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Molecular Cloning Reveals that the p160 Myb-Binding Protein Is a Novel, Predominantly Nucleolar Protein Which May Play a Role in Transactivation by Myb

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We have previously detected two related murine nuclear proteins, p160 and p67, that can bind to the leucine zipper motif within the negative regulatory domain of the Myb transcription factor. We now describe the molecular cloning of cDNA corresponding to murine p160. The *P160* gene is located on mouse chromosome 11, and related sequences are found on chromosomes 1 and 12. The predicted p160 protein is novel, and in agreement with previous studies, we find that the corresponding 4.5-kb mRNA is ubiquitously expressed. We showed that p67 is an N-terminal fragment of p160 which is generated by proteolytic cleavage in certain cell types. The protein encoded by the cloned p160 cDNA and an engineered protein (p67*) comprising the amino-terminal region of p160 exhibit binding specificities for the Myb and Jun leucine zipper regions identical to those of endogenous p160 and p67, respectively. This implies that the Myb-binding site of p160 lies within the N-terminal 580 residues and that the Jun-binding site is C-terminal to this position. Moreover, we show that p67* but not p160 can inhibit transactivation by Myb. Unexpectedly, immunofluorescence studies show that p160 is localized predominantly in the nucleolus. The implications of these results for possible functions of p160 are discussed.

It is becoming increasingly clear that c-*myb* plays an essential role in controlling the proliferation and differentiation of hematopoietic cells. This was first suggested on the basis of its preferential expression in immature hematopoietic cells and the subsequent decrease in expression on differentiation (25, 70). Confirmation has been provided by more recent loss-of-function studies involving targeted disruption of c-*myb*, which results in the abrupt failure of development of the fetal hematopoietic system (50), and by the ability of antisense oligonucleotides to inhibit the proliferation of both normal and transformed hematopoietic cells (1, 21). Furthermore, enforced expression of activated (23, 28) and normal (18) forms of c-*myb* can transform hematopoietic cells in vitro (but not, in general, other cell types) and inhibit the induced differentiation of certain leukemic cell lines (8, 11, 72). Taken together, these data suggest that one major function of c-*myb* is to maintain the proliferative state and immature characteristics of early hematopoietic cells.

The proteins encoded by normal and oncogenically activated *myb* genes (Myb) are transcription factors; i.e., they bind to specific DNA sequences (7) and can enhance transcription of genes and reporter constructs carrying Myb binding sites (53, 54, 71). These functions are also essential for the ability of *myb* oncogenes to transform hematopoietic cells (34, 43). Oncogenically activated forms of Myb differ from normal c-Myb in that they are truncated at either their amino termini, their carboxyl termini, or both (22, 64). Carboxyl truncation activates c-Myb by disrupting or deleting a region—termed the negative regulatory domain (NRD)—which appears to downmodulate transactivation, DNA binding, and transformation (15, 34, 58, 62).

One significant clue to how the NRD exerts its effects on Myb function comes from the observation that the NRD contains a leucine zipper-like motif (7) and that disruption of this motif by point mutations enhances transactivation and transformation (38). Because leucine zippers generally mediate protein-protein interactions, it seems likely that the Myb leucine zipper promotes association between c-Myb and another protein which inhibits Myb function. There is ample precedent for the existence of protein inhibitors of transcription factors, including Id (6), and IκB (5), which antagonize the function of MyoD and NF-κB, respectively. Alternatively, the Myb inhibitory protein could be c-Myb itself, since the leucine zipper is capable of mediating homodimerization and since Myb homodimers are ineffective in DNA binding or transactivation (55). In this scenario, dimerization may be modulated by competition with another protein capable of forming heterodimers; this latter protein would then function as an activator of Myb. In either case, understanding the regulation of Myb activity would clearly be aided by the identification and characterization of proteins which interact with the c-Myb leucine zipper.

We have previously described two murine proteins, termed p67 and p160 (17), that can bind to the c-Myb leucine zipper. These were identified by using a bacterially expressed fusion protein containing the Myb leucine zipper region as an affinity

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reagent to capture proteins from radiolabelled nuclear extracts. Their specificity was demonstrated by the observation that they do not bind to similar fusion proteins in which two of the critical leucine residues were replaced with proline or alanine residues. Although peptide mapping revealed that p67 and p160 are closely related, there are (at least) two important differences. First, p160, but not p67, can also bind to the c-Jun basic leucine zipper (bZip) region, suggesting that it may be involved in the regulation of other transcription factors in addition to c-Myb. Second, p160 is expressed in all the murine cell lines we have studied to date, whereas p67 was found only in a subset of early myeloid lines (17).

In this paper, we report the molecular cloning of cDNA sequences corresponding to murine p160. The predicted amino acid sequence indicates that p160 appears to be a novel protein. We have used the p160 cDNA to examine the relationship between p160 and p67; we find that p67 represents the amino (N)-terminal region of p160 and is generated by proteolytic cleavage. We further demonstrate that the cloned p160 can specifically associate with Myb and that a truncated form of p160, which retains its only N-terminal region, can inhibit transactivation by Myb. Immunofluorescence studies of the p160 protein show, surprisingly, that most of the protein is present in the nucleolus. This finding draws attention to some interesting parallels with other nucleolar proteins and, together with our other findings, suggests possible functions for p160 and p67.

MATERIALS AND METHODS

Purification of p67. Nuclear extracts were prepared from approximately 10^{10} FDC-P1 cells by a scaled-up version of the protocol described in reference 17 (see also reference 24). FDC-P1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 50 U of mouse granulocyte-macrophage colony-stimulating factor per ml (kindly supplied by Tracy Wilson, Walter and Eliza Hall Institute, Melbourne, Australia) to a density of 1×10^6 to 2×10^6 cells/ml. The nuclear extract was divided into two 50-ml aliquots and precleared successively with 2 ml of glutathione-Sepharose (Pharmacia), 1.25 ml of glutathione *S*-transferase (GST)-Sepharose (~8 mg of protein), and 1.25 ml of GST-L34P Myb-Sepharose (17) (~2 mg of protein), each with rotation for 1 h at 4°C. The precleared supernatant was incubated with 1.5 ml of GST-NRD2-Sepharose (17) (~2 mg of protein) as above to isolate p67. The Sepharose matrix and bound proteins were washed five times with 10 ml of cold NETN buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 [NP-40]) and twice with 10 ml of cold phosphate-buffered saline (PBS)–0.5% Triton X-100. Bound proteins were eluted with two washes of 1.5 ml of DOC buffer (100 mM NaCl, 50 mM Tris-HCl [pH 8.0], 1% deoxycholic acid, 1% NP-40, 5 mM dithiothreitol [DTT]) and concentrated (~100 μl) on a Centricon-30 column (Amicon). The concentrated protein was separated by electrophoresis in a sodium dodecyl sulfate SDS-8% polyacrylamide gel, and stained with CBR-250 to visualize proteins. The p67 protein band was excised from the gel and subjected to in-gel proteolysis.

In-gel protein digestion and amino acid sequence analysis. In-gel proteolysis of acrylamide gel-resolved proteins was performed essentially as described previously (49, 61). Tryptic peptides extracted from acrylamide gel pieces with trifluoroacetic acid-acetonitrile were fractionated by rapid reverse-phase high-pressure liquid chromatography on a Brownlee RP-300 cartridge (2.1 mm by 100 mm; Applied Biosystems, Foster City, Calif.) with a Hewlett-Packard 1090A liquid chromatograph fitted with a model 1040A diode-array detector as described previously (49). N-terminal amino acid sequence analysis of peptides was performed by automated Edman degradation with a Hewlett-Packard (model G1005A) protein sequencer operating with the routine 1.3 sequencer program. A Hewlett-Packard model HP1090M liquid chromatograph was used for phenylthiohydantoin amino acid analysis (49). The following five peptide sequences were obtained: PDQETRLAT(E) (peptide 1), VILRSPK (peptide 2), VLEEG (STGR) (peptide 3), VDHLHLEK (peptide 4), and ATPQIPETK (peptide 5); the residues shown in parentheses were ambiguous and in fact are not found in the predicted p67/p160 sequence (see Results and Fig. 1).

Degenerate oligonucleotides and library screening. Degenerate oligonucleotides were derived from four of the p67 partial tryptic peptide sequences (see Fig. 1B) and used in various combinations to amplify FDC-P1 cDNA by PCR. First-strand cDNA was synthesized from FDC-P1 poly(A)$^+$ RNA (4 μg) with an oligo(dT_{18}) primer and SuperScript reverse transcriptase (Gibco BRL) as described previously (60). Degenerate oligonucleotides corresponding to peptide 4 [5′-ATAGGATCCGA(C/T)CA(C/T)(C/T)T(C/T/G)CA(C/T)(C/T)T(C/T/G)GA(A/G)AA-3′], incorporating a *Bam*HI, site and peptide 5 [5′-AGGAATTCC TT(T/G/A)GT(T/C)TC(T/G/A)GG(G/A/T)AT(T/C)TG(T/G/A)GG-3′], incorporating an *Eco*RI site, yielded an amplification product of 114 bp under the following PCR conditions: 94°C for 1 min, 56°C for 2 min, and 72°C for 2 min for 35 cycles. The 114-bp product was digested with *Bam*HI-*Eco*RI, cloned into pGEM-4Z (Promega), and sequenced. DNA from a WEHI-3B cDNA Lambda Zap library (Stratagene) was used to obtain a 459-bp PCR product by using the T3 RNA polymerase promoter sequence and a primer derived from the initial 114-bp cDNA sequence: 5′-ATAGGATCCTCGTCTTAAAGAAGGCATG G-3′ (94°C for 30 s, 58°C for 2 min, and 72°C for 3 min for 35 cycles). The 459-bp product was digested with *Bam*HI, cloned into pBluescript SK (Stratagene), and verified by DNA sequencing. The same WEHI-3B cDNA Lambda Zap library (Stratagene) was screened by plaque hybridization with the 459-bp cDNA sequence as specified by the manufacturer. A positive plaque was identified, and a 3.1-kbp cDNA clone was obtained by excision of pBluescript SK from the UNI-ZAP XR vector as specified by the manufacturer. This cDNA clone was sequenced by first generating a unidirectional deletion series with the Erase-a-Base system (Promega) as specified by the manufacturer and then sequencing several of the deleted clones.

