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Prohibitin, an Evolutionarily Conserved Intracellular Protein That Blocks DNA Synthesis in Normal Fibroblasts and HeLa Cells

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Genes that act inside the cell to negatively regulate proliferation are of great interest because of their implications for such processes as development and cancer, but these genes have been difficult to clone. This report details the cloning and analysis of cDNA for prohibitin, a novel mammalian antiproliferative protein. Microinjection of synthetic prohibitin mRNA blocks entry into S phase in both normal fibroblasts and HeLa cells. Microinjection of an antisense oligonucleotide stimulates entry into S phase. By sequence comparison, the prohibitin gene appears to be the mammalian analog of *Cc*, a *Drosophila* gene that is vital for normal development.

The ability to negatively regulate cell proliferation is a necessity for all living organisms. Unicellular organisms must limit their replication to the times when adequate nutrients and other environmental factors are present, and multicellular organisms must accurately shape and maintain the architecture of their component tissues. The failure in a multicellular organism to provide adequate negative growth control in the developmental period may result in a malformation, which may be lethal; in the postdevelopmental period, such a failure may result in neoplasia. Because negative control is so critical, specific genes have evolved whose role is actively antiproliferative.

Tumor suppressor genes (reviewed in references 18, 21, 35, and 41) are a class of genes that have been identified on the basis of an association between neoplasia and the loss of function in both copies of the gene. While the existence of more than 10 tumor suppressor genes is predicted on the basis of such associations, only 4 such genes have been cloned to date: retinoblastoma (12, 13, 23), p53 (31), Wilms' tumor (3, 15, 37), and *dcc* (9). The recently cloned neurofibromatosis type 1 gene (5, 48) is likely to be a fifth member of this group. The retinoblastoma gene product (22) and p53 (7, 38) appear to be nuclear proteins. The Wilms' tumor gene product has a structure similar to that of other transcription factors (3, 15). The *dcc* gene product (9) resembles neural-cell adhesion molecules. The neurofibromatosis type 1 gene appears to be related to GTPase-activating proteins (2, 50).

Tumor suppressor genes may be only a subset of the important negative regulatory genes in the cell. A hypothetical second class of negative regulators would be antiproliferative genes whose loss of function kills the cell. A lethal outcome might occur for any number of reasons, such as when internal growth signals become too great for the maintenance of homeostasis. For example, the product of the *wee1* gene is a dose-dependent inhibitor of mitosis in the fission yeast *Schizosaccharomyces pombe* (40). In yeast cells that overproduce a mitotic inducer, the *cdc25* gene product (39), coexpression of the Wee1$^-$ phenotype is lethal. Negative regulators whose loss kills the cell would not produce the association between loss of function in both alleles and neoplasia that defines tumor suppressor genes, but their absence might cause death during embryogenesis. No member of this potential class of genes has yet been identified in mammalian cells.

In general, negative growth control genes that act within mammalian cells have been difficult to isolate. Despite intensive research in recent years, only a small number of genes have been cloned for which such an activity is likely. Of this subset, only four genes have been shown by expression in cells in tissue culture to be directly antiproliferative: the retinoblastoma gene product (19), p53 (1, 29), a *ras*-related transformation suppressor gene (20), and prohibitin (28).

The first cDNA for prohibitin was isolated by using a strategy (28) different from that used to isolate tumor suppressor genes. This cDNA was originally identified as one of a set of cDNAs corresponding to mRNAs more highly expressed in normal than regenerating liver. It was then shown that prohibitin mRNA enriched by hybrid selection could block DNA synthesis when microinjected into normal fibroblasts. No match was found between the partial cDNA clone and sequences in the GenBank database.

This report describes the cloning and analysis of a longer prohibitin cDNA with a complete open reading frame (ORF). It demonstrates the ability of prohibitin mRNA to block DNA synthesis in cancer cells and shows that the prohibitin gene is the mammalian analog of *Cc*, a *Drosophila melanogaster* gene required for normal development (8).

MATERIALS AND METHODS

Cloning and construction of Pro1 cDNA. Clones (6×10^5) from a rat intestine cDNA library in the LambdaZapII vector (Stratagene) were plated on *Escherichia coli* XL-1 Blue and screened by plaque hybridization by using standard tech-

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niques (26). The *Pst*I insert from the M5 cDNA (28) was labeled by random primer extension (10) and used as probe. Plasmids were derived from positive clones by using the in vitro excision procedure provided with the library. Clone I1 extends from nucleotide 162 of the sequence shown in Fig. 1 to a poly(A) tail of 30 bp. Clone I12 extends from nucleotide 1 to nucleotide 1480 of the Fig. 1 sequence. To construct the Pro1 clone, both plasmids were cut with *Hin*dIII. The fragment of I12 containing the Bluescript plasmid and the initial 970 bp of prohibitin and the fragment of I1 comprising the 3′ 718 bp of prohibitin and the poly(A) tail were gel purified and ligated to obtain the Pro1 cDNA.

Primer extension. Primer extension analysis of the transcript initiation site of prohibitin was performed essentially as described previously (6), except that vanadyl ribonucleoside complex was omitted from the reaction. A high-pressure liquid chromatography-purified synthetic oligonucleotide (Midland Certified Reagent Co.) complementary to nucleotides 131 to 159 of Pro1 (Fig. 1) was used to prime cDNA synthesis from 10 μg of poly(A) RNA prepared from rat intestine. Pro1 cDNA sequenced with the same primer was used as a molecular weight standard. Products were separated on a 6% sequencing gel and autoradiographed with Kodak XAR-2 film.

DNA sequencing. CsCl-purified preparations of plasmid DNAs were sequenced with the Sequenase 2.0 kit from U.S. Biochemicals and synthetic primers purchased from Midland.

Computer analysis of sequence data. The GenBank release current on 11 May 1990 was searched for DNA sequences homologous to Pro1 by using the FASTA algorithm (32). The amino acid sequence was searched for polypeptide motifs by using the QUEST program against the KEYBANK database on BIONET.

In vitro transcription. For transcription in the sense orientation, the Pro1 cDNA was linearized with *Apa*I and transcribed from the T3 promoter of the Bluescript vector. Transcription was performed with a kit purchased from Stratagene using the conditions for large-scale preparation of capped RNA given in the *Protocols and Applications Guide* (35a) available upon request from Promega Biotec. The 5′ cap analog was purchased from Boehringer Mannheim. Transcription was performed for 1 h at 37°C. The amounts of polymerase and nucleotide were then increased by 50%, and transcription continued for an additional 1 to 2 h. For the prolactin control mRNA, plasmid Pr1, containing a complete prolactin cDNA (46) was linearized with *Bgl*II and transcribed as described above but with SP6 polymerase.

