ANTICANCER RESEARCH *18:* 1409-1422 (1998)

Differential Gene Expression in Mammary Carcinoma Cell Lines: Identification of DRIM, a New Gene Down-Regulated in Metastasis

MARINA SCHWIRZKE[1], ANDREA GNIRKE[1], PEER BORK[2], DAVID TARIN[3] and ULRICH H. WEIDLE[1]

[1]*Boehringer Mannheim GmbH, Penzberg, Germany;* [2] *European Molecular Biology Laboratory, Heidelberg, Germany;* [3]*Cancer Center, San Diego, CA, U.S.A.*

Abstract. *Differential display technique was applied to a pair of cell lines derived from human breast carcinoma cell line MDA-MB 435 with metastatic and non-metastatic properties in the nude mouse system, with the objective to isolate genes involved in metastasis. DRIM (Down-Regulated In Metastasis) was the only gene found to be differentially expressed in this system. DRIM encodes a protein comprising 2785 amino acids with significant homology to a protein in yeast and C. elegans. The protein contains a conserved positively charged tail and several HEAT repeats, designated after four functionally characterized proteins in which the repeat was detected. Most of the hydrophobic regions of DRIM can be assigned to HEAT repeats. Expression of DRIM at the RNA level was investigated in several normal tissues and tumor cell lines.*

The discovery that mutant athymic mice do not reject heterotransplants of human tumor tissue (1) paved the way for the establishment of metastasizing and non-metastasizing tumor cell lines derived from each other as tools for the identification of genes involved in tumor progression and metastasis (2,3,4). From these experiments it was concluded that the capability to metastasize is an inherent property of individual tumor cells, which can be reproduced with substantial reliability by their progeny in immunologically compromised hosts.

With the advent of techniques allowing the investigation of genes differentially expressed in cell lines to be compared or in pathological *versus* non-pathological tissue, identification of genes involved in pathogenesis of disease became feasible. It is well documented now that the progression of cancer is brought about by the implementation of new patterns of expression of genes which mediate cell cycle control, adhesion, angiogenesis, invasion and finally metastasis (5,6). Clinical progression of breast cancer is known to be mediated by several defined molecular events, such as: estrogen-independent growth, tamoxifen resistance, acquisition of vimentin expression, increase of invasiveness and finally cross-resistance to a wide variety of chemothe-rapeutic agents referred to as multi-drug resistance (7, 8). In order to identify genes involved in metastasis of breast cancer we made use of cell line MDA-MB-435, which was isolated from a pleural effusion of a patient with breast cancer (9). We have derived a metastatic and a non-metastatic variant of this cell line and describe here the identification of a new gene which is almost exclusively expressed in the non-metastasizing variant as the only differentially expressed gene by applying differential-display techniques (10, 11, 12).

Materials and Methods

Animals. Athymic mice (MFINu) were obtained from the animal breeding facility at the John Radcliffe Hospital, Oxford University, UK. Mice were used at 6 - 8 weeks of age and were kept in filter-top boxes in a nude mouse isolation suite for the duration of the experiments.

Cell culture. 4A4 and 2C5 cells are cloned sublines of MDA-MB-435 which we isolated by the limiting dilution technique. These cells were maintained in Dulbeccos's Modified Eagle Medium (DMEM) (Flow Laboratories, Irvine, U.K.) supplemented with 5 % new born calf serum, sodium pyruvate, L-glutamine (2mM), non-essential amino acids and 2 x vitamin solution (GIBCO, Paisley, U.K.). The cultures were incubated at

Abbreviations: DRIM. Down-Regulated In Metastasis; mRNA, messenger RNA; nt, nucleotide(s); cDNA, complementary DNA; aa, amino acids; DD-PCR, Differential Display Polymerase Chain Reaction.

Correspondence to: Dr. Ulrich H. Weidle, Boehringer Mannheim, D-82372 Penzberg, Germany. Phone 498856602801, Fax 498856602659 E-Mail Ulrich_Weidle@bmg-boehringer-mannheim.com

Key Words: Breast cancer, metastasis, differential display, gene discovery.

0250-7005/98 $2.00+.40

37°C in a humidified atmosphere of 5 % CO_2-95 % air. For passaging tumor cells were harvested by washing the monolayer with phosphate buffered saline (PBS) and briefly incubating them in 0.25 % trypsin-0.02 % EDTA. The detached cells were washed by centrifugation and resuspended in DMEM ready for counting and inoculation.

All other mammary carcinoma cell lines referred to in this paper were grown under the same conditions.

From MDA-MB435, first MDA-MB-lung was established from pooled lung metastases arising in a nude mouse which had been injected with MDA-MB435. MDA-MB-lung is much more metastatic than the parent line. A series of clones were obtained by serial dilution of MDA-MB-lung in 96 well plates and subsequent clonal expansion. Clone 4A4 is metastatic, whilst clone 2C5 is not metastatic.

Tumourigenicity and metastasis formation in vivo. The tumourigenicity and spontaneous metastatic capability of the cells were determined by the injection of groups of nude mice with 1 x 10^6 cells in 0.1 ml DMEM into the lower right hind flank, either subcutaneously or in the right posterior mammary fat pad. The animals were monitored every 2 - 3 days for up to 5 months for their state of health and tumor growth. The rate of primary tumor growth of two of the MDA clones (4A4 and 2C5) was determined by plotting the means of two orthogonal diameters of the tumors, measured at 20 day intervals up to 100 days after injection. All animals were killed and autopsied 5 months after inoculation unless moribund earlier.

The experimental metastatic potential of the cell lines was assessed by intravenous injection of 1 x 10^5 cells in 0.1 ml serum-free DMEM into the lateral tail vein of nude mice. Recipient animals were killed and autopsied 2 months after injection.

Metastasis formation was assessed by macroscopic observation of all major organs for secondary tumors and confirmed by histological examination of organs and lymph nodes. Tissue samples for histological analysis were fixed in 10 % neutral formalin and embedded in paraffin wax for sectioning and staining.

Differential display PCR. Differential Display Polymerase chain reaction (DD-PCR) was performed following the method described by Liang and Pardee (10, 11, 12) using the RNAmap kits (GenHunter Corp., Brookline, MA) according to the manufacturer's recommendations.

Total RNA was isolated from 4A4 and 2C5 cells by the single step method described by Chomczynski and Sacchi (13) using the Total RNA Isolation System (Promega Corp., Madison, WI). Elimination of contaminating traces of DNA from total RNA samples was performed by digestion at 37°C for 30 minutes with RNase-free DNaseI using the MessageClean kit (GenHunter Corp., Brookline, MA).

DNA-free total RNA (0,2 µg) from 4A4 and 2C5 cells was used as a template for first strand synthesis in the presence of 10µM $T_{12}MG$, $T_{12}MC$, $T_{12}MA$ and $T_{12}MT$ anchored primers (where M is threefold degenerate for G, A and C), 1 x reverse transcriptase buffer [125 mM Tris-Cl, pH 8.3, 188 mM KCl, 7.5 mM $MgCl_2$, 25 mM dithiothreitol (DTT)] and 250 µM dNTP mix. The solution was heated to 65°C for 5 minutes and cooled to 37°C for 10 minutes, and then 200 units of Moloney murine leukemia virus (MMLV) reverse transcriptase were added. After incubation at 37°C for 1 hour the reaction was terminated by incubation at 95°C for 5 minutes.

PCR was performed in a mix containing 0.1 volume of reverse transcription reaction mixture, 10 µM of the respective $T_{12}MN$ anchored primer, 2 µM arbitrary 10-mer primer, 1 x PCR buffer [100 mM Tris-Cl, pH 8.4, 500 mM KCl, 15 mM $MgCl_2$, 0,01 % gelatin], 25 µM dNTP, 10 µCi [α-^{35}S]dATP, and 1 unit of AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, CT). PCR included a total of 40 cycles at 94°C for 30 seconds, 40°C for 2 minutes, 72°C for 30 seconds, and finally 5 minutes at 72°C.

After adding 2 µl loading buffer to 3.5 µl of each sample, the PCR products were heated at 80°C for 2 minutes and then loaded on a denaturing 5% polyacrylamide sequencing gel for electrophoresis. The dried gel was exposed to Kodak BioMax MR film for 48 hours at room temperature and the autoradiogram was analyzed with respect to differentially expressed genes. The reaction displaying unique fragments in one of the two cell lines was subsequently confirmed by repeating reverse transcription and PCR.

Unique bands reproducibly displayed in two independent DD-PCR reactions were excised from the dried gel and the cDNA was eluted from the gel by soaking the gel slice in 100 µl of H_2O for 10 minutes and subsequent boiling for 15 minutes. The cDNA was recovered by ethanol precipitation in the presence of 3 M NaOAc and 50 µg glycogen as a carrier and redissolved in 10 µl of H_2O.

Four µl of eluted cDNA were reamplified in a second PCR using the same 5' and 3' primers and conditions described above except for dNTP concentrations of 20 µM and no radioisotope included in the reaction.

The amplified PCR fragments obtained were analyzed on a 1.5 % agarose gel, then purified using the QIAquick Gel Extraction kit (Qiagen, Hilden) and used as probes for Northern analysis.

