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The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumors

(promoter/oncogene)

LYNN M. MATRISIAN*, G. TIM BOWDEN†, PETER KRIEG‡, GERHARD FÜRSTENBERGER§, JEAN-PAUL BRIAND¶, PIERRE LEROY*, AND RICHARD BREATHNACH*||

*Laboratoire de Génétique Moléculaire des Eucaryotes, Centre National de la Recherche Scientifique et U.184 de l'Institut National de la Santé et de la Recherche Médicale, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg Cédex, France; †Department of Radiation Oncology, University of Arizona Medical School, Tucson, AZ 85724; ‡Institute of Virus Research and §Institute of Biochemistry, German Cancer Research Center, Im Neuenheimer Feld 280, D-6900 Heidelberg, Federal Republic of Germany; and ¶Institut de Biologie Moléculaire et Cellulaire, Laboratoire d'Immunochimie, 15 rue Descartes, 67084 Strasbourg Cédex, France

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ABSTRACT **Transin RNA is a 1.9-kilobase RNA transcript induced by oncogenes in rat embryo fibroblast cell lines. We show that RNA species complementary to a cloned transin cDNA are present in mouse skin squamous cell carcinomas induced by a classical initiation–promotion protocol but not in premalignant, benign papillomas or in normal epidermis. A single application of a tumor promoting phorbol ester to normal epidermis elicits a transient increase in these RNA levels. Transin RNA encodes a secreted protease, an activity consistent with a functional role for enhanced expression of transin RNA in the progression of benign, encapsulated tumors to malignant, invasive carcinomas.**

During the process of either spontaneous or experimental carcinogenesis, the progression of normal cells through premalignancy to malignancy is accompanied by a variety of changes in morphological and biochemical properties. Some of these changes may result from alterations in cellular gene expression that are detectable as differences in the mRNA populations of transformed cells compared to their normal counterparts (1–7). For example, we have identified and sequenced a cloned cDNA corresponding to a 1.9-kilobase mRNA (transin RNA, formerly pTR1 RNA) that was present in significantly higher levels in rat cells transformed by polyoma virus, Rous sarcoma virus, and the activated cellular oncogene Ha-*ras* than in the normal parental cell lines (7). Transin RNA is also induced by epidermal growth factor in rat fibroblasts (7). A second RNA (transin-2 RNA) related to transin RNA and encoding a protein that is 71% homologous to transin is also induced by oncogenes in rat cells (L.M.M. and R.B., unpublished data). To investigate whether the expression of such transformation-specific transin RNAs occurs during the process of tumor progression *in vivo*, we have used the well-characterized mouse skin model of carcinogenesis (8–10), where it is known that invasive, malignant squamous cell carcinomas can be derived directly from benign, encapsulated, noninvasive papillomas (11) and that both tumor types are monoclonal in nature (12). We show that transin RNA can be detected in carcinomas and that transin is a secreted protease.

MATERIALS AND METHODS

RNA Analysis. Induction of tumors and preparation of RNA from papillomas, carcinomas, or skin samples was as described (6). RNA samples were subjected to gel electrophoresis, transferred to nitrocellulose, and hybridized to nick-translated pTR1 as described (7), except that the hybridization solution contained 40% (vol/vol) formamide and that filters were washed at 50°C in 2× SSC/0.1% $NaDodSO_4$. (1× SSC = 0.015 M sodium citrate/0.15 M NaCl, pH 7.0.)