Microinjection assay for antiproliferative activity. Microinjections were carried out and assayed as described previously (28). Human diploid fibroblasts from neonatal foreskin (CF-3) were grown on cover slips and then growth arrested by serum starvation (0.1%) for 1 week. Following microinjection (200 to 400 cells injected per experiment), the cells were stimulated with serum (10%), exposed to [^{3}H]thymidine for 24 h, and then fixed and processed for autoradiography. The concentration of transcript was 50 μg/ml unless otherwise noted. Oligonucleotides were injected at 1 mg/ml. HeLa cells were treated identically except that they were not placed in low serum. In a control experiment, 50 μg of sense prohibitin mRNA transcript per ml was treated with 100 μg of RNase A (Bethesda Research Laboratories) per ml for 30 min at 37°C prior to microinjection. In addition, the same final amount of RNase in buffer was microinjected into CF-3 cells to determine if RNase alone had any effect on thymidine uptake. RNasin (50 U/ml; Promega Biotec) was routinely used to protect the RNA transcripts from degradation. To control for any possible effect on entry into S phase, RNasin (50 U/ml) was microinjected into CF-3 cells. This resulted in no detectable effect on the ability of cells to enter S phase (data not shown). Percent inhibition was calculated as $[(U - I)/U] \times 100$, where U is the percentage of labeled nuclei in uninjected cells and I is the percentage of labeled nuclei in injected cells. A negative value therefore indicates stimulation.

In vitro translation. Synthetic mRNA transcribed as described above was translated by using a reticulocyte lysate kit, including canine pancreatic microsomes, purchased from Promega Biotec and by following the protocol explained in the kit. [^{35}S]methionine and [^{3}H]leucine were purchased from Amersham. Translation was allowed to proceed for 90 min at 30°C, and then RNase A was added to a final concentration of 0.1 mg/ml. Samples were digested with RNAse for 10 min and then made 10 mM in $CaCl_2$ and placed on ice. Samples (except for controls with no RNA) were halved. One half was diluted with 300 μl of boiling 0.1 M Tris (pH 8)–1% sodium dodecyl sulfate (SDS), let stand at 100°C for 5 min, diluted with 300 μl of 2× Laemmli gel buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.001% bromophenol blue), incubated for 5 min, and then frozen. The remaining half of each sample was digested with proteinase K at a final concentration of 50 μg/ml for 10 min on ice. The protease stock solution was prepared by predigesting a 0.5-mg/ml solution in 10 mM $CaCl_2$–10 mM Tris (pH 8.9) for 30 min at 37°C. Protease digestion was terminated by drawing the sample into a pipette tip containing 1 to 2 μl of 250 mM phenylmethylsulfonyl fluoride and diluting it immediately as described above. Protein samples were separated on precast 12% SDS-polyacrylamide gels (NOVEX). The gels were treated with a fluorographic enhancer (New England Nuclear) and exposed to Kodak XAR-2 film.

Northern (RNA) analysis of rat tissues and human fibroblasts. RNA was prepared from dissected rat tissues by disruption in guanidine isothiocyanate and CsCl ultracentrifugation and then enriched for poly(A) RNA by a single pass over oligo(dT)-cellulose, with only minor modifications of the standard procedures as described elsewhere (27). The RNA concentration was determined by A_{260}, and equal quantities were loaded on a gel for Northern blotting. A fragment of the Pro1 cDNA extending form nucleotides 1 to 543 (Fig. 1) was used as a probe. RNA was transferred to a GeneScreen Plus membrane and hybridized with the same fragment of Pro1 cDNA at 42°C overnight in 50% formamide–1% SDS–1 M NaCl–10% dextran sulfate–100 μg of herring sperm DNA per ml. The blot was washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 10 min at room temperature, given two 30-min washes at 60°C in 2× SSC–1% SDS, and then rinsed in 0.1× SSC for 30 min at room temperature.

Equivalent poly(A) content of the rat tissue samples was verified by analysis of replica dot hybridizations probed with ^{35}S-labeled poly(dT) (28) (data not shown). Autoradiography was performed with Du Pont WDR film. Densitometry was performed using a RAS video image analysis system (Amersham/Loats).

Human IMR-90 cells (Coriell Institute, Camden, N.J.) were grown at 37°C in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum. These normal diploid fibroblasts were harvested at a subconfluent density and at a population doubling level of 25. RNA was

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1   CAGAAGGAGTC ATG GCT GCC AAA GTG TTT GAG TCC ATC GGA AAG TTC GGC CTG GCC TTA GCA GTT GCA GGA GGC GTG GTG AAC TCT
              MET Ala Ala Lys Val Phe Glu Ser Ile Gly Lys Phe Gly Leu Ala Leu Ala Val Ala Gly Gly Val Val Asn Ser

87  GCT CTA TAT AAC GTG GAT GCC GGA CAC AGA GCT GTC ATC TTC GAC CGA TTC CGT GGC GTG CAG GAC ATC GTG GTA GGG GAA GGG
    Ala Leu Tyr Asn Val Asp Ala Gly His Arg Ala Val Ile Phe Asp Arg Phe Arg Gly Val Gln Asp Ile Val Val Gly Glu Gly

171 ACT CAC TTC CTC ATC CCC TGG GTA CAG AAG CCA ATC ATC TTT GAC TGC CGC TCT CGA CCA CGT AAT GTG CCG GTC ATC ACC GGC
    Thr His Phe Leu Ile Pro Trp Val Gln Lys Pro Ile Ile Phe Asp Cys Arg Ser Arg Pro Arg Asn Val Pro Val Ile Thr Gly

255 AGC AAA GAC TTG CAG AAT GTC AAC ATC ACA CTA CGT ATC CTC TTC CGG CCG GTG GCC AGC CAG CTT CCT CGT ATC TAC ACC AGC
    Ser Lys Asp Leu Gln Asn Val Asn Ile Thr Leu Arg Ile Leu Phe Arg Pro Val Ala Ser Gln Leu Pro Arg Ile Tyr Thr Ser

339 ATT GGC GAG GAC TAT GAT GAG CGG GTG CTG CCA TCT ATC ACC ACA GAG ATC CTC AAG TCG GTG GTG GCT CGA TTC GAT GCT GGA
    Ile Gly Glu Asp Tyr Asp Glu Arg Val Leu Pro Ser Ile Thr Thr Glu Ile Leu Lys Ser Val Val Ala Arg Phe Asp Ala Gly

