MOLECULAR AND CELLULAR BIOLOGY, Aug. 1997, p. 4801–4810 Vol. 17, No. 8
0270-7306/97/$04.00+0


# Identification of *drm*, a Novel Gene Whose Expression Is Suppressed in Transformed Cells and Which Can Inhibit Growth of Normal but Not Transformed Cells in Culture

LILIA Z. TOPOL,[1] MARIA MARX,[2] DANIELLE LAUGIER,[2] NATALIA N. BOGDANOVA,[3] NIKOLAI V. BOUBNOV,[3] PETER A. CLAUSEN,[4] GEORGES CALOTHY,[2] AND DONALD G. BLAIR[4]*

*Intramural Research Support Program, SAIC Frederick, NCI-FCRDC,[1] and Basic Research Laboratory, Division of Basic Sciences, National Cancer Institute,[4] Frederick, Maryland 21702-1201; Unité Mixte de Recherche 146 du Centre National de la Recherche Scientifique, Institut Curie, Centre Universitaire, 91405 Orsay Cedex, France[2]; and St. Louis University, St. Louis, Missouri 63104[3]*

Received 19 December 1996/Returned for modification 7 February 1997/Accepted 23 April 1997

**Using differential display analysis, we compared the expression of RNA in v-*mos*-transformed cells and their flat revertant and isolated a novel gene, *drm* (down-regulated in *mos*-transformed cells), whose expression is down-regulated in parental v-*mos*-transformed cells but which is expressed at a high level in the revertant and normal rat fibroblasts (REF-1 cells). Analysis of different oncogene-transformed cells revealed that *drm* gene expression was also suppressed in REF-1 cells transformed by v-*ras*, v-*src*, v-*raf*, and v-*fos*. The *drm* cDNA contains a 184-amino-acid-protein-encoding open reading frame which shows no significant homologies to known genes in DNA databases. Polyclonal antibodies raised against drm peptide detect a protein with the predicted size of 20.7 kDa in normal cells and under nonpermissive conditions in cells conditionally transformed by v-*mos* but not in parental v-*mos*-transformed cells. Northern analysis of normal adult tissues shows that *drm* is expressed as a 4.4-kb message in a tissue-specific manner, with high expression in the brain, spleen, kidney, and testis and little or no expression in the heart, liver, and skeletal muscle. In situ hybridization analysis in adult rat tissue reveals good correlation with this pattern and indicates that *drm* mRNA is most highly expressed in nondividing and terminally differentiated cells, such as neurons, type 1 lung cells, and goblet cells. Transfection of a drug-selectable *drm* expression vector dramatically reduced the efficiency of colony formation in REF-1 and CHO cells, and the *drm*-transfected REF-1 survivors expressed low or nondetectable levels of exogenous *drm* mRNA. The toxic effects of *drm* can be overcome by cotransfection with constructs expressing oncogenic *ras*; furthermore, cells expressing high levels of *drm* and conditionally transformed with *mos*-expressing Moloney murine sarcoma virus rapidly undergo apoptosis when shifted to the nonpermissive temperature. Taken together, our data suggest that cells expressing high levels of *drm* undergo apoptotic death in the absence of oncogene-induced transformation and that *drm* represents a novel gene with potential roles in cell growth control or viability and tissue-specific differentiation.**

Malignant transformation is characterized by alterations in the normal properties of cell growth, adhesion, motility and shape. The multistep nature of this process is now well defined in a number of systems, and it has been established that genetic changes in specific genes are responsible for both positive and negative contributions to that process. Analysis of the genes involved has identified those which act positively to induce aspects of the transformed state (oncogenes) and, more recently, has led to the identification of those which act to block or suppress the malignant phenotype, the so-called tumor suppressor genes (24). The importance of these genes in maintaining the normal phenotype was first implicated by the fact that in many human tumors, their functions have been lost as a consequence of deletion, rearrangement, or mutations of both alleles; indeed, the best-characterized members of this group, represented by Rb, p53, WTI, and DCC, were first identified and isolated following pedigree and genetic analyses (34). The frequent physical or functional loss of these tumor suppressor genes in specific human malignancies was strong evidence that these changes contribute to the development of the neoplastic phenotype.

Loss of function of a particular gene may occur by a variety of mechanisms, including the repression of its expression at the RNA level, and a large number of genes whose expression is repressed either in tumors or in cells transformed by positively acting oncogenes, such as v-*ras*, v-*src*, and the simian virus 40 T-antigen gene, have been identified. This group includes genes encoding the retinoic acid receptor (20), α-actinin (13), maspin (44), interferon regulatory factor I (19), and tropomyosin (31) as well as the DAN, 322, and *rrg* genes (8, 26, 28). Several of these were identified by subtractive hybridization or differential display techniques, which allowed the identification of RNA species whose expression was reduced in transformed cells. In gene transfer experiments, these genes exhibited tumor-suppressive and cell growth arrest activities, leading to the hypothesis that the reduced expression or function of certain genes was required for the expression of the transformed phenotype.

We previously reported the characterization of a flat (nontransformed) revertant cell line, F-1, which was isolated from rat fibroblasts (DTM) transformed by the serine/threonine kinase oncogene *mos* (41). F-1 cells express high levels of v-*mos*-specific RNA and kinase activity but fail to express character-

* Corresponding author. Mailing address: NCI-FCRDC, P.O. Box B, Bldg. 469, Rm. 102, Frederick, MD 21702-1201. Phone: (301) 846-1318. Fax: (301) 846-6164.

istic transformed properties, including colony formation in soft agar and tumor formation in nude mice. Moreover, the revertants are resistant to retransformation by v-*mos* and v-*raf*, while they can be efficiently transformed by v-*ras* and, with a somewhat lower efficiency, v-*src*. The reversion and resistance to retransformation correlated with the failure of the serine/threonine kinase oncogenes v-*mos* and v-*raf* to activate the mitogen-activated protein (MAP) kinase pathway due to their inability to activate MEK-1 or MEK-2, the immediate upstream activators of MAP kinase.

Since levels of MEK and MAP kinase were not changed in the revertant cells, and since growth factors and *ras* activated MEK and the MAP kinase cascade normally, our results suggested that the reversion could be the result of mutations affecting the expression or function of genes which contribute to the activation of MEK by v-*mos* or v-*raf* or could result from the expression in the revertant cells of genes which block this activation and which are down-regulated in DTM and other transformed cells. In an attempt to identify such transcriptional changes, we used differential display analysis to compare the expression of RNA in transformed and revertant cells. In this report, we describe the identification and characterization of a novel cDNA, designated *drm* (down-regulated in v-*mos*-transformed cells), which is expressed in the F-1 revertant and normal parental rat fibroblasts but is down-regulated in rat fibroblasts transformed by several retroviral oncogenes. The *drm* cDNA shows no significant homologies to known genes in DNA databases and contains an open reading frame (ORF) capable of encoding an 184-amino-acid, cysteine-rich protein with a calculated molecular weight of 20,682. Regions of the drm protein show significant sequence homologies with the rat and human DAN (NO3) gene products (10, 28–30), which have been shown to possess tumor- and growth-suppressing activities. The *drm* gene encodes a 20.7-kDa protein recognized by a specific antiserum in phenotypically normal rat cells. This protein was not detected in v-*mos*-transformed cells. Analysis of RNA from multiple tissues of the rat and in situ hybridization experiments in adult rats indicate that *drm* expression is regulated in a tissue-specific manner. In situ analysis also indicated that *drm* RNA is predominantly expressed in terminally differentiated, nondividing cells, such as neurons, type 1 cells of the lung, and goblet cells of the intestine.

Transfection analysis demonstrates that *drm* overexpression in normal rat fibroblasts blocks cell proliferation, while cotransfection with the *ras* oncogene reverses this inhibition. Furthermore, cells overexpressing *drm* and conditionally transformed with v-*mos*-expressing Moloney murine sarcoma virus (MSV) rapidly undergo apoptosis when shifted to the nonpermissive temperature. These results suggest that *drm* represents a newly identified gene which is likely to play a role in cell growth and tissue-specific differentiation.

## MATERIALS AND METHODS

**Cell culture.** The REF-1, DTM, F-1, and ST33c rat cell lines have previously been described (40–42). DTM and ST33c cell lines were maintained at 34°C in Dulbecco modified Eagle medium (Gibco) with 5% fetal calf serum; REF-1, as well as REF-1 transformed by different oncogenes, was grown at 37°C in Dulbecco modified Eagle medium (Gibco) with 5 or 10% fetal calf serum.

**DNA and RNA analyses.** High-molecular-weight DNA was purified by standard procedures (15) and analyzed by Southern blotting (35). Total RNA was extracted from culture cells by RNAzolB (Tel-Test, Inc., Friendswood, Tex.) (7), and 10 μg was used per lane in Northern analysis. Filters were prehybridized and hybridized at 42°C for 18 to 20 h in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaH_2PO_4$, and 1 mM $Na_2EDTA$ [pH 7.4]) containing 10× Denhardt's solution (9), 2% sodium dodecyl sulfate (SDS), 50% formamide, and 100 μg of heat-denatured salmon sperm DNA per ml. The filters were washed sequentially in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.05% SDS at room temperature for 30 min and in 0.1× SSC–0.1% SDS at 50°C for 40 min. Autoradiography was for 2 to 4 days at −70°C with an intensifying screen. Poly(A)$^+$ was isolated by using a Fast Track mRNA isolation kit (Invitrogen) as specified by the manufacturer. Multitissue Northern blotting (Clontech) was done as specified by the manufacturer.