Northern blot analysis. Poly A^+ RNA was isolated from total RNA using the PolyATtract III mRNA Isolation System (Promega Corp., Madison, WI). Parallel lanes of poly A^+ RNA from 4A4 and 2C5 cells (1 µg of each cell line) were size-separated on a denaturing 1 % agarose formaldehyde gel and then transferred to a positively charged nylon membrane (Boehringer Mannheim GmbH, Mannheim) by capillary blotting in 20 x SSC (3 M NaCl, 0.3 M Na_3citrate $2H_2O$, pH 7.0). After UV-crosslinking (Stratagene UV Stratalinker 1800) blots were hybridized to [α^{32}P]dCTP-labeled DD-PCR products prepared by random hexamer priming and labeled to a specific activity of 5 x 10^8 dpm/µg using the Random Primed DNA Labeling Kit (Boehringer Mannheim GmbH, Mannheim). Pre-hybridization (5 hours) and hybridization with radioactive probes overnight were performed in 50 % formamide, 5 x SSC, 5 x Denhardt solution, 1 % SDS and 100 µg/ml denatured salmon sperm DNA at 42°C. Membranes were washed with 1 x SSC, 0.1 % SDS at room temperature for 15 minutes twice followed by washing with 0.25 x SSC, 0.1 % SDS at 55 to 60°C for 15 to 30 minutes and exposed for autoradiography at -80°C for 48 to 72 hours. Equal loading and transfer of mRNA to the membrane was assessed by hybridizing the blots with ^{32}P-labeled ß-actin cDNA.

Cloning of DD-PCR fragments. Northern analysis was first performed using hybridization probes generated directly from PCR re-amplification. Those amplified PCR fragments detecting differentially expressed mRNAs on a Northern blot were subcloned into the PCRII vector by the TA Cloning System (Invitrogen, San Diego, CA). Subcloned fragments were isolated using the Qiagen plasmid kit (Qiagen, Hilden) and again used as probes for Northern analysis to verify differential mode of expression.

DNA sequencing of subcloned DD-PCR fragments. Those subcloned fragments corresponding to mRNAs with differential mode of expression were sequenced directly after subcloning into the TA cloning vector (see above) using the Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The nt sequence data were analyzed with respect to homology to known genes in the Genbank and EMBL DNA data bases using the computer program BLAST.

cDNA library screening. For isolation of cDNA, a 900 bp subcloned DD-PCR fragment which detected a differentially expressed mRNA was used as a probe to screen a HeLa cDNA library (Clontech, Palo Alto, CA) that had been constructed in a lambda gt10 vector. The isolated cDNA clones were sequenced in a lambda gt10 vector and compared to the subcloned DD-PCR-fragment. For isolation of full-length cDNA a 5'probe of the cDNA was prepared and used for rescreening of the library. This procedure was repeated for five times until the 5'end of the cDNA was isolated.

5'RACE PCR. To identify the 5'-extended region of the cDNA showing differential mRNA expression a 5'RACE (Rapid Amplification of

cDNA Ends) PCR was performed following the method as described in (14) with some modifications (15).

For amplification of the 5'-cDNA end anti-sense gene-specific 24-mer primers were used. The obtained 5'RACE PCR products were sequenced on both strands and compared to the cDNA clone isolated from cDNA library screening.

Multiple Tissue Northern blots. To examine the tissue-specific expression of DRIM, the distribution of DRIM mRNA in different human tissues was analyzed by *Northern* blot analysis using Multiple Tissue *Northern* blots (Clontech, Palo Alto, CA). The MTN blots containing size-fractionated mRNA extracted from various human tissues were probed with an [α^{32}P]dCTP-labeled cDNA probe derived from the 3 '-coding region of DRIM. Equal loading of mRNA was verified by rehybridizing the blots with [α^{32}P]dCTP-labeled ß-actin cDNA.

Results

Characteristics of clones 4A4 and 2C5. As described in the Materials and Methods section both clones were derived from the mammary carcinoma cell line MDA-MB-435 resulting in a metastatic (4A4) and a non-metastatic (2C5) variant. The *in vitro* growth characteristics of clones 4A4 and 2C5 are almost identical, generally retaining the characteristics of the parent cell line, which consists of mononucleated cells with a spindle-shaped appearance, a low cytoplasm to nuclear ratio and visible nucleoli. At confluence around 5 - 10 % the population consists of multinucleated "giant cells". We have noticed that cell line 2C5 is exhibiting a slightly higher cytoplasm to nucleus ratio. *In vivo* their behavior is totally different. After a latent period of approximately 6 weeks both clones begin to produce tumors in nude mice (16, 17). Tumors derived from clone 4A4 grow more rapidly than 2C5, but more importantly, clone 4A4 is metastatic, whilst clone 2C5 is not metastatic.

Differential expression of a 10kb mRNA in cell lines 4A4 and 2C5. Both cell lines were grown to confluence before RNA was extracted for gene expression studies. Northern blotting experiments revealed the expression of vimentin (8) in both cell lines, the estrogen-receptor and p53 displayed signals of the same intensity in both cell lines (data not shown). Both cell lines were negative with respect to expression of metalloproteinases MMP2 and MMP9 (18); however, interstitial collagenase (Collagenase type IV) was equally expressed in both cell lines (data not shown). Urokinase and urokinase receptor (19, 20) messenger RNAs were expressed at equivalent levels in both cell lines, whereas E-Cadherin (21) messenger RNA was undetectable in both cell lines (data not shown). Both cell lines scored positive with respect to erbB2 receptor (22) mRNA and negative with respect to EGF receptor (22) mRNA (data not shown). Extensive Differential Display experiments as outlined in the Materials and Methods section revealed only one species of messenger RNA being differentially expressed in clones 4A4 and 2C5. The steady state level of a 10 kb mRNA was increased 10 fold in the non-metastatic variant 2C5 (Figure 1). In the following this mRNA and its corresponding cDNA will be referred to as DRIM (Down-Regulated In Metastasis).

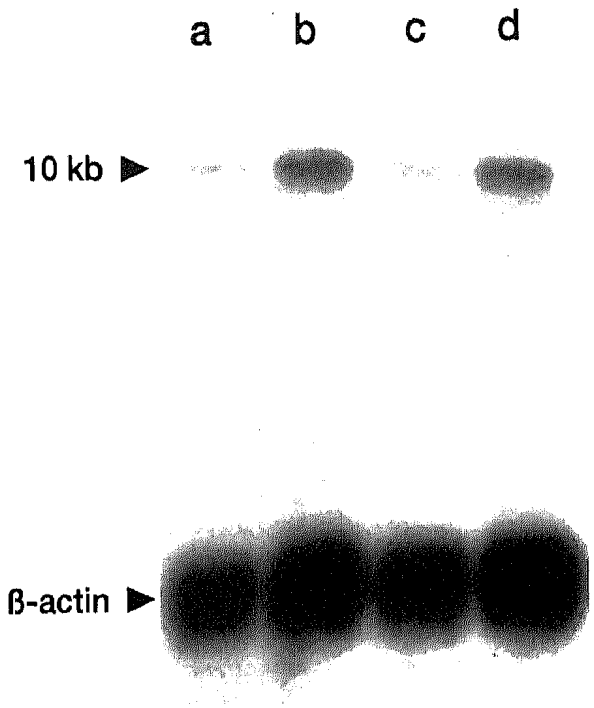

Figure 1. *Differential expression of DRIM messenger RNA in cell lines 4A4 and 2C5 displayed by Northern blot analysis. RNA derived from different cell culturing experiments (lanes a and b versus lanes c and d) are displayed. PolyA$^+$RNA from cell lines 4A4 (lanes a and c) and 2C5 (lanes b and d) was electrophoresed on a denaturing 1 % agarose formaldehyde gel, transferred to a positively charged nylon membrane and hybridized to an [α^{32}P]dCTP-labeled fragment covering part of the 3' coding region of DRIM cDNA. The blot was rehybridized to a human ß-actin probe as an internal reference. Size of marker is indicated.*