423 GAA TTG ATT ACC CAG CGA GAG CTG GTC TCC AGG CAG GTG AGT GAT GAC CTC ACA GAG CGA GCA GCA ACA TTC GGG CTC ATC CTG
    Glu Leu Ile Thr Gln Arg Glu Leu Val Ser Arg Gln Val Ser Asp Asp Leu Thr Glu Arg Ala Ala Thr Phe Gly Leu Ile Leu

507 GAT GAC GTG TCC CTG ACA CAT CTG ACC TTC GGG AAG GAG TTC ACA GAG GCG GTG GAA GCC AAA CAG GTG GCT CAG CAG GAA GCA
    Asp Asp Val Ser Leu Thr His Leu Thr Phe Gly Lys Glu Phe Thr Glu Ala Val Glu Ala Lys Gln Val Ala Gln Gln Glu Ala

591 GAG AGA GCC AGA TTT GTG GTG GAA AAG GCT GAG CAG CAG AAG AAG GCG GCC ATC ATC TCT GCT GAG GGT GAC TCC AAA GCG GCT
    Glu Arg Ala Arg Phe Val Val Glu Lys Ala Glu Gln Gln Lys Lys Ala Ala Ile Ile Ser Ala Glu Gly Asp Ser Lys Ala Ala

675 GAG CTG ATC GCC AAC TCA CTG GCC ACC GCC GGG GAT GGC CTG ATC GAG CTG CGA AAG CTG GAA GCT GCT GAG GAC ATT GCT TAT
    Glu Leu Ile Ala Asn Ser Leu Ala Thr Ala Gly Asp Gly Leu Ile Glu Leu Arg Lys Leu Glu Ala Ala Glu Asp Ile Ala Tyr

759 CAG CTC TCC CGC TCT CGG AAC ATC ACC TAC CTG CCA GCA GGG CAG TCC GTG CTC CTC CAG CTC CCC CAG TAA    GGCCAGCCAG
    Gln Leu Ser Arg Ser Arg Asn Ile Thr Tyr Leu Pro Ala Gly Gln Ser Val Leu Leu Gln Leu Pro Gln  *

841 CCAGGGCCTC CATCGCTCTG AATGACGCCT TCCTTCTGCC CCACCCCAGA AATCACTGTG AAATTTAATG ATTGGCTTAA CATGAAGGAA ATAAAGGTAA

941 AATCACTTCA TATCTCTAAT TATCAAATGA AGCTTTTATT GTTACACTTT TTGCCCACTT TCATAACAAA ATTGCCAAGT GCCTATGCAG ACTGGCCTTC

1041 CACCCTGGGT GCTGGCAGTC GGCGGAAGAA AGGCAGGGCA GTGTGTGTGG TGGACGGGGA GCCAGCTGGC AGCCTGAGTA GACCTTGAGC CTCCATTCTG

1141 CCATATATTG AAGATTTACA GACAGTGGTG CACACACGTG AACCAAAAGC AAGCCCTCAA TTTTTCCAGC CATACGAACC CGGACAGATG CAGCTGAGGA

1241 GGGCCTGAGG AAGTGGTCTG TCTTAACTGT AAGGCCATTC CCTCTTAACC GTGACCAGCG GAAGCAGGTG TGTGCGTGCG ACTAGGGCAT GGAGTGAAGA

1341 ATCTGCCCAT CACGGTGGGT GGGCCTAATT TTGCTGCCCC CACCAGAGAC CTAAACTTTG GATAGACTTG GATAGAATAA GAGGCCTGGA CTGAGATGTG

1441 AGTCCTGTGG AAGACTTCCT GTCCACCCCC CACATTGGTC CTCTCAAATA CCAATGGGAT TCCAGCTTGA AGGATTGCAT CTGCCGGGGC TGAGCACACC

1541 TGCCAAGGAC ACGTGCGCCT GCCTTCCCGC TCCCTCTCTT CGAGATTGCC CTTCCTTCCC AAGGGCTGTG GGCCAGAGCT CCGAAGGAAG CAATCAAGGA

1641 AAGAAAACAC AATGTAAGCT GCTGTCAATA AATGACACCC AGACCCTC(AAA)n
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FIG. 1. Nucleotide sequence of Pro1 cDNA and translation of the longest ORF. The common *Hin*dIII site used to create Pro1 from cDNAs I12 and I1 begins at nucleotide 970. The I12 cDNA sequence runs from nucleotide 1 to 1480, and the I1 sequence runs from 162 to the poly(A) tail. The numbering is in base pairs. The AATAAA polyadenylation signals are boxed, as are the ATTTA mRNA stability motifs. The portion of Pro1 identical to M5 (28) is overlined. Amino acids identical in the predicted gene products of the rat prohibitin cDNA and the *Drosophila Cc* cDNA are shown in boldface italic type. The nucleotides of the region used to define oligonucleotides for microinjection are also shown in boldface italic type.

then extracted and analyzed as for rat tissues, except that total RNA rather than poly(A)-enriched material was used.

Western (immunoblot) analysis of prohibitin from cell cultures and rat liver. All separations were performed on precast 12% SDS-polyacrylamide gels (NOVEX), and all transfers were to nitrocellulose membrane in 12 mM Tris–96 mM glycine (pH 8.3) in 20% methanol. For analysis of cell lysates and culture supernatant (see Fig. 4), IMR-90 cells were grown to confluence over a 4-day period in DMEM supplemented with fetal bovine serum to 2%. Medium was removed and concentrated in a Centricon-10 microconcentrator (Amicon). Cells were washed with phosphate-buffered saline, scraped from the dish, pelleted, and lysed in 10 mM Tris (pH 7.5)–10 μg of leupeptin per ml–1 mM phenylmethylsulfonyl fluoride–0.2 μg of DNase I (Boehringer) per ml (lysis buffer) for 10 min at room temperature. SDS was added to 0.1%, and the extract was heated to 65°C for 10 min. The extract was spun for 10 min at 13,000 × *g* in a microfuge, and the supernatant was stored at −20°C. Aliquots representing 0.5% of the total material of each preparation (lysed cells versus supernatant) were compared.