The murine recombinant retrovirus expressing v-*src* was a generous gift of S. M. Anderson. The vector expressing activated *ras* is pEJ-*ras* (38) containing the $Val^{12}$-mutated fragments of human c-*ras* in pBR322.

**Identification and isolation of *drm* cDNA.** mRNAs expressed differentially in DTM and F-1 cells were displayed as described by Liang and Pardee (25). First-strand cDNAs were synthesized on 1.5 μg of polyadenylated RNA extracted from either cell line, using a cDNA cycle kit for reverse transcription-PCR (Invitrogen) and specific primers T12VA and T12VC (V is either A, C, or G). cDNAs were then amplified by PCR using [α-$^{35}$S]dATP and combinations of 3′ specific primers and arbitrary 5′ primers (AGCCAGCGAA, GACCGCTTGT, AGGTGACCGT, GGTACTCCAC, and GTTGCGATCC). PCR products were separated on a 6% polyacrylamide gel and visualized by autoradiography.

**Screening of a cDNA library.** An oligo(dT)-primed cDNA library of rat embryo fibroblasts constructed in the λZAP XR vector was screened with the 691-bp *drm* cDNA isolated from F-1 mRNA by the differential display technique as described previously (35). Three independent clones (Cl3ZAP, Cl7ZAP, and Cl10ZAP) were isolated and further analyzed. 5′ sequences of the Cl7ZAP absent from the other clones were used as probes to screen a rat kidney 5′-stretch λgt11 cDNA library (Clontech). Two clones (Cl7gt and Cl10gt) were isolated, further amplified, and analyzed. cDNA clones were sequenced on both strands by the dideoxy-chain termination method using a T7 sequencing kit (Pharmacia Biotech) (36). Portions of the sequencing data were compiled and analyzed by using the University of Wisconsin Genetics Computer Group package (11).

**Protein analysis. (i) In vitro transcription and translation.** The 2.1-kb *Eco*RI fragment of Cl10gt and the *Bam*HI/*Kpn*I fragment from this insert, both containing the putative *drm* coding region, were inserted into the Bluescript KS vector. Plasmid DNAs were transcribed and translated by using the TNT T7 and T3 reticulocyte lysate system (Promega) with L-[$^{35}$S]cysteine (1,200 Ci/mmol; Amersham). Translation products were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and processed for fluorography. T7 polymerase produces a sense message, while T3 produces an antisense product. Luciferase DNA was used as a positive control.

**(ii) Construction of a tagged *drm* protein expression vector.** The coding region of *drm* cDNA was fused in frame at its 3′ end with the DNA fragment encoding the nine-residue epitope of influenza virus hemagglutinin (HA1) by PCR. The primers used were 5′ (5′-CCGCTCGAGGTGACAGAATGAATCGC-3′) and 3′ (5′-CCCGTTAACTTAGGCGTAGTCGGGCACGTCGTAGGGGTAATC CAAGTCGAT-3′). The 5′ primer introduces an *Xho*I restriction site, while the 3′ primer removes the stop codon from the *drm* and introduces another one downstream from the inserted HA1 sequence. It also introduces an *Hpa*I site downstream from the stop codon. The PCR product was digested with *Xho*I and *Hpa*I and inserted into the pSVL expression vector (39) between the *Xho*I and *Sma*I sites.

**(iii) Preparation and characterization of antibodies.** Two peptides based on the predicted sequence of drm protein were selected to raise rabbit polyclonal antibodies. An N-terminal cysteine residue was added to the first peptide (peptide 990), which corresponds to amino acids 79 to 92, to enable coupling of the peptide to keyhole limpet hemocyanin carrier protein prior to immunization. The second peptide, 987, corresponding to amino acids 158 to 172, was coupled to the carrier protein through a natural cysteine residue on its N-terminal end. The polyclonal antipeptide antibody 990 gave the strongest immune response and was the only one used in this study.

**(iv) Immunoprecipitation and Western blotting.** Either cell lysates prepared under denaturing conditions were first immunoprecipitated with *drm*-specific antiserum 990 or anti-HA1 monoclonal antibody (Babco), followed by separation on SDS-PAGE and Western blotting, or total lysates were analyzed by SDS-PAGE and Western blotting.

For immunoblotting, proteins were electrophoretically transferred to nitrocellulose at 60 mA for 2 h. Filters were incubated first with the appropriate primary antibody and then with horseradish peroxidase-labeled secondary antibodies (Amersham). Antibodies were detected by using the ECL detection system (Amersham) or the Super Signal CL-HRP Substrate system (Pierce) and visualized by using Kodak XAR-5 X-ray film.

Western blots were stripped for reprobing with other primary antibodies as specified by the manufacturer (Amersham).

**Transfection of *drm* expression vectors.** For stable transfection experiments, cDNA containing the full-length *drm* ORF was inserted into the *Bam*HI and *Kpn*I restriction sites of the pMEXneo expression vector (21). In this construct, *drm* and the *neo*-selectable marker were under the control of a murine leukemia virus long terminal repeat and a simian virus 40 promoter, respectively. For colony formation assays, $5 \times 10^5$ cells were overlaid with a mixture consisting of 5 μg of pMEX*drm* or pMEX and 30 μl of DOTAP (Boehringer Mannheim). After 6 h, this mixture was replaced with regular medium and the cultures were maintained for another 48 h. Cells were then split 1:3 and grown in the presence of G418 (effective concentration, 400 μg/ml; Life Technologies) for 2 weeks, and colonies resistant to G418 were counted and isolated. Growth temperatures for transfected cells were as follows: for REF-1 and CHO, 37°C; for DTM, 34°C; and for ST33c, 34 and 39°C. Transient transfections of Cos-7 cells were performed by

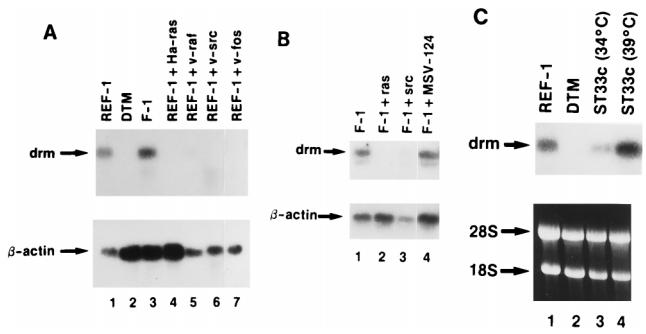


FIG. 1. Expression of the *drm* gene in normal and oncogene-transformed rat fibroblasts. (A) Northern blot of total RNA (10 μg) hybridized with *drm* cDNA and exposed to X-ray film for 3 days. Lanes: 1, REF-1 (immortalized normal rat fibroblasts); 2, DTM; 3, F-1 revertant; 4, REF-1 transformed by Ha-MSV (v-*ras*); 5, REF-1 transformed by 3611 MSV (v-*raf*); 6, REF-1 transformed by a murine retroviral construct expressing v-*src* (MRSV); 7, REF-1 transformed by FBR MSV (v-*fos*). (B) Northern blot of total RNA (10 μg) hybridized with *drm* cDNA. Lanes: 1, F-1 revertant; 2, F-1 superinfected with Ha-MSV; 3, F-1 superinfected with MRSV; 4, F-1 superinfected with MSV-124. (C) Northern blot of total RNA (10 μg) was hybridized with *drm* cDNA. Lanes: 1, REF-1 at 34°C; 2, DTM at 34°C; 3, ST33c constantly maintained at 34°C; 4, ST33c at 39°C (after 48 h). Levels of a β-actin probe (A and B) and ethidium bromide staining of rRNA (C) confirmed similar loading of the lanes.

using the pSVL vector expressing a HA1-tagged *drm* and LipofectAMINE (Life Technologies, Gaithersburg, Md.) as specified by the manufacturer.

**In situ hybridization.** Tissues from Sprague-Dawley rats were processed and analyzed by in situ hybridization as described by Sassoon and Rosenthal (37). A nonradioactive riboprobe containing 1.9 kb of the 3′ end of *drm* was generated by using a digoxigenin RNA labeling kit (SP6/T7; Boehringer Mannheim), and the concentration of the labeled probe was determined by using a SIG nucleic acid detection kit (Boehringer Mannheim). Detection was performed by using an antidigoxigenin antibody conjugated with alkaline phosphatase (nucleic acid detection kit; Boehringer Mannheim). Sections were counterstained with methyl green (1%) and mounted in aqueous mounting medium (Signet Laboratories). Analysis was performed on a Nikon Labophot 2 microscope.

