0 (Genetics Computer Group, Madison, Wisc.).

RNA analyses. For Northern blot analysis, samples of total RNA (10 μg) were electrophoresed on a 1% agarose/2.2 M formaldehyde gel alongside the 0.24-9.5 kb RNA ladder (Gibco/BRL), transferred to a nylon membrane and hybridized to a ^{32}P-labelled full-length p18 cDNA probe. Stringent hybridization and washing conditions were used as previously described (19).

Ribonuclease protection assay. The DNA fragment containing nucleotides −129 to +170 relative to the putative start site of p18 clone 40 cDNA was amplified from human p18 genomic subclone λ4S4 by PCR using a 5′ primer of sequence 5′-GTAGGATCCGAGAGCGGGAGGCGGGAATGAGG-3′ and a downstream primer of sequence 5′-GTATCTAGATTTTCGCTGAAACAATTGCTGCT-3′. This amplicon was subcloned as a *BamHI*/*Xba*I insert in the pcDNA3 plasmid vector and sequenced. Radiolabelled cRNA was synthesized using Sp6 RNA polymerase as indicated in the manufacturer's protocol (Promega Riboprobe *in vitro* transcription systems). Hybridization of 2 μg of K562 polyA+ RNA with the antisense probe was performed using the RPA II Kit (Ambion), following the supplier's protocol. Assay products were resolved on a 5% denaturing polyacrylamide gel and revealed by autoradiography.

Mutational analysis of p18 in breast cancer tissue. Samples remaining from 35 breast tumors after determination of hormone receptor content were used for the mutational analysis. DNA fragments for mutation analysis were generated from total RNA by amplifying p18 coding nucleotides +189 to +375 by RT-PCR (Fig. 4). The 5′ primer corresponded to nucleotides +189 to +213 and contained an A instead of a T at nucleotide 212 (5′-CGATTTGAAAGACCGAACTGGTTAC-3′, mismatch underlined). This substitution was designed to add an internal *Bst*EII restriction site to p18 PCR fragments containing the codon 72 substitution (14). The C-terminal oligonucleotide was complementary to nucleotides + 375 to +353 of p18 (5′-GTGCTTCACCAGGAACTCCACCA-3′). ^{33}P-labeled dATP was incorporated during the PCR which was carried out at an annealing temperature of 62 °C in a reaction volume of 20 μl. Half of the PCR product was then digested with 10 units of the restriction enzyme *Bst*EII for 3 hours after which 10 μl of formamide loading buffer was added to each sample. A 4 μl aliquot was heat-denatured and run on an 6% denaturing polyacrylamide gel. To confirm the specificity of the PCR, the 188-bp product was purified, subcloned and sequenced.

Direct sequencing of RT-PCR products was performed as described (20) using oligonucleotides complementary to bases 114-136 and 507-483 for the PCR and an oligonucleotide complementary to nucleotides 288-266 for sequencing.