5′ RACE PCR. 5′ rapid amplification of DNA ends (RACE) PCR (19) was performed by the method used with the 5′-AmpliFINDER kit (Clontech). First-strand cDNA was synthesized from FDC-P1 poly(A)$^+$ RNA (2 μg) with a p160 cDNA-derived oligonucleotide (5′-TTGTAAAGGCCACCATCATAGTCAAC TGCC-3′) and SuperScript reverse transcriptase (Gibco BRL) as described previously (60). 5′ RACE PCR was undertaken with a p160 gene-specific primer, incorporating a *Bam*HI site (5′-CGGATCCGGAAGGTACCCACGTATG-3′) and the AmpliFINDER anchor primer (Clontech) under the following conditions: 94°C for 45 s, 60°C for 1 min, and 72°C for 2 min for 30 cycles. The resulting PCR product was digested with *Eco*RI-*Bam*HI, cloned into pBluescript SK (Stratagene), and verified by DNA sequencing.

Construction of plasmids. A full-length p160 cDNA clone was constructed in pGEM-3Z (Promega) by using the unique *Kpn*I site present in both the 5′ RACE PCR product and the 3.1-kbp partial p160 cDNA clone (nucleotides 992 to 4126). Partial p160 cDNA in pBluescript (Stratagene) was digested with *Eco*RI-*Xho*I and subcloned into the *Eco*RI-*Sal*I sites of pGEM-3Z. This construct was subsequently digested with *Eco*RI-*Kpn*I and ligated with the 5′ RACE PCR *Eco*RI-*Kpn*I fragment to yield a full-length p160 cDNA clone (pGEM3Z-160). The p67* cDNA construct was derived from p160 cDNA by introducing a termination codon after nucleotide position 1755 by PCR to yield a protein with a predicted molecular mass of approximately 67 kDa. The following PCR primers were used to generate the p67* cDNA clone: 5′-TATCGAATTCGTGACG TGTTTGGCTCAGC-3′ and 5′-ACGTGGATCCTCATTCCTTCAGAGTACT C-3′, incorporating a *Bam*HI site (94°C for 45 s, 60°C for 1 min, and 72°C for 90 s for 30 cycles). The resulting PCR product was digested with *Eco*RI-*Bam*HI and cloned into pGEM-3Z to yield pGEM3Z-67*. Two GST fusion constructs containing 5′ or 3′ p160 sequences were made as follows. The 5′ p160 fragment (nucleotides 1 to 1083) was generated by digestion of pGEM3Z-160 with *Eco*RI-*Kpn*I, blunt ended, and ligated into the *Sma*I site of pGEX-4T-1 (Pharmacia). The 3′ p160 fragment (nucleotides 3151 to 4101) was generated by digestion of partial p160 cDNA in pBluescript (Stratagene) with *Bam*HI-*Ssp*I, blunt ended, and ligated into the *Sma*I site of pGEX-3X (Pharmacia). The in-frame GST-p160 sequences were verified by DNA sequencing.

Site-directed mutagenesis and construction of expression vectors. The FLAG epitope (DYKDDDDK) was introduced into the amino termini of p160 and p67* by in vitro site-directed mutagenesis with the pALTER-1 system (Promega) as specified by the manufacturer. The mutagenic oligonucleotides for both p160 and p67* cDNAs incorporated the FLAG octapeptide sequence downstream of a Kozak consensus initiation site, with the original translation initiation site replaced. The presence of the mutagenized sequences was verified by DNA sequencing.

The FLAG epitope-tagged p160 and p67* expression plasmids were constructed from the pact-c-*myb* expression vector (described in reference 54) by first excising the c-*myb* cDNA by digestion with *Nco*I-*Xba*I. This was replaced by an *Nco*I-*Xba*I fragment containing FLAG p67* cDNA to yield pact-67*FLAG. pact-160FLAG was constructed by generating a fragment containing FLAG p160 cDNA by PCR from the pALTER-1-p160FLAG plasmid with the SP6 RNA polymerase promoter sequence and 5′-ACGTACTAGTTCAAGGTGTCTGCA CTCTC-3′ (94°C for 45 s, 50°C for 1 min, and 72°C for 4 min for 30 cycles), digestion with *Nco*I-*Spe*I, and replacement of the c-*myb* gene between the *Nco*I/*Xba*I sites of pact-c-*myb*.

The retroviral construct containing p160FLAG cDNA was generated by digestion of the pALTER-1-p160FLAG plasmid with *Eco*RI-*Sal*I, blunt-ended, and ligated into the *Hpa*I site of pRUF*neo* (60).

In vitro transcription-translation. Proteins were synthesized in vitro by using the TNT coupled transcription-translation rabbit reticulocyte lysate kit (Promega). Briefly, 1 μg each of pGEM3Z-160 and pGEM3Z-67* was incubated at 30°C for 90 min in a 50-μl reaction volume containing the recommended quantities of kit reagents, including T7 RNA polymerase and 4 μl of translation grade [^{35}S]methionine (10.2 mCi/ml; NEN Dupont). Labelled proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and detected by autoradiography with Hyperfilm-MP (Amersham).

Proteolysis by cell extracts. Extracts were made by suspending cells at 10^8/ml in 25 mM HEPES (pH 7.4)–1 mM EDTA–0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)–10% sucrose–5 mM DTT. The cells were lysed with three cycles of freezing in liquid nitrogen and thawing at 37°C and then centrifuged at 1,000 rpm (Heraeus Biofuge 13) for 5 min at 4°C. The supernatant was clarified by centrifugation at 13,000 rpm (Heraeus Biofuge 13) for 15 min and stored at −20°C. Proteolysis reactions were carried out with 10 μg of extract, 10 μl of in vitro-translated [^{35}S]methionine-labelled p160, plus buffer in a total volume of 30 μl, and the reaction mixtures were incubated at 37°C for various periods before the reactions were terminated by adding 2× SDS loading buffer; the products were analyzed by SDS-PAGE.

Southern blotting analysis. Genomic DNA was isolated from FDC-P1 cells as described previously (2), except that the DNA was spooled onto a Pasteur pipette rather than pelleted. DNA was digested with restriction enzymes, fractionated by electrophoresis in a 1% agarose gel, and transferred to Hybond-N nylon membrane (Amersham) as specified by the manufacturer. Southern blots were probed with 5′ (nucleotides 1 to 1083) and 3′ (nucleotides 3151 to 4101) p160 cDNA sequences and labelled with [α-^{32}P]dATP by using a random primer labelling kit (Amersham). Hybridizations were performed as described previously (63) at the following stringency: 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 42°C. The blots were washed with 2× SSC–0.1% SDS at 60°C.

Chromosomal localization. Interspecific mouse backcross mapping was used to determine the chromosomal locations of p160 sequences. Backcross progeny were generated by mating (C57BL/6J × *Mus spretus*)F_1 females and C57BL/6J males as described previously (12). A total of 205 N_2 mice were used to map the *p160* loci (see Fig. 2B). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described previously (37). All the blots were prepared with Zetabind nylon membrane (AMF Cuno). The 3′ p160 cDNA probe used is the same as that described in the previous section and was labelled with [α-^{32}P]dCTP by using a nick translation labelling kit (Boehringer Mannheim). Washing was done to a final stringency of 0.8× SSC, 0.1% SDS at 65°C. Fragments of 19.0 and 5.0 kb were detected in *Hin*dIII-digested C57BL/6J DNA, and fragments of 15.0, 12.0, 7.3, and 5.0 kb were detected in *Hin*dIII-digested *M. spretus* DNA. The presence or absence of the 15.0-, 12.0-, and 7.3-kb *Hin*dIII *M. spretus*-specific fragments, which segregated independently, was monitored in the backcross mice.

A description of the probes and restriction fragment length polymorphisms (RFLPs) for the loci used to position the *p160* loci in the interspecific backcross has been reported. These include *Ren1* and *Fasl*, chromosome 1 (66); *Myhsf1*, *Glut4*, and *Nf1*, chromosome 11 (9, 32); and *Hfhbf1* and *Sos2*, chromosome 12 (3). Recombination distances were calculated as described previously (29) with the computer program SPRETUS MADNESS. The gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RNA isolation and Northern blot analysis. Total RNA was isolated from adult mouse tissues or e14 fetuses as described in reference 10. Poly(A)$^+$ RNA was isolated directly from cultured cells as described previously (26). Total RNA (30 μg) or poly(A)$^+$ RNA (2 μg) was fractionated by formaldehyde-agarose gel electrophoresis (2). RNA was then transferred to a Hybond-N nylon membrane (Amersham) as specified by the manufacturer. Northern blots were probed with a 1.1-kb fragment representing the 5′ end of the p160 cDNA (nucleotides 1 to 1083). For probe labelling, hybridization, and washing conditions, see the section on Southern blotting analysis, above. The blots were stripped and reprobed with a murine glyceraldehyde-3-phosphate dehydrogenase cDNA fragment.

Cell transfection and infection. The 293T cell line, a derivative of the human embryonic kidney 293 cell line (16), was used to express p160FLAG, p67*FLAG, and/or Myb. 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Before transfection, the cells were seeded at 1.4×10^6 in 60-mm culture dishes and left to adhere overnight, after which the medium was changed. Four hours later, calcium phosphate precipitates prepared as described previously (48), containing 7 μg of pact-p160FLAG or pact-p67*FLAG DNA, were added for 4 h, after which the cells were trypsinized and replated onto 100-mm culture dishes. After incubation for 40 to 48 h, total-cell extracts were prepared. Cotransfections of pact-p160FLAG and pact-WTMyb or L3,4P Myb (38) were performed similarly, except that 3 μg of each DNA was used, with the total amount of DNA transfected made up to 10 μg with pact vector.