DRIM mRNA encodes a new protein. The nt as well as the aa sequence of the new protein are displayed in Figure 2. The open reading frame encodes a protein of 2785 aa. Significant homology to a yeast protein and to a protein from *C. elegans* was identified. Homology comparisons are displayed in Figure 3. It reveals that DRIM contains long hydrophobic patches, but also regions with clusters of positively charged residues. Homologies to proteins in yeast and *C. elegans* confirm the correctness of the sequence, a match to a thale cress EST indicates a wide phylogenetic distribution among eukaryotes. The multiple alignment in Figure 3 reveals a conserved positively charged tail which appears to be functionally relevant. There are a few other conserved clusters of charged aa (Figure 3) suggesting unknown binding functions. Most of the hydrophobic regions can be explained by the presence of divergent HEAT repeats, segments comprising approximately 40 aa each of which appears to consist of a helix-loop-helix structure (30). The HEAT repeats seem to cover almost the entire sequence although only part of them could be assigned with confidence (Figure 3). The absence of a signal sequence and the presence of HEAT repeats suggest an intracellular localization of DRIM.

```
   1  cccaggggctcaagccgcacgtgagaaagtctgggcatctgggattcggagagtatagcc   60
  61  tgtgagccgtttcccctcttactgtcggttgcatcccttcgacactcccgaggccgtc   120
 121  gcgggccactggcccctctgcagcc

                                    ATGAAGACAAAGCCCGTTTCCCACAAGACCGAGAAC   180
   1                                 M  K  T  K  P  V  S  H  K  T  E  N
 181  ACCTACCGGTTTCTTACATTTGCTGAACGACTGGGGAATGTTAATATTGATATTATTCAC   240
  13  T  Y  R  F  L  T  F  A  E  R  L  G  N  V  N  I  D  I  I  H
 241  CGGATTGATAGAACTGCAAGCTATGAGGAGGAGGTTGAAACCTACTTTTTTGAGGGTCTG   300
  33  R  I  D  R  T  A  S  Y  E  E  E  V  E  T  Y  F  F  E  G  L
 301  CTGAAATGGAGAGAATTAAACCTCACAGAACACTTCGGAAAATTTTACAAAGAAGTTATT   360
  53  L  K  W  R  E  L  N  L  T  E  H  F  G  K  F  Y  K  E  V  I
 361  GACAAATGCCAATCATTCAATCAGTTGGTGTATCACCAAAACGAGATAGTTCAGAGTTTG   420
  73  D  K  C  Q  S  F  N  Q  L  V  Y  H  Q  N  E  I  V  Q  S  L
 421  AAGACTCACCTGCAAGTTaAGAACAGTTTTGCCTATCAACCCCTTTTGGATTTGGTTGTA   480
  93  K  T  H  L  Q  V  K  N  S  F  A  Y  Q  P  L  L  D  L  V  V
 481  CAGTTGGCACGAGATCTGCAGATGGATTTCTACCCACACTTTCCAGAGTTTTTTTTGACT   540
 113  Q  L  A  R  D  L  Q  M  D  F  Y  P  H  F  P  E  F  F  L  T
 541  ATCACCTCGATCCTGGAGACTCAGGACACAGAGTTGTTAGAATGGGCTTTCACCTCGTTA   600
 133  I  T  S  I  L  E  T  Q  D  T  E  L  L  E  W  A  F  T  S  L
 601  TCATATCTTTATAAGTACCTGTGGAGACTGATGGTGAAGGACATGTCCAGTATATACAGC   660
 153  S  Y  L  Y  K  Y  L  W  R  L  M  V  K  D  M  S  S  I  Y  S
 661  ATGTACAGCACACTCCTGGCTCATAAAAAACTACATATAAGAAATTTTGCTGCTGAAAGT   720
 173  M  Y  S  T  L  L  A  H  K  K  L  H  I  R  N  F  A  A  E  S
 721  TTTACTTTTTTGATGAGAAAGGTCTCTGATAAAAACGCACTTTTCAATTTAATGTTTCTT   780
 193  F  T  F  L  M  R  K  V  S  D  K  N  A  L  F  N  L  M  F  L
 781  GATCTTGATAAACATCCAGAAAAAGTTGAAGGTGTTGGACAGTTGCTCTTTGAAATGTGC   840
 213  D  L  D  K  H  P  E  K  V  E  G  V  G  Q  L  L  F  E  M  C
 841  AAAGGAGTTAGAAATATGTTTCACTCCTGTACAGGCCAGGCAGTGAAGCTAATTTTGCGA   900
 233  K  G  V  R  N  M  F  H  S  C  T  G  Q  A  V  K  L  I  L  R
 901  AAGCTAGGACCAGTCACTGAAACAGAAACTCAACTACCATGGATGTTAATTGGAGAAACA   960
 253  K  L  G  E  V  T  E  T  E  T  Q  L  P  W  M  L  I  G  E  T
 961  CTCAAAAACATGGTCAAATCCACTGTATCCTACATCTCCAAGGAACATTTTGGTACATTT  1020
 273  L  K  N  M  V  K  S  T  V  S  Y  I  S  K  E  H  F  G  T  F
1021  TTTGAATGTTTGCAAGAATCGCTCTTGGATCTACACACAAAAGTAACAAAAACTAACTGT  1080
 293  F  E  C  L  Q  E  S  L  L  D  L  H  T  K  V  T  K  T  N  C
1081  TGTGAAAGTTCTGAACAGATTAAAAGGTTGTTGGAAACATACCTTATACTTGTAAAACAT  1140
 313  C  E  S  S  E  Q  I  K  R  L  L  E  T  Y  L  I  L  V  K  H
1141  GGAAGTGGGACAAAGATACCCACGCCTGCTGATGTCTGTAAGGTGTTATCTCAAACACTG  1200
 333  G  S  G  T  K  I  P  T  P  A  D  V  C  K  V  L  S  Q  T  L
1201  CAAGTAGCCAGTCTCTCCACATCTTGCTGGGAGACCCTCTTGGATGTAATTTCTGCTTTG  1260
 353  Q  V  A  S  L  S  T  S  C  W  E  T  L  L  D  V  I  S  A  L
1261  ATCCTGGGTGAAAATGTTTCCTTGCCGGAGACCCTCATCAAAGAAACCATAGAAAAAATA  1320
 373  I  L  G  E  N  V  S  L  P  E  T  L  I  K  E  T  I  E  K  I
1321  TTTGAGAGCAGATTTGAAAAACGTTTAATTTTCAGTTTTTCTGAAGTCATGTTTGCCATG  1380
 393  F  E  S  R  F  E  K  R  L  I  F  S  F  S  E  V  M  F  A  M
1381  AAGCAGTTTGAGCAGCTTTTTCTACCAAGCTTTCTGTCATATATTGTGAATTGCTTCTTA  1440
 413  K  Q  F  E  Q  L  F  L  P  S  F  L  S  Y  I  V  N  C  F  L
1441  ATTGATGATGCTGTAGTCAAAGATGAAGCTCTGGCCATTCTGGCCAAGCTCATTCTGAAC  1500
 433  I  D  D  A  V  V  K  D  E  A  L  A  I  L  A  K  L  I  L  N
1501  AAAGCAGCACCTCCCACTGCTGGCTCGATGGCAATTGAAAAGTACCCTCTGGTTTTCTCA  1560
 453  K  A  A  P  P  T  A  G  S  M  A  I  E  K  Y  P  L  V  F  S
1561  CCGCAGATGGTGGGATTCTATATAAAGCAGAAGAAGACTAGATCCAAGGGAAGAAACGAA  1620
 473  P  Q  M  V  G  F  Y  I  K  Q  K  K  T  R  S  K  G  R  N  E
1621  CAGTTTCCAGTATTGGACCATCTTTTATCTATAATTAAGTTACCCCCAAATAAAGATACT  1680
 493  Q  F  P  V  L  D  H  L  L  S  I  I  K  L  P  P  N  K  D  T
1681  ACTTACCTTTCACAATCTTGGGCAGCCCTCGTGGTGTTACCTCATATTAGACCTCTTGAG  1740
 513  T  Y  L  S  Q  S  W  A  A  L  V  V  L  P  H  I  R  P  L  E
1741  AAAGAGAAGGTGATACCACTCGTCACCGGCTTCATAGAGGCACTCTTCATGACTGTTGAC  1800
 533  K  E  K  V  I  P  L  V  T  G  F  I  E  A  L  F  M  T  V  D
1801  AAAGGAAGCTTTGGGAAAGGAAACTTATTTGTTCTTTGTCAAGCTGTAAATACTCTACTA  1860
 553  K  G  S  F  G  K  G  N  L  F  V  L  C  Q  A  V  N  T  L  L
1861  AGTTTGGAAGAATCTTCTGAACTTCTTCATTTGGTTCCTGTGGAACGTGTGAAGAATTTA  1920
 573  S  L  E  E  S  S  E  L  L  H  L  V  P  V  E  R  V  K  N  L
1921  GTATTAACCTTTCCCCTGGAGCCATCTGTGTTGCTGTTGACTGATCTCTATTATCAGAGA  1980
 593  V  L  T  F  P  L  E  P  S  V  L  L  L  T  D  L  Y  Y  Q  R
1981  TTAGCCTTGTGTGGCTGCAAAGGGCCACTTTCCCAGGAGGCTTTAATGGAATTATTTCCC  2040
 613  L  A  L  C  G  C  K  G  P  L  S  Q  E  A  L  M  E  L  F  P
2041  AAGTTACAAGCAAACATTTCAACTGGTGTATCCAAGATTCGGCTTTTGACAATAAGGATC  2100
 633  K  L  Q  A  N  I  S  T  G  V  S  K  I  R  L  L  T  I  R  I
2101  CTAAACCATTTTGATGTCCAGCTTCCAGAATCAATGGAGGATGATGGGCTCTCAGAGCGG  2160
 653  L  N  H  F  D  V  Q  L  P  E  S  M  E  D  D  G  L  S  E  R
2161  CAGTCTGTCTTTGCTATATTACGCCAGGCAGAACTTGTTCCAGCAACTGTGAATGATTAT  2220
 673  Q  S  V  F  A  I  L  R  Q  A  E  L  V  P  A  T  V  N  D  Y
2221  AGAGAGAAGCTTCTTCATTTGAGAAAACTAAGACATGATGTGGTACAGACTGCTGTCCCT  2280
```