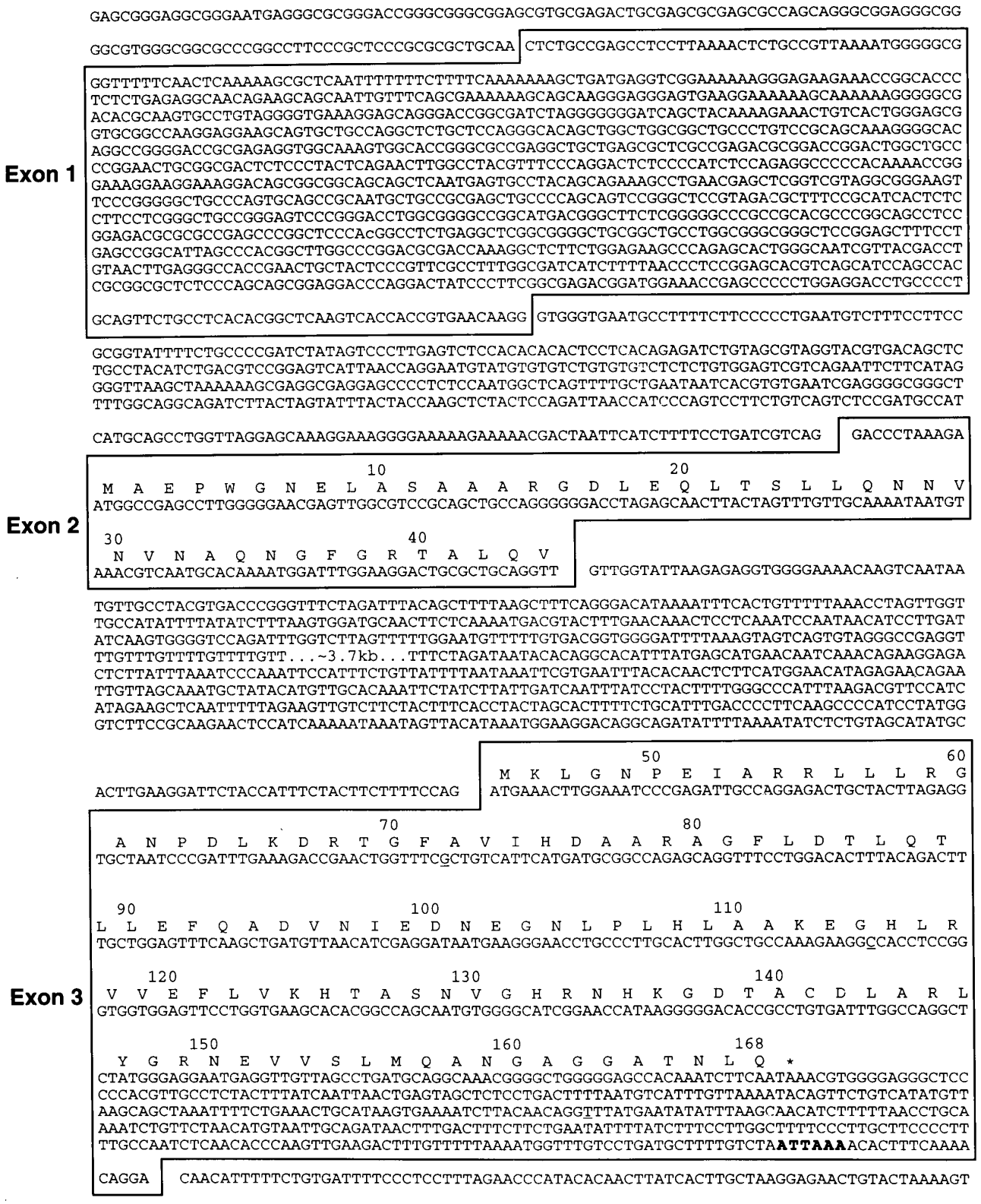
RESULTS

Characterization of a Full-length p18 cDNA

To isolate full-length p18 cDNAs we screened a BT-20 human breast cancer cell cDNA library with a probe comprised of the coding region of p18. Nine positive clones were obtained and six of these were characterized in detail. The sequence of the p18 transcript is shown in Figure 1. The longest p18 cDNA isolated from the BT-20 cell cDNA library, designated clone 40, contained 2103 nucleotides, including 1215 nucleotides upstream of the ATG codon and a 364-basepair (bp) 3′-untranslated sequence. The polyadenylation signal (ATTAAA) is located 23 nucleotides upstream from the poly(A) tail.

We had previously determined by SSCP and direct sequencing of RT-PCR products that BT-20 cells express two variants of p18 mRNA: a mutant form of p18 containing a G to C change at coding nucleotide 214 (codon 72) and a transcript containing a silent polymorphism at codon 114 (14). The codon 114 variant was identified in cDNA clone 40. The codon 72 variant was identified in cDNA clone 13, which contained 1093 nucleotides upstream of the ATG. Sequencing of the 3′ untranslated region of clone 13 revealed a T to C change 151 nucleotides downstream of the stop codon. This substitution did not introduce a restriction site.

Human p18 gene



These results indicate that the codon 72 and 114 variants are expressed from different alleles.

Characterization of the p18 Gene

The p18 gene was isolated using conventional screening techniques. Four positive clones were obtained by screening a λEMBL-3 human leukocyte genomic DNA library with a full-length p18 cDNA probe. All four clones were found to contain the entire coding sequence of the p18 gene. These clones and subclones thereof were plaque-purified for further analysis by restriction mapping and sequencing.