**Analysis of apoptosis.** ST33c cells were transfected with the control vector or with the vector containing *drm* at 34°C, and pools of G418-resistant colonies were selected, expanded, and analyzed for expression of *drm*-specific mRNA. ST33c cells expressing *drm* were shifted to 39°C for 24 h, and cells were fixed in 3.7% formaldehyde in phosphate-buffered saline (10 min, room temperature), washed three times, stained in 4′,6-diamidino-2-phenylindole (DAPI) (10 min, room temperature), and examined with a Nikon inverted microscope under UV illumination. DNA fragmentation analysis was performed as previously described (1).

**Nucleotide sequence accession number.** The *drm* sequence has been assigned GenBank/EMBL accession no. Y10019.

## RESULTS

**Identification of an mRNA expressed in revertant cells but repressed in v-*mos*-transformed rat fibroblasts.** To identify genes expressed in F-1 revertant cells but not in v-*mos*-transformed parental cells (DTM cells), we performed differential display analysis (25) using oligo(dT)-selected RNA isolated from rapidly growing DTM and F-1 cells. Eight cDNAs differing in intensity between DTM and F-1 mRNAs were identified and used to probe Northern blots containing poly(A)$^+$ RNA from DTM and F-1 cells. Only one exhibited differential mRNA expression; this probe detected a 4.4-kb RNA expressed in F-1 cells but absent in DTM cells (Fig. 1A, lanes 2 and 3). Analysis of this cDNA, designated *drm*, revealed a 691-bp sequence, which included a consensus polyadenylation signal (AATAAA) located 20 bp upstream from the poly(A) tail, as well as the 5′ and 3′ primers used for PCR. A search of nucleotide sequences compiled in the GenBank database showed no significant similarities to known genes.

**Repression of *drm* mRNA expression following cell transformation.** To establish a correlation between repression of *drm* gene expression and the transformed cell phenotype, we analyzed the hybridization of *drm* cDNA to RNA from normal and transformed REF-1 cells. We found that *drm* was expressed at similar levels in REF-1 and revertant F-1 cells, but its expression was completely repressed in REF-1 cells transformed by the v-*ras*, v-*raf*, v-*src*, and v-*fos* oncogenes (Fig. 1A). These results demonstrated that repression of *drm* expression was not restricted to transformation induced by v-*mos*.

Since the initial identification of *drm* was based on its expression in the F-1 revertant, and we had previously shown that F-1 cells could be transformed by v-*ras* and v-*src*, we analyzed the effect of expression of these oncogenes in F-1 cells on *drm* expression. F-1 cells expressing and transformed by v-*ras* and v-*src* did not contain *drm* transcripts detectable by Northern blot analysis (Fig. 1B, lanes 2 and 3); in contrast, F-1 cells infected with the v-*mos*-expressing MSV-124 show levels of *drm* RNA essentially identical to those in uninfected F-1 cells or REF-1 parental cells (Fig. 1B, lane 4). Since we had previously shown that superinfection of F-1 cells with additional copies of v-*mos* did not induce transformation (41), these results are consistent with the hypothesis that *drm* expression is down-regulated following oncogene-mediated transformation.

To further analyze the correlation between *drm* expression and the transformed phenotype, we used REF-1 cells transformed by a temperature-sensitive (*ts*) isolate of Moloney MSV (*ts*110) (3). These cells (ST33c) are transformed at 34°C but express a phenotypically normal, nontransformed phenotype at 39°C (42). Analysis of RNA extracted from cells maintained at both temperatures indicated that *drm* RNA was synthesized at 39°C in the absence of the v-*mos* protein and was markedly decreased at 34°C (Fig. 1C, lanes 3 and 4). Taken together, these results further indicate that in REF-1 cells, repression of the *drm* RNA expression correlates with the transformed phenotype. The results with *ts* MSV-transformed cells and the F-1 revertant indicate that *drm* expression is directly or indirectly modulated by the v-Mos oncoprotein and its transforming functions.

***drm* is a novel gene.** To fully characterize the *drm* gene and its product, we screened rat fibroblast and rat kidney cDNA libraries and isolated five independent overlapping cDNA clones, which covered ~3,820 bp of *drm* mRNA (Fig. 2). Southern blot analysis (data not shown) indicated that the *drm* sequence is derived from a single gene spanning at least 12 kb and is not rearranged either in DTM, which does not express *drm*, or in the F1 revertant.

The cloned cDNA of 3,820 nucleotides is shorter than the RNA identified in REF-1 cells, suggesting that the isolated clone may not include the entire *drm* mRNA sequence. However, this cDNA does contain a single long ORF beginning at nucleotide 130 and terminating with an in-frame stop codon at nucleotide 693. Translation is predicted to start at the first in-frame methionine at nucleotide 139 within a favorable translation initiation context (A at −3, C at −4, G at −6, and A at +4) (22, 23). Thus, the characterized *drm* cDNA consists of 138 bp of 5′ untranslated sequence (65% GC), a 552-bp coding region, and 3,130 bp of 3′ untranslated sequence containing a consensus polyadenylation signal, AATAAA, located 21 nucleotides upstream from the poly(A) tail.

The major ORF contained in the *drm* cDNA would be predicted to encode a 184-amino-acid polypeptide with a calculated molecular weight of 20,682. The presumptive *drm* gene product is highly basic (7.61% arginine, 8.7% lysine, and 2.17% histidine), with the $NH_2$-terminal half containing a leucine-rich hydrophobic domain located between amino acids 4 and 24, whereas the carboxy-terminal moiety is characterized by the presence of nine cysteines. The presence of an amino-terminal

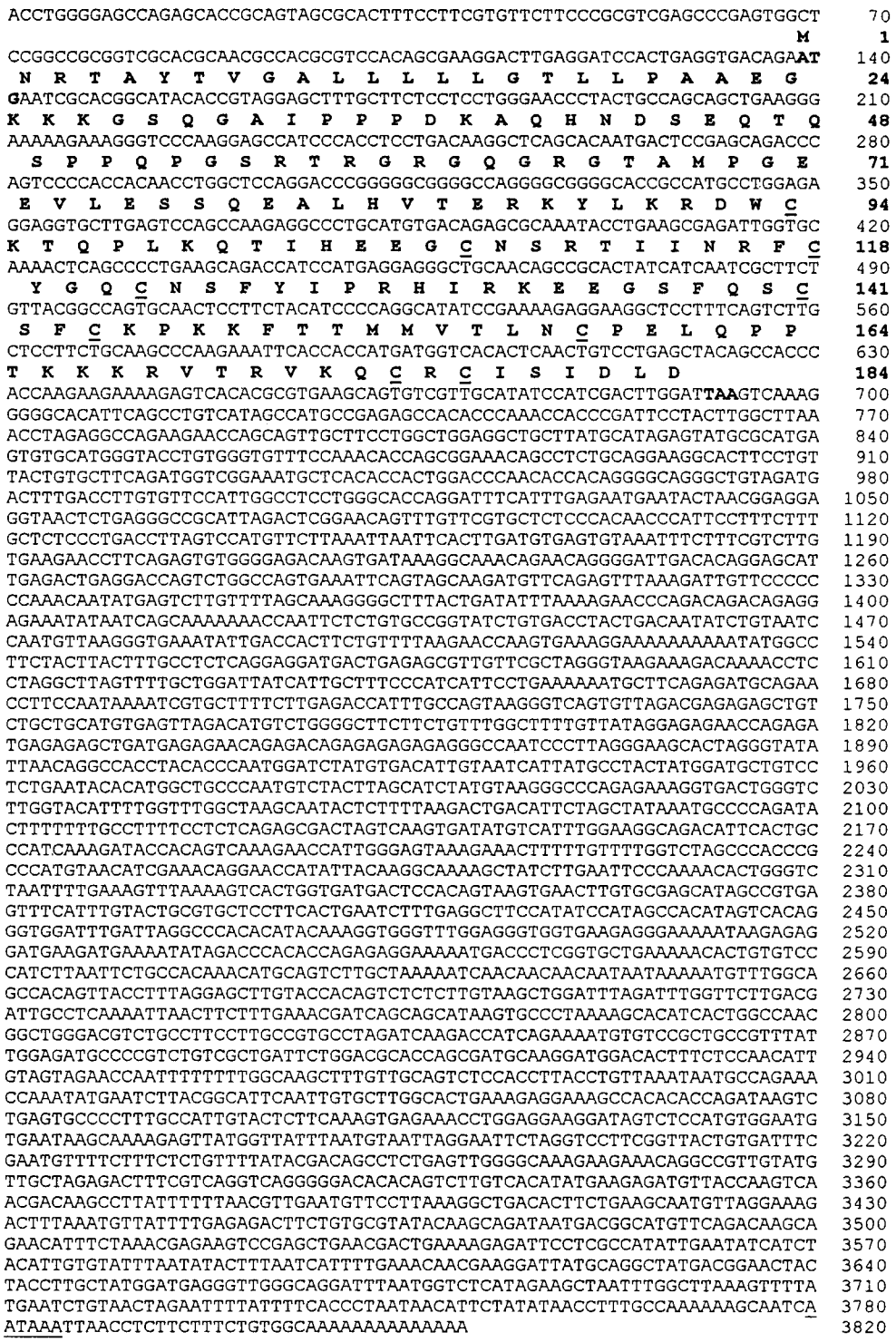


FIG. 2. Nucleotide and deduced amino acid sequences of the *drm* cDNA. The ATG and TAA are in boldface. The polyadenylation signal is underlined, as are the cysteine residues making up the potential motif described in the text.