Protein Analysis. Antibodies against a synthetic peptide were generated in rabbits by repeated injections of peptide in Freund's complete adjuvant. For immunoprecipitation studies, Rat-1 (± epidermal growth factor at 20 ng/ml), B-77, and Rat-1 1.2 cells (see ref. 7 for origins) in 60-mm culture dishes were placed in 2 ml of methionine-free Dulbecco's modified Eagle's medium (DMEM) and incubated for 24 hr in the presence of 115 μCi of [^{35}S]methionine (1 Ci = 37 GBq; Amersham). The conditioned medium was collected and clarified by centrifugation. Intracellular proteins were isolated by Nonidet P-40 lysis as described (13). For Fig. 3*A*, amounts of each sample corresponding to 10^6 cpm incorporated into protein were boiled in 1% $NaDodSO_4$/25 mM EDTA and diluted in immunoprecipitation buffer (50 mM Tris/acetate, pH 7.5/1% Triton X-100/0.4 M NaCl/1 mM dithiothreitol). Following preincubation of the samples with 4 mg of protein A-Sepharose (Pharmacia), the supernatants were incubated at 4°C for 3–4 hr with 5 μl of either immune or preimmune rabbit serum. Another 4 mg of protein A-Sepharose was added, and the samples were agitated overnight at 4°C. The pellets were washed seven times in immunoprecipitation buffer/0.2% $NaDodSO_4$, and eluted material was examined by electrophoresis on a 10% $NaDodSO_4$/polyacrylamide gel (14). The gels were fixed in 40% (vol/vol) methanol/7% (vol/vol) acetic acid and prepared for fluorography by soaking in 60 g of 2,5-diphenyloxazol/340 ml of dimethyl sulfoxide for 90 min following equilibration in dimethyl sulfoxide. Hydrated gels were dried and exposed to x-ray film at −80°C. Size markers were ^{14}C-labeled standards (Bio-Rad, high molecular weight). For Fig. 3*B*, amounts of medium samples corresponding to 2×10^4 cpm incorporated in protein were used.

Protease Activity. pTR2 and pTR3 were prepared by introduction of a full-sized transin cDNA (7) into the unique *Eco*RI sites of pKCR3 and pKCR6, respectively. pKCR3 was prepared from pKCR2 (15) by replacing pBR322 vector sequences by those of pAT153. pKCR6 was prepared by ligation of a *Pst* I/*Bgl* II digest of pKCR2 (15) with a *Pst* I/*Bam*HI digest of pMTVdhfr (16). Transfection of COS cells (17) and Chinese hamster ovary (CHO) cells deficient in dihydrofolate reductase, clone DXB11 (18), was performed by the calcium phosphate precipitation procedure (19). Cells (3×10^5 cells per 60-mm dish) were exposed to 10 μg of DNA. Selection of CHO cells stably maintaining pTR3 was done

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Abbreviations: CHO, Chinese hamster ovary; SV40, simian virus 40.

||To whom reprint requests should be addressed.

using selection medium (20) supplemented with 10 nM methotrexate. For collection of conditioned medium, CHO cells, CHO cells stably maintaining pTR3 and COS cells 2 days after transfection with pTR2 or a β-globin control plasmid pEP1 (21), were placed in methionine-free DMEM (2 ml per 60-mm dish) containing either unlabeled or ^{35}S-labeled methionine (Amersham, 1075 Ci/mmol, 105 μCi/2 ml) for 24 hr. For Fig. 4*B*, 50 μl of ^{35}S-labeled medium was immunoprecipitated as described above and electrophoresed on an 8% NaDodSO$_4$/polyacrylamide gel. Medium samples represent 20 μl of ^{35}S-labeled medium. For casein digestion assays, unlabeled conditioned medium from cells described above was concentrated 10 times, and the buffer was changed to 20 mM Tris·HCl, pH 7.5/5 mM $CaCl_2$ by ultrafiltration (Amicon YM30 filter). The concentrated samples (40 μl) were incubated with ≈5 μg of [*methyl*-^{14}C]α-casein (New England Nuclear; 0.0029 mCi/mg, 10–15 nCi/50 μl, solubilized by treatment with NaOH and brought to pH ≈7 with HCl) in 0.1 M Tris·HCl, pH 7.5, and 1.0 mM aminophenylmercuric acetate at 37°C for 6 hr. Casein digestion products were analyzed on a 12% NaDodSO$_4$/polyacrylamide gel and visualized by fluorography as described above.