For comparison of cell culture lysates and the in vitro translation products (see Fig. 5A), 2.5% of the IMR-90 lysate described above was run adjacent to 7% of the in vitro translation product. For comparison of prohibitin in liver tissue and lysates from various cell cultures (see Fig. 5C), Rat-1, HeLa, and IMR-90 cells were grown in DMEM plus 10% fetal bovine serum, and lysates from confluent cultures were prepared as described above. One gram of fresh liver tissue was minced and Dounce homogenized in 1 ml of ice-cold lysis buffer. One-tenth of this material was diluted 20-fold into lysis buffer, SDS was added to 0.1%, and the extract was heated and centrifuged as described above. The protein contents of the samples were determined by a dye-binding assay (BioRad). Twenty micrograms of protein was loaded on each lane.

Antibody (Hazelton Research Products, Denver, Pa.) was raised against selected peptides of prohibitin (Multiple Peptide Systems, San Diego, Calif.) conjugated to keyhole limpet hemocyanin. Nine serum samples with antibodies raised against three peptides were pooled. Preimmune sera were from the same animals before peptide injection. An-

tiprohibitin antibody bound to the filter was detected by using biotinylated second-antibody–streptavidin–alkaline phosphatase complex (Vector Laboratories) under conditions suggested by the supplier except that components of the complex were diluted 1:5.

RESULTS

As previously reported (28), the strategy used to isolate the first prohibitin cDNA clone (named M5) was based on the observation that mRNA from various nondividing cell types could block DNA synthesis when microinjected into dividing cells. This had been demonstrated for senescent fibroblasts (24), lymphocytes (34), and liver from humans (33) and rats (25). The peak of activity in rat liver copurified with mRNAs of about 2 kb on a sucrose centrifugation gradient (25). A cDNA library made from this 2-kb gradient fraction was therefore screened with radioactive cDNAs complementary to mRNAs from either normal or regenerating liver. M5 was isolated as one of the cDNAs that hybridized more strongly to cDNAs corresponding to normal liver. It was then shown that mRNA enriched by hybrid selection with M5 DNA could block DNA synthesis when microinjected into serum-stimulated fibroblasts in culture (28).

Cloning and sequencing of a prohibitin cDNA with a complete ORF. To identify the protein-coding region of prohibitin mRNA, M5 was used as a hybridization probe to screen a cDNA library from rat intestine constructed in the LambdaZapII vector (Stratagene). Two clones, I1 and I12 (Fig. 1), with identical DNA sequences over most of their lengths, were isolated. I12 had more of the 5′ sequence of the mRNA, while I1 encoded a poly(A) tail. The two clones were cleaved at their common *Hin*dIII site, and the 5′ portion of I12 was joined to the 3′ portion of I1. The resulting construction, named Pro1, encodes a complete ORF of 272 amino acids and a long 3′ untranslated region. This region contains two potential polyadenylation sites, two ATTTA motifs that have been implicated in the control of mRNA stability (44), and a 30-base poly(A) tail. The Pro1 cDNA lies between a T7 and a T3 promoter in the vector, so it can be transcribed in either orientation in vitro. To rule out any cloning artifacts in the construction of Pro1, the complete sequences of I1, I12, and Pro1 were obtained from both strands.

Nucleotide sequence and predicted amino acid sequence of Pro1. The predicted amino acid sequence of the largest ORF was determined from the nucleotide sequence (Fig. 1). Using the QUEST program of BIONET, this amino acid sequence was searched for a variety of protein motifs, including ATP-binding sites, nuclear localization signals, transcription factors (leucine zipper, helix-turn-helix, homeobox), and signal sequences. No matches better than those for random sequences were found. A hydrophobic region was found at the N terminus of the protein, but it is only 12 amino acids long, probably too short for a signal sequence (47). No other hydrophobic regions characteristic of transmembrane regions were seen. There are two potential glycosylation sites (Asn-X-Thr or Asn-X-Ser), but in the case of prohibitin, these are apparently not utilized (see Fig. 3).

Primer extension analysis of prohibitin mRNA. To determine how much 5′ untranslated mRNA was missing from the hybrid cDNA clone, primer extension studies were performed. A 29-base oligonucleotide complementary to prohibitin mRNA (positions 131 to 159; Fig. 1) was used to prime rat intestine poly(A) RNA (Fig. 2). Four primer extension products 28, 40, 49, and 62 bases longer than the 5′ end of the Pro1 cDNA were identified.

Because the Pro1 cDNA is incomplete, the prohibitin ORF might in principle be larger than we have proposed, initiating at an ATG 5′ of the ATG we designate here. However, we have recently found an in-frame terminator codon (TGA) 24 nucleotides 5′ to our designated protein start, with no initiator codon in between, in a third prohibitin cDNA isolate (data not shown).

In vitro translation of a synthetic prohibitin mRNA. The predicted ORF of Pro1 encodes a protein product of approximately 30 kDa that has two potential N-glycosylation sites. To verify the existence of this reading frame, prohibitin RNA synthesized in vitro from Pro1 was translated in vitro (Fig. 3). Translation of the lysate with no added synthetic RNA produced a control protein band slightly larger than 46 kDa (lanes 9 and 10); presumably this reflects translation of mRNA endogenous to the lysate and is unrelated to prohibitin. Translation of a prohibitin sense transcript (lane 1) produced one additional protein band of approximately 30 kDa, as expected. To test the use of the putative N-linked glycosylation sites, canine microsomes were added during a set of reactions translating prohibitin sense RNA (lane 2). This did not result in a change in mobility of the 30-kDa band, as might have been expected from sugar addition or signal peptide cleavage. More rigorous evidence that prohibitin is not translocated into the microsomes was obtained by digestion of the prohibitin reaction products with proteinase K following translation in the absence (lane 3) and presence (lane 4) of the microsomal preparation. Protease digestion completely degraded the prohibitin protein in both cases. A known secreted protein, mating factor α, was analyzed in equivalent reaction mixtures to show that the microsomal preparations used in these experiments were capable of posttranslational modification and protein internalization. Results with microsomes (lane 6) show that the protein made without microsomes (lane 5) can be modified. The modified protein (lane 6) is protected from protease digestion, whereas the protein not exposed to microsomes (lane 5) is not (lane 8). These data indicated that prohibitin does not behave as a secreted protein in this system; they therefore support the concept that prohibitin is an intracellular protein.