hydrophobic domain suggested a possible membrane localization of the protein, and analysis of the drm deduced amino acid sequence by using the TMbase database of transmembrane proteins (Lausanne, Switzerland) indicated a high probability that this protein could form a transmembrane helix in this region. Examination of the predicted sequence also identified two potential nuclear localization signals which fulfill the motif K(R/K)X(R/K) (KPKK [amino acids 145 to 148] and KKKR [amino acids 166 to 169]), two protein kinase C phosphorylation sites (TER [amino acids 84 to 86] and TKK [amino acids

```
DRM   93 WCKTQPLKQTIHEEGCNSRTIINRFCYGQCNSFYIPRHIRKEEG  136
DAN   29 WCEAKNITQIVGHSGCEAKSIQNRACLGQCFSYSVPNTFPQSTE   72
CER  168 ACKTLPFTQNIVHENCDRMVIQNNLCFGKC----ISLHVPNQQD  207
MUC2 879 PCSTVPVTTEVSYAGCTKTVLMN-HCSGSCGTF-VMYSA-KAQA  919

DRM  137 SFQSCSFCKPKKFTTMMVTLNCPEL-QPPTKKKRVTRVKQCRC   178
DAN   73 SLVHCDSCMPAQSMWEIVTLECPGHEEVPRVDKLVEKIVHCSC   119
CER  208 RRNTCSHCLPSKFTLNHLTLNCTGSKNVVKV---VMMVEECTC   247
MUC2 920 LDHSCSCCKEEKTSQREVVLSCPNG-GSLTHT--YTHIESCQC   959
```

FIG. 3. Comparison of the amino acid sequences of the cysteine-rich regions of drm, DAN, CER, and MUC2 proteins. Identical or similar residues are indicated in boldface; hyphens indicate gaps.

165 to 167]), and three cyclic AMP and cyclic GMP-dependent protein kinase phosphorylation sites (KKGS [amino acids 26 to 29], KKFT [amino acids 147 to 150], and KRVT [amino acids 168 to 171]).

Comparison of the drm amino acid sequence to entries in the GenBank and EMBL databases by using FASTA program showed that the drm protein exhibits an overall similarity of 30% with the rat and human DAN gene products, which express tumor-suppressive properties (28, 29). Using the BLAST program, we detected a 52% similarity between the carboxy-terminal cysteine-rich half of drm, the central region of the DAN protein, and the carboxy-terminal region of the *Xenopus* protein Cerberus (CER), a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer (4). Further analysis also revealed similarity to the carboxy-terminal cysteine-rich end of the human intestinal mucin MUC2 (16) (Fig. 3). Interestingly, the nine cysteines of the drm are also present in DAN, CER, and MUC2 proteins at similar amino acid intervals. This alignment generated the cysteine motif $CX_{13}CX_{8-9}CX_3CX_{14-18}CX_2CX_{13}CX_{15-18}CXC$. Within this motif, several amino acids are conserved, suggesting that proteins containing this domain could be members of a related family.

**Characterization of the *drm* gene product.** In vitro transcription and translation of the ORF-containing 2.1-kb *Eco*RI fragment and 730-bp *Bam*HI/*Kpn*I fragment of *drm* cDNA confirmed that the presumptive ORF could express a protein of approximately the expected size (data not shown). To further characterize the *drm* product, we generated an antipeptide polyclonal rabbit antibody directed against amino acids 79 to 92 of the rat drm protein. To assess the specificity of the antiserum, we constructed an expression vector synthesizing an HA1 epitope-tagged drm protein by introducing a DNA fragment encoding the HA1 epitope at the 3′ end of the coding region. The pSVL expression vector containing this fusion was used to transfect Cos-7 cells; cell lysates were prepared 48 h later, immunoblotted on a nitrocellulose filter, and incubated with the drm antiserum. We detected a band with a predicted molecular mass of ~21.4 kDa (Fig. 4A, lane 2), and the same band was revealed with the monoclonal antibody against the HA1 tag (Fig. 4A, lane 6). It was not detected when lysates were exposed to antiserum 990 preincubated with peptide against which this antiserum was raised (Fig. 4A, lane 4) or in lysates of cells transfected with an empty vector (Fig. 4A, lane 1). A protein of the same molecular mass was detected in HA1-*drm*-transfected Cos-7 lysates immunoprecipitated with antiserum 990 and blotted with anti-HA1 serum, and this precipitation could be blocked by the homologous 990 peptide (data not shown).

To identify the endogenous drm protein, we analyzed total lysates from various cells by Western blotting (Fig. 4B). We detected low levels of a 20.7-kDa protein in primary embryonic rat fibroblasts and in REF-1 cells (Fig. 4B). Analysis of drm

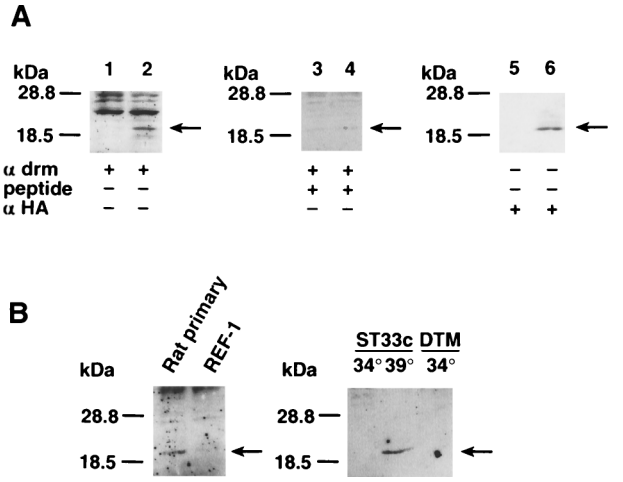


FIG. 4. Expression of HA1-tagged and endogenous drm proteins. (A) Characterization of HA1-tagged drm protein. Lysates of Cos-7 cells transfected with the pSVL expression vector alone (lanes 1, 3, and 5) or with HA1-tagged *drm* cDNA (lanes 2, 4, and 6) were separated by SDS-PAGE and transferred to a nitrocellulose filter. The same filter was probed sequentially with the antibodies against drm (lanes 1 and 2), drm antibodies preincubated with homologous competing peptide (lanes 3 and 4), and HA1-tagged specific antibodies (lanes 5 and 6). In each case, the filter was stripped (Materials and Methods) prior to probing with the next antibody. (B) Detection of endogenous drm protein in different cells. Lysates from the indicated cells were analyzed for drm expression by Western blot analysis as described in Materials and Methods. ST33c and DTM lysates were prepared from cells grown at either 34 or 39°C, as indicated. The positions of the molecular weight markers are indicated.

protein expression in ST33c cells, conditionally transformed by v-*mos*, showed good correlation with *drm*-specific RNA expression. The protein was not detected in lysates of transformed cells at 34°C (Fig. 4B) but could be seen in cell lysates prepared 48 h after the cultures were shifted to the nonpermissive temperature (Fig. 4B). We could not detect drm protein in lysates of v-*mos*-transformed DTM cells (Fig. 4B).

***drm* RNA is expressed in a tissue-specific fashion in adult rats.** To further characterize the *drm* gene and its possible function, we examined the expression pattern of *drm* in rodent tissues. Northern blot analysis of poly(A)$^+$ RNA extracted from adult rat tissues (Sprague-Dawley) showed that the *drm* gene was expressed in the brain, kidney, spleen, testis, and lung but not in the heart and skeletal muscle (Fig. 5). Highest levels

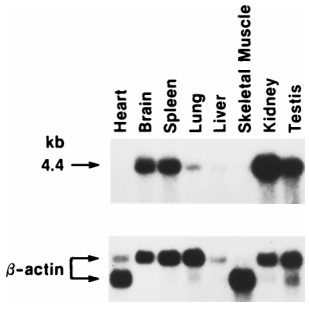


FIG. 5. Expression of the *drm* gene in normal rat tissues. Multitissue Northern blot analysis of poly(A)$^+$ RNA from rat tissues. A multitissue poly(A)$^+$ RNA Northern blot (Clontech) from adult rat tissues was hybridized with a $^{32}$P-labeled 1.9-kb *drm* cDNA fragment. After the initial 3-day exposure, the probe was stripped off and the blot was rehybridized with a human β-actin probe and exposed to X-ray film for 20 h. The position of *drm* is indicated as a 4.4-kb transcript.