NIH 3T3 cells were infected essentially as described by Pear et al. (57). Briefly, BOSC 23 retroviral packaging cells were transiently transfected with 10 μg of pRUF*neo*-160FLAG by the calcium phosphate procedure. After 24 h, supernatants were collected and used to infect NIH 3T3 cells seeded the previous day at 3×10^5 cells/60-mm dish. After an additional 24 h, cells were selected with G418 at 400 μg/ml.

Preparation of cell extracts. Total-cell extracts for GST fusion protein-binding assays were prepared by lysing the cells in 450 μl of NP-40 lysis buffer (10 mM Tris-HCl [pH 8.0], 25 mM KCl, 5 mM $MgCl_2$, 0.5% NP-40, 2 mM phenylmethylsulfonyl fluoride, 40 μl of protease inhibitor cocktail [Boehringer Mannheim] per ml, 5 mM DTT) plus 500 mM NaCl. Extracts were incubated on ice for 10 min and centrifuged at 45,000 rpm (TLA-45 rotor; Beckman TL-100 ultracentrifuge) for 20 min, and the supernatants were removed, aliquoted, and stored at −70°C. Nuclear and cytoplasmic extracts for immunoblotting were prepared from FDC-P1 and NIH 3T3 cells as described previously (17). Total-cell extracts for immunoprecipitations were prepared essentially as described by Harlow et al. (30), with the addition of 10% glycerol.

Binding reactions and immunoprecipitation. 293T total-cell extracts containing overexpressed p160FLAG and p67*FLAG were used in binding reactions with GST fusion proteins. These reactions were performed essentially as described previously (17). Similarly, for coimmunoprecipitation experiments, 293T cells were transfected with pact vectors encoding p160FLAG, WT Myb, or L3,4P Myb as appropriate. Immunoprecipitation from total-cell extracts was performed essentially as described by Harlow et al. (30), except that the washing buffer contained 143 mM NaCl. The proteins were analyzed by immunoblotting with the M2 FLAG monoclonal antibody (IBI) or with anti-Myb monoclonal antibody 1.1 (59). Control immunoprecipitations were carried out with irrelevant monoclonal antibodies.

Antibodies and immunoblotting. After SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) and blocked in TBS-T (50 mM Tris-HCl [pH 7.4], 135 mM NaCl, 0.1% Tween 20) containing 5% skim milk. Epitope-tagged proteins were detected by using the M2 FLAG monoclonal antibody (1:500; IBI), peroxidase-linked anti-mouse immunoglobulin (Ig) (1:1,000; Amersham), and enhanced chemiluminescence (Pierce).

The GST-p160 5′ and 3′ fusion constructs (see above) were used to express amino-terminal and carboxyl-terminal p160 fusion proteins, respectively, and were purified essentially as described previously (17). The purified GST fusion proteins, containing p160 amino-terminal residues 1 to 326 or carboxyl-terminal residues 1046 to 1344, were used to raise polyclonal antisera in rabbits, termed anti-160N and anti-160C, respectively. The p160 antiserum was used at 1:200 (anti-160N) or 1:500 (anti-160C) for immunoblotting with peroxidase-linked anti-rabbit Ig (1:1,000) and enhanced chemiluminescence.

Immunofluorescence. NIH 3T3 cells stably infected with pRUF*neo*-160FLAG or uninfected cells were seeded at 3×10^4 cells/well of an eight-well chamber glass slide and left to adhere overnight. After being washed with PBS, the cells were fixed with ice-cold 50% (vol/vol) methanol-acetone for 30 s and washed with cold PBS containing 1% bovine serum albumin. The cells were incubated on ice with primary antibody (anti-FLAG M2 monoclonal antibody [IBI] at 1:300 or anti-160C [see above] at 1:300) for 45 min. Fluorescein isothiocyanate-conjugated secondary antibodies were incubated with the cells on ice for 30 min at the following dilutions: 1:160 for affinity purified anti-mouse IgG (Silenus) and 1:80 for sheep anti-rabbit Ig F(ab′)$_2$ (Silenus). The cells were mounted with 1% propylgallate–86% glycerol and viewed under a BioRad MRC 600 confocal microscope. Digitized images were manipulated with Adobe Photoshop software.

Transactivation analysis. Myb transactivation experiments were performed essentially as described previously (54). Briefly, CV-1 cells were cotransfected with 4 μg of the chloramphenicol acetyltransferase reporter plasmid, p*myc*CAT (51), 4 μg of each of the effector plasmids pact-c-*myb* or pact-c-*myb*L34P (38), various amounts (0 to 5 μg) of pact-160FLAG or pact-p67*FLAG, and 1 μg of the internal control plasmid pact-β-gal (44). After 48 h, cell extracts were normalized for β-galactosidase activity and assayed for CAT activity.

Nucleotide sequence accession number. The sequence reported here has been submitted to the GenBank database under accession no. U63648.

RESULTS

Isolation of a p160 cDNA clone. We have described the detection of p67 and p160 as radiolabelled nuclear proteins that bound to a bacterially expressed GST fusion protein containing the wild-type Myb leucine zipper region but that failed to bind to similar fusion proteins bearing a mutated leucine zipper (17). By scaling up and slightly modifying this procedure, we were able to isolate sufficient p67 from FDC-P1 cells to allow the generation, purification, and partial amino acid sequencing of five tryptic peptides (24) (see Materials and Methods). Comparison of these sequences with protein and DNA sequence databases indicated that they represented a novel protein. Degenerate oligonucleotides corresponding to four of these peptides were used in various combinations as PCR primers for the amplification of potential p67 coding sequences from FDC-P1 cDNA (Fig. 1A; also see Materials and Methods). Nucleotide sequencing of a number of the resultant products identified a 114-bp fragment, generated with primers corresponding to peptides 4 and 5, that contained an open reading frame. This sequence encoded residues from peptide 4 that were not present in the corresponding PCR primer and also placed a lysine residue immediately preceding

the N- terminus of peptide 5, which is consistent with its generation by trypsin cleavage.

An oligonucleotide from within the 114-bp fragment was then used as a PCR primer to obtain a 459-bp sequence from a WEHI-3B cDNA library. This fragment also contained an open reading frame and was used to screen the same library by hybridization. One clone containing a 3.1-kbp insert was isolated, and the insert was sequenced. This clone contained an open reading frame encoding 1,010 amino acids which started with a good match (nucleotides 1115 to 1121) to the Kozak consensus (A/GNNATGG [40]). However, the predicted molecular mass of 114 kDa and the in vitro translation product of ~120 kDa (data not shown) were both much larger than p67 but smaller than p160. We therefore explored the possibility that this clone represented a partial cDNA clone of the p160 mRNA by performing 5′ RACE PCR (19) to extend the cloned sequences. This resulted in an additional ~1 kb of sequence being identified which, when joined to the 3.1-kb clone, could encode a predicted protein of 152 kDa, again with sequence surrounding the initiation codon (nucleotides 13 to 19) conforming to the Kozak consensus. The relationship between the various clones described above is illustrated in Fig. 1A, and the nucleotide and predicted amino acid sequences are shown in Fig. 1B. The sequence shown in Fig. 1B may include the 3′ terminus of the mRNA since a consensus polyadenlylation signal (AATAAA; beginning at nucleotide 4106) is present 15 nucleotides before the end of the cloned sequence. To confirm that the complete sequence does encode p160, the joined clones were translated in vitro and compared to p160 (and p67) from FDC-P1 nuclear extracts. The in vitro-translated protein comigrated with in vivo-labelled p160 on an SDS-polyacrylamide gel, and the two proteins showed similar peptide patterns on V8 protease digestion (data not shown).

Features of the predicted p160 sequence. It is apparent from the predicted p160 amino acid sequence (Fig. 1B) that all five p67 tryptic peptides are present in the predicted p160 protein sequence. Inspection of this predicted sequence also indicated the presence of a highly acidic region in the middle of the protein and seven short, highly basic sequences embedded in a proline-serine-rich region at the carboxyl terminus (Fig. 1B). These basic repeats conform to a consensus Lys-Lys-Xaa-Xaa-Lys, although some repeats contain arginine instead of lysine at one position and the Xaa residues are also frequently basic. Searches for functional motifs indicated the presence of numerous potential phosphorylation sites (not shown), including those for casein kinase II [(S/T)XX(E/D)] protein kinase C [(S/T)X(R/K)], $p34^{cdc2}$ [(S/T)PX(K/R)] (reviewed in reference 39), and MAP kinase [PX(T/S)P] (27). Because p160 and p67 bind to Myb via the leucine zipper region of Myb (17), we particularly looked for potential leucine zippers within the predicted p160 sequence. Two regions, residues 307 to 335 and 900 to 928, resemble sequences that could conceivably function as leucine zippers (see Discussion). In addition, p160 contains several copies of a recently described motif termed a leucine-charged domain (LCD) that is involved in interactions between nuclear hormone receptors and coactivators, possibly by forming a short amphipathic helix (31, 68). Five copies of the core motif (LXXLL) are present, as well as four possible copies in which one of the last two leucines is replaced by another aliphatic amino acid (Fig. 1B).

Searching of nucleic acid and protein sequence databases revealed that several partial p160 cDNAs have been obtained by others using essentially random sequencing approaches (i.e., as expressed-sequence tags). In addition, the deduced sequence of a protein which is almost certainly the rat homolog of p160 has recently appeared in the GenPept database (accession no. U83590). This sequence corresponds to a protein termed PIP, which was apparently identified as interacting with PAR transcription factors (see Discussion). Other than this, homology to sequences of proteins with known functions is very limited. The highest-scoring sequences are those of the *Drosophila lethal 1B(i)* protein (GenBank accession no. U20542 [69]) and a hypothetical yeast protein of unknown function (Yelo55cp; GenPept accession no. U18795). Most of the other (relatively) highly scoring matches, including upstream binding factor (UBF) (36), show homology mainly within the acidic region of p160, suggesting that the database search has preferentially identified a subset of proteins with highly acidic regions (see Discussion).

