Identification of Genes Overexpressed in Tumors through Preferential Expression Screening in Trophoblasts

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ABSTRACT

Early trophoblastic cells share several features with neoplastic cells. Based on that observation, we attempted to identify genes overexpressed in tumors by analyzing genes preferentially expressed in trophoblasts. A subtracted library enriched in complementary DNA from early cytotrophoblasts was constructed, and the expression level of selected recombinants was analyzed on a large panel of normal and tumor tissues. The library was prepared using a polymerase chain reaction-based complementary DNA subtraction method with 6-week amenorrhea cytotrophoblast endoplasmic reticulum-bound RNA as target, and a mixture of complementary DNA prepared from terminal placenta and activated T-lymphocytes as driver. Two rounds of screening were performed to isolate clones preferentially expressed in early placenta. From a total number of recombinant clones estimated at 32,000 in the subtracted library, 594 inserts were analyzed by Southern blot and 21 sequences were isolated as corresponding to genes highly expressed in early placenta. Eleven encoded known molecules, such as carcinomembryonic antigen, human chorionic gonadotropin, and mitochondrial rRNAs. Ten sequences represented novel genes. Northern blot analysis confirmed that most of these genes were preferentially expressed in early trophoblast in comparison to terminal placenta. Three clones that gave detectable hybridization signals on total RNA were extensively studied and were found to be overexpressed in various tumors. Two of these clones, designated B9 and E4, were later identified as corresponding to genes coding for the putative ribosomal protein S18 and the bifunctional enzyme ADE2H1 involved in purine biosynthesis, respectively. Expression of the third clone, E9, was increased up to 10-fold in breast cancer tissues in comparison with normal counterparts. Present results confirm that many genes expressed in the trophoblast are overexpressed in malignant cells. This approach could provide a general targeted method for the identification of genes overexpressed in various neoplastic cell types.

INTRODUCTION

In the search for molecules preferentially expressed in tumors and implicated in the neoplastic process, the placenta represents a model of particular interest. Although the placenta is of normal tissue, its constituent cells share several common features with neoplastic cells, associated in particular with highly mitotic, invasive cytotrophoblasts able to penetrate the maternal tissue. Trophoblasts are plentiful sources of growth factors, hormones, and growth factor receptors, and several pertinent observations suggest autocrine growth control (1–5). Furthermore, most known protooncogenes have also been found to be expressed in placenta (6, 7). Their invasiveness, high cell proliferative activity, immune privilege, and lack of cell contact inhibition, particularly during the first trimester of pregnancy, have led to the definition of the trophoblast as a pseudomalignant type of tissue (8, 9). Taken together, these features suggest that genes preferentially expressed in trophoblasts might also be preferentially expressed in neoplastic cells. Furthermore, while the placenta resembles a locally invasive tumor, trophoblast invasion remains under strict control during normal pregnancy (9). Trophoblastic molecules involved in this control are potential candidates as tumor suppressors and might be absent or modified in tumor cells. These observations led us to investigate gene expression in trophoblasts as a source of genes generally implicated in cell growth control and tumor development.

We report here the isolation of genes expressed in early trophoblasts using a PCR3-based cDNA subtraction method. Analysis of their distribution on a large panel of normal and tumoral cell lines and tissues has demonstrated that this approach provides a suitable method for identifying genes overexpressed in various neoplastic cell types. Among these genes, we identified gene E9, which is preferentially expressed in tumoral breast.

MATERIALS AND METHODS

Tissues. Early placentae (5 to 12 weeks of pregnancy) from women undergoing legal abortions, normal term placentae from uncomplicated caesarean section, and surgical samples of normal and tumoral tissues (maximal size, 1 cm³) were immediately frozen (within 10 min after surgical intervention) and stored in liquid nitrogen until RNA preparation. This tissue collection was obtained and used in accordance with protocols previously approved by the human studies committees of each contributing hospital (Institut Gustave-Roussy, Hôpital Antoine Béclère, Hôpital St. Vincent de Paul, and Hôpital Bichat).