a

Figure 2. *Nucleotide and amino acid sequence of DRIM. Nucleotides of the 5'-and 3'-untranslated regions are displayed by small letters, nucleotides of the coding region are displayed by capital letters. HEAT repeats are boxed.*

```
 693    R  E  K  L  L  H  L  R  K  L  R  H  D  V  V  Q  T  A  V  P
2281    GATGGGCCGTTACAGGAGGTGCCGCTTCGTTATTTGTTAGGCATGCTATATATTAATTTC  2340
 713    D  G  P  L  Q  E  V  P  L  R  Y  L  L  G  M  L  Y  I  N  F
2341    AGTGCACTCTGGGATCCTGTTATTGAACTCATAAGTTCTCATGCACACGAAATGGAAAAT  2400
 733    S  A  L  W  D  P  V  I  E  L  I  S  S  H  A  H  E  M  E  N
2401    AAGCAATTTTGGAAAGTCTACTATGAGCATCTAGAAAAAGCAGCTACGCATGCTGAGAAG  2460
 753    K  Q  F  W  K  V  Y  Y  E  H  L  E  K  A  A  T  H  A  E  K
2461    GAACTACAGAATGATATGACAGATGAGAAGTCCGTTGGAGATGAAAGTTGGGAGCAGACC  2520
 773    E  L  Q  N  D  M  T  D  E  K  S  V  G  D  E  S  W  E  Q  T
2521    CAGGAAGGAGATGTTGGAGCTCTTTATCATGAGCAGTTAGCATTGAAAACTGACTGTCAG  2580
 793    Q  E  G  D  V  G  A  L  Y  H  E  Q  L  A  L  K  T  D  C  Q
2581    GAAAGACTTGACCACACCAACTTCAGATTCCTGCTCTGGAGAGCTCTGACCAAATTCCCA  2640
 813    E  R  L  D  H  T  N  F  R  F  L  L  W  R  A  L  T  K  F  P
2641    GAAAGAGTAGAGCCACGGTCCAGGGAGCTTTCCCCGCTTTTCTTGAGATTTATCAACAAT  2700
 833    E  R  V  E  P  R  S  R  E  L  S  P  L  F  L  R  F  I  N  N
2701    GAGTATTACCCAGCAGATCTGCAAGTTGCTCCAACCCAGGATCTACGGAGAAAAGGCAAA  2760
 853    E  Y  Y  P  A  D  L  Q  V  A  P  T  Q  D  L  R  R  K  G  K
2761    GGGATGGTGGCAGAGGAAATCGAAGAGGAACCTGCCGCAGGAGATGATGAAGAGTTGGAG  2820
 873    G  M  V  A  E  E  I  E  E  E  P  A  A  G  D  D  E  E  L  E
2821    GAAGAGGCAGTGCCCCAAGATGAATCCTCACAGAAGAAAAAGACGAGGAGAGCTGCAGCA  2880
 893    E  E  A  V  P  Q  D  E  S  S  Q  K  K  K  T  R  R  A  A  A
2881    AAGCAATTAATTGCTCATTTGCAAGTTTTCTCTAAATTTTCAAATCCACGGGCCTTATAT  2940
 913    K  Q  L  I  A  H  L  Q  V  F  S  K  F  S  N  P  R  A  L  Y
2941    CTGGAATCCAAACTATATGAGTTATATCTTCAGTTGTTGCTACACCAAGATCAAATGGTG  3000
 933    L  E  S  K  L  Y  E  L  Y  L  Q  L  L  L  H  Q  D  Q  M  V
3001    CAAAAAATAACCTTGGATTGCATAATGACATATAAACATCCTCATGTCCTCCCTTACAGG  3060
 953    Q  K  I  T  L  D  C  I  M  T  Y  K  H  P  H  V  L  P  Y  R
3061    GAAAACTTACAAAGGTTGCTTGAAGACAGAAGCTTTAAGGAAGAGATAGTGCATTTTAGC  3120
 973    E  N  L  Q  R  L  L  E  D  R  S  F  K  E  E  I  V  H  F  S
3121    ATTTCAGAAGATAATGCTGTAGTGAAAACAGCCCACCGAGCAGATCTATTTCCTATTCTG  3180
 993    I  S  E  D  N  A  V  V  K  T  A  H  R  A  D  L  F  P  I  L
3181    ATGAGAATTTTGTATGGGCGAATGAAGAATAAGACTGGGAGTAAAACTCAGGGGAAATCT  3240
1013    M  R  I  L  Y  G  R  M  K  N  K  T  G  S  K  T  Q  G  K  S
3241    GCTTCAGGCACCCGCATGGCCATTGTCCTGCGGTTCCTGGCCGGGACCCAACCTGAGGAG  3300
1033    A  S  G  T  R  M  A  I  V  L  R  F  L  A  G  T  Q  P  E  E
3301    ATCCAGATATTCTTAGACCTGCTGTTTGAACCTGTGAGGCATTTCAAGAATGGAGAGTGC  3360
1053    I  Q  I  F  L  D  L  L  F  E  P  V  R  H  F  K  N  G  E  C
3361    CATTCTGCAGTCATTCAAGCAGTAGAAGACTTGGATTTGTCTAAAGTTCTTCCTTTAGGT  3420
1073    H  S  A  V  I  Q  A  V  E  D  L  D  L  S  K  V  L  P  L  G
3421    CGTCAGCACGGTATCTTAAACAGCCTTGAGATAGTATTGAAAAACATTAGTCATCTGATC  3480
1093    R  Q  H  G  I  L  N  S  L  E  I  V  L  K  N  I  S  H  L  I
3481    AGCGCATACCTGCCGAAGATTTTGCAGATACTGCTCTGTATGACAGCAACCGTATCACAC  3540
1113    S  A  Y  L  P  K  I  L  Q  I  L  L  C  M  T  A  T  V  S  H
3541    ATCCTTGACCAACGAGAAAAGATACAGCTGAGATTTATTAATCCATTGAAAAATTTAAGA  3600
1133    I  L  D  Q  R  E  K  I  Q  L  R  F  I  N  P  L  K  N  L  R
3601    CGTCTTGGAATCAAAATGGTAACTGATATCTTTTTGGACTGGGAATCATATCAGTTTAGA  3660
1153    R  L  G  I  K  M  V  T  D  I  F  L  D  W  E  S  Y  Q  F  R
3661    ACAGAAGAAATTGATGCTGTGTTTCATGGTGCAGTTTGGCCCCAGATCAGCAGGCTTGGA  3720
1173    T  E  E  I  D  A  V  F  H  G  A  V  W  P  Q  I  S  R  L  G
3721    TCTGAGAGTCAATATTCTCCTACTCCTCTGCTGAAACTGATCAGTATCTGGAGCAGAAAC  3780
1193    S  E  S  Q  Y  S  P  T  P  L  L  K  L  I  S  I  W  S  R  N
3781    GCAAGATATTTCCCTTTGCTGGCTAAACAGAAGCCTGGGCACCCAGAATGTGATATCCTG  3840
1213    A  R  Y  F  P  L  L  A  K  Q  K  P  G  H  P  E  C  D  I  L
3841    ACCAATGTTTTTGCAATTCTCTCAGCGAAGAATCTTTCTGATGCCACAGCCAGTATTGTA  3900
1233    T  N  V  F  A  I  L  S  A  K  N  L  S  D  A  T  A  S  I  V
3901    ATGGACATAGTTGATGACCTTCTTAACCTTCCAGATTTCGAGCCTACAGAAACAGTTTTG  3960
1253    M  D  I  V  D  D  L  L  N  L  P  D  F  E  P  T  E  T  V  L
3961    AACTTGCTGGTAACTGGATGTGTATACCCTGGCATAGCAGAAAACATCGGTGAGTCTATC  4020
1273    N  L  L  V  T  G  C  V  Y  P  G  I  A  E  N  I  G  E  S  I
4021    ACAATAGGAGGAAGATTAATTCTACCTCATGTACCTGCAATTCTTCAGTATCTCAGCAAA  4080
1293    T  I  G  G  R  L  I  L  P  H  V  P  A  I  L  Q  Y  L  S  K
4081    ACCACAATAAGCGCAGAAAAGGTGAAAAAGAAAAAGAATAGAGCACAAGTCAGTAAAGAG  4140
1313    T  T  I  S  A  E  K  V  K  K  K  K  N  R  A  Q  V  S  K  E
4141    CTTGGCATTCTTTCAAAGATCAGCAAGTTTATGAAAGACAAAGAACAAAGTTCTGTACTC  4200
1333    L  G  I  L  S  K  I  S  K  F  M  K  D  K  E  Q  S  S  V  L
4201    ATTACGCTTCTCCTTCCATTCCTCCACCGTGGCAATATTGCTGAGGATACAGAGGTTGAT  4260
1353    I  T  L  L  L  P  F  L  H  R  G  N  I  A  E  D  T  E  V  D
4261    ATTCTGGTGACAGTACAAAACTTGTTAAAGCATTGTGTGGACCCTACAAGCTTCCTCAAG  4320
1373    I  L  V  T  V  Q  N  L  L  K  H  C  V  D  P  T  S  F  L  K
4321    CCTATAGCAAAACTTTTCTCAGTTATTAAGAACAAATTGTCAAGAAAATTGCTTTGTACG  4380
1393    P  I  A  K  L  F  S  V  I  K  N  K  L  S  R  K  L  L  C  T
4381    GTTTTTGAGACTCTTTCTGATTTTGAGAGTGGGTTAAAATATATTACTGATGTTGTCAAG  4440
1413    V  F  E  T  L  S  D  F  E  S  G  L  K  Y  I  T  D  V  V  K
4441    CTTAACGCCTTCGATCAAAGACATCTTGATGATATCAACTTCGACGTTCGCTTTGAGACT  4500
```