Western blot analysis of prohibitin secretion. To further test the idea that prohibitin is an intracellular protein, prohibitin levels were assayed by antibody (Fig. 4) in total protein from cultured IMR-90 cells (lanes C) or their supernatants (lanes S). Equal fractions of total cell lysate and total cell supernatant were compared. Although the amount of material that could be analyzed in this way was limited by the large amount of bovine serum protein present in culture supernatant, a clear band of the correct size for prohibitin (arrow) can be seen in the cell lysate material stained with the antiprohibitin antibody (C, lane I); there is no such band in the cell supernatant lane (S, lane I) or in the lanes visualized with preimmune serum (lane P). Protein bands are seen in the supernatant lanes, but they are not specifically visualized with antiprohibitin antibody (S, lane I versus lane P). These data are inconsistent with secretion of prohibitin in amounts approaching or greater than the amount retained in cells; they therefore further support the concept that prohibitin is an intracellular protein.

Comparison of prohibitins from in vitro and in vivo sources. The primer extension studies presented above show that the Pro1 cDNA is not complete. Therefore it is important to show that the prohibitin protein made in vitro corresponds in

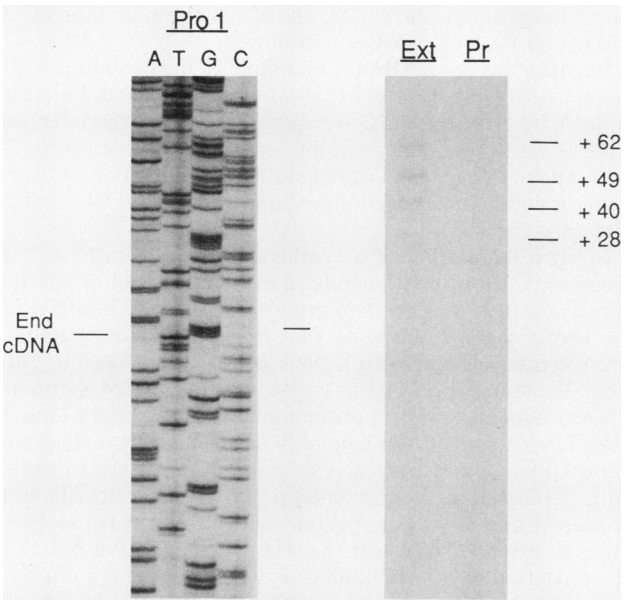

FIG. 2. Primer extension analysis of transcript initiation. The primer extension products shown were produced by priming rat intestine poly(A) RNA with a 29-base oligonucleotide complementary to the Pro1 cDNA (lane Ext). The number of bases that these products extend beyond the 5′ end of the Pro1 cDNA is indicated at the right. On the left (lanes Pro1) are sequencing reactions using Pro1 cDNA as the template and the same 29-mer as the primer. The latter was included as a size standard, and the 5′ end of the Pro1 cDNA is noted; beyond this point is vector sequence. An additional control was the labeled primer alone (lane Pr) to show that no material of large molecular weight was artifactually labeled.

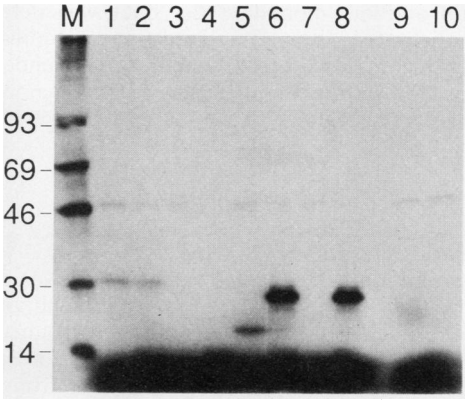

FIG. 3. In vitro translation of synthetic Pro1 mRNA. Shown is an autoradiogram of the products of several in vitro translation reactions. Lane M, ^{14}C molecular weight markers (Amersham); the sizes are indicated at the left in kilodaltons. Lanes 1 through 4 contain the products of translation of the sense transcript of the Pro1 template. In lanes 1 and 3, microsomes were not added; in lanes 2 and 4, microsomes were added. The products in lanes 3 and 4 were digested after translation with proteinase K. Lanes 5 through 8 show the products of translation of yeast mating factor α mRNA. Lanes 5 and 7 are translations in the absence of microsomes; lanes 6 and 8 were run in the presence of microsomes. Lanes 7 and 8 are the results of posttranslation digestion with proteinase K. Lanes 9 and 10 are the products observed without the addition of exogenous RNA in the absence (lane 9) and presence (lane 10) of microsomes.

size to that made in vivo. This is shown by Western analysis (Fig. 5A). Lanes marked N indicate the protein products made in the rabbit reticulocyte lysate system when no exogenous RNA was added, i.e., when the only proteins and mRNAs were those endogenous to the rabbit lysate. Two prohibitin bands were revealed among these products by the antiprohibitin antiserum; we speculate that these bands are due to rabbit prohibitin proteins present in the lysate. They are unlikely to be the freshly translated products of rabbit prohibitin mRNAs, since they are not radioactively labeled during the translation process (Fig. 3). When rat prohibitin mRNA synthesized from the Pro1 cDNA was added to this system (lanes R), a new band appeared between the putative rabbit bands, which is therefore the rat protein. This protein is indistinguishable in size from the protein produced in vivo by IMR-90 fibroblasts (lanes F).

Figure 5B shows that these fibroblasts have mRNAs for prohibitin, as expected from the protein results seen in panel A.

However, it is still theoretically possible that the human prohibitin protein from the IMR-90 cells is a different size than rat prohibitin and only coincidentally matches the size of the rat prohibitin protein produced in vitro. To rule out this idea, we compared the sizes of two human prohibitins, from IMR-90 fibroblasts (Fig. 5C, lane F) and HeLa cells (lane H), to those of two rat prohibitins, from Rat-1-transformed fibroblasts (lane 1) and rat liver (lane L). Within the limits of the gel resolution, these prohibitins were not different in size.

Comparison with the *Drosophila Cc* gene. The nucleotide sequence of Pro1 is approximately 67% identical throughout the predicted protein-coding region with the *Cc* cDNA (8) of *D. melanogaster* (Fig. 6), discounting a central 57-base region that is found only in rat cDNA. The homology is much lower outside the protein-coding region. *Cc* is a gene of unknown function that was discovered during a chromosome walk in the region of the dopa decarboxylase gene. Flies homozygous for nonfunctional alleles of *Cc* die during the larva-to-pupa metamorphosis (8).

Three potential ORFs were found in sequencing the *Cc* cDNA (8). The first one shares the same protein start as the rat ORF, is 27 amino acids long, and shows 22% identity with the rat ORF over this region (Fig. 6). The second *Cc* ORF is 96 amino acids long and out of frame with the rat ORF. The third *Cc* ORF is 203 amino acids long, begins at nucleotide 201 of the *Cc* sequence shown in Fig. 6, and shares 55% homology with the rat ORF.