Cells. Human cell lines used during this study and their histological origin are: gestational choriocarcinoma (JAR and JEG-3); hepatocellular carcinoma (PLC/PRF/5 and Hep G2); colon adenocarcinoma (LS180); ovarian carcinoma (OV1/p and OV1/VCR; the OV1/VCR cell line was derived from OV1/p and was resistant to vincristin); epidermoid carcinoma (A431); lung carcinoma (A427); cervix epithelioid carcinoma (HeLa); mammary carcinoma (MCF7, MDA-MB-361, SK-BR-3, BT-20, and BT-474); normal breast transformed by SV-40 (HBL-100); a neuroblastoma cell line (SH-SYSY-5); and a normal fibroblast cell line (CCL-137). All cell lines were obtained from the American Type Culture Collection (Rockville, MD), with the exception of IGR/OV1 (OV1/p; Ref. 10) and OV1/VCR cell lines, kindly provided by Dr. Jean Bénard (Institut Gustave Roussy, Villejuif, France), and SH-SYSY and CCL-137, kindly provided by Dr. Pierre-Olivier Couraud (Institut Cochin de Génétique Moléculaire, Paris, France). Cell lines were grown in Dulbecco’s modified Eagle’s medium or RPMI 1640 (GIBCO-BRL Laboratories, Gaithersburg, MD) supplemented with heat-inactivated 10% fetal calf serum (GIBCO-BRL Laboratories, Gaithersburg, MD), 10 μM nonessential amino acids, 4 mM glutamine, 100 units/ml penicillin, and 100 μM streptomycin.

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μg/ml streptomycin at 37°C with 5% CO₂. Ov1/VCR derived from the Ov1/p cell line was grown as described (11).

Isolation of Cytotrophoblasts. Cytotrophoblasts were purified as described by Kliman et al. (12). Briefly, villous tissue from first trimester placenta was dispersed with trypsin and deoxyribonuclease I. The dispersed cells were then purified on a 5–70% Percoll step gradient (Pharmacia, Uppsala, Sweden). The band at 1.040–1.060 g/ml density was collected. Microscopic examination revealed it to be comprised of cytotrophoblasts with less than 5% contamination by nontrophoblastic cells such as macrophages, fibroblasts, and endothelial cells.

RNA Preparation. Total RNA was prepared from preconfluent cell cultures or frozen tissues using guanidinium isothiocyanate and cesium chloride gradient centrifugation as adapted from the protocol of Chirgwin et al. (13). Endoplasmic reticulum MB-RNA was purified as described previously (14). After trypsinization and purification on Percoll, cytrophoblasts were homogenized in a glass dounce, and MB-RNA was isolated on a sucrose gradient including vanadyl ribonucleoside complexes as ribonuclease inhibitors.

Synthesis of cDNA and Amplification by PCR. A total of 0.1 μg of MB-RNA dissolved in 5 μl diethyl pyrocarbonate-treated water was denatured with 0.1 M NaOH and β-mercaptoethanol. First-strand cDNA was synthesized with the avian myeloblastosis virus reverse transcriptase (Copy kit; In vitrogen, San Diego, CA), using the modified dT-primer shown below (15). RNA-cDNA heteroduplexes ranging from 0.5 to 2 kilobases were electroeluted after migration in 2% agarose. A tail of oligodeoxyguanosine was added to the 3’-end of the first-strand cDNA with terminal deoxynucleotidyl transferase (IBI, New Haven, CT), and the RNA was eliminated by alkali treatment. The cDNAs were amplified with nonspecific primers, including restriction sites for NotI and SalI. The sequence of the “T-primer” used for reverse transcription and PCR was 5’-GACTCGAGTCGACATCGA11ITT 1F1T1T111FF 1-3’. The “C-primer” was 5’-GCATCGCCGC GGCGAGGCCCCCCCCCCC-3’ as described by Loh et al. (16).

The reaction mixtures consisted of 1.25 mM each of the four deoxyribonucleotide triphosphates, 0.5 μM of each primer, and 2.5 units of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3.5 mM MgCl₂, and 0.01% gelatin. Amplification was performed in a Hybaid thermocycler for 25 cycles consisting of 20 s at 94°C, 30 s at 55°C, and 1 min at 72°C. The products were loaded onto a 1% low-melting agarose gel (FMC BioProducts, Rockland, ME). Then, the 0.5–2-kilobase region was excised and reapplied under the same conditions. The products were precipitated, digested with NotI and SalI, size selected, and electroeluted as above.

Total RNA from terminal placenta and from T-lymphocytes activated with phytohemagglutinin and cultivated for several days in the presence of IL2 was prepared using a double-stranded cDNA synthesis system (In vitrogen).