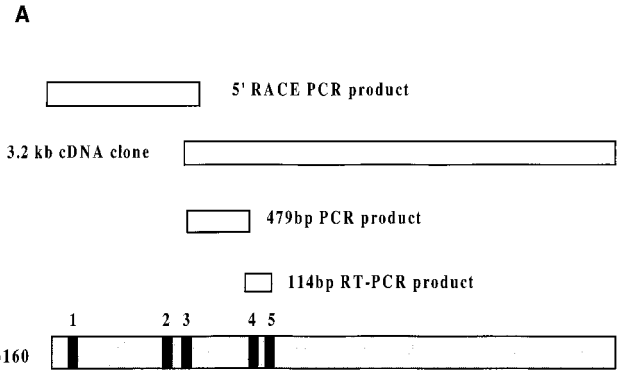
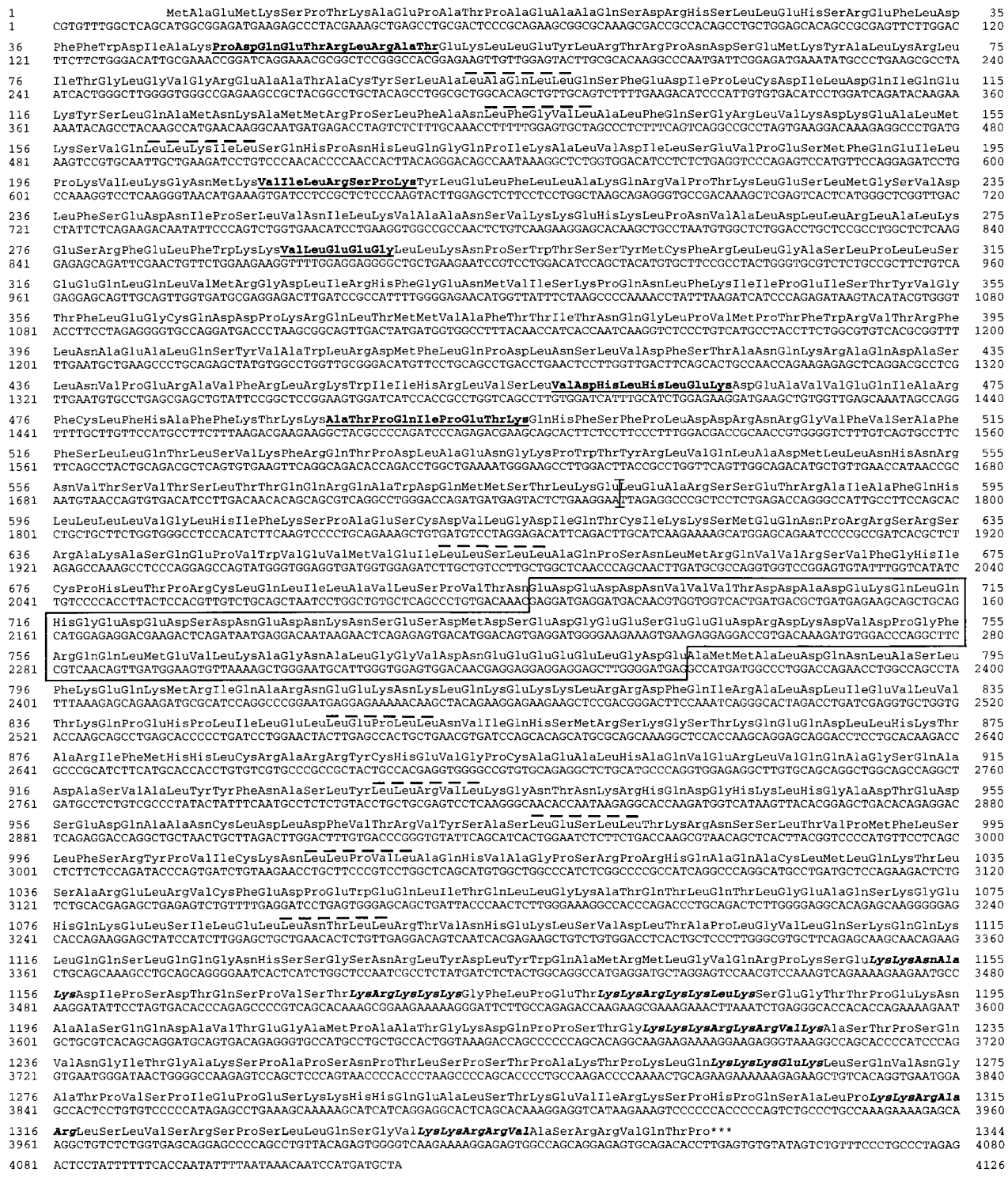


FIG. 1. Molecular cloning, nucleotide sequence, and predicted amino acid sequence of p160 (see Materials and Methods for details). (A) Overlapping cDNA clones that comprise the p160 cDNA and location of p67-derived peptide sequences. The top four (open) boxes represent cDNA clones derived by PCR, library screening, or 5′-RACE PCR as indicated. The bottom box (shaded) represents the entire sequence and shows the locations of the sequences corresponding to the five p67-derived tryptic peptides (solid boxes). (B) Nucleotide sequence of p160 cDNA and predicted amino acid sequence of p160. The p67-derived tryptic peptide sequences are underlined and bold, the acidic region is boxed, and the basic repeats are italicized and bold (Note that two of these sequences contain overlapping repeat motifs, while that starting at residue 1332 is incomplete.) Potential LCD motifs are indicated by dashed overlining. The symbol ⌶ indicates the carboxyl terminus of p67*.

Chromosomal localization of the p160 gene and related sequences. Since the p160 cDNA sequence appeared to represent a novel gene, it was of interest to determine its chromosomal location and thus to determine whether it maps to a locus corresponding to a previously identified mouse mutant. This in turn could provide a clue to the function of the gene. Initial Southern analysis of genomic p160 sequences carried out with probes corresponding to both the 5′ and 3′ ends of the p160 cDNA (Fig. 2A) detected three or more hybridizing bands with each probe, suggesting that there may be more than one p160-related gene.

Chromosomal localization was carried out by Southern hybridisation of the 3′ probe to DNA from a panel of interspecific (*Mus musculus* × *M. spretus*) backcrosses (12). RFLPs between the two species were used to monitor the segregation of p160-related *M. spretus* sequences in the backcross mice. The results of this analysis, illustrated in Fig. 2B, indicate that there are three loci which contain p160-related sequences on chromosomes 1, 11, and 12. Although three (or more) fragments could be detected with the 5′ probe with most restriction enzymes (Fig. 2A), only one RFLP could be identified, which allowed the corresponding fragment to be mapped to chromosome 11 (data not shown). Since this band did not show recombination with the chromosome 11 fragment detected with the 3′ probe (data not shown), both fragments presumably

B



represent the same locus. Moreover, we have found that an intron probe, derived from genomic clones that correspond in sequence to at least 2,067 bp of the p160 cDNA, also maps to chromosome 11 and cosegregates with the locus identified with the other probes (data not shown). We have therefore termed the chromosome 11 locus *P160*, since it appears to represent the entire p160 cDNA sequence, and have termed the loci on chromosomes 1 and 12 *P160-rs1* and *P160-rs2* (where *rs* stands

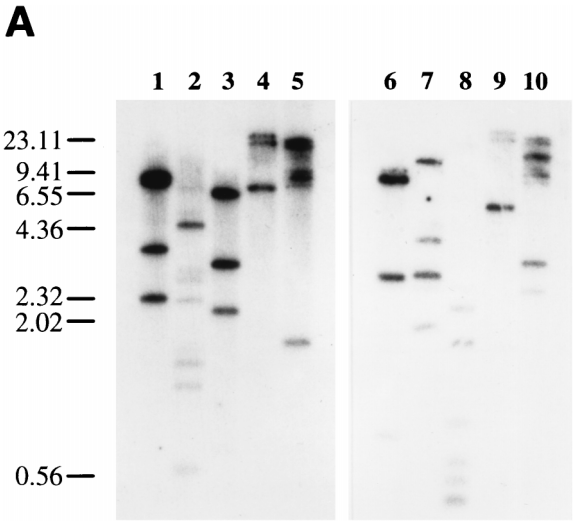
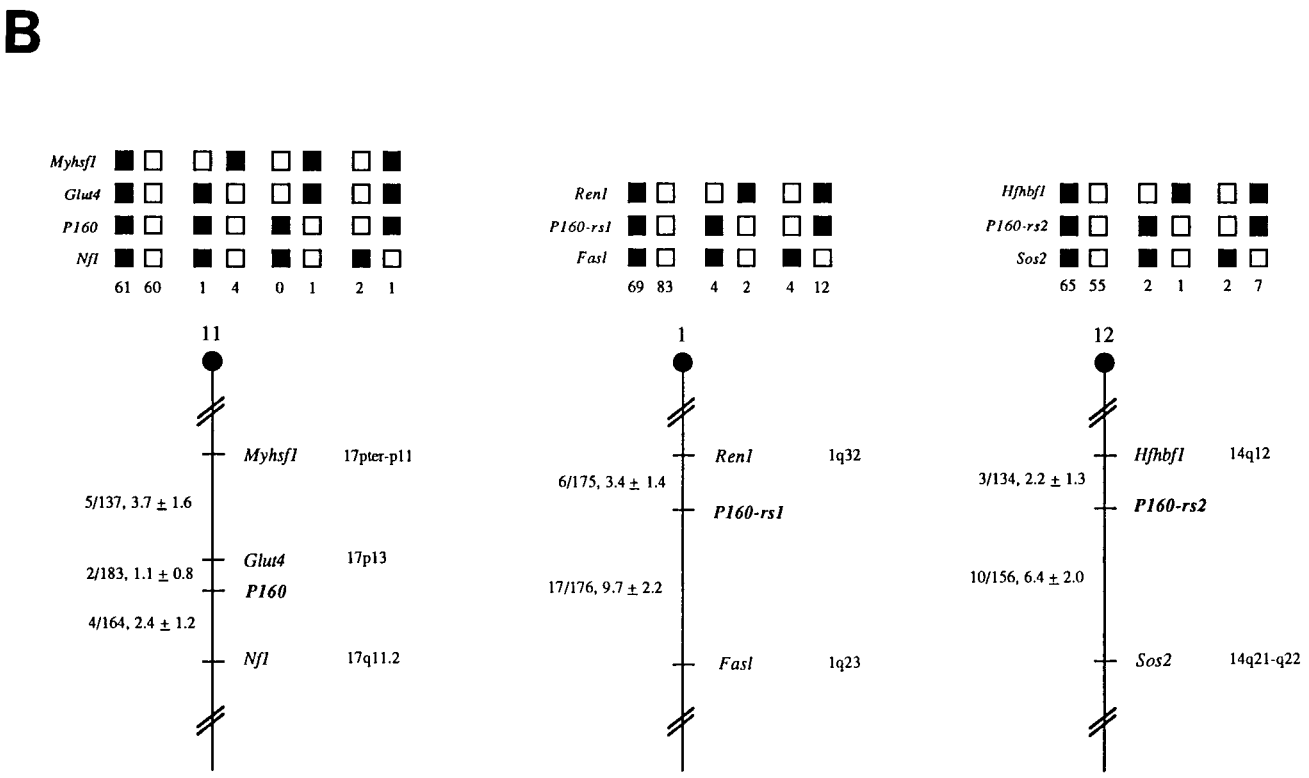