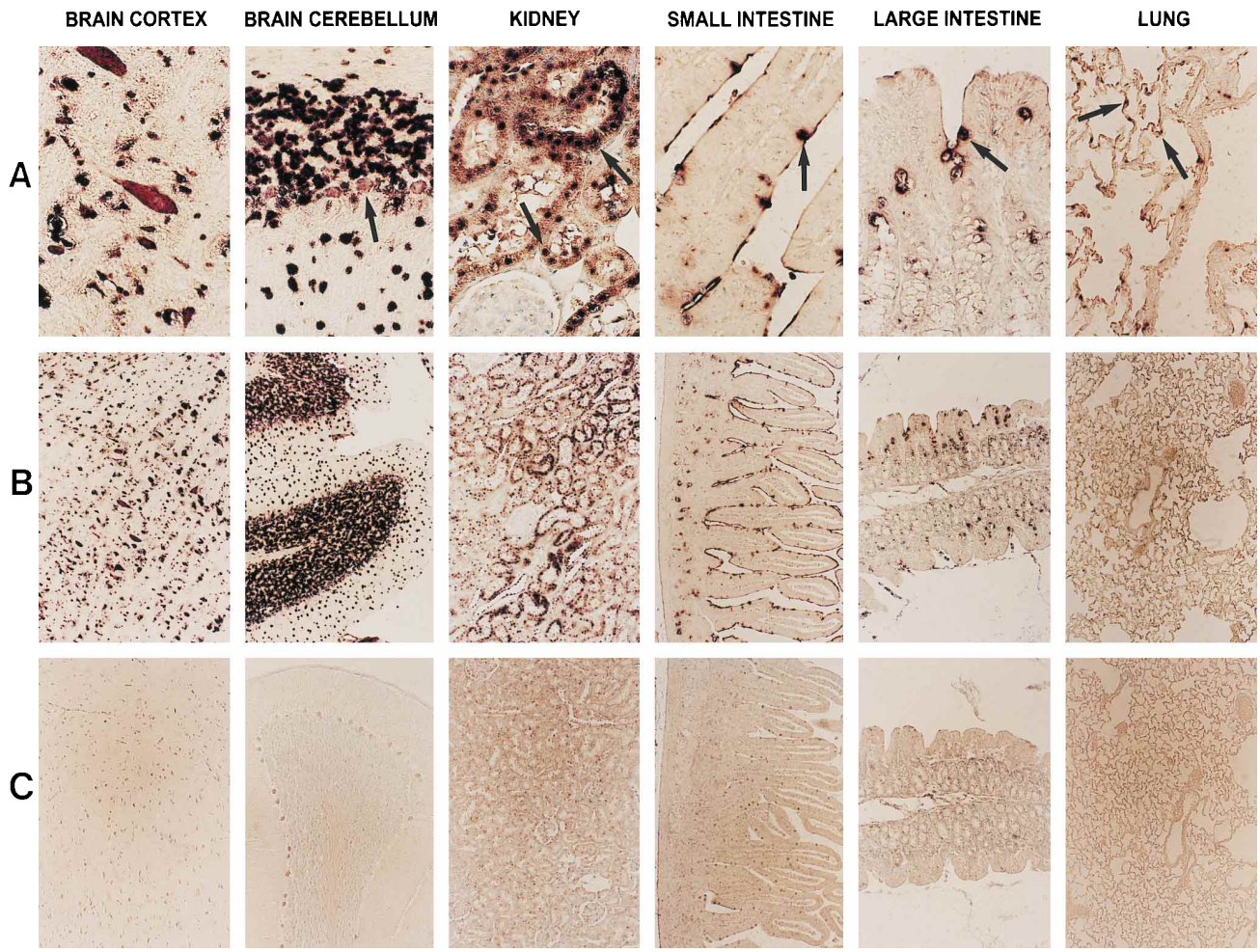


FIG. 6. In situ analysis of *drm* expression in rat tissues. Adjacent sections (5 μm) were hybridized with a *drm* riboprobe in antisense (A and B) and sense (C) orientations. (A) High magnification. Arrows show the positions of specific features discussed in the text. (B) Lower magnification showing an overview of the tissues. (C) Negative control; same magnification as panel B.

were seen in the kidney, testis, brain, and spleen; levels in the liver and lung were significantly lower.

To investigate whether *drm* expression was specific for any particular cell type, we analyzed tissues from the same strain of rat by in situ hybridization using sense and antisense *drm* riboprobes (Fig. 6). In situ expression patterns in general correlated well with the Northern analysis, but interestingly, we found that *drm* RNA appeared to be predominantly expressed in differentiated cells (e.g., neurons in brain, type 1 cells in lung, and goblet cells in intestine). In all cases, the control sense probe showed no detectable hybridization (Fig. 6C).

The brain exhibited ubiquitous expression of *drm* RNA. High levels of *drm* expression were found in both neurons and glial cells of the brain cortex; in the cerebellum, *drm* RNA was strongly expressed in all cells of molecular and granular layers. Its expression was significantly weaker in Purkinje cells (Fig. 6A).

In the kidney, *drm* RNA was found in epithelial cells of the proximal and distal tubules in the cortex, medullae, and papillae. Very strong signals appeared to be localized in the nuclei of the epithelial cells (Fig. 6A).

In the small and large intestine, the *drm* gene was predominantly detected in goblet cells, and specifically in the most differentiated goblet cells (on the tip of the villi in the small intestine and the base and neck of the crypt in the large intestine) (Fig. 6A). However, some goblet cells in the crypt of the small intestine were also found to be positive for *drm* expression.

In the lung, *drm* expression was localized to the nuclei of type 1 epithelial cells lining the alveoli (Fig. 6A). Type 1 cells are known to be terminally differentiated from their precursor type 2 cells (6). *drm* was not expressed in every type 1 cell, which could indicate a possible correlation of *drm* expression with the stage of cell differentiation. A few endothelial cells of the airways and a number of macrophages also expressed *drm* RNA; in the spleen, *drm* RNA was detected only in megakaryocytes. In agreement with the results of Northern blot analysis, we could not detect *drm* hybridization in the liver, heart, and skeletal muscle (data not shown).

***drm* blocks colony formation by normal but not transformed cells.** To determine the biological effect of *drm* overexpression in vivo, a portion of the *drm* cDNA containing the full-length ORF was inserted into the *neo*-containing pMEX expression vector (21). This construct, as well as the empty vector, was introduced into REF-1 and DTM cells, and G418-resistant colonies were counted after 2 to 3 weeks. Our results indicated that colony formation was inhibited 30-fold when REF-1 cells were transfected with the *drm* expression vector (Fig. 7). *mos-*

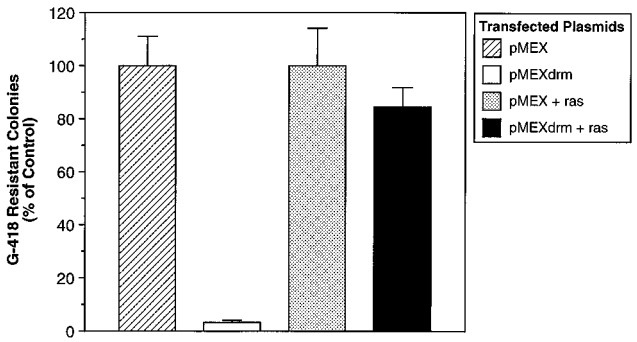


FIG. 7. Effects of *drm* alone and together with activated *ras* on the growth of REF-1 cells. REF-1 cells were transfected or cotransfected with the indicated plasmids. Following 3 weeks of selection in G418, macroscopic colonies were counted. Results are expressed as percentages of colony formation in cultures transfected with control vector lacking the *drm* insert and are the means of three or more independent experiments.

transformed DTM cell colony formation was not affected (data not shown). Results were similar for CHO cells, indicating that inhibition of colony formation is not specific to REF-1 cells. Analysis of independent, *drm*-transfected G418-resistant clones of REF-1 cells showed that all surviving clones expressed very low or undetectable levels of exogenous *drm* mRNA (Fig. 8A, lanes 3 to 6), suggesting that survival may select for cells expressing low levels of *drm*. In contrast, DTM cells, which showed no inhibition of colony formation, exhibited high levels of exogenous *drm* expression (Fig. 8A, lane 1). In some cases, expression of endogenous *drm* RNA was also increased in DTM cells expressing exogenous *drm*, suggesting a possible autoregulation loop of *drm* expression.

Since oncogene-transformed stable cell lines had shown down-regulation of *drm* expression (see above), we further investigated the interactions between transforming oncogenes and *drm* by cotransfecting REF-1 and CHO cells with *drm* and the activated (38) *ras* oncogene. Consistent with our previous results with DTM cells, cotransfection of *drm* with the *ras* oncogene did not suppress morphological transformation. However, cotransfection of *ras* with *drm* reversed the *drm*-dependent inhibition of colony formation both in REF-1 cells (84% of the control [Fig. 7]) and in CHO cells (data not shown). The level of exogenous *drm* RNA in five of six G418-resistant clones cotransfected with pMEX*drm* and *ras* was increased (Fig. 8B). These data are consistent with the hypothesis that high levels of *drm* inhibit the growth or viability of normal cells but that transformed cells are resistant to this inhibitory effect.