Construction of the Subtracted Library. Subtractive hybridization was performed basically as described by Klickstein (17): 0.2 μg of PCR-amplified early cytrophoblast cDNA digested with NotI and SalI was mixed with 8 μg of activated T-lymphocyte cDNA and 8 μg of terminal placenta cDNA digested with AluI and RsaI (New England Biolabs, Beverly, CA), and then dissolved in 40 μl of hybridization buffer [50% deionized formamide, 10 mM sodium phosphate buffer (pH 7), 5X SSC, 0.1% sodium dodecyl sulfate, and 10 mM EDTA], denatured 5 min at 98°C, and incubated 24 h at 37°C. Common sequences between target and driver were reannealed to form duplexes between short (AluI/RsaI, driver) and long (NotI/SalI, target) fragments, inhibiting formation of cohesive ends. Uninhibited reannealing of complementary strands of cDNAs specifically expressed in early cytrophoblasts allowed the regeneration of NotI and SalI cohesive ends for unidirectional cloning into respective sites in the pBSK II + phagemid vector (Strategene, La Jolla, CA).

Screening of the Library. The subtracted cDNA library was plated at low density, and colonies were picked for growth in duria-Bertani medium and PCR amplification. Plasmid DNA prepared by the boiling miniprep method (18) and digested with NotI and SalI or PCR products of inserts amplified using the original primers were analyzed by Southern blot from 1.2% agarose gels. The gels were soaked in 0.4 M NaOH and sandwiched between two nylon gels. The gels were soaked in 0.4 M NaOH and sandwiched between two nylon (18) and digested with NotI and SalI or PCR products of inserts amplified with the avian myeloblastosisvirus reverse transcriptase (Copy kit; In vitrogen, San Diego, CA), using the modified dT-primer shown below (15). RNA-CDNA heteroduplexes ranging from 0.5 to 2 kilobases were electroeluted after migration in 2% agarose. A tail of oligodeoxyguanosine was added to the 3’-end of the first-strand cDNA with terminal deoxynucleotidyl transferase (IBI, New Haven, CT), and the RNA was eliminated by alkali treatment. The cDNAs were amplified with nonspecific primers, including restriction sites for NotI and SalI. The sequence of the “T-primer” used for reverse transcription and PCR was 5’-GACTCGAGTCGACATCGA11ITT 1F1T1T111FF 1-3’. The “C-primer” was 5’-GCATCGCCGC GGCGAGGCCCCCCCCCCC-3’ as described by Loh et al. (16).

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Preparation of the subtracted cDNA library

Preparation of target cDNA

Isolation of cytrophoblasts on Percoll gradient

Purification of MB-RNA

1st strand cDNA synthesis

dG tailing

PCR amplification with NotI and SalI primers

Synthesis of double-stranded cDNA

NotI and SalI digestion

NotI and SalI digestion

Denaturation and reannealing

Ligation into pBSKI, II digested by NotI and SalI

Transformation into X11-blue host bacteria

Fig. 1. Schematic outline of the procedure for the construction of the subtracted cDNA library enriched for sequences expressed in early cytrophoblasts (6-week amenorrhea). A detailed explanation of the subtracted cDNA library screening is provided in the text. MB-RNA, endoplasmic reticulum membrane-bound polysomal RNA.

RESULTS

Isolation of cDNA clones. We used 6-week amenorrhea placenta as the richest source of highly proliferative, invasive cytrophoblasts for synthesis of target cDNA used in the subtraction experiment described here. Fig. 1 shows the schematic outline of the cloning procedure. cDNA was synthesized from both terminal placenta and
activated T-lymphocyte polyadenylated RNA and used as a driver in an attempt to diminish genes expressed at all stages of placental development and those encoding molecules such as cytokines previously identified in T-lymphocytes. The total number of recombinant clones was estimated at 32,000. The size of the inserts ranged between 500 and 1,000 base pairs. Two rounds of screening were performed to isolate clones preferentially expressed in early placenta. In the first screening, 5,000 colonies were plated. A total of 341 randomly chosen inserts were analyzed by Southern blot. Among these inserts, 72 recombinant clones which showed weak or no hybridization signals with radiolabeled total cDNA probes from terminal placenta (T probe) and activated T-lymphocytes (A probe) were selected. Clones were sibling grouped to 13 discrete sequence populations by matrix cross-hybridization of dot blots. The frequency of clone duplication ranged from 1 to 48. Following this first step of screening, we plated 2,500 colonies of the subtracted cDNA library and performed a second screening. In light of the redundancy of clones isolated during the first screening, we modified our screening procedure. A cDNA probe which encompassed each previously selected clone was synthesized (M probe); 253 inserts from white colonies were amplified by PCR and analyzed on duplicate Southern blots. The two blots were hybridized with the M probe and a mixture of the A+T probes, respectively. Eighteen clones which did not hybridize with these probes were isolated. Sibling grouping defined eight new discrete sequence populations. The frequency of duplication for these clones ranged from one to four.