FIG. 2. Genomic organization and chromosomal localization of *P160* and the related genes, *P160-rs1* and *P160-rs2*. (A) Southern blot analysis of murine DNA with probes corresponding to the 5′-most 1.0 kb (left) and the 3′-most 1.0 kb (right) of the p160 cDNA sequence. DNA was digested with the following restriction endonucleases: lanes 1 and 6, *Xba*I; lanes 2 and 7, *Sac*I; lanes 3 and 8, *Pst*I; lanes 4 and 9, *Hin*dIII; lanes 5 and 10, *Eco*RI. The positions and sizes (in kilobases) of DNA markers are shown at the left. (B) Summary of chromosomal localization of p160-related loci in the mouse genome, as determined by interspecific backcross analysis. The grids above each chromosome map represent the possible genotypes of the backcross mice, with solid squares representing the *M. spretus* alleles and open squares representing the *M. musculus* alleles; the numbers of recombinants of the indicated genotypes are shown below the grid. The number of recombinant N2 animals over the total number of N2 animals is shown at the left of each chromosome map. The recombination frequencies between each *P160*-related locus and the adjacent markers, expressed as genetic distance in centimorgans (±1 standard error), are also shown. The positions of corresponding loci on human chromosomes, where known, are shown at the right of each chromosome map.



for "related sequence"), respectively. The lack of further RFLPs with the 5′ probe did not allow us to determine whether or not the *P160-rs1* and *P160-rs2* loci also contain 5′ p160-related sequences.

Expression of p160 mRNA. The cloned p160 cDNA sequence was used to identify and examine the expression of p160 mRNA. Northern blotting of polyadenylated RNA from a number of cell lines with a probe corresponding to the 5′-most 1 kb of p160 (Fig. 3A) or with the entire p160 cDNA (data not shown) revealed a single mRNA species of ~4.5 kb in each murine cell line. The size of this transcript is clearly adequate to include all of the cloned sequences (4.1 kb) and a poly(A) tract. Furthermore, the ubiquitous expression of the 4.5-kb mRNA corresponds to the previously determined distribution of p160 (17). Importantly, no additional mRNA species were present in cells (such as FDC-P1 and WEHI-3B) that also expressed p67 (17). Therefore, there does not appear to be a separate transcript that could encode p67 (see below). Note also that the p160 cDNA did not detect a transcript in RNA from the human HL-60 cell line; this is consistent with our previous failure to detect p160 or p67 in human cells (including HL-60) by our standard binding assay (17).

We also examined the expression of p160 mRNA in a number of different tissues and organs of the mouse. Figure 3B shows that p160 is expressed in most tissues and organs; moreover, in other experiments, expression was detected in the

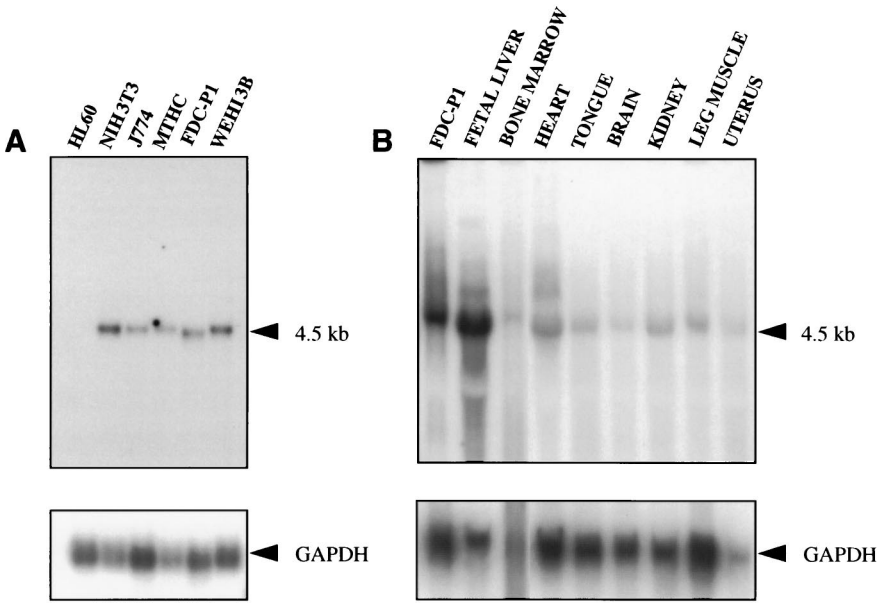


FIG. 3. Northern blot analysis of p160 mRNA expression with the p160 cDNA probe. In the upper panels, 2 μg of poly(A)$^+$ RNA from the indicated cell lines was loaded in each lane (MTHC, *myb* transformed hemopoietic cells) (A) or 30 μg of total RNA from the indicated mouse tissues (or FDC-P1 cells) was loaded in each lane (B). The size of the p160 mRNA was determined by comparison with RNA markers (not shown). In the lower panels, the Northern blots shown in the upper panels were stripped and reprobed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as a control for mRNA loading; only the relevant area of each blot is shown.

spleen, liver, intestines, and lungs (data not shown). Interestingly, the level in fetal liver is particularly high; this probably does not indicate preferential expression in hematopoietic tissue, since expression in bone marrow is much lower. Moreover, expression is not substantially greater in hematopoietic cell lines than in fibroblasts (Fig. 3A).

Generation of antisera that recognize p160 and p67. To generate a reagent for the detection of p160 (and p67), antisera were raised against GST fusion proteins containing the N-terminal 326 residues and C-terminal 298 residues of p160. These were then used to probe nuclear and cytoplasmic extracts of FDC-P1 and NIH 3T3 cells. Figure 4 shows that, as expected from our previous work (17), the N-terminal antiserum (anti-160N) recognized proteins of 160 and 67 kDa in nuclear but not cytoplasmic extracts from FDC-P1 cells. Also as expected from our previous report (17), p160 but not p67 was detected in NIH 3T3 nuclear extracts. In contrast, the C-terminal antiserum (anti-160C) detected only p160 in nuclear but not cytoplasmic extracts from both cell types. Moreover, each antiserum detected the same proteins following binding of nuclear extracts to GST fusion proteins containing the Myb NRD (data not shown). Other bands (of ~97 and ~110 kDa) detected by the antisera may be due to nonspecific

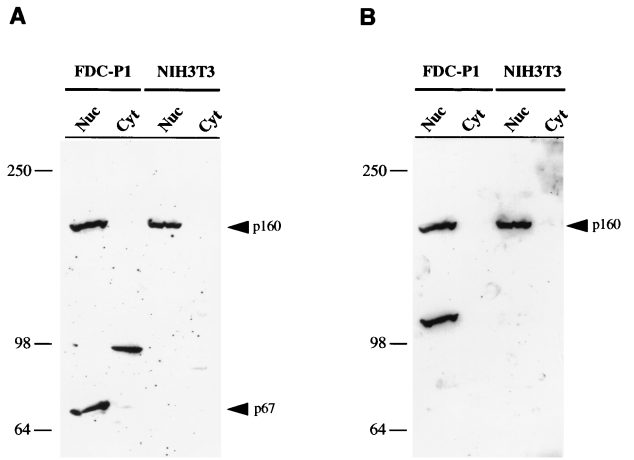


FIG. 4. Proteins detected by antisera raised to the N and C termini of p160. Cytoplasmic (Cyt) and nuclear (Nuc) extracts from FDC-P1 and NIH 3T3 cells, as indicated, were fractionated by SDS-PAGE and immunoblotted with antisera against the N-terminal (anti-160N) (A) or the C-terminal (anti-160C) (B) regions of p160. The positions of p160 and p67 are indicated by arrows, and those of size standards (in kilodaltons) are indicated at the left.