Microinjection studies. To demonstrate the antiproliferative activity of prohibitin mRNA, Pro1 was transcribed in vitro and the synthetic mRNA was microinjected into normal human fibroblasts (Fig. 7). The number of nuclei labeled with tritiated thymidine was 69% less than in an uninjected population. Dose-response experiments (Fig. 7, bars 1, 5, 10, and 50) demonstrated a half-maximal antiproliferative effect upon injection of approximately 240 RNA molecules per cell (calculated from amount injected, concentration injected, and molecular weight). A synthetic rat prolactin mRNA (46) caused an 11% increase (bar PR), indicating that inhibition is not produced by every mRNA. Prohibitin mRNA also caused a 34% decrease in labeled HeLa cell nuclei (bar HL), indicating that prohibitin can inhibit replication in these cancer cells.

Control experiments with RNAse-digested sense transcript (bar RT) showed that, as expected, the antiproliferative effect was dependent on intact prohibitin mRNA. Microinjection of RNase alone (bar R) had no effect on

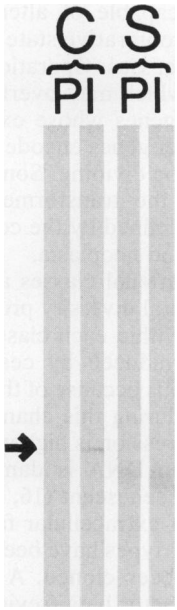

FIG. 4. Prohibitin in cultured cells versus supernatant. A Western blot analysis with antibody against synthetic prohibitin peptides was performed on lysates of IMR-90 normal human fibroblasts (lanes C) and supernatant (lanes S) from the same culture. Lanes I, Antiprohibitin antibody used; lanes P, preimmune serum from the same animals before peptide injection used. The arrow indicates the size of the prohibitin protein expected on the basis of the Pro1 ORF (~30 kDa).

proliferation, making it clear that the RNase acted by degrading prohibitin mRNA rather than by providing a canceling proliferative stimulus.

To demonstrate a physiologic antiproliferative role for prohibitin, we microinjected an 18-base oligonucleotide to bind endogenous prohibitin mRNA and block its activity. This antisense oligonucleotide (bar AO) caused a 22% increase in the number of nuclei incorporating thymidine (Fig. 7). Control injection of the corresponding sense oligonucleotide (bar SO) produced a 3% decrease in labeled nuclei. These data suggest that prohibitin and its mRNA are normally present in fibroblasts and play a role in regulating proliferation.

In principle, the antiproliferative effect produced by microinjecting prohibitin mRNA might be due to an effect of this mRNA as RNA, rather than via the activity of its protein. However, the microinjection data shown here and previously published (28) show that not just any microinjected RNA is antiproliferative. Furthermore, to our knowledge there is no example of an mRNA that acts other than through its protein, with the exception of self-splicing RNAs.

Microinjected cells showed no evidence of toxicity. No abnormal morphologic changes such as rounding or detachment were observed. Viability of microinjected cells as assessed by cell numbers 24 h after injection was over 95%. These viable microinjected cells could all reenter S phase (not shown).

Expression of prohibitin mRNA in vivo. One might expect that a gene playing a central role in the regulation of cell proliferation would be expressed in most adult-cell types. Indeed, this result was obtained when the M5 partial prohibitin cDNA was used to analyze a variety of adult rat tissues by Northern blot (28). In those experiments, however, only a single mRNA of 1.9 kb was observed, whereas in the experiments reported here, both a 1.9- and a 1.2-kb mRNA are seen in human fibroblasts (Fig. 5B). The Pro1 cDNA has two AATAAA polyadenylation cleavage signals at appropriate locations to produce both a 1.2- and a 1.9-kb mRNA, allowing for a poly(A) tail of about 200 bases (Fig. 1); the reason for this discrepancy is therefore that the M5 probe is part of a region expressed only in the 1.9-kb mRNA (Fig. 1).

It seems likely that both the 1.9- and the 1.2-kb mRNAs encode the same protein, since we have recently cloned and sequenced a cDNA corresponding to the 1.2-kb clone as well (not shown). To show that both mRNAs are expressed in different cell types, mRNA was extracted from a number of rat tissues and analyzed by Northern hybridization (Fig. 8).

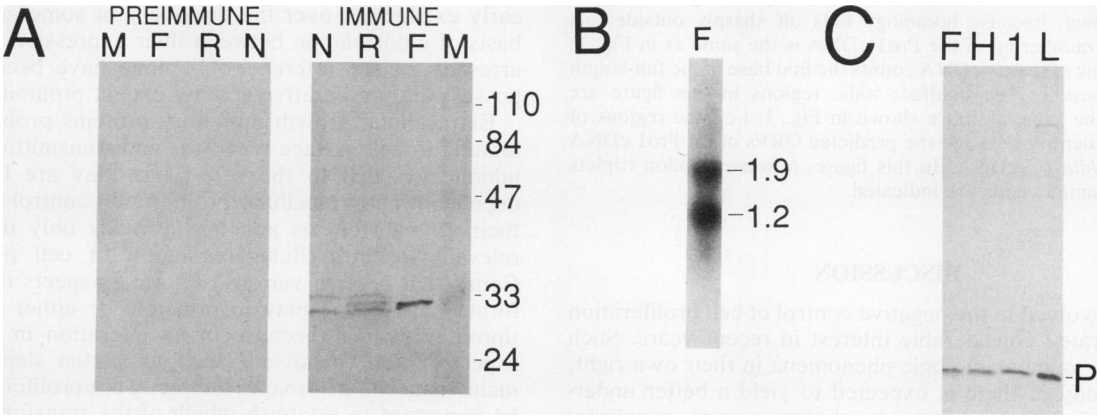

FIG. 5. In vitro versus in vivo prohibitins. (A) Western blot analysis of synthetic and natural prohibitins. Preimmune and immune sera were used to visualize bands. Lanes: N, Products of an in vitro translation reaction carried out with no mRNA added (i.e., any proteins seen were made in vivo before translation or were produced in vitro from mRNAs present in the rabbit reticulocyte lysate); R, products of in vitro translation when rat prohibitin mRNA transcribed in vitro from the Pro1 cDNA was added; F, proteins made in vivo in IMR-90 fibroblasts; M, molecular weight standards, with sizes at the right in kilodaltons. (B) Northern analysis of prohibitin mRNAs found in IMR-90 human fibroblasts (F). (C) Cell lysates from different in vivo sources are compared by Western analysis as for panel A. Lanes: F, IMR-90 human fibroblasts; H, HeLa cells; 1, Rat-1 cells; L, rat liver tissue. The prohibitin band is marked with a P.