**Conditionally transformed cells expressing exogenous *drm* undergo apoptosis at the nonpermissive temperature.** Since transfection of nontransformed rat and hamster cells with *drm* expression vectors leads to the inhibition of cell growth, we could not obtain stable cell lines expressing high levels of *drm* for molecular and biological analysis. To overcome this problem, we used conditionally transformed ST33c cells to investigate the effects of *drm* overexpression. As shown in Fig. 9A, when v-*mos* is functional (34°C) and ST33c cells are transformed, transfection of the pMEX*drm* vector does not affect the efficiency of colony formation in comparison to the control vector. These results are consistent with our data for DTM cells and for REF-1 cells cotransfected with pMEX*drm* and *ras* (Fig. 7), showing that the presence of transforming oncogene blocks the inhibitory effect of *drm*. In contrast, at 39°C the percentage of colonies that survived following pMEX*drm* transfection was significantly lower than that observed in control vector-transfected ST33c cells (Fig. 9A).

To analyze how *drm* overexpression blocks cell growth and colony formation, G418-resistant colonies of transfected ST33c cells were isolated at 34°C and tested for the expression of *drm*. Pools of G418-resistant cells expressed elevated levels of *drm* RNA similar to those seen in transfected DTM (Fig. 8A, lane 1) or *ras*-transformed (Fig. 8B) cells. These transfected pools grew like the parental ST33c cells at 34°C, when v-*mos* is expressed, but rapidly lost viability after a shift to 39°C, and colony-forming ability was significantly reduced. This finding is consistent with the fact that, as previously shown, v-*mos* is not expressed in these cells at 39°C and thus cannot neutralize the effects of the high level of exogenous *drm* in these cells. The morphological changes seen in these cells at 39°C, including cell shrinkage, cell membrane blebbing, and loss of cell-cell contact and adhesion to the substrate, resemble those of cells undergoing apoptosis. Furthermore, *drm*-expressing ST33c cells exhibited nuclear fragmentation and condensation within 24 h of a shift to 39°C, while no such fragmented nuclei were observed in these cells cultured at 34°C or in REF-1 cells cultured at either 34 or 39°C (Fig. 9B). We observed that 15 to 30% of the ST33c cells expressing *drm* at 39°C exhibited fragmented, condensed nuclei, while only 5 to 6% of the control ST33c cells manifested similar changes following a shift to 39°C. DTM cells transfected with *drm* and containing two copies of v-*mos* (*ts* and wild-type v-*mos*) also showed 5 to 7% fragmented nuclei at 39°C, which could represent the background level for *ts* v-*mos*-transformed cells shifted to 39°C. Apoptosis of *drm*-expressing ST33c cells at 39°C was also confirmed by agarose gel electrophoresis of genomic DNA, which showed significant fragmentation only in the cells shifted to 39°C (Fig. 9C). Furthermore, the proportion of cells undergoing apoptosis was seen to correlate with the level of *drm* expression in a series of individual clones of ST33c cells trans-

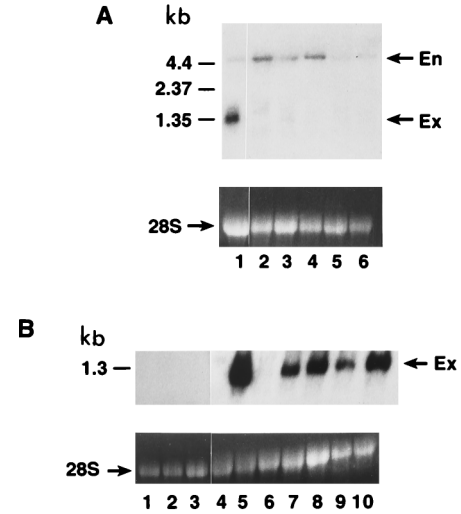


FIG. 8. Northern blot analysis of *drm* transcripts in REF-1 cells transfected with *drm* alone (A) or cotransfected together with activated *ras* (B). (A) DTM (lane 1) and REF-1 (lanes 3 to 6) cells were transfected with the pMEX*drm* (the pMEX expression vector containing the *drm* insert). As a control, REF-1 cells were transfected with vector alone (lane 2). Arrows show the positions of endogenous (En) and exogenous (Ex) *drm*. (B) REF-1 cells were cotransfected with the pMEX expression vector and the vector containing activated *ras* (lanes 1 to 4) or with *ras* and pMEX*drm* (lanes 5 to 10). An arrow shows the position of exogenous (Ex) *drm*. Hybridization of Northern blots was performed with a radiolabeled *drm* probe, and ethidium bromide-stained 28S rRNA was used to indicate the amount of RNA loaded in each lane.

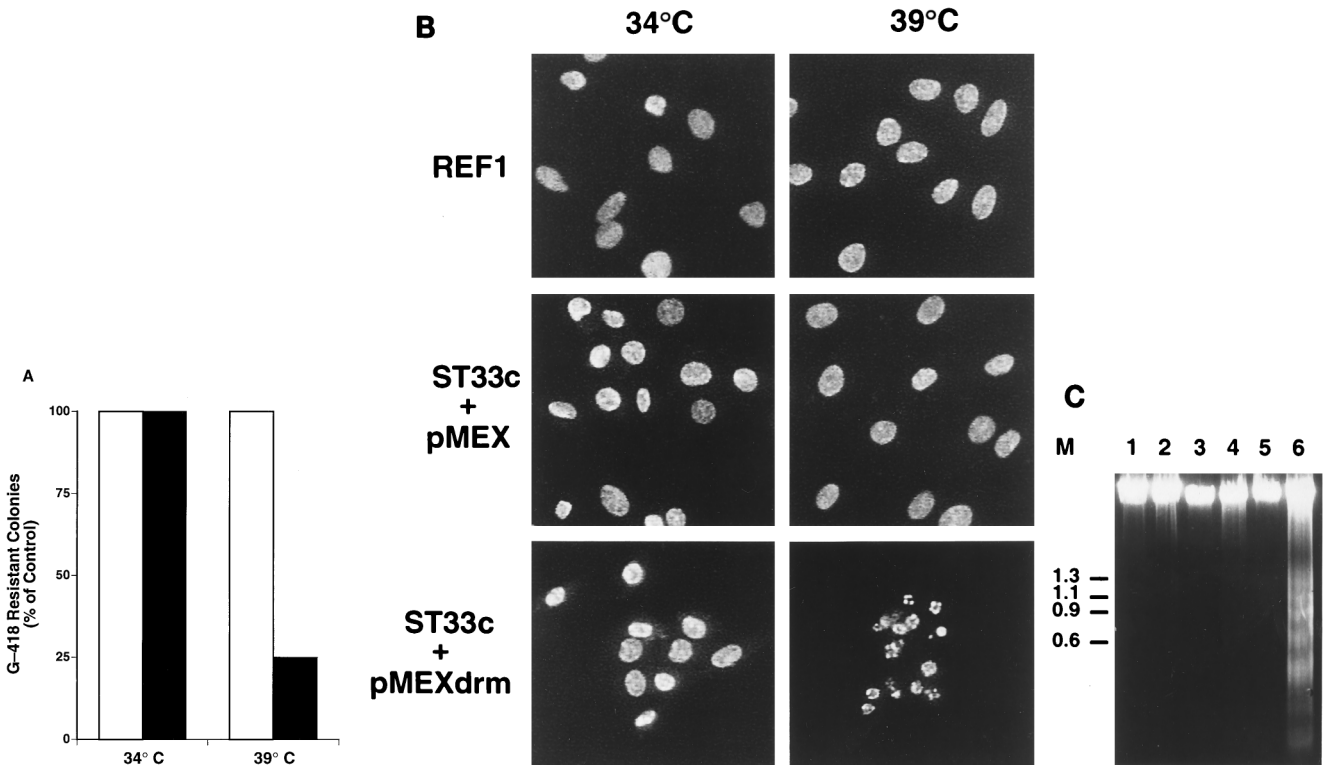


FIG. 9. Analysis of conditionally transformed ST33c cells transfected with the expression vector pMEX*drm*. (A) ST33c cells were transfected with the vector pMEX (□) or pMEX containing the *drm* insert (■) at 34 or 39°C. Results are expressed as percentages of G418-resistant colony formation in cultures transfected with control vector and are the means of two independent experiments. (B) DAPI staining of transfected ST33c and untransfected REF-1 cells. ST33c cells were transfected with the indicated constructs and selected at 34°C for G418 resistance. Pools of colonies were plated at 34°C and then shifted to 39°C. Twenty-four hours after the shift, cells were fixed and stained as described in Materials and Methods and examined with a Nikon inverted microscope under UV illumination. (C) Fragmentation of genomic DNA in cells shifted at 39°C. Purified genomic DNA was fractionated in a 1.5% agarose gel. Lanes: 1 and 2, DNA from REF-1 cells at 34 and 39°C, respectively; 3 and 4, DNA from ST33c cells transfected with the control vector at 34 and 39°C, respectively; 5 and 6, DNA from ST33c cells expressing *drm* at 34 and 39°C, respectively. Positions of molecular weight markers (M; *Hin*dIII-digested λ DNA and *Hin*cII-digested ϕX174 DNA) are indicated in kilobases.

fected with *drm* (data not shown). Taken together, our data suggest that cells expressing high levels of *drm* undergo apoptotic death in the absence of oncogene-induced transformation.