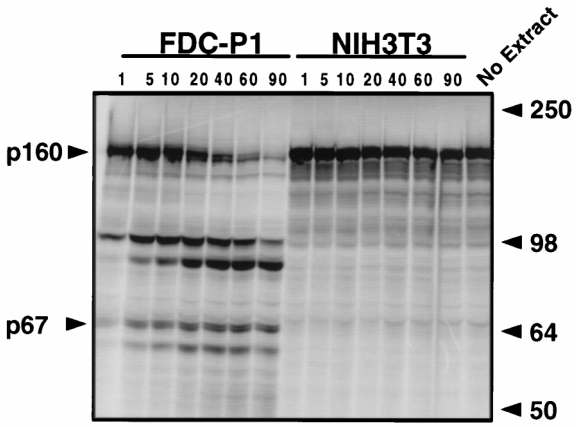


FIG. 5. Proteolysis of p160 by cell extracts. In vitro-translated p160 labelled with [^{35}S]methionine was incubated with extracts from FDC-P1 or NIH 3T3 cells for the indicated times (in minutes), after which the products were analyzed by SDS-PAGE. The positions and molecular masses of size standards are indicated at the right, and the bands corresponding to p160 and the 67-kDa product (p67) are indicated at the left.

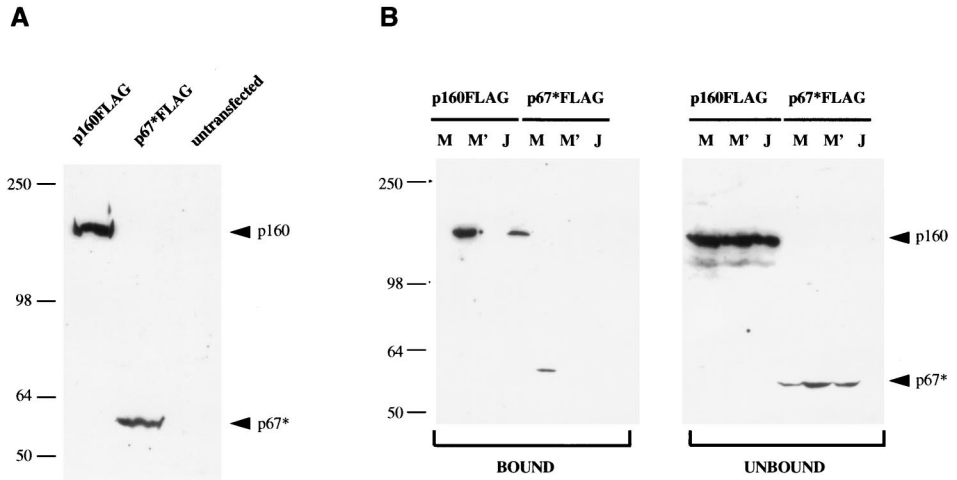


FIG. 6. Expression and binding properties of p160FLAG and p67*FLAG following transfection into 293T cells. In each panel, proteins were detected by immunoblotting with anti-FLAG monoclonal antibody M2. (A) SDS-PAGE of extracts from cells transfected with pact expression vectors containing p160FLAG, p67*FLAG constructs, or mock-transfected cells, as indicated. (B) Analysis of bound and unbound p160FLAG and p67*FLAG following incubation of the cell extracts shown in panel A with Sepharose beads carrying the GST-NRD2 (lanes M), GST-L3,4P (lanes M′), or GST-Jun (lanes J) fusion proteins. Note that only 50% of each unbound fraction was loaded.

reactivities; however, it is also possible that they represent other p160-related proteins.

p67 represents an N-terminal proteolytic cleavage product of p160. The clones described here (Fig. 1) were isolated on the basis of peptide sequences derived from p67, but in fact they encode p160 (see also below). While this is compatible with the observation that the two proteins are related, as judged by V8 protease peptide mapping (17), it is apparent from the sequence that the N-terminal portion of p160 contains all five of the tryptic peptides that were derived from p67 (Fig. 1B). Moreover, we show above that the anti-160N antiserum but not the anti-160C antiserum also reacts with p67 (Fig. 4). These observations led us to suspect that p67 is the N-terminal proteolytic cleavage product of p160. The existence of a single mRNA species corresponding to p160 in cells such as FDC-P1 and WEHI-3B that express both p160 and p67 (Fig. 3A) is also consistent with this notion; if p67 were derived from an alternatively spliced transcript or another gene, we might expect to find an additional mRNA species.

If p67 is generated by proteolysis, cells in which p67 is detected should contain a proteolytic activity capable of cleaving p160, while this activity should be absent from cells that do not contain detectable p67. This prediction was tested by incubating radiolabelled in vitro-translated p160 with FDC-P1 and NIH 3T3 cell extracts. Figure 5 shows that the FDC-P1 extract generated a number of cleavage products, including one with an apparent molecular mass of ca. 67 kDa. Moreover, this protein represents the N-terminal portion of p160 since it could be immunoprecipitated with the anti-160N antiserum described above (data not shown). In contrast, the NIH 3T3 extract failed to cleave p160 even after extended incubation (Fig. 5).

Binding properties of full-length and truncated forms of p160. To examine the binding properties of the protein(s) encoded by the p160 cDNA clone, we constructed a carboxyl-truncated form of p160, which we term p67*, by inserting a termination codon after nucleotide 1755 (Fig. 1B), resulting in a 580-amino-acid protein with a predicted molecular mass of ca. 67 kDa (this construct was initially made to approximate the endogenous p67 protein). Epitope-tagged versions of p160 and p67* were then constructed by adding a sequence encoding the FLAG epitope (33) to the 5′ ends of each cDNA construct. These constructs were inserted into the pact expression vector (54) and transiently transfected into human embryonic kidney 293T cells. Cell extracts were then analyzed directly by immunoblotting with anti-FLAG antibodies (Fig. 6A)

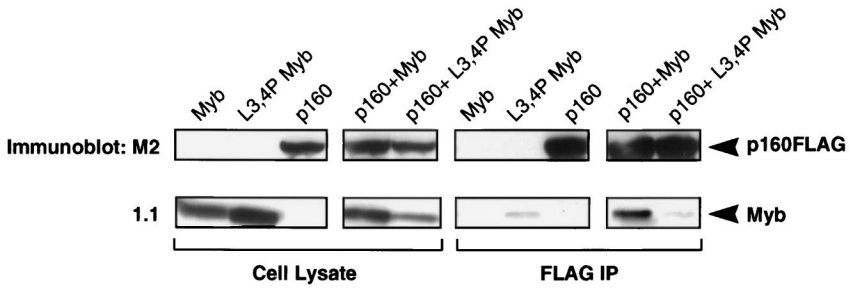


FIG. 7. Coimmunoprecipitation of Myb and p160. Extracts from 293T cells cotransfected with expression vectors encoding p160FLAG, wild-type Myb (Myb), L3,4P mutant Myb (L3,4PMyb), or combinations of these as indicated were subject to immunoprecipitation with anti-FLAG antibodies. The resultant immunoprecipitates were then analyzed by SDS-PAGE and immunoblotting with anti-Myb monoclonal antibody 1.1. The immunoblots were then stripped and reprobed with M2 anti-FLAG antibodies. Also shown is a direct immunoblot of a portion of each cell lysate used for immunoprecipitation (Cell Lysate).

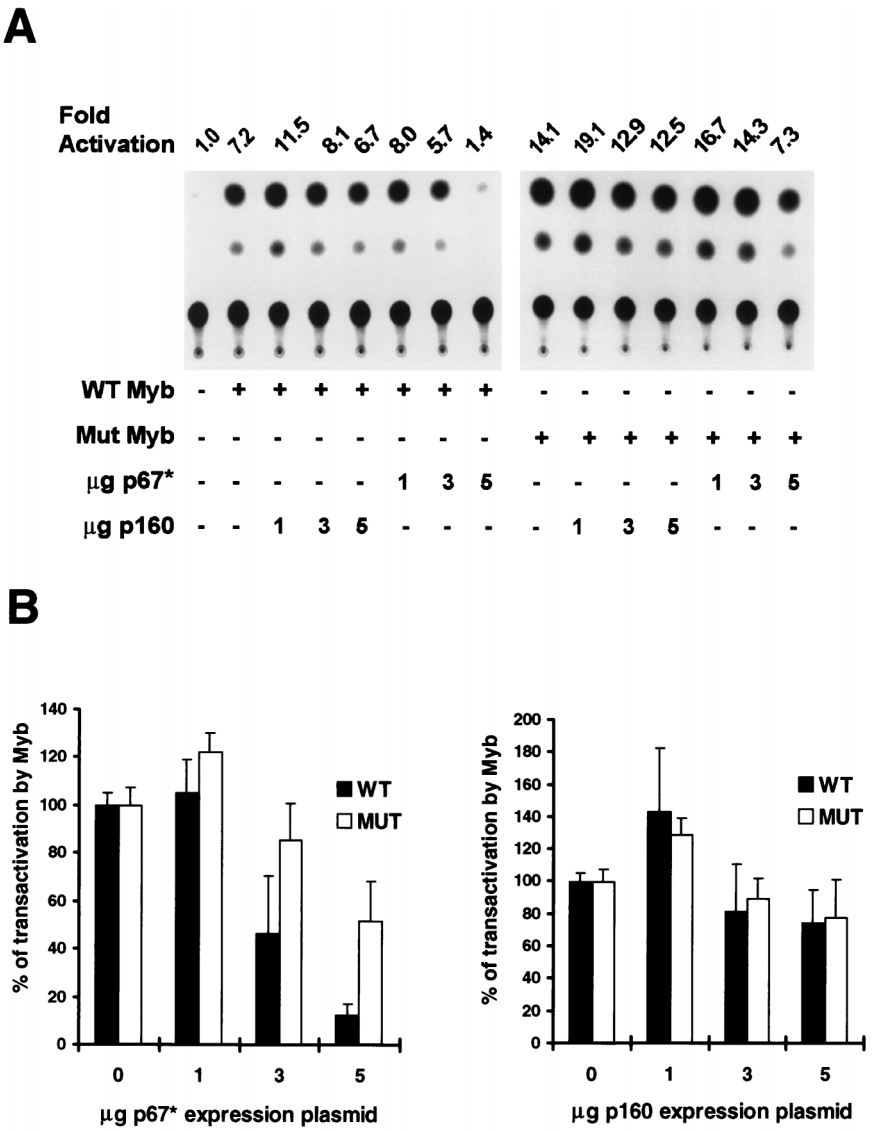


FIG. 8. Effect of p160 and p67* on transactivation by wild-type (WT) and L3,4P mutant (Mut) Myb. (A) CAT activity (measured by thin-layer chromatography) in CV-1 cells transfected with the pc-myc-CAT reporter plasmid, pact-β-galactosidase, and pact expression vectors encoding Myb, p160FLAG, or p67*FLAG, as indicated at the bottom of the chromatograms. Where the p160FLAG or p67*FLAG vector was included, the amount of plasmid used (in micrograms) is shown. The degree of activation, expressed as the ratio of CAT activity obtained in each transfection compared to that in the control transfection with no Myb vector (leftmost lane), is shown above each lane; these values are normalized with respect to β-galactosidase activity to correct for differences in transfection efficiency. See Materials and Methods for details. (B) Representation of the data from panel A to indicate the degree of inhibition of Myb-stimulated transactivation by p160 and p67*, which is taken as 100% for each form of Myb (WT or MUT, as indicated). Error bars represent the standard error as determined from triplicate transfections.