The p18 gene is comprised of three exons and spans greater than 7.5 kbp of genomic DNA. The first exon is noncoding and contains a 1204-nucleotide long 5′-untranslated region. The second exon contains the last 11 nucleotides of the 5′-untranslated region and the complete nucleotide sequence of the first 44 amino acids of p18. Exon 3 encodes the last 124 amino acids, the stop codon and contains the 364 nucleotides of 3′ untranslated region.

Exons 1 and 2 are separated by an intron of 458 nucleotides. Intron 1 was sequenced in its entirety (Figure 1). Exons 2 and 3 are separated by intron 2 which contains approximately 4500 bp, approximately 750 of which were sequenced. The size of intron 2 was estimated by restriction analysis of λ clones and PCR amplification of genomic DNA (data not shown). To confirm the deduced structure of the p18 gene, Southern blot analysis of *Sac* I-digested λ phage DNA and human genomic DNA was performed using exon-specific probes and the full-length p18 cDNA. The band pattern observed with the p18 probes was in agreement with the restriction map of the p18 gene (data not shown). Furthermore, the exon-specific probes detected single strongly hybridizing bands, suggesting that p18 gene is a single-copy gene and that highly homologous genes do not exist in the human genome.

The genomic clone that we isolated contains approximately 5 kb of DNA upstream of exon 1. Approximately 129 bp of 5′ flanking DNA were sequenced (Fig. 1). The putative p18 promotor did not contain a recognizable TATA box but the region adjoining the putative transcription start site was GC-rich. A search for the presence of known transcription factor binding sites using the FINDPATTERNS function of the Wisconsin GCG Version 9.0 sequence analysis package and the tfsites.dat database identified a number of potential Sp1 binding sites. The 5 kb of putative p18 promotor DNA was capable of directing the expression of the luciferase reporter gene in transiently transfected cells (data not shown).

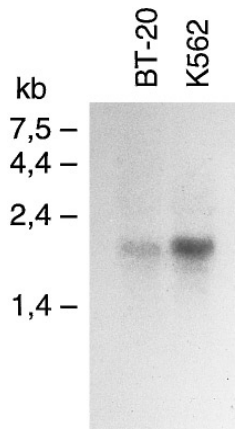


FIG. 2. RNA blot analysis of p18 mRNA in BT-20 and K562 cells. Total RNA (10 μg per lane) from BT-20 and K562 cells was size-separated on a 1% agarose/2.2 M formaldehyde gel, immobilized on a nylon membrane and incubated with a radiolabelled full-length p18 cDNA probe. Hybridized membranes were autoradiographed by exposure to Kodak X-OMAT AR film at −80°C for 3 days. The positions of the RNA size markers are indicated.

Characterization of p18 mRNA

A previous report proposed the existence of multiple p18 transcripts (12). Since the different p18 cDNAs that we isolated did not vary significantly in length, we assessed the size of the p18 transcript(s) expressed in human breast BT-20 cells and leukemia K-562 cells by Northern blot analysis. As illustrated in Figure 2, the ^{32}P-labeled p18 cDNA detected a predominant 2.2 kb mRNA present in total RNA prepared from BT-20 and K562 cells. Though a few much fainter bands could be identified, the size of the major transcript is in agreement with that of the longest cDNA that we isolated. Similar results were obtained using polyadenylated RNA.

To verify that the longest cDNA (clone 40) truly corresponded to the longest transcript, we performed ribonuclease protection assays (RPA) using polyadenylated

FIG. 1. Nucleotide sequence of the human p18 gene. Exons 1 to 3 are boxed. The beginning of exon 1 marks the 5′ end of the longest cDNA clone we isolated. Amino acids corresponding to p18 are indicated in the single-letter code and are numbered above the DNA sequence. An asterisk (*) denotes the stop codon. The underlined nucleotides at positions 214 and 342 of the coding region and 151 nucleotides downstream of the stop codon in the 3′-untranslated region correspond to the different p18 variants. A putative polyadenylation signal in the 3′-untranslated region is represented by bold letters. The sequence of the human p18 gene includes 129 nucleotides of 5′ flanking DNA and 215 nucleotides of 3′ flanking DNA. The complete sequence of the first intron is shown as well as approximately 750 nucleotides of the larger intron 2. The nucleotide sequences reported in this paper have been deposited in GenBank under accession numbers AF041248, AF041249 and AF041250.