**Sequence Analysis of Selected cDNA Clones.** Partial DNA sequencing was performed on the 21 selected cDNA clones. In general, 250–350 bases were sequenced for each, sufficient for determining identity to known genes. Database interrogation originally revealed 10 novel sequences. The presence of long open reading frames and polyadenylation signals suggested functional transcripts. The other 11 cDNA clones corresponded to known molecules, as summarized in Table 1. Clones B9 and E4, originally among the 10 novel sequences, are listed with their determined identities. Clones of interest corresponding to novel sequences were completely sequenced from both strands.

### Table 1  Identification of cDNA clones

<table>
<thead>
<tr>
<th>Clone symbol</th>
<th>Frequency</th>
<th>cDNA insert (base pairs)</th>
<th>Homologous gene</th>
<th>mRNA transcript(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4</td>
<td>2</td>
<td>430 Novel</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>E5</td>
<td>1</td>
<td>400 Novel</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>E9</td>
<td>1</td>
<td>580 Novel</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>2</td>
<td>308 Novel</td>
<td>0.8;1.2</td>
<td></td>
</tr>
<tr>
<td>S9D8</td>
<td>1</td>
<td>900 Novel</td>
<td>0.6;1.5</td>
<td></td>
</tr>
<tr>
<td>7B6</td>
<td>1</td>
<td>300 Novel</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>8A6</td>
<td>1</td>
<td>500 Novel</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>8D1</td>
<td>1</td>
<td>600 Novel</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>S5A1</td>
<td>1</td>
<td>1000 Cytochrome C oxidase 1</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>A10</td>
<td>16</td>
<td>430 12S mitochondrial RNA</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>53</td>
<td>610 16S mitochondrial RNA</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>F11</td>
<td>2</td>
<td>203 9S mitochondrial RNA</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>E4</td>
<td>1</td>
<td>780 ADE2-1</td>
<td>1.53;1.1</td>
<td></td>
</tr>
<tr>
<td>F10</td>
<td>1</td>
<td>470 a-tubulin</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>7D5</td>
<td>1</td>
<td>580 CEA/psg1</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>8B6</td>
<td>1</td>
<td>800 Fibronectin</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>E8</td>
<td>1</td>
<td>545 Nucleophosmin</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>B9</td>
<td>1</td>
<td>550 Ribosomal protein S18</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>B10</td>
<td>1</td>
<td>330 Ubiquitin</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>E6</td>
<td>1</td>
<td>435 Vinculin</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* Number of siblings.
* Insert size as determined by sequencing or electrophoresis of PCR products of inserts.
* Transcript size as determined from Northern blots.
* Not determined; CEA, carcinoembryonic antigen.

**Northern Blot Analysis of Tissue Distribution.** The enrichment attained by subtractive hybridization was confirmed by comparing the...
level of expression of the transcripts in early placenta versus both terminal placenta and activated T-lymphocytes by Northern blotting, as shown for clones B9, E4, and E9 taken as representative (Fig. 2). While B9 and E4 expression was also high in lymphocytes, a general survey of several clones, including mitochondrial sequences, revealed mean densitometric scan ratios of 2.2:1:1.4 between hybridization signals on equal amounts of early placenta, terminal placenta, and lymphocyte total RNA, respectively. We then analyzed the expression level of B9, E4, and E9 on a large panel of normal and corresponding tumoral tissues, in order to determine the potential implication of these genes in the neoplastic process.