or following binding reactions with GST fusion proteins containing Myb or Jun leucine zipper regions (Fig. 6B). In agreement with the properties of endogenous p160 and p67 (17), FLAG-tagged p160 and p67* both bound to the GST-NRD2 protein, which contains a wild-type Myb leucine zipper region, but not to the GST-L3,4P (17) protein, which has a mutated leucine zipper. Interestingly, it appears that p67* binds more efficiently than p160 to GST-NRD2 (compare the "bound" and "unbound" lanes in Fig. 6B). Also in agreement with studies of the endogenous proteins, p160FLAG bound to the GST-Jun protein which carries the leucine zipper and basic regions of c-Jun whereas p67*FLAG did not. These observations further validate the authenticity of the p160 cDNA clone and show that a truncated version of p160, i.e., p67*, has similar binding properties to p67.

To determine whether full-length p160 and c-Myb proteins could interact in the intracellular milieu, we used expression vectors encoding wild-type c-Myb or the L3,4P leucine zipper mutant and p160FLAG to coexpress these proteins in 293T cells. Cell extracts were then subjected to immunoprecipitation with anti-FLAG antibodies, and the immunoprecipitates were analyzed by immunoblotting with anti-FLAG and anti-Myb antibodies. Figure 7 shows that anti-FLAG immunoprecipitates from cells expressing both p160 and c-Myb contained WT c-Myb protein while those from transfections of p160FLAG or Myb alone did not. Neither protein was precipitated when an irrelevant control antibody was used (data not shown). Moreover, Fig. 7 shows that, in contrast to WT c-Myb, the leucine zipper mutant did not interact specifically with p160FLAG. The small amount of Myb detected in the immunoprecipitate

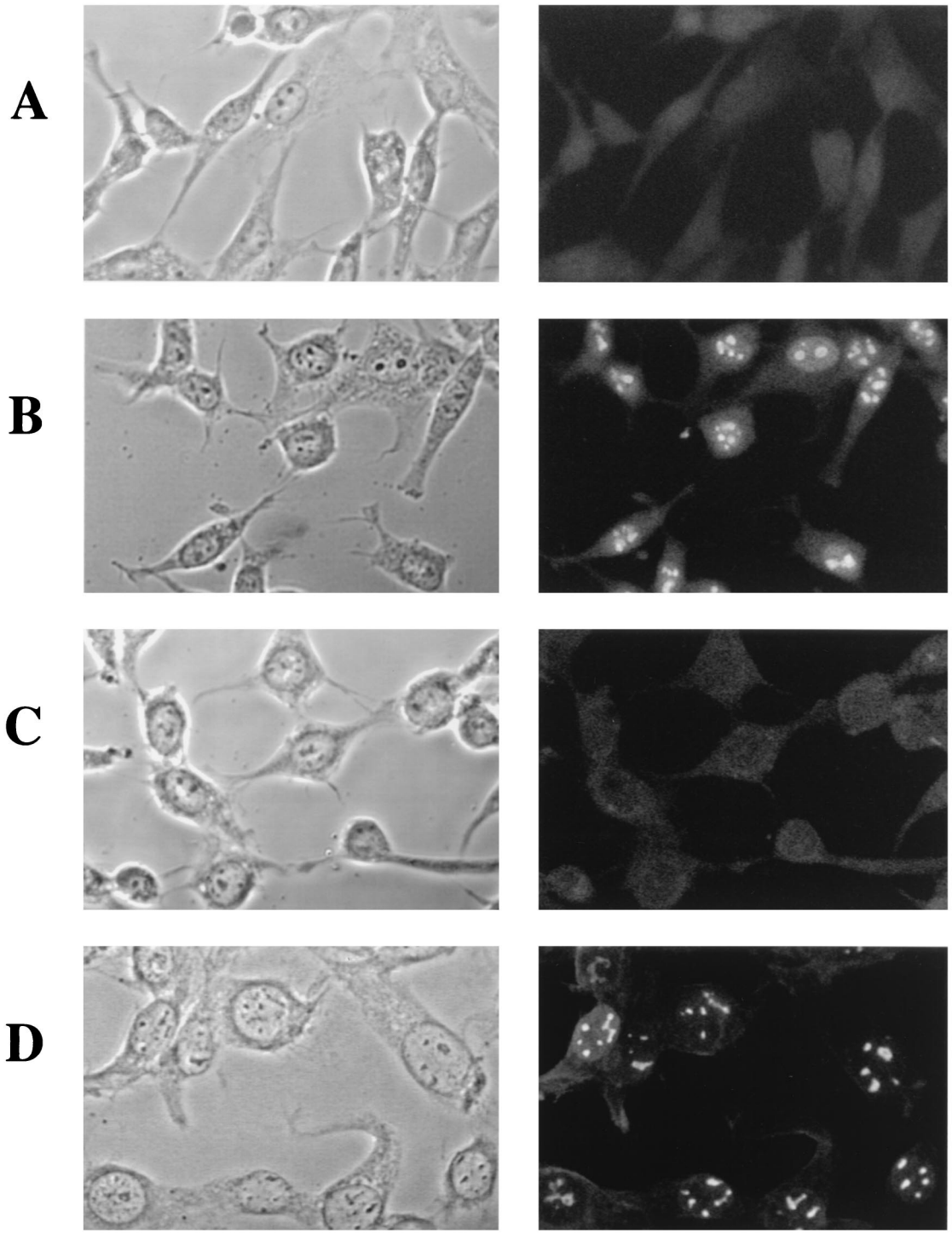


FIG. 9. Immunofluorescence analysis of the subcellular localization of p160. Each panel shows phase-contrast (left) and fluorescence (right) images of the same field of cells. The cells shown in panels A to C are NIH 3T3 fibroblasts, and those shown in panel D are NIH 3T3 fibroblasts expressing p160FLAG (see the text for details). These were stained with pre-immune rabbit serum (A), 160C rabbit antiserum (B), and anti-FLAG monoclonal antibody M2 (C and D).

from the p160FLAG-plus-L3,4P Myb cotransfection probably reflects a degree of "stickiness" of the latter protein, since a similar amount was observed in precipitates from cells transfected with L3,4P Myb alone. These data confirm that full-length c-Myb and p160 can indeed associate specifically in vivo and that their association requires the c-Myb leucine zipper motif.

p67* but not p160 inhibits transactivation by Myb. Since the original rationale for isolating proteins that interact with the Myb leucine zipper was that such proteins may be inhibitors of

Myb function, we examined the effects of p160 on one of the key activities of Myb, i.e., its ability to enhance transcription from promoters containing Myb-binding sites. p67* was also tested in this assay, since although it can interact with the Myb leucine zipper (Fig. 6), it presumably lacks other functional domains of p160 and thus may have had the potential to act as a dominant negative antagonist of p160 function. For this purpose, we used the c-*myc* promoter region coupled to the chloramphenicol acetyltransferase reporter gene (51, 54). This construct was cotransfected into CV-1 cells along with expression vectors for either wild-type or leucine zipper-mutated (L3,4P) Myb and with various amounts of p67* or p160 expression vector. Note that as expected (38), a greater degree of transactivation was observed when cells were transfected with the leucine zipper-mutated Myb (Fig. 8A). Figure 8B shows that increasing amounts of p67* substantially inhibited transactivation by wild-type Myb, down to a level of just 12% of that obtained in the absence of p67*. On the other hand, p160 had relatively little effect, giving a slight stimulation at low levels and a slight (25%) inhibition at the highest level. Most of the inhibition by p67* was dependent on the Myb leucine zipper, although nearly 50% inhibition of transactivation by L3,4P Myb was obtained with the highest level of p67*. Again, p160 had little effect on transactivation by the mutant form of Myb (Fig. 8). These results are unlikely to reflect a nonspecific suppression of transcriptional activity, because expression of the control reporter construct (pact-β-galactosidase) showed no inhibition by p67* or p160 (data not shown).

p160 is a predominantly nucleolar protein. The subcellular localization of p160 was further examined by using the p160 antisera described above in immunofluorescence studies on NIH 3T3 fibroblasts. To our surprise, staining with the 160C antiserum revealed that p160 is located predominantly in the nucleolus (Fig. 9B), although, significantly (see Discussion), some nucleoplasmic staining was also evident. To independently confirm this observation, we examined NIH 3T3 fibroblasts that had been infected with a retroviral vector encoding p160FLAG. Again, staining with monoclonal anti-FLAG antibody showed predominantly nucleolar localization (Fig. 9D).

DISCUSSION

Features of the p160 protein. We report here the molecular cloning of cDNA corresponding to p160, one of two nuclear proteins that were identified by their ability to bind to the c-Myb-negative regulatory domain and, specifically, to the leucine zipper motif within this domain (17). Although the p160 cDNA was isolated on the basis of peptide sequences derived from p67, evidence based on size, peptide mapping, mRNA expression pattern, and the binding specificity of its translation product demonstrates that the cDNA represents p160.