b

Figure 2. *b.*

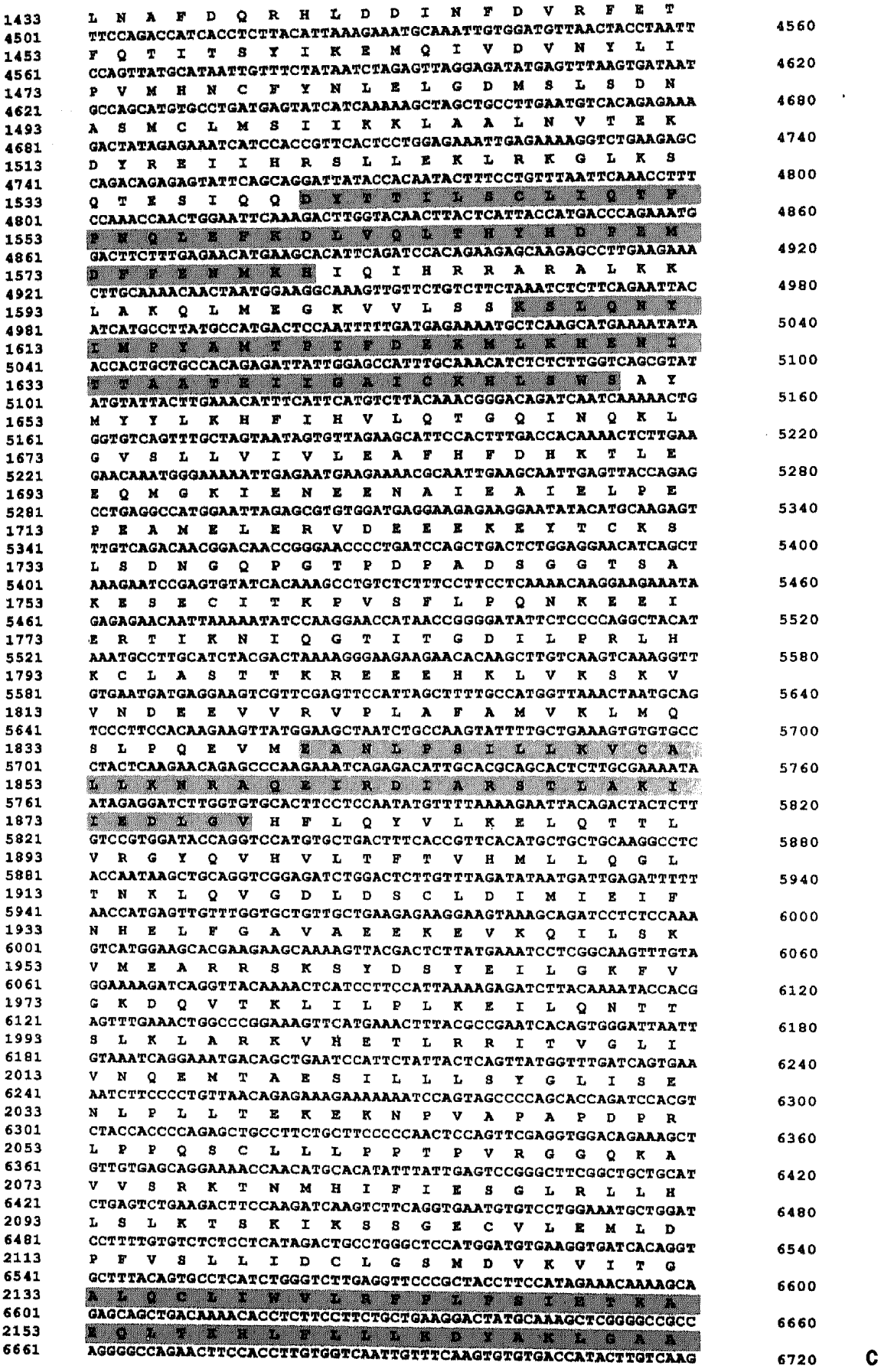

Figure 2. *c.*

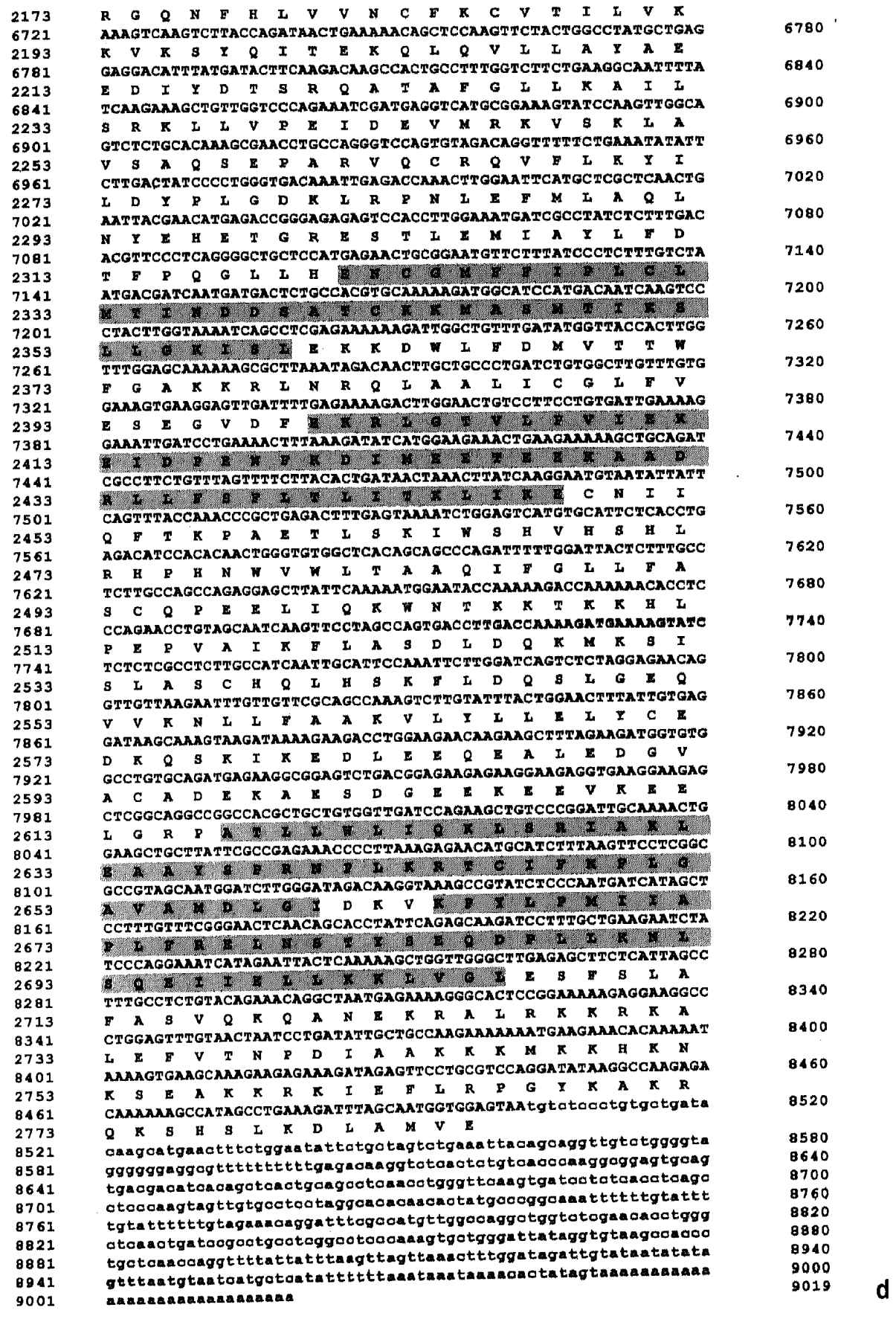

Figure 2. *d.*

Expression pattern of DRIM. Steady state levels of DRIM mRNA in different organs revealed by Northern blotting are displayed in Figure 4. Strong expression was noted in tissues such as heart, skeletal muscle, pancreas, testis and ovary (Figure 4a, f, h, l and m). Moderate levels of DRIM mRNA were found in placenta, spleen, thymus, prostate, small intestine, appendix and fetal liver (Figure 4c, i, j, k, n, p and s). Very low levels of transcripts were identified in brain, colon and bone marrow (Figure 4b, o and r). No DRIM transcripts were found in lung, liver, kidney and peripheral blood leucocytes (Figure 4d, e, g and q).

Inspection of several human tumor cell lines revealed a broad pattern of expression of DRIM mRNA (Figure 5) such as in HL60 cells, a promyelocytic leukemia cell line (lane a), Hela cells (lane b), K562 cells derived from chronic myelogenous leukemia (lane c); Raji cells, derived from Burkitt's lymphoma (lane e); SW-480 cells, representing colorectal adenocarcinoma (lane f) and G361 melanoma cells (lane h). Only very weak expression of DRIM mRNA was noted in MOLT4 cells representing lymphoblastic leukemia (lane d) and lung carcinoma A549 cells (lane g).

In addition/DRIM transcript levels were scored in several mammary carcinoma cell lines (Figure 6). Low levels of DRIM mRNA were identified in cell lines which are invasive and/or are metastasizing in nude mice as xenografts such as MDA-MB 231 (lane b) (23), MDA-MB 435 (9) (lane c), MDA-MB 436 (9) (lane d), Hs 578 T (24) (lane g) and cell lines LCC-I, LCC-2 and LCC-9 (25, 26) (lanes j, k and l) and T47D (lane f) (27). High levels of DRIM mRNA were detected in the non-metastasizing pair of cell lines MCF-7 and MCF-7_{ADR} (28) (Figure 5, lanes h and i) and in cell line ZR-75-1 (29) derived from malignant effusions of breast cancer patients (Figure 5, lane e). Very low levels of DRIM transcripts were identified in normal mammary gland tissue (Figure 5, lane a).

Discussion

DRIM (Down-Regulated In Metastasis), a new protein composed of 2785 aa was identified as the only protein differentially expressed in the metastatic (4A4) and non-metastatic (2C5) sublines of human breast carcinoma cell line MDA-MB 435 (Figure 1).

Investigation of the steady-state mRNA level revealed expression of DRIM in a broad spectrum of tissues with strong expression in heart, skeletal muscle, pancreas, testis and ovary; no expression was found in lung, liver, peripheral blood leucocytes, moderate to low levels were detected in the rest of organs evaluated (Figure 4). Examination of DRIM expression in several human tumor cell lines of different origin revealed a broad pattern of expression, with weak expression only in a lymphoblastic leukemia cell line (MOLT4) and cell line A549 derived from human lung carcinoma (Figure 5). Since these cell lines have not been investigated in xenograft murine models of metastasis it is not possible to correlate expression of DRIM with metastatic capacity. Investigation of a panel of human mammary carcinoma cell lines indicated an inverse correlation between metastatic properties in nude mice and expression of DRIM (Figure 6). However, these investigations have to be extended to a larger panel of cell lines to correlate decreased expression of DRIM with the metastatic phenotype in a conclusive manner.