RESULTS

Tumor production in female NMRI mice was initiated with a single dose of 100 nmol of 7,12 dimethylbenz[a]anthracene, followed with multiple applications of the potent phorbol ester tumor promoter phorbol 12-myristate 13-acetate (PMA; 10 nmol, twice weekly). Benign papillomas, ≈10 per animal in all animals, were apparent after ≈15 weeks. Conversion of 5–7% of the papillomas to squamous cell carcinomas occurred after 25–30 weeks. Blots of total RNA from the tumors were hybridized with the nick-translated rat transin probe pTR1 (7). This probe will detect both transin and transin 2 RNAs. No RNA hybridizing to this probe was detectable in normal mouse epidermis or in any of the six papillomas tested (Fig. 1, lanes 1–5, and Fig. 2). A 1.9-kilobase transcript was detected, however, in five out of six carcinomas tested (Fig. 1, lanes 6–10, and Fig. 2). (The smear observed under the carcinoma RNA is due to slight degradation.) In addition, three out of three carcinomas produced by repeated application of the carcinogen *N*-methyl *N*-nitroso-*N'*-nitroguanidine contained RNA species hybridizing to pTR1 (data not shown). Screening of λgt10 cDNA libraries prepared from carcinoma RNA with the rat transin probe pTR1 allowed us to estimate that transin RNAs represent ≈0.002% of poly(A)$^+$ RNA transcripts in carcinomas. No pTR1 hybridizing clones were detected in 200,000 phage from a papilloma cDNA library. Partial nucleotide sequence analysis of a cloned pTR1-cross-hybridizing carcinoma cDNA allowed its identification as the mouse homologue of rat transin cDNA (data not shown).

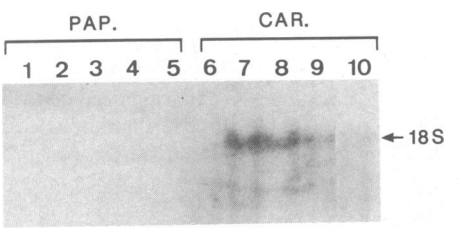

FIG. 1. Transin RNA expression in carcinomas. Total RNAs (10 μg) from each sample were analyzed simultaneously by gel electrophoresis, transferred to nitrocellulose, and hybridized with the transin probe pTR1. Lanes 1–5: papillomas (PAP). Lanes 6–10: carcinomas (CAR). The blot was also hybridized to a nick-translated probe corresponding to 7S RNA (22), which demonstrated roughly equivalent levels of RNA in each lane (data not shown). The location of 18S rRNA is indicated.

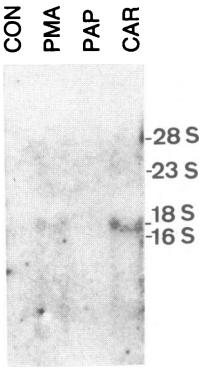

FIG. 2. Tumor promoter induction of transin RNA. The back skin of mice was treated with 17 nmol of phorbol 12-myristate 13-acetate (PMA) dissolved in acetone or acetone alone (CON), dissected 4 hr later, and frozen immediately on a cold table. The epidermis was scraped off and collected in liquid nitrogen, and RNA was analyzed as in Fig. 1. Size markers represent *Escherichia coli* and mouse rRNA species. Papilloma (PAP) and carcinoma (CAR) RNA are also shown for comparison. All lanes contained equivalent amounts of 7S RNA (data not shown).

It has been suggested that tumor promoters can bring about the transient expression of certain malignant phenotypes in normal cells (23, 24). Consistent with a link between the expression of transin family RNAs and malignancy, a single application of phorbol 12-myristate 13-acetate (17 nmol) to normal mouse epidermis induced a transient increase in RNA species detectable with the pTR1 probe that was maximal at 4 hr and decreased rapidly thereafter (Fig. 2 and data not shown).

The above results raise the possibility that the proteins corresponding to the carcinoma- and tumor promoter-induced RNAs may be involved in the progression from a benign to a malignant tumor. We, therefore, undertook the characterization of the protein transin. We have published (7) the sequence of a full-length transin cDNA and the deduced sequence of the 475-amino acid protein transin. To examine transin produced *in vivo*, we prepared a synthetic peptide (25) corresponding to 23 amino acids located near the carboxyl terminus of the protein (residues 445–468, ref. 7). This peptide was injected into rabbits for preparation of an antiserum. Cellular proteins and conditioned medium labeled with ^{35}S-methionine were collected from Rat-1 cells in the presence or absence of epidermal growth factor, Rous sarcoma virus-transformed Rat-1 cells (B-77), and polyoma middle-sized tumor oncogene-transformed Rat-1 cells (Rat-1 1.2) and immunoprecipitated with the anti-peptide antiserum. No immunoprecipitable radioactivity was observed in the cellular extracts from any of the samples (data not shown). In the conditioned medium samples, however, the anti-peptide antiserum precipitated a doublet of 58–60 kDa from B-77, Rat-11.2, and Rat-1 cells treated with epidermal growth factor, but not from untreated Rat-1 cells (Fig. 3*A*). Addition of the synthetic peptide to the immunoprecipitation reaction blocked precipitation of the doublet (data not shown). We conclude that transin is a secreted protein. Examination of the profile of total ^{35}S-labeled proteins secreted into the medium revealed that transin is a minor component of the proteins secreted by these cells (Fig. 3*B*). A similar experiment following labeling of cells with [^{32}P]orthophosphate indicated that transin is not a phosphoprotein (data not shown). The slight difference in the apparent molecular weight of transin produced *in vitro* and *in vivo* is likely due to a difference in glycosylation or some other post-translational modification.