**Identification of B9, E4, and E9 Clones.** Clone B9 hybridized with a 0.7-kilobase transcript abundantly expressed in early trophoblast. Moreover, expression of B9 was increased in tissue specimens from colon, rectum, stomach, breast, thyroid, and bladder cancers in comparison with their normal counterparts (Fig. 3). Finally, B9 was expressed sparsely in terminal placenta and at varying levels in most other normal tissues. Sequence analysis revealed 549 nucleotides containing a polyadenylation signal and an open reading frame of 185 codons. Initially, the 580-base pair insert included a polyadenylation signal and an open reading frame of 152 amino acids. In a first attempt, database comparison using the FASTA program revealed a recent entry by a match of 125 of 130 amino acids. PROSITE analysis revealed an N-glycosylation site and potential phosphorylation sites. Comparison of both nucleic acid and the predicted polypeptide sequences with GENBANK and SWISSPROT databases, respectively, revealed no significant homology with previously known sequences. The E9 sequence has been submitted to GENBANK (GSDB) with the accession number L34839. In order to investigate its potential association with the tumoral phenotype, we analyzed its distribution in various normal and tumoral tissues by Northern blotting (Fig. 3). E9 was overexpressed in tumoral rectum and bladder, and more strikingly, in tumoral breast tissues in comparison with their normal counterparts. When comparing the expression level of E9 in tumor and in the adjacent normal tissue from five patients, we found it elevated 7-, 5.2-, 3.2-, and 330-fold in patients A, B, D, and E, respectively (Fig. 4). Northern blot analysis of its expression in normal and tumor cells also revealed a high level of transcripts in tumoral breast cell lines (Fig. 5).

**DISCUSSION**

In an attempt to identify genes preferentially expressed in both early trophoblastic cells and tumors, we constructed a subtracted cDNA library and analyzed the expression level of the cDNA clones selected after differential screening on a large panel of normal and tumoral tissue samples. Throughout recent decades, numerous techniques based on tumor cells as sources of investigation have been used in the search for either tumor-associated antigens or genes involved in neoplastic growth. Immunological approaches have led to the finding of a small number of highly immunogenic antigens usually abundantly present in normal tissues (25). Molecular biology methods led to the characterization of a limited set of genes differentially expressed in tumors of a given histological origin (26, 27). In the search for an approach enabling characterization of classes of molecules
involved in either cell growth control or tumor development, we chose
neoplastic cells, which display a pseudomalignant phenotype,
as a source of genes encoding such molecules. Indeed, the placenta, at
an early stage of development, shares several common features with
neoplastic cells.

Endoplasmic reticulum-bound RNA was used in order to enrich for
transcripts encoding membrane or secreted proteins, and clones

Fig. 4. Relative abundance of E9 mRNA in normal and tumoral breast samples. Five
µg of total RNA prepared from five pairs (A-E) of adjacent normal human breast tissues
(N) and human breast cancer tissues (T) were hybridized with 32P-labeled cDNA probe
for clone E9 (each paired sample was obtained from the same patient). Autoradiograms
were quantified by scanning densitometry. Values were normalized to the intensity of each
respective 28S rRNA band after scanning densitometry on photograph of ethidium
bromide-stained nitrocellulose membranes, and ratios were given in relation to the lowest
value. The results were confirmed by normalization to β-actin hybridization signals.

Breast cancer types and tumor stage are: A, intraductal comedocarcino (stage T1/N0/M0); B, infiltrating ductal carcinoma, (stage T2/ N0/ M0); C, infiltrating ductal carcinoma (stage T3 N0/ M0); D, infiltrating ductal carcinoma (stage T4 N0/M0); E, infiltrating ductal carcinoma (stage T4 N0/M0).

From a methodological point of view, our PCR-based DNA-driven
cDNA subtraction method was neither exhaustive nor meant to be
representative of the entire RNA population. Subtraction with double-
stranded cDNA has been described as giving enrichment of up to 50%
(17), and PCR amplification of heterogeneous molecule populations
tends to differ for each molecular species and is irreproducible be-
tween identical samples due to template size, sequence, and abun-
dance. Amplification may favor the presence of some cDNA species
to the detriment of others. While some sequences may be lost, rare
molecular species of potential interest might be amplified; among the eight clones, the sequences of which did not reveal any significant homology to sequences in the databases, it is noteworthy that transcripts of four clones were not detected by Northern blot of total RNA. This observation, as well as the presence of polyadenylation signals, suggests that these clones correspond to rare transcripts.