The function of the newly identified gene DRIM (Figure 2 and 3) and its gene product are presently unknown. Comparison of the sequence with the homologous yeast and *C. elegans* proteins reveals a conserved positive carboxy-terminal tail which seems to be functionally relevant and hydrophobic regions covered by HEAT repeats. These are approximately 40 aa comprising segments which appear to consist of helix-loop-helix structures (30). Systematic analysis of multidomain disease proteins revealed that a considerable fraction of huntingtin contains tandem arrays of heat repeats (30). They are designated according to four functionally characterized proteins in which the repeat was detected: huntingtin, elongation factor 3 (EF3), the regulatory A subunit (65 kD) of protein phosphatase 2A (PP2A) and TOR1, a target of rapamycin that seems to be essential for progression of the G1 phase of the cell cycle (30).

All proteins of the HEAT family seem to be very large, most of them are part of protein complexes and the functionally characterized proteins containing HEAT repeats are eukaryotic cytoplasmic proteins, most of them seem to be involved in cytoplasmic transport processes (30,31). Experiments designed to investigate the biochemical functions of DRIM and its role in metastasis are in progress.

References

1 Rygaard J and Povlsen CD: Heterotransplantation of a human malignant tumor to nude mice. Acta Pathol Microbiol Scand *77*: 758-769, 1969.

2 Fidler IJ: Rationale and methods for the use of nude mice to study the biology and therapy of human cancer metastasis. Cancer Metastasis Rev *5*: 29-49, 1986.

3 Fidler IJ: Critical factors in the biology of human cancer metastasis. Cancer Rep *50*: 6130-6138, 1990.

4 Giavazzi R, Campbell DE, Jessup JM, Cleary K and Fidler IJ: Metastatic behavior of tumor cells isolated from primary and metastatic human colorectal carcinomas implanted in different sites in nude mice. Cancer Res *46*: 1928-1933, 1986.

5 Pardee AB: Growth dysregulation in cancer cells. Advances in Cancer Res *65*: 213-227, 1994.

6 Ponta H, Sleeman J and Herrlich P: Tumor metastasis formation: cell-surface proteins confer metastasis-promoting or suppressing properties. Biochem Biophys Acta *1198*: 1-10, 1994.

7 Clarke R, Brünner N, Thompson EW, Glanz P, Katz D, Dickson RB and Lippman ME: The inter-relationships between ovarian-independent growth, tumorigenicity, invasiveness and antiestrogen resistance in the malignant progression of human breast cancer. J Endocrinol *122*: 331-340, 1989.

8 Sommer CL, Heckford SE, Sherker JM, Worland P, Torri JA, Thompson EW, Byess SW and Gelman EP: Loss of epithelial markers and acquisition of vimentin expression in Adriamycin- and

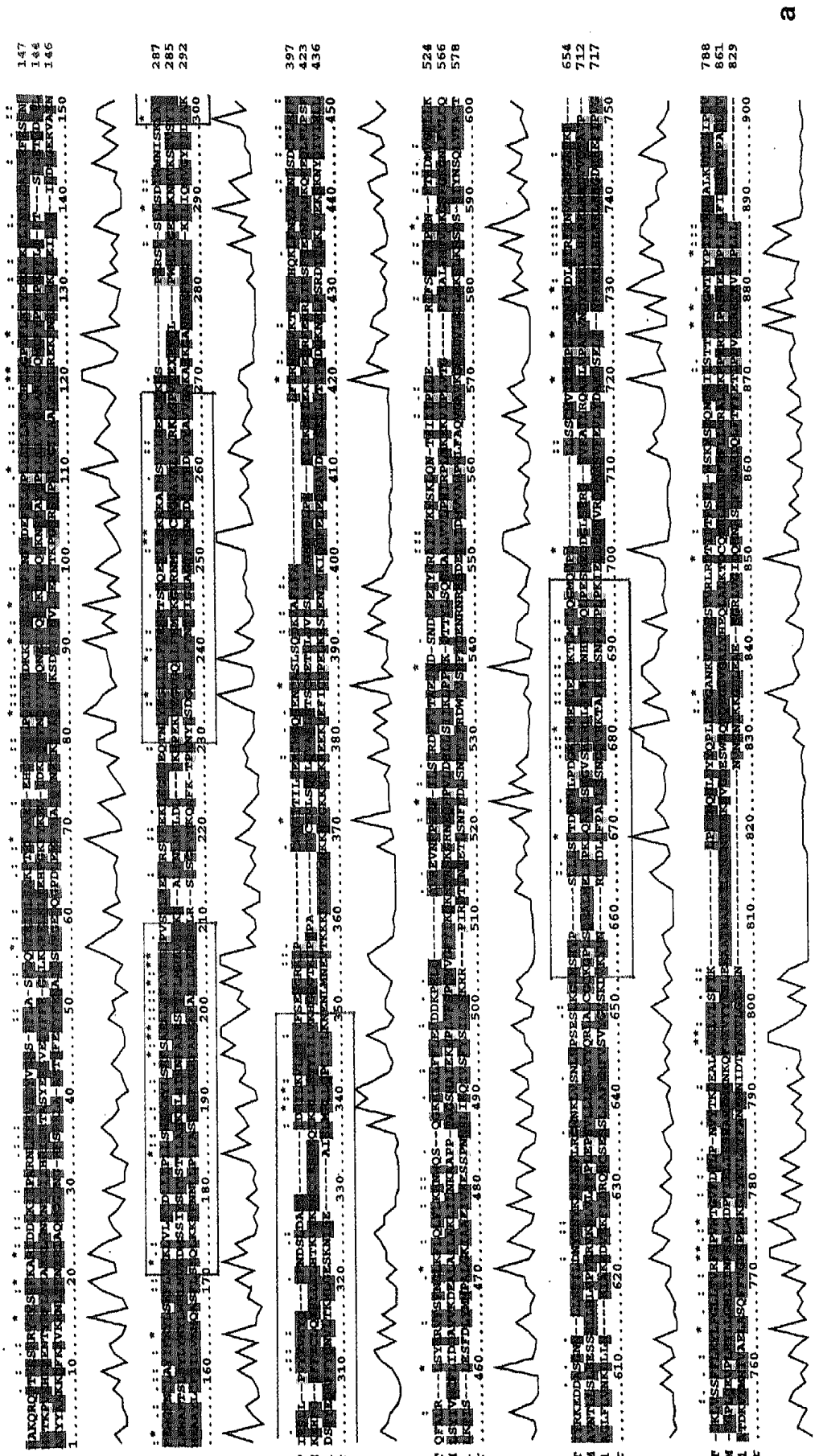

Figure 3. *Homology alignment at the amino acid level. Alignment of DRIM with yeast YBL004W (YBA4, accession number P 35 194) and a hypothetical C. elegans protein that was reconstructed by fusing the C-terminal part of the predicted ORF CEC36E6_5 (accession number U 39996) with the succeeding ORF CEF 18C5_1 (accession number U 29007). The consensus line above the alignment denotes conserved positions (* for identity.: and . denote conserved aa properties that are also highlighted in the color scheme as provided by the CLUSTAX program (Thompson et al, submitted). The bottom line shows the conservation profile. Predicted HEAT repeats are boxed. Color code: yellow = P, blue = LAVMFW, green = STQN, light blue = YH, magenta = C, red = RK, pink = DE, orange = G.*