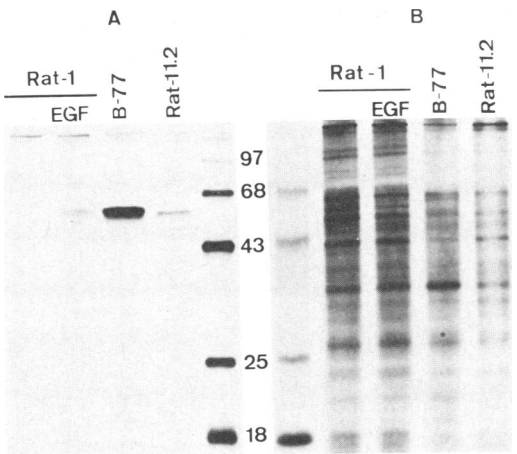

FIG. 3. Immunoprecipitation of transin produced *in vivo*. Rat-1 [(± epidermal growth factor (EGF) at 20 ng/ml], B-77, and Rat-1 1.2 cells were incubated for 24 hr in the presence of [^{35}S]methionine. The conditioned medium was collected and clarified by centrifugation. Samples of conditioned medium were analyzed by electrophoresis on 10% $NaDodSO_4$/polyacrylamide gels either before (*B*) or after (*A*) immunoprecipitation using an anti-peptide antiserum. The positions of migration of ^{14}C-labeled marker proteins (Bio-Rad, high molecular weight) are shown.

The observation that transin is secreted by malignant cells suggested that it could be a protease. To test this hypothesis, full-length transin cDNA was introduced into vectors suitable for transient (pTR2) or permanent (pTR3) expression in eukaryotic cells (Fig. 4*A*). pTR2 is designed to express transin cDNA under control of the simian virus 40 (SV40) early gene promoter with use of splice and polyadenylylation signals from a β-globin gene. It also contains the SV40 origin of replication and will replicate to high copy numbers in COS cells (17). Conditioned medium from COS cells transfected with pTR2 contained significant levels of transin and was capable of digesting the protein casein (Fig. 4 *B* and *C*). It was, however, necessary to activate the conditioned medium by treatment with *p*-aminophenylmercuric acetate before casein digestion took place, suggesting that the protease activity is latent. Conditioned medium from COS cells transfected with a control recombinant expressing β-globin sequences did not contain detectable levels of transin and showed little activity in the casein digestion assay (Fig. 4 *B* and *C*).

pTR3 contains two transcription units, one for expressing transin cDNA under control of the SV40 early gene promoter with use of splice sites from a β-globin gene and a polyadenylylation site from the SV40 late gene region and one for expressing a dihydrofolate reductase cDNA under control of a weak mouse mammary tumor virus long terminal repeat promoter, with use of splice and polyadenylylation signals from the SV40 early gene region. The recombinant pTR3 was introduced into CHO cells deficient in dihydrofolate reductase (18), and transformants were selected in the absence of nucleosides (20) but in the presence of methotrexate in an attempt to amplify expression of transin (20). Conditioned medium from CHO cells transfected with pTR3 and resistant to 10 nM methotrexate contained significant levels of transin and was much more active in the casein digestion assay than conditioned medium from the parent CHO line (Fig. 4 *B* and *C*). (It is not possible to compare the protease activities of COS and CHO cells as we cannot determine their relative transin contents. These lines also probably secrete different levels of protease inhibitors.) We also tested conditioned medium from COS and CHO cells expressing transin for its ability to degrade type I collagen. However, we were unable to detect any collagenase activity using ^{3}H-labeled collagen