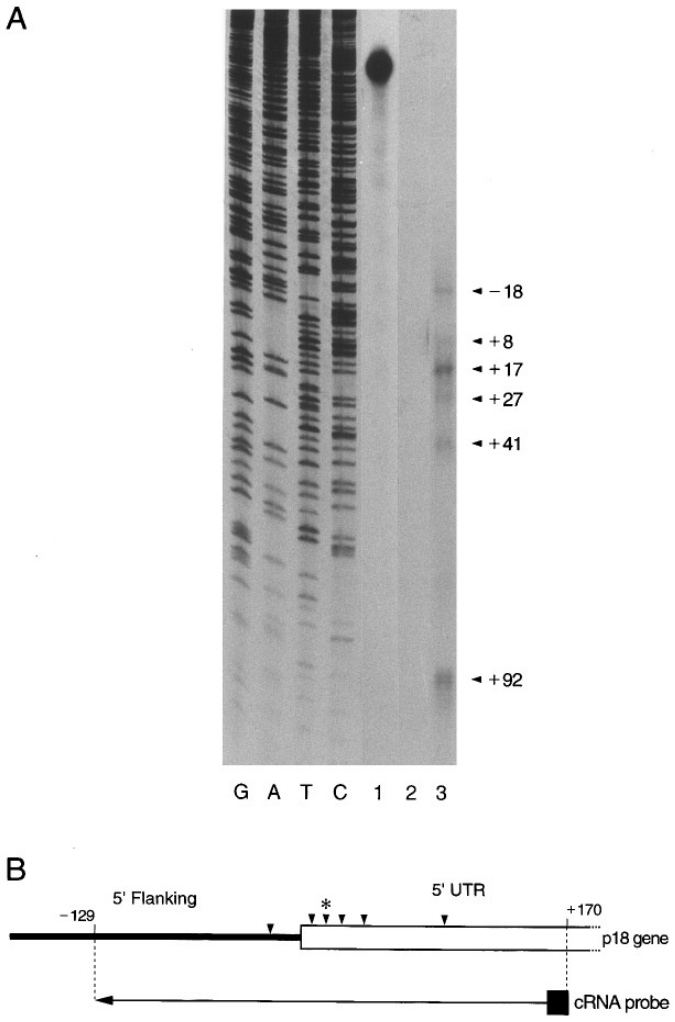


FIG. 3. Ribonuclease protection analysis of human p18 mRNA. A, Polyadenylated RNA (2 μg) from K562 cells was hybridized with a human p18 cRNA probe (lane 3), as described in Materials and Methods. Lane 1 contains the undigested probe whereas lane 2 contains the tRNA control. The approximate positions of the 5′ end of each protected fragment (relative to the 5′ end of the longest cDNA) are indicated on the right. B, Schematic representation of the human p18 gene and the cRNA probe. The 5′-flanking region of the gene is represented by a solid line whereas exon 1 is represented by an open box. The cRNA probe produced from the Sp6 promotor (black square) is shown as an arrow below the gene. Nucleotide positions relative to the 5′ end of exon 1 are indicated. The arrowheads correspond to the start sites shown in the autoradiogram above. The major start site at +17 is indicated by an asterisk (*). The gel was exposed to film for 5 days. The undigested probe (Lane 1) was exposed to film overnight in this composite figure.

mRNA from K562 cells which express higher levels of p18 mRNA (Fig. 2). A cRNA probe was generated from a genomic p18 fragment comprised of 129 bp of 5′ flanking DNA contiguous with 170 bp of the 5′ end of exon 1. As shown in Figure 3, this probe detected a group of 5 start sites clustered between approximately −18 and +41 relative to the 5′ end of the longest cDNA we isolated. The most abundant start site mapped to approximately +17 relative to the 5′ end of cDNA clone 40. In addition, a sixth start site was detected around +92 relative to the 5′ end of cDNA clone 40. Rapid amplification of cDNA ends yielded products with 5′ ends in the vicinity of the +17 and +92 start sites (data not shown). Taken together, the results of the RPA and Northern analysis indicate that transcription of the p18 mRNA initiates in the vicinity of the 5′ end of cDNA clone 40.