## DISCUSSION

We have used differential display analysis of RNA to screen for genes either up- or down-regulated in a revertant (F-1) of *mos* oncogene-transformed rat embryo fibroblast cells (DTM cells). We have identified a gene, *drm*, whose expression was high in the revertant cell line and REF-1, the nontransformed parent of the DTM cell line, but was not detectable in DTM cells or in REF-1 cells transformed by either *ras* or *src*. We have cloned 3.8 kb of the *drm* cDNA which can encode a 184-amino-acid protein and shown that introduction of a drug-selectable construct containing this sequence into nontransformed cells significantly reduced their colony-forming ability. In contrast, cotransfection of normal cells with *drm* and *ras*, or transfection of *drm* into stably transformed cells, generated colonies with efficiencies essentially equivalent to those seen with vector controls. Cells conditionally transformed by v-*mos* and expressing high levels of transfected *drm* grew stably at the permissive temperature but rapidly underwent apoptosis when cells were shifted to the nonpermissive temperature. Furthermore, the expression pattern of *drm* seen upon in situ hybridization in rat tissues suggested that the *drm* expression occurs in nonproliferating, terminally differentiated cells (Table 1). Taken together, these results demonstrate that *drm* represents a previously unidentified gene and suggest that *drm* functions in the regulation of cell growth or viability.

The *drm* cDNA contains a 552-bp ORF capable of encoding a 20.7-kDa protein, and an antibody raised against a peptide contained in this predicted sequence recognizes a protein of similar size in cells expressing the *drm* message, including primary rat fibroblasts, REF-1 cells, and *ts* v-*mos*-transformed ST33c cells at the nonpermissive temperature. This specific protein was not detected in DTM cells and other transformed cells which did not express *drm*-specific RNA. Analysis of the 184-amino-acid predicted protein sequence encoded by the 552-bp ORF reveals several potential structural features, including a potential transmembrane domain, possible nuclear localization signals, and multiple potential sites for phosphor-

TABLE 1. *drm* is preferentially expressed in terminally differentiated cells

| Tissue | Cell type | Proliferation/ differentiation |
|---|---|---|
| Brain | Neuron | None/terminally |
| | Glial | Low/differentiated |
| Kidney | Tubular epithelial | Low/differentiated |
| Lung | Type 1 epithelial | None/terminally |
| Intestine | Goblet | Low/differentiated |
| Spleen | Megakaryocyte | Differentiated |

ylation by protein kinases, whose functional significance requires further analysis. A domain of homology shared by drm, the tumor suppressor DAN (NO3), the morphogenic protein CER, and the human intestinal mucin MUC2 was also noted. This domain contains a well-conserved array of nine cysteines, with only small differences of spacing between cysteines 4 and 5 and between cysteines 7 and 8. The fact that this motif is found in only four proteins suggests that it is highly specific and may represent a novel structural domain. It is also possible that these apparently unrelated proteins are derived from a common ancestral gene or interact with related or identical partners.

In addition, the homology between drm and DAN is interesting in view of the fact that drm was initially identified on the basis of its down-regulation in transformed cells, which was suggestive of a potential tumor-suppressing activity. Expression of *drm* RNA and protein correlated with the nontransformed phenotype in REF-1 and the F-1 revertant of *mos*-transformed cells and was seen in conditionally transformed cells at the nonpermissive temperature. The observations that (i) *drm* expression was down-regulated by oncogenes, such as v-*raf*, v-*mos*, v-*src*, and v-*ras*, which are known to activate MAP kinase and (ii) *drm* is up-regulated in the F-1 revertant in which *mos*-dependent activation of MAP kinase is blocked suggest that *drm* may be regulated by the MAP kinase cascade. This is also consistent with our observation that transformation by activated MKK or v-*fos*, the viral homolog of *fos* and a known downstream target of the MAP kinase cascade, also down-regulated *drm* (data not shown), supporting the hypothesis that the MAP kinase cascade is involved in *drm* regulation. Several tumor suppressor genes are known to be down-regulated in response to transformation by specific oncogenes. In particular, DAN (28) is down-regulated by v-*src*, v-*mos*, and v-*raf* but not v-*ras*, while 322 (26) is down-regulated by v-*src*, v-*ras*, v-*fos*, and v-*myc* but not v-*raf* and v-*mos*. Drm, however, exhibited a unique regulation pattern which nevertheless is consistent with that seen in other transformation-sensitive genes.

Although analysis of the predicted drm peptide sequence failed to reveal its biological function, the results of in situ hybridization in normal rat tissue, together with the effects of introducing *drm*-expressing constructs into cells in culture, suggest a possible role for *drm* in the control or maintenance of cell growth and viability. We observed high levels of expression in terminally differentiated, nondividing cells of specific tissues of adult rat (Table 1), such as goblet cells of the colon and the small intestine. Goblet cells, which have a unique morphology and can be easily identified, enter the proliferative zone of the crypt, differentiate as they migrate up the villus, and undergo apoptosis as they reach the top (14, 17). The majority of *drm* expression is seen in goblet cells on the neck and top of the villus, corresponding to differentiated cells and cells undergoing apoptosis. Similarly, correlations between *drm* expression and differentiation were also seen in the lung, where the *drm* message was seen in the nuclei of type 1 cells, epithelial cells lining the alveoli which are known to be terminally differentiated from their precursor type 2 cells (6). Not every type 1 cell expresses *drm*, suggesting a possible correlation with the stage of differentiation, and significantly, we failed to detect *drm* expression in proliferating type 2 precursor cells. Similarly, *drm* expression in the brain was seen in terminally differentiated neurons, while in the kidney, *drm* RNA was detected in epithelial cells of the proximal and distal tubules, which are present in a differentiated state, with only 0.1% of these cells dividing at any given time (2, 32). These findings are highly suggestive that in multiple rat tissues, *drm* expression is seen in terminally differentiated, nondividing cell populations.

This pattern seen in situ seems even more interesting in view of the fact that the introduction of *drm* expression constructs into normal REF-1 cells significantly reduced their colony-forming ability, indicating an effect on cell growth or viability. This inhibiting effect of *drm* in normal cells could be overcome by cotransfection with the *ras* oncogene, and no effect of *drm* transfection was seen in cells stably transformed with either v-*mos* or *ras*. While the precise mechanism of *drm* inhibition on REF-1 colony formation has not been determined, we were able to use cells conditionally transformed by v-*mos* to analyze the effects of *drm* overexpression on normal cell growth and viability. These cells, which expressed high levels of *drm* and proliferated normally at the permissive temperature, underwent apoptosis within 24 h following a shift to the nonpermissive temperature.

The results with cells conditionally expressing the v-*mos* oncogene suggest that the transforming function is able to inactivate or block the apoptotic response to *drm*. This type of protective effect in response to oncogene expression may be a common response, and it has been shown that *src* transformation involves the up-regulation of nr-13, a new member of the *bcl* family (12) which protects cells from apoptosis. Furthermore, studies of transformation by DNA tumor viruses showed that the 19-kDa adenovirus E1B transforming protein plays a direct role in the suppression of apoptosis induced by E1A and that its effects resemble those of the proto-oncogene product Bcl-2 (5, 27, 33, 43). Alternatively, the level of *drm* expression in v-*mos*- and *ras*-transformed cells may be insufficient to interfere with cells in which proliferation is driven by these oncogenes. The experimental model allowing the conditional expression of the apoptotic response in transformed cells should prove useful in dissecting the mechanism of *drm* function in growth control and apoptosis.

Taken together, our in vitro and in vivo results suggest that high levels of *drm* expression lead to a loss of growth potential and/or apoptosis, which is normally associated with terminal differentiation, and that these effects can be mediated by interactions between *drm* and the proteins or genes involved in these processes. Experiments are in progress to investigate the effects of *drm* expression on the functions of genes known to be involved in growth control. Furthermore, our results demonstrating down-regulation of *drm* in cells transformed by dominant oncogenes suggest a possible role for *drm* in the oncogenic transformation of specific cell lineages. We hypothesize that oncogene expression in differentiating cells expressing or potentially expressing *drm* leads to *drm* down-regulation, in order to abrogate the apoptotic response and allow progression toward malignancy. Studies are in progress to determine if this model is consistent with analyses of specific human neoplasms.

## ACKNOWLEDGMENTS

L.Z.T. and M.M. made equal contributions to this work.

We are grateful to Terry Copeland (SAIC) for preparing the peptides, Draginja Djurickovic (LASP, SAIC) for preparation of the antisera, and Karen Cannon for preparation of the manuscript. We thank G. Craescu and F. Gibrat for help with drm protein analysis. N.N.B. expresses sincere appreciation to Emily Garabedian for sharing expertise in the analysis of intestine and to M. Moxley and R. Fleming for help in the analysis of lung.