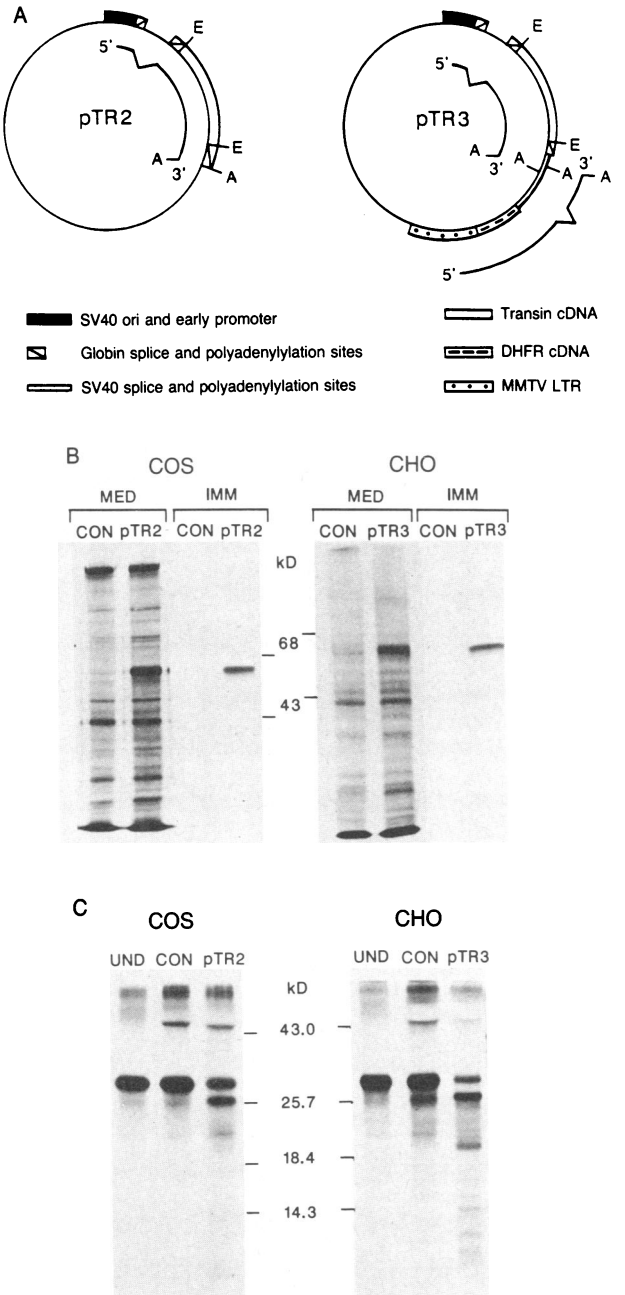

FIG. 4. Transin expression correlates with enhanced proteolytic activity. (*A*) Recombinants used for transin expression. The structures of spliced polyadenylylated mRNAs from each transcription unit are shown. Drawings are not to scale. E, *Eco*RI; A, polyadenylylation site. DHFR, dihydrofolate reductase. MMTV LTR, mouse mammary tumor virus long terminal repeat. (*B*) Production of transin protein. CHO cells deficient in dihydrofolate reductase (CON), CHO cells stably maintaining pTR3 and grown in 10 nM methotrexate (pTR3), and COS cells 2 days after transfection with pTR2 (pTR2) or the β-globin control plasmid pEP1 (CON) were placed in methionine-free DMEM containing either unlabeled or ^{35}S-labeled methionine for 24 hr. ^{35}S-labeled medium was electrophoresed on an 8% $NaDodSO_4$/polyacrylamide gel before (MED) or after (IMM) immunoprecipitation using anti-peptide antiserum. (*C*) Casein digestion assay. Unlabeled conditioned medium from cells described above was concentrated 10 times, and the buffer was changed to 20 mM Tris·HCl, pH 7.5/5 mM $CaCl_2$ by ultrafiltration (Amicon YM30 filter). The concentrated samples were incubated with [*methyl*-^{14}C]α-casein and 1.0 mM aminophenylmercuric acetate at 37°C for 6 hr. Casein digestion products were analyzed on a 12% $NaDodSO_4$/polyacrylamide gel. Undigested casein (UND) was incubated in the same buffer. Results are representative of three separate experiments. The positions of molecular size standards are shown in kDa.