Analysis of p18 Status in Human Breast Tumors

We previously reported the existence of an inactivating mutation of p18 in BT-20 breast cancer cells (14). Preliminary results obtained in tumor specimens using SSCP analysis suggested that this p18 variant might occur frequently in breast tumors. We therefore designed a sensitive mismatch PCR/restriction fragment length polymorphism assay to detect the codon 72 variant. Radiolabelled DNA fragments were generated by RT-PCR from a second series of 35 breast tumors using a 5′ oligonucleotide primer that was designed to introduce a *BstE*II site in fragments harboring the codon 72 substitution (Fig. 4A). Following digestion with *BstE* II, p18 PCR products containing the codon 72 variant are cleaved to a 167-bp fragment. This assay allowed us to detect both the codon 72 and codon 114 substitutions simultaneously since the codon 114 polymorphism introduces a *BstE* II site in the p18 sequence which generates a 157-bp fragment following digestion of the corresponding RT-PCR product with *BstE* II. The *BstE*II restriction fragments were resolved from the nondigested 188-bp PCR product on polyacrylamide gels.

Figure 4B illustrates the results of the PCR/RFLP analysis obtained in five of the 35 breast tumor specimens analyzed. BT-20 cells, which express the codon 72 and codon 114 variants, were used as a positive control whereas MCF-7 cells, which express wild-type p18 mRNA, were used as negative control. Plasmids containing the wild-type p18 cDNA or the codon 72 variant are shown as additional controls. PCR/RFLP analysis of RNA or DNA samples from BT-20 cells produced the expected 167- and 157-bp *BstE*II restriction fragments. The *BstE*II restriction fragment corresponding to the codon 72 substitution was detected in three of the 35 breast tumor RNA samples examined (Tumor nos. 2, 3 and 4). These results were confirmed by direct sequencing of PCR products amplified by RT-PCR of tumors 2, 3 and 4 (Fig. 4C). The existence of the codon 72 substitution was also confirmed in DNA samples from 2 positive tumors for which DNA samples were available. All three tumors also expressed the codon 114 polymorphism (Fig. 4B). On the other hand, tumor no. 1 contained wild-type p18 mRNA only whereas both the codon 114 variant and wild-type p18

