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Identification of Genes Differentially Expressed in B16 Murine Melanoma Sublines with Different Metastatic Potentials[1]

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ABSTRACT

B16-F10 and B16-BL6 are B16 mouse melanoma sublines that preferentially metastasize to the lung following i.v. and s.c. injections, respectively. To study molecular mechanisms underlying the different metastatic behaviors exhibited by the B16 melanoma sublines, we performed differential hybridization of the genes transcribed in these cells and compared their expression levels. We isolated four genes that were highly expressed in B16-F10 cells but not in B16-BL6 cells: TI-225 (polyubiquitin), TI-229 (pyruvate kinase), TI-241 (LRF-1 homologue), and TI-227 (novel gene). Triosephosphate isomerase, 10-formyltetrahydrofolate dehydrogenase, tyrosinase-related protein 2, cytochrome *c* oxidase, ATP synthetase α subunit, RNA helicase, and ribosomal protein (L37, J1, acidic phosphoprotein), however, showed higher expression in B16-BL6 cells than in B16-F10 cells. Among these clones, transfection of TI-241 into the low metastatic clone F1 converted the parental cells from low- into high-metastatic cells. TI-241 may regulate the expression of various genes as a transcription factor in the complex process of metastasis.

INTRODUCTION

In spite of the rapid progress in cancer therapy, tumor metastasis is still the major cause of death in patients with solid malignant tumors since metastatic foci are generally resistant to surgical treatment and chemotherapy. Recently, the molecular mechanisms of tumor metastasis have been studied, and practical applications of the results for cancer prognosis and therapy are expected.

Blood-borne tumor metastasis is composed of complicated sequential steps: growth at a primary site, vascularization, invasion into blood vessels, circulation, attachment to capillary endothelial cells, extravasation, invasion, and growth in target organs (1, 2). Several metastatic cell lines that can be used for *in vivo* study were established. To date, studies with those cells have revealed the existence of genes expressed in association with metastatic potential. However, the critical genetic events that would control those whole sequential steps are still unknown. Various methods have been developed to clarify the cellular events and the underlying molecular mechanisms involved in cancer metastasis. Differential screening is one of the methods to identify previously unknown genes that are highly expressed in certain cell lines (3). Several novel genes were found by comparing the expression levels of low- and high-metastatic cell lines, and such studies have helped interpret metastasis as a consequence of molecular events (4–7).

In this study, we used well-known B16 melanoma sublines established from C57BL/6 mouse melanoma because of the variety of metastatic potentials and behaviors, despite the same origin (8–10). The low-metastatic subline B16-F1 was established from lung metastatic foci of i.v. injected B16 melanoma cells, and highly metastatic B16-F10 cells were established by 10 successive selections for lung metastasis following i.v. injection (8). B16-BL6 cells were established from B16-F10 cells that penetrated the mouse bladder membrane (10). Although B16-F10 and B16-BL6 cells are both malignant and have similar characteristics, B16-BL6 cells implanted into the subcutis are capable of spontaneously metastasizing to the lung, whereas B16-F10 cells colonize in the lung only by direct inoculation into the blood vessels. There are some critical differences in the expression levels of certain genes that may confer the metastatic potential through i.v. and s.c. injections. In this study, we isolated genes that correlated with the experimental metastatic potential of B16 melanoma cells.

MATERIALS AND METHODS

Cell Lines. The parental line and sublines of B16 melanoma (B16, B16-F1, B16-F10, B16-BL6 cells) were kindly provided by Dr. I. J. Fidler and Dr. G. L. Nicolson (University of Texas, M. D. Anderson Cancer Center, Houston, TX). The cells were cultured in DMEM containing 10% fetal bovine serum, 100 μg/ml kanamycin, and 2 mM glutamine. All cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Constuction of cDNA Library. Poly(A)$^+$ RNA was prepared from 8×10^7 exponentially growing cells using a Fast-Track mRNA isolation kit (Invitrogen, San Diego, CA), according to the instruction manual. Randomly primed and oligo(dT)-primed cDNA libraries of B16-F10 cells were prepared from 5 μg of each poly(A)$^+$ RNA using a Uni-ZAP XR2 kit (Stratagene, La Jolla, CA) and recloned into Uni-ZAP XR vectors according to the supplied instructions. The titers of both libraries were approximately 5×10^5 plaque-forming units before amplification, and the average length of the cDNA was 2 kb. The clones isolated by differential screening were transformed into Bluescript vectors (Stratagene) using the *in vivo* excision method according to the manufacturer's protocol.