(*N*-[*propionate*-2,3-^{3}H]propionylated-collagen, 0.74 mCi/mg).

The above results show that transin expression leads to enhanced proteolytic activity. The simplest conclusion is that transin is a protease, either acting directly on casein or via proteolytic activation of another latent protease.

This conclusion was confirmed by two other pieces of data. (*i*) Conditioned medium from Rous sarcoma virus-transformed Rat-1 cells (B-77) was immunoprecipitated using anti-peptide antibodies linked to protein A-Sepharose. The resulting complex of transin–antibody–protein A-Sepharose was able to degrade casein, producing fragments similar to those obtained with transin produced in COS cells. When preimmune antibodies were used, no degradation of casein was detected (data not shown). (*ii*) The amino acid sequence of transin shows some homology to that of proteases known to degrade extracellular matrix components (see below).

DISCUSSION

We have shown elsewhere that oncogene expression leads to an increase in the levels of two related mRNAs (transin and transin-2 mRNAs) in cultured rat embryo fibroblast cells (ref. 7 and L.M.M. and R.B., unpublished data). We show here that this effect has a counterpart *in vivo*: chemically induced skin carcinomas express RNA species hybridizing to a cloned rat transin cDNA at a higher level than normal skin or chemically induced papillomas. The hybridization probes we have used here did not permit a discrimination between transin and transin-2 mRNAs. However, we have used nucleotide sequence analysis to show that a cloned cDNA obtained from carcinoma RNA is the mouse version of rat transin cDNA. We show here that transin is a secreted protease (the high extent of homology between transin and transin-2 strongly suggests that transin-2 is also a secreted protease). One of the differences between the papillomas and eight out of the nine carcinomas tested here is thus the enhanced expression of a secreted protease.

An extrapolation from the results obtained with cultured rat cells (7) suggests that the expression of transin RNAs in carcinomas but not in normal skin is a consequence of oncogene activation. However, activation of *ras* oncogenes has already occurred in papillomas (26), which do not express high levels of transin RNAs. In this respect it is interesting to note that the expression of transin RNA in Rat-1 cells transformed by Ha-*ras* oncogenes can be repressed by serum factors (7). It seems reasonable to suggest that transin RNA expression in papillomas is repressed in a similar way by serum factors and that progression to the carcinoma state is accompanied by loss of part of the repression mechanism (e.g., the receptor for the serum factor). Transin RNA expression in carcinomas would thus be due to a two-stage mechanism: first, oncogene activation, then lifting of negative control.

What are the implications of the expression of proteins like transin in carcinomas? The production of degradative enzymes by transformed cells in culture and in tumor tissue has been well documented (27–30). Among the best characterized of these enzymes are collagenases and plasminogen activators. The amino acid sequence of transin or transin-2 shows no homology with the sequences of identified plasminogen activators (31, 32) or other proteases that are available in data bases. The amino acid sequence of a human skin collagenase has been determined (33) and shows ≈48% homology to the transin sequence. However, a comparison of a partial amino acid sequence of rat skin collagenase with the transin sequence shows that transin is not the rat homologue of human skin collagenase (33), a finding consistent with our inability to detect proteolytic activity of transin on type I collagen. The transin–collagenase sequence homology suggests that the proteolytic activity of transin may be directed against other matrix components, perhaps other types of collagen, and could be responsible for one of the major differences between papillomas and carcinomas, namely the absence (and presumed disintegration) of the connective tissue matrix that encapsulates the benign tumor. It is thought that proteases intervene at the transition from *in situ* to invasive carcinoma (34, 35), where local dissolution of the basement membrane is observed microscopically. The availability of transin cDNA and anti-transin antibodies should allow us to investigate the role played by transin in the processes of invasion and metastasis.

A possible indication that transin may play a role in invasion is the observation that the C-terminal half of transin is distantly related to the similar halves of the serum heme-binding protein hemopexin (36), a protein that interacts with receptors on liver cells. The N-terminal half of transin contains its proteolytic domain (L.M.M. and R.B., unpublished data). Transin may thus have a catalytic domain and a binding domain. The latter domain could bind substrate. Alternatively, as in the case of plasminogen activator (37), the binding domain might interact with cell-surface receptors. Cell-surface binding of proteases could be an important aid to tumor invasion. In this case transin would provide a direct link between oncogene expression and tumorigenicity.

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