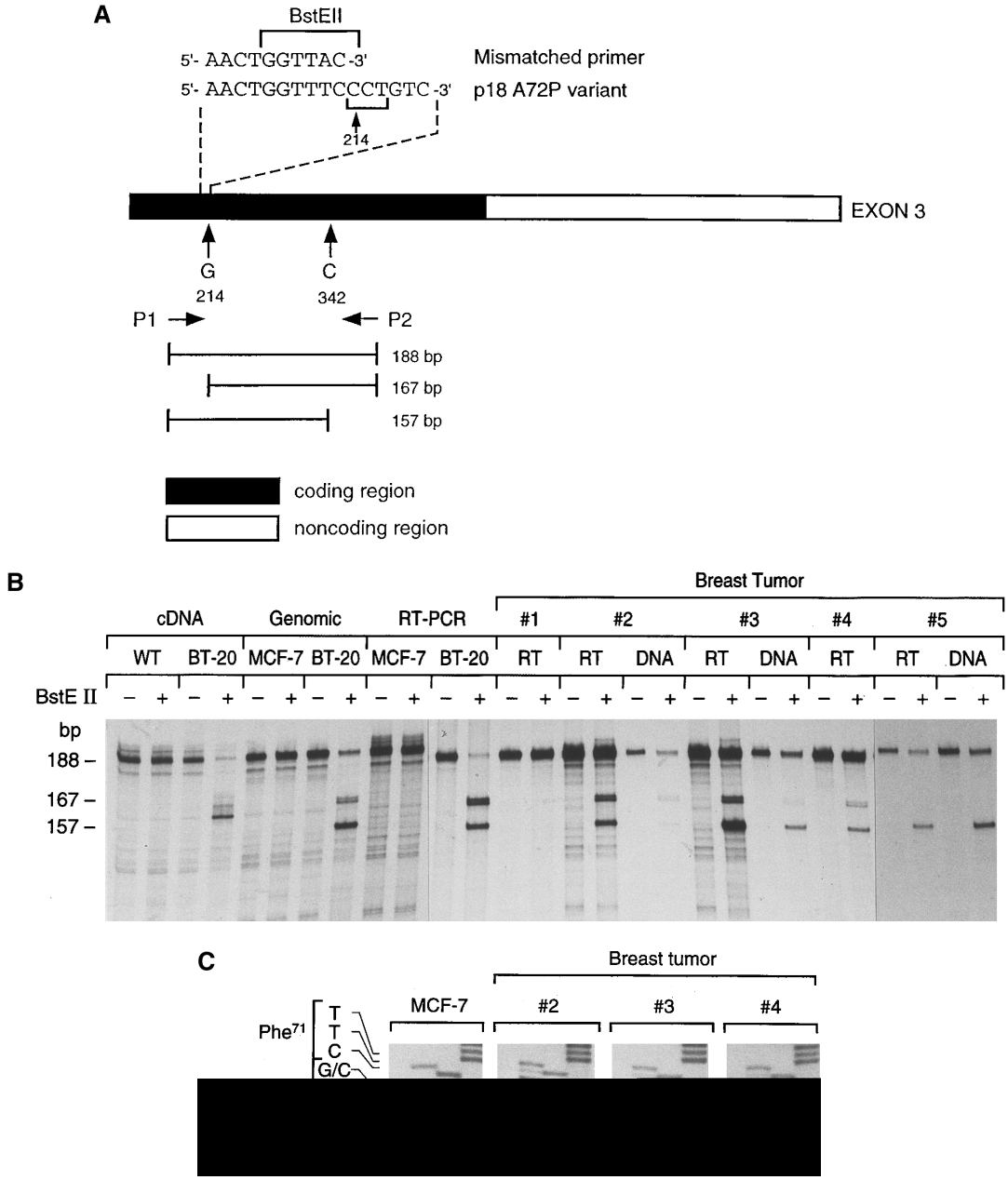


FIG. 4. Analysis of p18 status in human breast tumors. A, Schematic diagram depicting the mismatch PCR/RFLP assay for the p18 variants at coding nucleotides 214 and 342. The arrows designated P1 and P2 correspond to the upstream and downstream primers used to generate the full-length PCR product (188 bp). The upstream primer (incorporating a single mismatch at nucleotide 212) lies 2 nucleotides 5′ to codon 72 sequences. The presence of a C at nucleotide 214 (codon 72 variant) associated with the mismatched nucleotide at position 212 creates a *BstE*II restriction site thereby generating a fragment of 167 bp after *BstE*II digestion of the corresponding PCR product. The C to T change at nucleotide 342 (codon 114 variant) creates a natural *BstE*II restriction site to generate a fragment of 157 bp following digestion. B, PCR products before (−) and following (+) *BstE*II digestion. The wild type p18 cDNA (WT) as well as MCF-7 genomic DNA and RT-PCR products were used as negative controls whereas the p18 codon 72 variant cDNA as well as BT-20 genomic DNA and RT-PCR products were used as positive controls. *BstE*II digests of PCR amplicons from 5 unrelated breast tumor RNA (RT) and, where indicated, their corresponding genomic DNA (DNA), are shown. Restriction fragment length analysis of the PCR products after digestion with *BstE*II was performed on a denaturing 6% polyacrylamide gel. C, Direct sequencing of p18 RT-PCR amplification products from MCF-7 cells and human breast tumors #2, 3 and 4.

were detected in tumor no. 5 (Fig. 4B). The silent polymorphism at codon 114 was detected in 26 (74%) of the 35 tumors analyzed. In contrast, neither *BstE*II restriction fragment was detected in MCF-7 cell samples that were intercalated between tumor samples and processed concurrently (Fig. 4B and data not shown).

DISCUSSION

We report here the structure and sequence of the gene encoding the human cyclin-dependent kinase inhibitor p18. The p18 gene consists of three exons, the first of which is entirely noncoding. Exons 1 and 2 are separated by a relatively short 0.45 kb intron whereas exons 2 and 3 are separated by approximately 4.5 kb. We have sequenced the entire p18 gene with the exception of 3.7 kb in intron 2. The sequence of the genomic DNA and the deduced structure of the p18 gene were in agreement with the sequence of the longest cDNA clone that we isolated from a human BT-20 breast cancer cell cDNA library.