A portion of this work was supported by the Centre National de la Recherche Scientifique, the Institut Curie, and the Association Pour la Recherche sur le Cancer and by a NATO Collaborative Research Grant (to G.C.).

## REFERENCES


1. **Athanasiou, M., G. Mavrothalassitis, C. C. Yuan, and D. G. Blair.** 1996. The *gag-myb-ets* fusion oncogene alters the apoptotic response and growth factor dependence of interleukin-3 dependent murine cells. Oncogene **12:**337–344.
2. **Barnes, J. L., and S. Milani.** 1995. In situ hybridization in the study of the kidney and renal diseases. Semin. Nephrol. **15:**9–28.
3. **Blair, D. G., M. A. Hull, and E. A. Finch.** 1979. The isolation and preliminary characterization of temperature sensitive transformation mutants of Moloney sarcoma virus. Virology **95:**303–316.
4. **Boowmeester, T., S. H. Kim, Y. Sasai, B. Lu, and E. M. De Robertis.** 1996. Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. Nature **382:**595–601.
5. **Boyd, J. M., S. Malstrom, T. Subramanian, L. K. Venkatesh, U. Schaeper, B. Elangovan, C. D'Sa-Eipper, and G. Chinnadurai.** 1994. Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins. Cell **79:**341–351.
6. **Brody, J. S., and M. C. Williams.** 1992. Pulmonary alveolar epithelial cell differentiation. Annu. Rev. Physiol. **54:**351–371.
7. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
8. **Contente, S., K. Kenyon, D. Rimoldi, and R. M. Friedman.** 1990. Expression of gene rrg is associated with reversion of NIH3T3 transformed by LTR-c-H-ras. Science **249:**797–798.
9. **Denhardt, D. T.** 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23:**641–646.
10. **Enomoto, H., T. Ozaki, E. I. Takahashi, N. Nomura, S. Tabata, H. Takahashi, N. Ohnuma, M. Tanabe, J. Iwai, M. Yoshida, T. Matsunaga, and S. Sakiyama.** 1994. Identification of human DAN gene, mapping to the putative neuroblastoma tumor suppressor locus. Oncogene **9:**2785–2791.
11. **Genetics Computer Group.** 1994. Program manual for the Wisconsin GCG package. Version 8.0. University of Wisconsin, Madison, Wis.
12. **Gillet, G., M. Guerin, A. Trembleau, and G. Brun.** 1995. A BCL-2 related gene is activated in avian cells transformed by the Rous sarcoma virus. EMBO J. **14:**1372–1381.
13. **Glück, U., D. J. Kwiatkowski, and A. Ben-Ze'ev.** 1993. Suppression of tumorigenicity in simian virus 40-transformed 3T3 cells transfected with α-actinin cDNA. Proc. Natl. Acad. Sci. USA **90:**383–387.
14. **Gordon, J. I., and M. L. Hermiston.** 1994. Differentiation and self-renewal in the mouse gastrointestinal epithelium. Curr. Opin. Cell Biol. **6:**795–803.
15. **Gross-Bellard, M., P. Oudet, and P. Chambon.** 1973. Isolation of high-molecular-weight DNA from mammalian cells. Eur. J. Biochem. **36:**32–38.
16. **Gum, J. R., J. W. Hicks, N. W. Toribara, E-M. Rothe, R. E. Lagace, and Y. S. Kim.** 1992. The human MUC2 intestinal mucin has cysteine-rich subdomains located both upstream and downstream of its central repetitive region. J. Biol. Chem. **267:**21375–21383.
17. **Hall, P. A., P. J. Coates, B. Ansam, and D. Hopwood.** 1994. Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. J. Cell Sci. **107:**3569–3577.
18. **Hamelin, R., B. L. Brizzard, M. A. Nash, E. C. Murphy, and R. B. Arlinghaus.** 1985. Temperature-sensitive viral RNA expression in ts110 Moloney murine sarcoma virus-infected cells. J. Virol. **50:**478–488.
19. **Harada, H., M. Kitayawa, N. Tanaka, H. Yamamoto, K. Horada, M. Ishihara, and T. Taniguchi.** 1993. Anti-oncogenic and oncogenic potentials of interferon regulation factors-1 and -2. Science **259:**971–974.
20. **Houle, B., C. Rochette-Egly, and W. E. C. Bradley.** 1993. Tumor suppressive effect of the retinoic acid receptor β in human epidermoid lung cancer cells. Proc. Natl. Acad. Sci. USA **90:**985–989.
21. **Katzov, S., D. Martin-Zanca, and M. Barbacid.** 1989. Vav, a novel human oncogene derived from a locus ubiquitously expressed in hematopoietic cells. EMBO J. **8:**2283–2290.
22. **Kozak, M.** 1987. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. **15:**8125–8133.
23. **Kozak, M.** 1992. Regulation of translation in eukaryotic systems. Annu. Rev. Cell Biol. **8:**197–225.
24. **Levine, A.** 1993. The tumor suppressor genes. Annu. Rev. Biochem. **62:**623–651.
25. **Liang, P., and A. B. Pardee.** 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science **247:**967–971.
26. **Lin, X., P. J. Nelson, B. Frankfort, E. Tombler, R. Johnson, and J. H. Gelman.** 1995. Isolation and characterization of a novel mitogenic regulatory gene, 322, which is transcriptionally suppressed in cells transformed by *src* and *ras*. Mol. Cell. Biol. **15:**2754–2762.
27. **Nuygen, M., P. E. Branton, P. A. Walton, Z. N. Oltvai, S. J. Korsmeyer, and G. C. Shore.** 1994. Role of membrane anchor domain of Bcl-2 in suppression of apoptosis caused by E1B-defective adenovirus. J. Biol. Chem. **269:**16521–16524.
28. **Ozaki, T., and S. Sakiyama.** 1993. Molecular cloning and characterization of a cDNA showing negative regulation in v-src-transformed 3Y1 rat fibroblasts. Proc. Natl. Acad. Sci. USA **90:**2593–2597.
29. **Ozaki, T., and S. Sakiyama.** 1994. Tumor-suppressive activity of NO3 gene product in v-src-transformed Rat 3Y1 fibroblasts. Cancer Res. **54:**646–648.
30. **Ozaki, T., Y. Nakamura, H. Enomoto, M. Hirose, and S. Sakiyama.** 1995. Overexpression of DAN gene product in normal rat fibroblasts causes a retardation of the entry into the S phase. Cancer Res. **55:**895–900.
31. **Prasad, G. L., R. A. Fuldner, and H. L. Cooper.** 1993. Expression of transduced tropomyosin 1 cDNA suppresses neoplastic growth of cells transformed by the ras oncogene. Proc. Natl. Acad. Sci. USA **90:**7039–7043.
32. **Preisig, P. A., and H. A. Franch.** 1995. Renal epithelial cell hyperplasia and hypertrophy. Semin. Nephrol. **15:**327–340.
33. **Rao, L., M. Debbas, P. Sabbatini, D. Hockenbery, S. Korsmeyer, and E. White.** 1992. The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19 kDa and Bcl-2 proteins. Proc. Natl. Acad. Sci. USA **89:**7742–7746.
34. **Sager, R.** 1989. Tumor suppressor genes: the puzzle and the promise. Science **246:**1406–1412.
35. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Sanger, F.** 1981. Determination of nucleotide sequences in DNA. Science **214:**1205–1210.
37. **Sassoon, D., and N. Rosenthal.** 1993. Detection of messenger RNA by in situ hybridization. Methods Enzymol. **225:**389–403.
38. **Shih, C., and R. A. Weinberg.** 1982. Isolation of a transforming sequence from a human bladder carcinoma cell line. Cell **29:**161–169.
39. **Sprague, J., J. H. Condra, H. Arnheiter, and R. A. Lazzarini.** 1983. Expression of a recombinant DNA gene coding for the resiculor stomatitis virus nucleocapsid protein. J. Virol. **45:**773–781.
40. **Topol, L. Z., A. G. Tatosyan, D. Blair, and F. L. Kisselov.** 1991. A new recipient line for the transfection of biologically active oncogenes. Mol. Biol. **25:**541–551. (Translated.)
41. **Topol, L. Z., M. Marx, G. Calothy, and D. G. Blair.** 1995. Transformation-resistant mos revertant is unable to activate MAP kinase kinase in response to v-mos or v-raf. Cell Growth Differ. **6:**27–38.
42. **Topol, L. Z., and D. G. Blair.** 1995. Activation of the mitogen-activated protein kinase cascade in response to the temperature inducible expression of v-mos kinase. Cell Growth Differ. **6:**1119–1127.
43. **White, E., P. Sabbatini, M. Debbas, W. S. M. Wold, D. I. Kusher, and L. Gooding.** 1992. The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor α. Mol. Cell. Biol. **12:**2570–2580.
44. **Zou, Z., A. Anisowicz, M. J. C. Hendrix, A. Thor, M. Neveu, S. Sheng, K. Rafidi, E. Seftor, and R. Sager.** 1994. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science **263:**526–529.