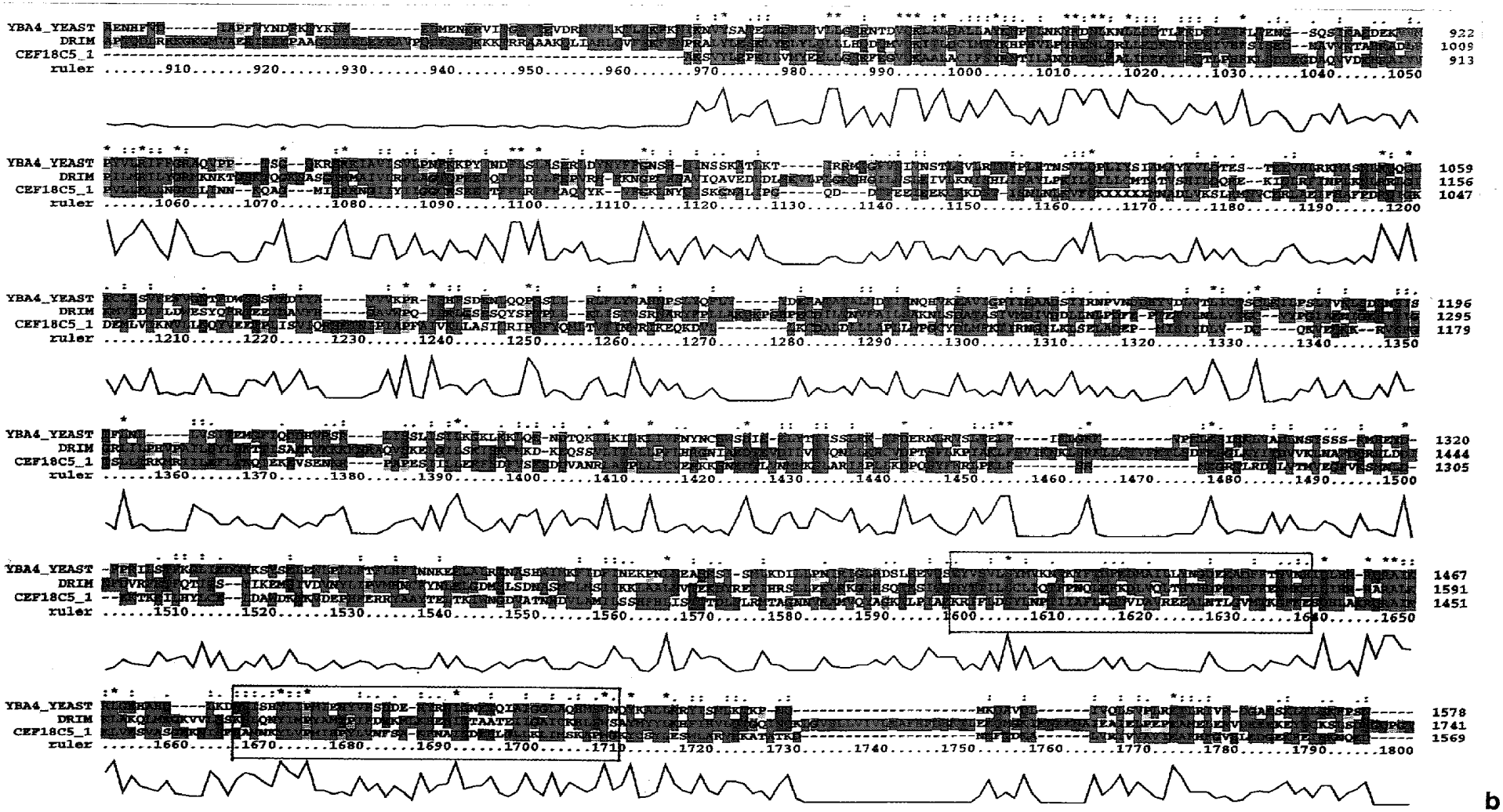

Figure 3. *b*

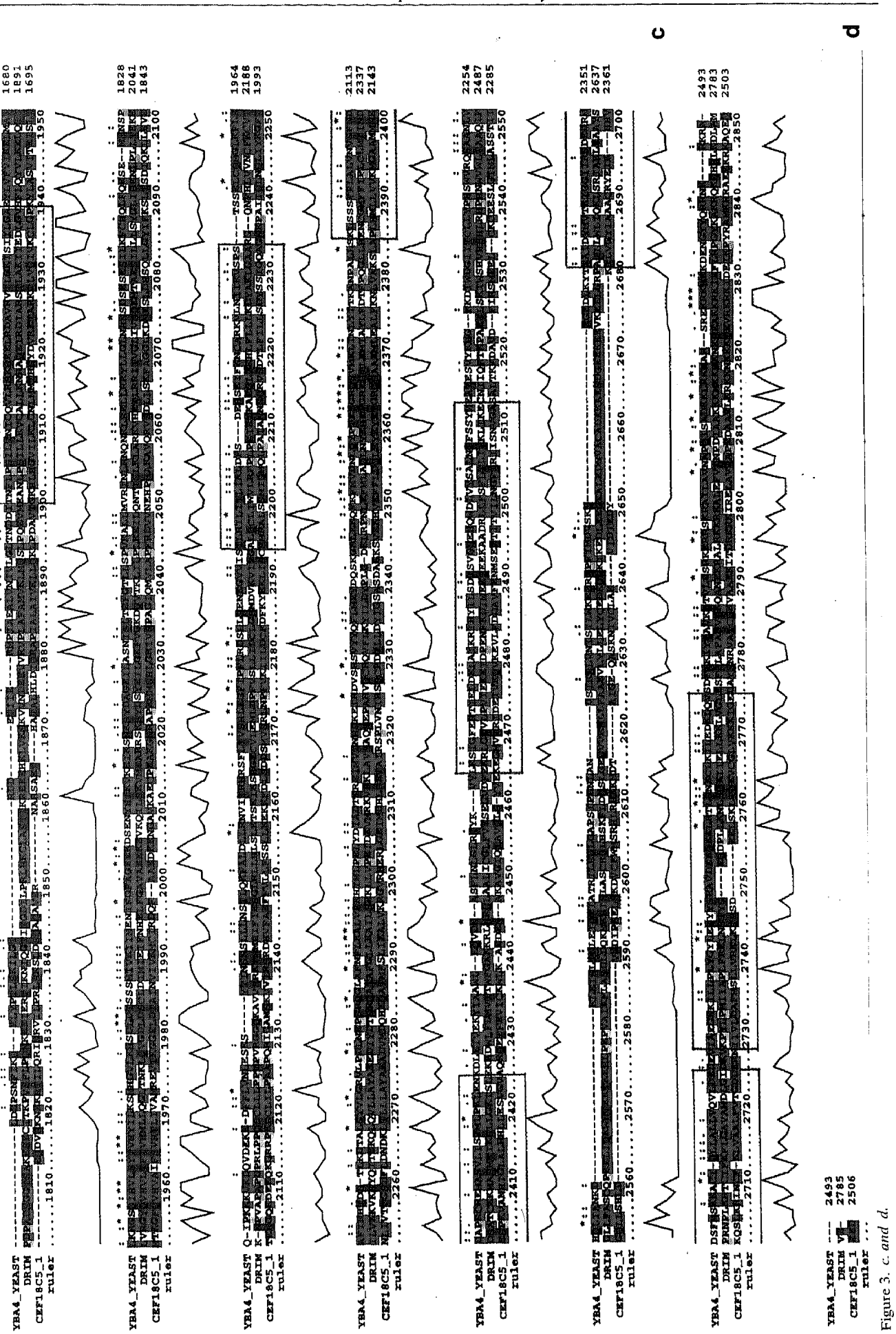

Figure 3. *c. and d.*

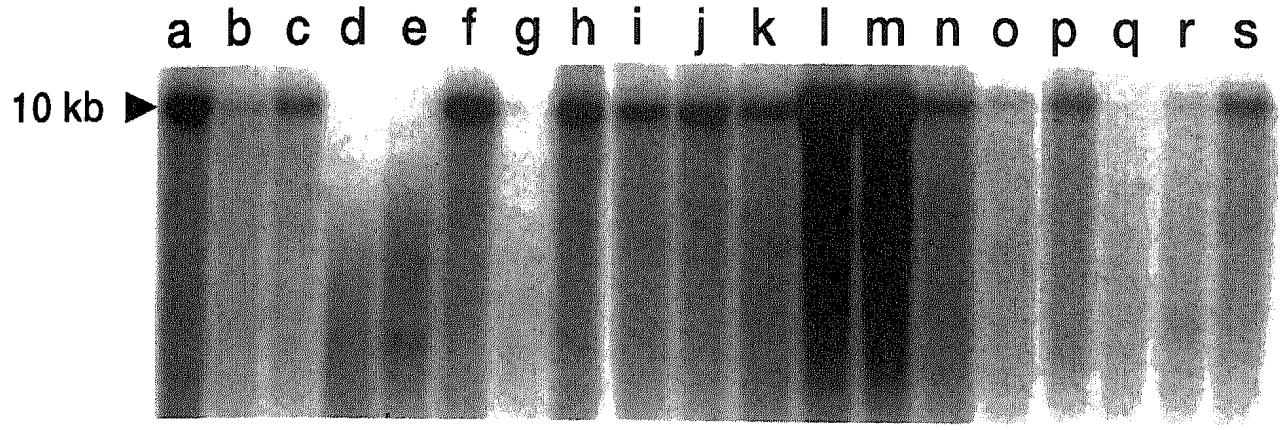

Figure 4. *Expression of DRIM messenger RNA in selected tissues. Clontech filters with immobilized polyA$^+$RNA were hybridized with an [α^{32}P]dCTP-labeled probe covering part of the 3'coding region of DRIM cDNA. Lanes: a, heart; b, brain; c, placenta; d, lung; e, liver; f, skeletal muscle; g, kidney; h, pancreas; i, spleen; j, thymus; k, prostate; l, testis; m, ovary; n, small intestine; o, colon; p, appendix; q, peripheral blood leucocytes; r, bone marrow; s, fetal liver.*