Partial sequence analysis of the selected cDNA clones, which revealed their identity, enabled us to further evaluate the efficacy of the subtraction. Among the 21 cDNA clones, 11 were first identified as corresponding to known molecules. One-half of these known genes were mitochondrial. The abundance of mitochondrial sequences in subtracted libraries is a well-known nuisance. In this case, the particularly high numbers of mitochondrial sequences may have been due to entrapment of mitochondria along with the membrane in the sucrose gradient performed to isolate RNA. In addition, it is striking that the abundance of mitochondrial sequences in early trophoblasts used as the target cDNA in comparison to terminal placenta used as the driver cDNA, as assessed by Northern blot analysis. The most likely explanation is that variations in expression of mitochondrial sequences are related to energy metabolism in the trophoblastic tissue. It is also noteworthy that mitochondrial genes have been implicated in cancer development, not only by insertion into the nuclear genome, disrupting normal genes, but also through increased expression (29, 30). Among several alterations in chemically induced rat hepatomas, very high levels of mitochondrial transcripts have been noted. Interestingly, overexpression of ND5 transcripts in rat hepatomas occurs during the early steps of carcinogenesis and therefore does not result simply from ultrastructural changes following neoplasia. Thus, an increased level of expression of mitochondrial transcripts in early trophoblasts could correlate with its pseudomalignant pattern.

Among the known genes found in the present study, several have been described as being related to cancer. In addition to the obvious carcinoembryonic antigen/pregnancy-specific protein family of cell adhesion molecules (31), ubiquitin mRNA is known to be one of the major stress-induced molecules (32), and ubiquitin immunoreactivity has been found to be increased in malignancy (33).

E4, first considered as an unknown gene expressed in early placenta, was finally identified as encoding an enzyme involved in de novo purine biosynthesis, ADE2H1. The observed sequence differences between ADE2H1, cloned from HeLa, and E4 might stem from cloning artifacts or sequencing errors, although PCR-induced sequence artifacts were not noticed when comparing other known sequences. Denaturing-gradient gel electrophoresis analysis performed after reverse transcription-PCR on RNA samples from both early trophoblast and HeLa cells may not have been adequate to differentiate between the different transcripts expressed in each cell type, and very low levels of a highly homologous transcript cannot be excluded. The high level of expression of an enzyme implicated in nucleotide synthesis correlates to the highly mitotic and proliferative status of cytrophoblasts of first trimester placenta. Its overexpression in various cancer tissues might also reflect the highly proliferative phenotype of neoplastic cells.

The B9 gene product has been defined as human ribosomal protein S18, and its expression level is significantly increased in some tumors. Interestingly, numerous ribosomal proteins have been demonstrated to be overexpressed in tumors. These include mRNA for ribosomal proteins S3 and S19, which were shown to be expressed at increased levels in colon carcinomas and polyps (34, 35). Since the index of proliferation of colorectal carcinomas is not significantly different from that of normal mucosa, a higher percentage of dividing cells in tumor tissue might not be the only factor. A high level of expression of ribosomal protein L19 has also been described in breast tumors that overexpress erbB-2 (36). In the case of ribosomal protein S18, the homologous mouse KE3 gene belongs to a group of six genes (KEI–5 and SET) located in the H-2K region of murine major histocompatibility complex (37). A homologue of this H-2K region has also been localized within the human major histocompatibility complex region known to contain several genes encoding proteins with important biological activities (38). This possible localization, in addition to pattern homologies of B9 to the fos and jun gene families, should stimulate further studies so as to explore a possible complementary role of the B9 gene in transcriptional regulation.

Finally, the study of clone E9 confirmed that this experimental approach enabled the identification of new genes overexpressed in tumor tissues. Indeed, the higher expression of E9 in tumoral tissues in comparison with their normal counterparts was particularly striking in breast carcinomas. The high level of expression of the E9 gene in six tumoral breast cell lines suggested that it might be expressed by neoplastic cells themselves rather than by stromal cells surrounding the tumor. Recently, a new member of the family of metalloproteinase enzymes which degrade the extracellular matrix, stromelysin-3, was found to be overexpressed in breast adenocarcinomas by a subtractive hybridization method (39). In contrast to E9, its expression was restricted to fibroblasts immediately surrounding neoplastic cells of the invasive component of the tumor. The identification of the E9 gene, overexpressed by neoplastic breast cells, will enable us to investigate its function with regard to malignant transformation. Experiments are in progress to characterize the complete genomic sequence of the E9 gene and its translated product.

In conclusion, the PCR-based DNA-driven cDNA subtraction method described here appears to be suitable for construction of a cDNA subtraction library from small amounts of RNA. This study demonstrates that the trophoblast is a source of genes preferentially expressed in various tumor cells and confirms the pseudomalignant nature of early placenta.

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