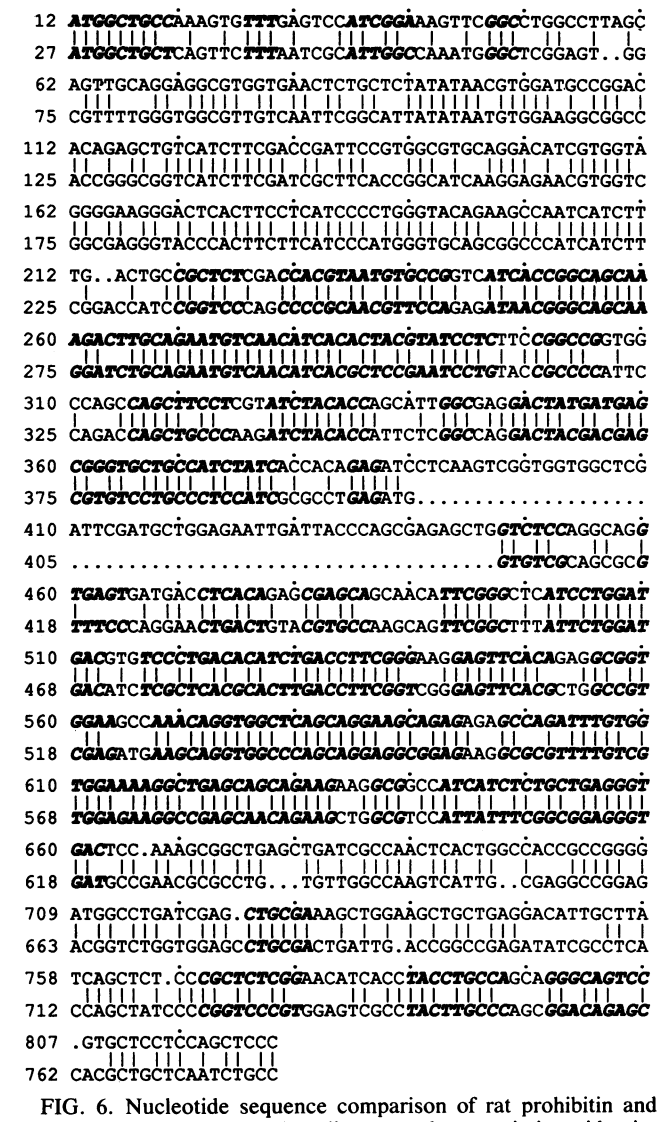

FIG. 6. Nucleotide sequence comparison of rat prohibitin and Drosophila *Cc* cDNAs. The alignment that maximizes identity between Pro1 cDNA and *Drosophila Cc* cDNA is shown. The rat sequence is on the top of each strand pair. Only the region of the rat ORF is shown, because homology falls off sharply outside this region. The numbering of the Pro1 cDNA is the same as in Fig. 1; the numbering of the *Cc* cDNA counts the first base of the full-length cDNA as base 1. The boldface italic regions in this figure are essentially the same as those shown in Fig. 1, i.e., the regions of amino acid identity between the predicted ORFs of rat Pro1 cDNA and *Drosophila Cc* cDNA. In this figure, however, codon triplets rather than amino acids are indicated.

DISCUSSION

Genes involved in the negative control of cell proliferation have generated considerable interest in recent years. Such genes have value as biologic phenomena in their own right, and the study of them is expected to yield a better understanding of differentiation, development, and neoplasia. They promise to be powerful tools for manipulating cell growth both positively and negatively in studies examining the control of proliferation. Ultimately, they may be useful in designing strategies for the treatment of cancer.

Many different types of genes are expected to have some connection to decreased proliferation, because numerous external signals are capable of altering proliferation and because a change in proliferative state requires alterations in many intracellular processes. Operationally, these genes fall into several classes, which may overlap. For example, one group includes those genes whose expression is greater in nondividing cells. Other genes encode secreted proteins that signal other cells to stop dividing. Some can reverse various abnormal aspects of the transformed phenotype. Tumor suppressor genes are defined by the connection between the loss of their activity and neoplasia.

Each of these operational classes appears to have many members. This structural diversity probably reflects a broad diversity of function within each class. For example, genes whose expression is induced by cessation of growth are expected to be numerous because of the many processes that are probably altered during this change of state; groups of such genes whose expression is high when serum concentration is low (43), when DNA is damaged (11), and when cultured cells become senescent (16, 49) have been cloned. Similarly, a variety of extracellular factors that can inhibit growth of different cell types have been described, and some of these factors have been cloned. A few examples include transforming growth factor beta (reviewed in references 30, 36, and 45), interferon gamma (17), Muellerian inhibiting substance (4), leukemia inhibitory factor (14), and oncostatin M (51). A number of laboratories are currently cloning genes that reverse the abnormal transformed phenotype of *ras*-transformed cells; thus far, two have been reported (20, 42). The existence of more than 10 tumor suppressor genes has been suggested on the basis of associations between the mutation of both alleles at a specific chromosomal locus and neoplasia. Several have already been cloned, and it is clear from the diversity of their structures that they cause neoplasia by acting at very different sites. For example, the retinoblastoma gene product is a nuclear protein (23), while the *dcc* gene product resembles known proteins that span the plasma membrane (9).

For an understanding of negative growth control, some of these genes will be much more informative than others. Among genes more highly expressed in nondividing cells, the important genes will be those that perform a regulatory function, but controlling antiproliferative genes are likely to represent only a small subset of all growth arrest-associated genes. Experience has fulfilled this prediction: in spite of early excitement over the discovery of some genes on the basis of a correlation between their expression and growth arrest (e.g., see reference 43), none have been shown to possess antiproliferative activity except prohibitin.

Extracellular growth inhibitory proteins probably act by binding to cell surface receptors and transmitting a growth inhibitory signal to the cell. Thus they are likely to be dependent on intracellular proliferation control proteins for their ultimate effects and may provide only indirect data relevant to intracellular regulation of cell proliferation. Genes that reverse various abnormal aspects of the transformed phenotype may in principle be either directly antiproliferative or, because of an alteration in some other aspect of cell behavior, such as serum dependence or maintenance of cell shape, indirectly antiproliferative. It will be important to establish which of the transformation suppressor genes that have been and are likely to be cloned will have an antiproliferative effect separate from their effects on such behavior. In contrast, prohibitin has now been shown to exert its antiproliferative effect on normal cells, at standard serum levels, and without a concomitant change in cell shape.