The predicted p160 amino acid sequence is novel and shows no close homology to other sequences present in databases other than the presumptive rat homolog (GenPept accession no. U83590); this sequence may represent a partial clone since it lacks the first 67 residues of the mouse protein. Many of the other higher-scoring matches show homology only to the acidic region of the protein (data not shown), which casts some doubt on the biological significance of such similarities. However, the similarities between p160 and the highest-scoring match, the product of the *Drosophila lethal 1B(i)* gene, extend beyond this region and may be of more significance (see below).

Inspection of the predicted p160 sequence reveals some potentially interesting features (see Results and Fig. 1). As mentioned above, there are two leucine zipper-like sequences, starting at amino acids 307 and 900, with the following residues in a possible heptad repeat: L-L-L-I-M and L-L-Q-L-L. However, mutagenesis of this region, which replaced two leucine residues in each of these sequences with prolines, had no effect on the ability of p160 to bind to the Myb or Jun leucine zipper regions (39a). Thus, the structures that mediate the interaction of p160 with Myb and Jun remain to be identified; one intriguing possibility is that these interactions involve some of the LCD motifs. Our results do show, however, that the Myb leucine zipper- and Jun bZip domain-binding regions of p160 are distinct. The Myb-binding region must lie within the first 580 amino acids, since p67*, like endogenous p67, efficiently and specifically binds to the Myb leucine zipper; similarly, the Jun-binding region is carboxy-terminal of residue 580 because p67* fails to bind the Jun leucine zipper region. The functions of the other structurally distinguishable regions of p160, i.e., the highly acidic region in the center of the protein and the basic repeats at the C terminus, are currently uncertain. It is possible that the acidic region indicates a function in transcriptional regulation, since many transcription factors have acidic transactivation domains (65). It is also interesting that the basic repeats bear a close similarity to nuclear localization signals (reviewed in references 20 and 35), and, indeed, preliminary data suggest that their deletion results in the cytoplasmic localization of at least a proportion of the protein, which, as we show here (Fig. 4), is normally exclusively nuclear (20a).

Relationship between p160 and p67. Earlier data clearly showed that p160 and p67 are functionally and structurally related since they both bind the c-Myb leucine zipper and share many peptides generated by partial digestion with V8 protease (17). The present work extends those results and provides several lines of evidence that p67 is generated by proteolysis of p160. This raises the possibility that, despite its reproducibility and strict cell type specificity, the detection of p67 in nuclear extracts is a consequence of the nuclear isolation procedure. In fact, little of this protein is detected when whole cells are lysed directly in the presence of SDS (66a). Nevertheless, it is conceivable that there may be physiological conditions that also activate or release the protease which cleaves p160. This, in turn, could have biological consequences, since p67, like the p67* construct, may similarly function as an inhibitor of transactivation by Myb. Examination of the effects of various cell treatments, e.g., growth factor deprivation or induction of differentiation, on the generation of p67 will be required to resolve this issue.

Similarities of p160 to other nucleolar proteins and possible implications for p160 function. An unexpected finding from this study was the nucleolar localization of p160. Although this was not anticipated for a potential regulator of c-Myb activity, since Myb is not detected in the nucleolus (4), this observation is consistent with the homology between p160 and the product of the *Drosophila lethal 1B(i)* gene (69) (GenBank accession no. U20542). Unfortunately, little information is available about the function of this gene, but, interestingly, its product is reportedly also a nucleolar protein (cited in the GenBank entry), as is PIP, the presumptive rat homolog of p160 (again, as cited in the GenPept entry). It may also be of significance that one of the other higher-scoring matches from BLAST searches of protein databases is UBF (36). UBF is a polymerase I transcription factor and thus is also a nucleolar protein. As remarked above, homology to p160 is mostly within the acidic region, but it is of interest that the acidic region of UBF is essential for its transactivating/antirepressing capacity and is involved in displacing histones from DNA (41, 42). Another nucleolar protein, Nopp140 (45, 46), also shows some other general similarities to p160, although there is no significant

sequence homology. Like p160, Nopp140 also has an acidic region containing multiple casein kinase II phosphorylation sites and multiple, basic nuclear localization signal-like sequences toward its C terminus (46). Although Nopp140 is predominantly nucleolar, it was noted that a small proportion of Nopp140 can be detected in the nucleoplasm (46), as also seen here with p160. The significance of this has been highlighted by a recent report (47) that Nopp140 can function as a transcriptional coactivator of the bZip transcription factor C/EBPβ in stimulating the transcription of a polymerase II-transcribed gene. Thus, the similarities between p160 and Nopp140 (and between p160 and UBF) raise the possibility that p160 plays a role, e.g., as a coactivator, in the transcription of both polymerase I- and some polymerase II-transcribed genes. In this light, the predominantly nucleolar localization of p160 can be reconciled with its interaction with Myb, Jun, and possibly other transcription factors; i.e., the small fraction of p160 in the nucleoplasm may be involved in such interactions. This notion is further supported by the reported (i.e., as stated in the GenPept entry) identification of the presumptive rat homolog of p160, PIP, by its interaction with members of the PAR family of transcription factors. Moreover, the presence of several LCD motifs, which have recently been reported to mediate the interaction between certain coactivators and nuclear hormone receptors (31, 68), is also consistent with a role for p160 in the regulation of polymerase II-mediated transcription.

Effects of p67* and p160 on transactivation by Myb. Although overexpression of p160 had no significant effect on transactivation by Myb, our findings remain consistent with a role for p160 as a potential cofactor in Myb-regulated transcription. It may simply be that the levels of endogenous p160 in CV-1 cells are sufficiently high as not to be rate-limiting, so that excess p160 has little or no effect (a similar observation was made for p/CIP [68]). On the other hand, inhibition by a truncated form of p160, i.e., p67*, supports a role for p160 in transcriptional regulation by Myb. One possible mechanism is, as we envisaged, that p67* is acting as a dominant negative antagonist of a coactivator function of (endogenous) p160, since it lacks many of the potential functional domains of the latter. Alternatively, truncation may unmask or enhance an inhibitory function of p160 that is normally subject to regulation (e.g., by phosphorylation or by other protein-protein interactions). In any case, while the precise mechanism of inhibition remains to be defined, it almost certainly involves binding to Myb via the leucine zipper motif of Myb, since inhibition of transactivation by p67* is largely dependent on the integrity of this motif. (A small [~50%] inhibition of transactivation by mutant [L3,4P] Myb was consistently observed upon cotransfection with p67*. Two possible explanations for this are that p67* can interact with another region of c-Myb in addition to the leucine zipper, and/or that it has a more general transcriptional repressor activity, due for example to interactions with other transcription factors.)

If p160 does function as a coregulator of c-Myb-mediated transcription, it would represent a third such protein known to interact with Myb, since CREB-binding protein (CBP) (13, 56) and p100 (14) have already been shown to do so. These may function together as part of a transcription complex; alternatively, there may be some exclusivity in these interactions. The net effect of all of the possible interactions may be complex and difficult to predict. For example, p100 appears to repress transactivation by Myb (14), yet it was originally identified as a transcriptional coactivator (67). Moreover, while p160 has at least some characteristics of a transcriptional coactivator (see above), it binds to a region of c-Myb that is involved in negative regulation. It is possible that in vivo, these potential regulators differentially affect transcription of various Myb "target genes"; for instance, p160 may be required for Myb to enhance the transcription of a subset of target genes involved in the function of mature hemopoietic cells but not required for its transforming effects (see the discussion of target genes in reference 52).

Expression of p160. The expression of p160 mRNA corresponds to that of p160 as detected by binding to GST-Myb fusion protein in a series of cell lines (17). Our present work extends the analysis of expression to a range of tissues and organs; together, these data suggest that p160 is present in most or all cell types. Therefore, we might suspect that p160 serves a function or functions required in all cell types. This is consistent with a role in rRNA synthesis, as suggested by its nucleolar localization, and also with its potential to interact with c-Jun, which is expressed more widely than in the limited range of cells that express c-Myb. The high level of expression in fetal liver, which is a very active site of cell proliferation, is similarly consistent with a role in rRNA synthesis. Neither of these possibilities excludes a role in interacting with c-Myb; in fact, the high p160 level in fetal liver, which is enriched for hematopoietic progenitor cells that express c-Myb, is also compatible with this role.

Chromosomal localization of the p160 gene. Finally, we should consider the potential significance of the presence in the mouse genome of three loci that hybridize with the p160 cDNA. We have termed the chromosome 11 locus *P160* because it contains sequences related to both the 5′ and 3′ ends of the p160 cDNA and because genomic clones that map to this locus correspond in sequence to the cDNA. Furthermore, by exploiting the weak cross-reaction of mouse p160 cDNA with human genomic sequences (which was not sufficient to allow detection of human p160 mRNA [Fig. 3A]), we have identified a human *P160* homolog located in a syntenic region of the human genome (39b). Thus, it is highly unlikely that one of the other loci (*P160-rs1* or *P160-rs2*) contains the sequence which we have cloned. It is somewhat surprising that we did not detect other transcripts that could correspond to the other *P160*-related loci; this might indicate that all the transcripts are of the same size or that expression of the related genes is much more restricted. However, it should also be borne in mind that one (or both) of these loci could represent a pseudogene(s).

The existence of the three *P160*-related genes complicates the identification of potential correlations between these genes and loci corresponding to mutant phenotypes. Without knowing the precise functions of p67 and p160, it is unclear what kinds of phenotypes one might expect. However, the ability of p160 and p67 to interact with the NRD of c-Myb raises the possibility that *P160* potentially acts as a tumor suppressor gene. To the best of our knowledge, there are no obvious murine mutants which exhibit neoplastic or preneoplastic phenotypes that map close to any of the three *P160* loci. Current studies on the human homolog of *P160* (39a), together with the studies on the activities of murine p160 with the reagents described in this report, should help to determine whether lesions in *P160* play a role in human disease.

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