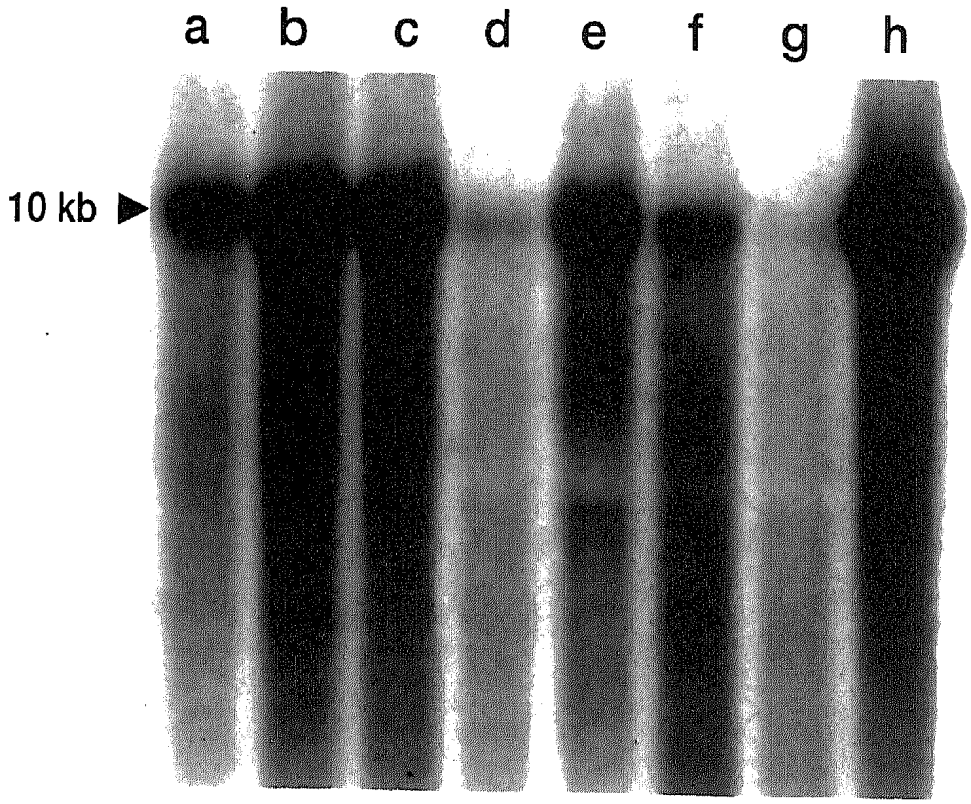

Figure 5. *Expression of DRIM messenger RNA in selected tumor cell lines Clontech filters with immobilized polyA$^+$RNA were hybridized with an [α^{32}P]dCTP-labeled probe covering part of the 3'coding region of DRIM cDNA. Lanes: a, HL60 cells; b, HeLa cells; c, K562 cells; d, MOLT-4 cells; e, Raji cells; f, SW-480 cells; g, A 540 cells; h, G 361 cells.*

Vinblastine-resistant human breast cancer cell lines. Cancer Res *52:* 5190-5197, 1992.

9 Caillou R, Olive M and Cruciger QVJ: Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In vitro 14:* 911-915, 1978.

10 Liang P and Pardee AB: Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science *257:* 967-970, 1992.

11 Liang P, Averboukh L and Pardee AB: Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. Nucleic Acids Res *21:* 3269- 3275, 1993.

12 Liang P, Averboukh L, Keyomassi K, Sager R and Pardee AB: Differential display and cloning of messenger RNAs from human breast cancer versus mammary epithelial cells. Cancer Res *52:* 6966-6968, 1992.

13 Chomczynski P and Sacchi N: Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem *162:* 156-159, 1987.

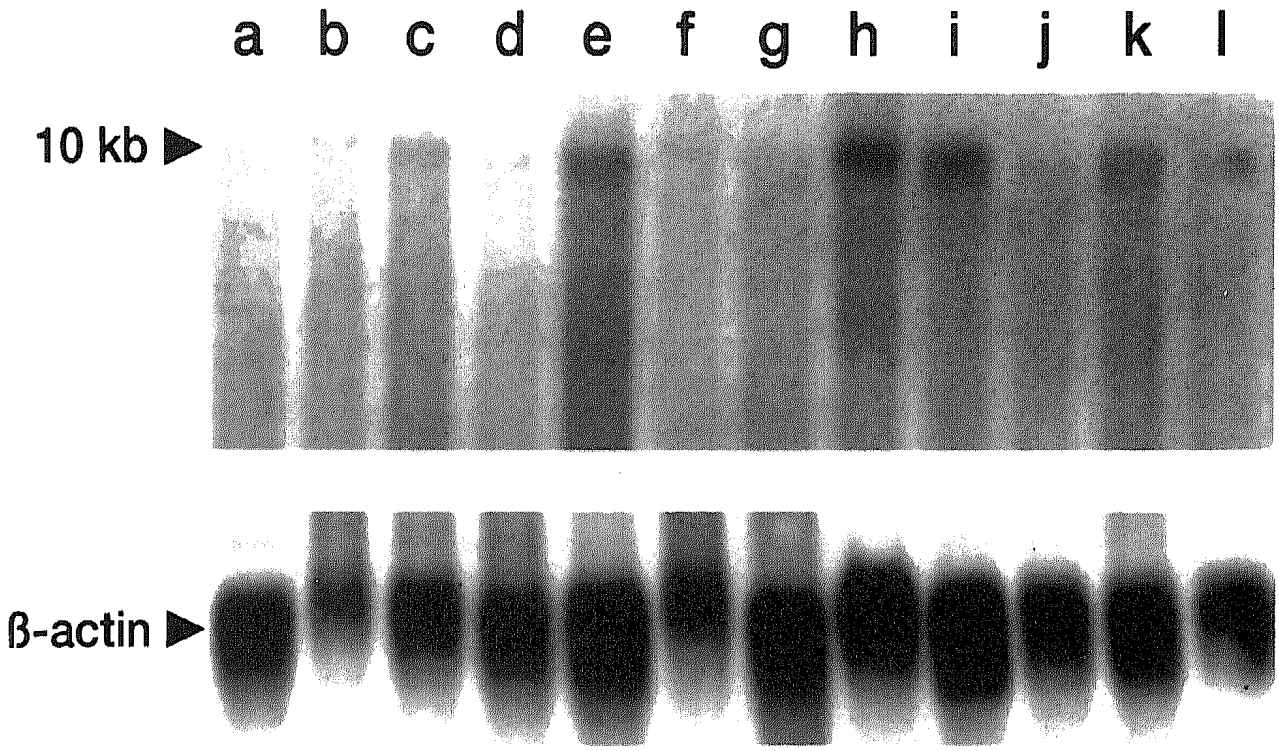

Figure 6. *Northern blot analysis of DRIM messenger RNA expression in selected mammary carcinoma cell lines. PolyA$^+$RNA was extracted from confluent cell lines, separated on a denaturing 1 % agarose formaldehyde gel, transferred to a positively charged nylon membrane and hybridized to an [α^{32}P]-labeled probe derived from the 3' coding region of DRIM. Lanes: a, mammary gland; b, MDA-MB 231; c, MDA-MB 435; d, MDA-MB 436; e, ZR-75- 1; f, T47D; g, Hs578T; h, MCF-7; i, MCF-7ADR; j, LCC-1; k, LCC-2; l, LCC-9.*

14 Frohmann M: On beyond classic RACE (Rapid Amplification of cDNA Ends). PCR Meth. Appl *4:* 40-48, 1994.

15 Schaefer B: Revolutions in rapid amplification of cDNA ends: new strategies for polymerase chain reaction cloning of full-length cDNA ends. Anal Biochem *227:* 255- 273, 1995.

16 Bao L, Pigott R, Matsumura Y, Babon D and Tarin D: Correlation of VLA-4 integrin expression with metastatic potential in various human tumor cell lines. Differentiation *52:* 239-246, 1993.

17 Bao L, Matsumura Y, Baban D, Sun Y and Tarin D: Effects of inoculation site and Matrigel on growth and metastasis of human breast cancer cells. Eur J Cancer *70:* 228- 232, 1994.

18 Muneau C, Noel A, Weidle UH, Krell HW and Foidart JM: Modulation of the expression of interstitial and type IV collagenases in coculture of HT 1080 fibrosarcoma cells and fibroblasts. Inv Metastasis *15:* 169-178, 1995.

19 Duffy MJ: Urokinase-type plasminogen activator and malignancy. Fibrinolysis *7:* 295- 302, 1993.

20 Blasi F: Surface receptors for urokinase plasminogen activator. Fibrinolysis *2:* 73-84, 1988.

21 Birchmeier W and Behrens J: Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. Biochem. Biophys. Acta *1198:* 11-26, 1994.

22 Aaronson SA: Growth factors and cancer. Science *254:* 1146-1153, 1991.

23 Cailleau R, Young R, Olive M and Reeves WJ Jr: Breast tumor cell lines from pleural effusions. J Natl Cancer Inst *53:* 661-674, 1974.

24 Hackett AJ, Smith HS, Springer EL, Owens RB, Nelson-Rees WA, Riggs JL and Gardner MB: Two syngeneic cell lines from human breast tissue: the aneuploid mammary epithelial (Hs 578 T) and the diploid myoepithelial (Hs 578 Bst) cell lines. J Natl Cancer Inst *58:* 1795-1806, 1977.

25 Brünner N, Boulay V, Fojo A, Freter CE, Lippman ME and Clarke R: Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. Cancer Res *53:* 283-290, 1993.

26 Brünner N, Frandsen TL, Holz-Hansen C, Bei M, Thompson EW, Wakeling AE, Lippman ME and Clarke R: MCF 7/LCC 2: a 4-hydroxytamoxifen resistant human breast cancer variant that retains sensitivity to the steroidal antiestrogen ICI 182, 780. Cancer Res *53:* 3229-3232, 1993.

27 Freake HC, Marcocci C, Iwasaki J and MacIntyre I: 1,25-dihydroxy-vitamin D3 specifically binds to a human breast cancer cell line (T47D) and stimulates growth. Biochem Biophys Res Commun *101:* 1131 -1138, 1981.

28 Schiemann S, Rückels M, Engelholm LA, Schwirzke M, Brünner N and Weidle UH: Differential gene expression in human mammary carcinoma cells: identification of a new member of a receptor family. Anticancer Research *17:* 13-20, 1997.

29 Engel LW, Young NA, Tralka TS, Lippmann ME, O'Brian SJ and Joyce MJ: Establishment and characterization of three new continuous cell lines derived from human breast carcinomas. Cancer Res *38:* 3352-3364, 1978.

30 Andrade MA and Bork P: Heat repeats in the Huntingtons's disease protein. Nature Genet *11:* 115-116, 1995.

31 Görlich D, Dabrowski M, Bischoff FR, Kutay U, Bork P, Hartmann, E, Prehn S and Izauralde E: A novel class of RanGTP binding proteins. J Cell Biol *138:* 65-80, 1997.

Received January 2, 1998
Accepted February 9, 1998