The INK4c gene is a member of the cyclin D-dependent kinase inhibitor family which is comprised of p16/INK4a, p15/INK4b, p19/INK4d and p18. The importance of the INK4a gene in human cancer has been well established, particularly in familial melanoma (21). Moreover, disruption of the INK4a locus in mice led to the development of spontaneous tumors such as sarcomas and lymphomas (22). These results indicate that INK4a plays a major role as a regulator of retinoblastoma protein function in mice, at least in the affected cell lineages. This also signifies that INK4 functions are not totally redundant in all cells. However, the role of the other INK4 genes in human cancer is not as well understood. We identified a mutant form of p18 in BT-20 human breast cancer cells that abrogates the ability of p18 to bind CDK6 (14). This mutation, which was originally identified by SSCP analysis and direct sequencing of RT-PCR products from BT-20 cells, was confirmed by the isolation of full-length cDNA clones harboring the codon 72 substitution (this report).

Using a mismatch PCR/RFLP assay, we have identified this p18 variant in three of 35 breast tumors examined. The occurrence of the same mutation in different individuals suggests that the codon 72 variant may be a polymorphism. However, more detailed genetic studies will be required to confirm this possibility. It is also of interest to note that the codon 114 substitution was identified in approximately 75% of the tumors examined. This polymorphic site was detected in 19% of 31 healthy Caucasian volunteers (23) and in 17% of 71 lung cancer specimens (24). The higher incidence observed in our series may be attributed to characteristics of the sample population which was composed of residents of Québec province.

Mismatch PCR/RFLP strategies that have been described in the literature usually utilize non-radioactive DNA and agarose gel electrophoresis. To enhance the sensitivity of this technique and to augment the resolution between different restriction fragments, we have used radiolabelled DNA and polyacrylamide gels (25). This made it possible to discriminate between DNA fragments ranging from 157 to 188 bp in size, thereby allowing the simultaneous detection of two p18 variants. This modification of an established technique should prove useful in similar applications.

The results obtained by Northern blot analysis using total as well as polyadenylated RNA isolated from BT-20 and K-562 cells indicate that the most abundant p18 transcript expressed in these cells is approximately 2.2 kb long. The size of the major transcript approximates that of the longest cDNA that we isolated from BT-20 cells and transcription start sites in the vicinity of the 5′ end of the cloned cDNA were identified by ribonuclease protection assay. The 2.2 kb transcript likely corresponds to the 2.4 kb message observed in an RNA blot prepared from normal human tissues (26). Normal tissues were also found to express a 1.2 kb transcript that we have not detected in K562 and BT-20 cells. Depending on the cDNA probe used, up to 5 p18 transcripts have been identified in normal tissues (12). Other investigators have identified p18 transcripts of 1.5 and 0.7 kb in breast cancer cells but no 2.2-2.4 kb mRNA (27). Such differences in the expression pattern of p18 transcripts may be attributable to cell-specific expression patterns, differences between normal and cancerous tissue and/or differences in probe selection and hybridization conditions.

The levels of p18 mRNA are regulated in a cell cycle-dependent manner. In murine macrophages stimulated with CSF-1, p18 mRNA levels peak in S phase, as do p19 mRNA levels (28). These induction kinetics are similar to that of p16β in human lymphocytes stimulated with phytohemaglutinin and IL-2 (29). However, in human leukemic HL-60 cells, treatment with phorbol esters caused a decrease in p18 and p19 mRNA and an increase in p15 mRNA (30). In addition, p18 is induced during myogenic and B cell differentiation (31, 32). It will be of interest to characterize the human p18 promotor and study its regulation by various regulators of cell growth. The availability of the human p18 gene will allow more detailed studies of the control of p18 expression in both normal and cancerous cells, and will allow further studies into the involvement of p18 in human cancer.

ACKNOWLEDGMENTS

The authors wish to thank D. Monté and J. Simard for helpful discussions, P. Couture for tips on the RFLP analysis, F. Labrie for his interest in this work, and the Illustration Service of our research center for artwork. This work was supported by a grant from the Canadian Breast Cancer Research Initiative to C. L. Additional support was provided by Endorecherche. C. L. was supported in part by a fellowship from the Fonds de la recherche en santé du Québec.

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