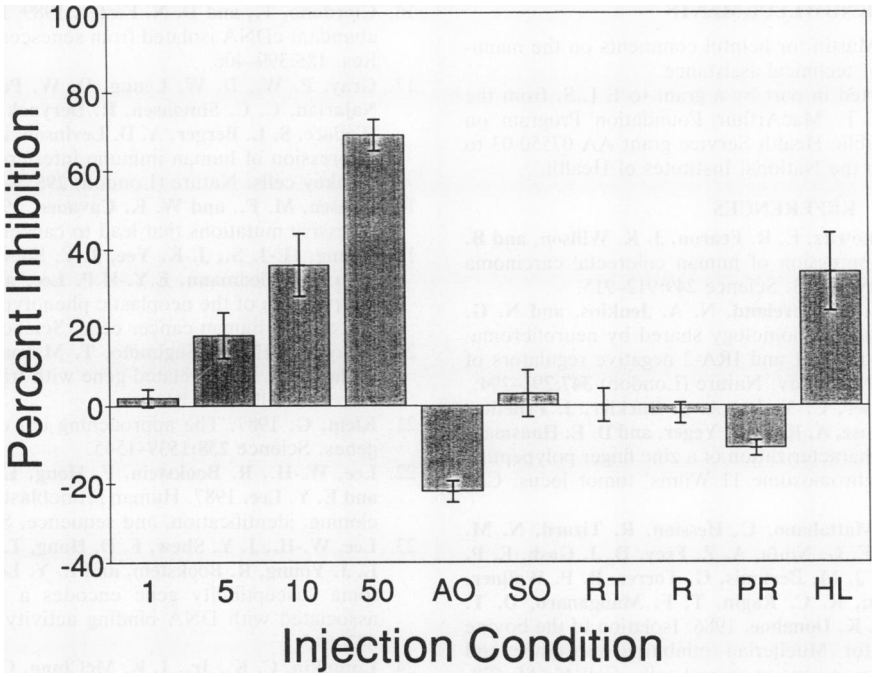

FIG. 7. Microinjection assays results, showing the effects of microinjecting a variety of synthetic transcripts and oligonucleotides into normal human fibroblasts and HeLa cells. Percent inhibition was calculated as $[(U - I)/U] \times 100$, where U is the percentage of labeled nuclei in uninjected cells and I is the percentage of labeled nuclei in injected cells. A negative value therefore indicates stimulation of proliferation. The error bars show the standard errors of the means for three or more experiments. Bars: 1, 5, 10, and 50, sense transcript of Pro1 injected at 1, 5, 10, and 50 μg/ml, respectively (all other transcripts were injected at 50 μg/ml); AO, antisense oligonucleotide (both oligonucleotides were injected at 1 mg/ml); SO, sense oligonucleotide; RT, RNase-treated sense transcript; R, RNase alone; PR, prolactin sense transcript; HL, injection of Pro1 sense transcript into HeLa cells (all other bars refer to normal fibroblasts).

The retinoblastoma gene product and p53 have been directly shown to be antiproliferative by expressing them in cultured cells (1, 19, 26), and it is expected that other tumor suppressor genes will also be shown to directly block proliferation. However, the diversity of structure already apparent among cloned tumor suppressor genes suggests that some of these genes may ultimately be shown to block other aspects of the malignant phenotype, such as invasiveness.

A point which has not received much consideration is that tumor suppressor genes may constitute only a subset of the most important negative regulatory genes in the cell. This is because their definition, which absolutely requires the production of a tumor in the null state, has the effect of excluding negative regulatory genes whose complete loss of function kills the cell. It is certainly reasonable that cell death might be the effect of the absence of such a gene product; one obvious mechanism would be the generation of a hyperproliferative state incompatible with homeostasis. Prohibitin may in fact be such a gene. Loss of function in both alleles of the *Drosophila* homolog, *Cc*, results in death during the larva-to-pupa transition rather than tumor formation in the adult fly (8).

This report demonstrates that prohibitin is an intracellular protein with antiproliferative activity. It does not enter microsomes and is not found in culture supernatants, microinjection of its mRNA blocks DNA synthesis, and interference with endogenous prohibitin mRNA by means of an antisense oligonucleotide stimulates DNA synthesis. These findings indicate that prohibitin is a powerful negative regulator of cell growth. Prohibitin can even shut off the replication of HeLa cells, showing that in vitro it can override alterations that make these cells lethal in vivo. It is thus a potential tool for the analysis and perhaps ultimately the treatment of malignancy. Prohibitin is widely expressed in different tissues and highly conserved in evolution. These data suggest that the antiproliferative processes controlled by prohibitin and its evolutionary cognates are fundamental to an extremely wide range of cells.

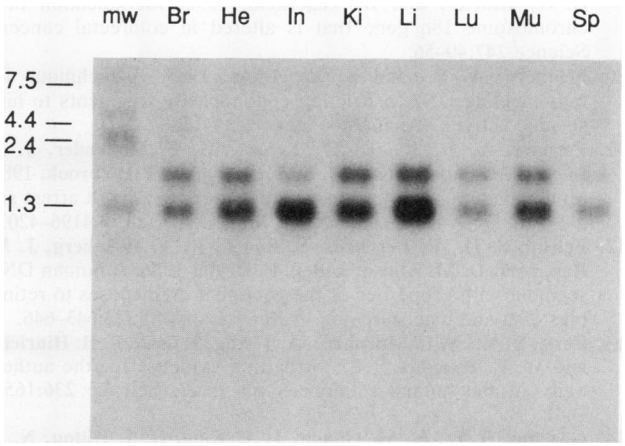

FIG. 8. Steady-state expression of prohibitin mRNAs in rat tissues. Northern hybridization of prohibitin gene expression is shown for eight rat tissues. Poly(A) RNA from each organ (1.5 μg) was sized by electrophoresis, transferred to a filter, and hybridized as described in Materials and Methods. Lanes: mw, RNA molecular weight standards (sizes given in kilobases at the left); Br, brain; He, heart; In, intestine; Ki, kidney; Li, liver; Lu, lung; Mu, skeletal muscle; Sp, spleen.

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