Library Screening. Recombinant clones were plated at a density of 13,000 plaques/90-mm plate and transferred to Hybond N(+) nylon filters (Amersham, Buckinghamshire, United Kingdom) according to the manufacturer's instruction, except baking was at 120°C for 20 min. cDNA probes were prepared using the nucleotide labeling mixture containing digoxygenin-labeled dUTP (Boehringer Mannheim, Mannheim, Germany). Duplicate filters were hybridized with 26 ng/ml digoxygenin-labeled F10 and BL6 cDNA probes in hybridization buffer [50% formamide, 5× SSC, 0.05 M sodium phosphate (pH 7.0), 7% SDS, 0.005% yeast RNA, 0.1% lauroylsarcosine, and 2% blocking reagent] at 39°C for 16 h. Hybridized filters were washed twice in 2× SSC-0.1% SDS at room temperature for 5 min and twice in 0.5× SSC-0.1% SDS at 65°C for 15 min. Filters were hybridized with antialkaline phosphatase antibody and disodium 3-(4-methoxyspiro{1,2-dioxyetane-3,2′-tricyclo-[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate according to the digoxigenin detection kit instructions (Boheringer Mannheim), and then exposed to X-ray films at room temperature for 1 to 60 min.

Northern Blot Analysis. Five-tenths to 2.0 μg poly(A)$^+$ RNA were fractionated by electrophoresis on 1% agarose gel plates containing formaldehyde and transferred to nylon membranes (Amersham). Membranes were hybridized with ^{32}P-labeled probes in a solution containing 50% formamide, 4× saline-sodium phosphate-EDTA, 1% SDS, 0.5% Irish cream (R and A Bailey and Co., Dublin, Ireland), and 125 μg/ml salmon testis DNA at 42°C for 24 h, washed in 2× SSC-0.1% SDS at 42°C four times, and autoradiographed. The membranes were dehybridized at 100°C for 5 to 60 min and rehybridized with mouse actin cDNA probes to confirm the equivalent amounts of poly(A)$^+$ RNAs in the fractions.

Sequencing of cDNA Clones. Plasmid DNAs of the isolated clones were prepared by the alkaline-lysis method and purified with Qiagen-tip 20 (Qiagen, Hilden, Germany) according to the instruction manual. Sequencing of cDNA

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Table 1 *Sequence analysis of the isolated cDNA clones*

Clone	Putative identification (accession no.)[a]	% identity (bp)
TI-1	Mouse domesticus hydrophobic protein (L07096)	100.0 (112)
TI-2	*Xenopus* ribosomal protein L1a (X05216)	77.3 (300)
TI-3	Human casein kinase 2 α′ subunit (M55268)	93.0 (299)
TI-4	Mouse laminin receptor (J02870)	100.0 (174)
TI-32	Mouse elongation factor 1α (M22432)	98.6 (495)
TI-35	Rat 10-formyltetrahydrofolate dehydrogenase (M59861)	74.7 (178)
TI-37	Mouse ferritin light chain (J04716)	99.2 (244)
TI-41	Mouse heat shock protein 70 (M19141)	99.3 (151)
TI-57	Mouse triosephosphate isomerase (X53333)	100.0 (253)
TI-59	Mouse RNA helicase (X65627)	100.0 (178)
TI-62	Mouse tyrosinase-related protein 2 (X63349)	99.8 (517)
TI-72	Mouse ubiquitin (X51703)	97.7 (349)
TI-102	Mouse ATP synthetase α subunit (L01062)	100.0 (259)
TI-115	Mouse glucose-regulated protein 78 (M30779)	100.0 (228)
TI-201	Murine J1 protein (Y00225)	99.7 (293)
TI-216	Mouse acidic ribosomal phosphoprotein (X15267)	100.0 (188)
TI-225	Mouse polyubiquitin (S40697)	99.6 (227)
TI-229	Rat pyruvate kinase (X15800)	97.7 (130)
TI-238	Rat ribosomal protein L 37 (X66369)	95.7 (280)
TI-240	Mouse subunit I of cytochrome *c* oxidase (X57780)	100.0 (163)
TI-241	Rat LRF-1 (M63282)	92.1 (229)

[a] Genbank/European Molecular Biology Laboratory sequence data base accession number for the indicated sequences.

clones was performed using double-stranded templates and plasmid-specific primers in an automatic DNA sequencer (Applied Biosystems Model 373A, Version 2.0). The homology search was performed using Genbank (release no. 87.0) and European Molecular Biology Laboratory (release no. 41) data bases.

DNA Transfection. Transfection of the TI-241 expression vector into low-metastatic clone F1 was performed using the electroporation technique. Control transfection was done with the hygromycin B resistance plasmid pDR-2 (Clontech, Palo Alto, CA). pDR-2 is an EBV-derived mammalian expression vector. The expression of cDNA cloned into pDR-2 is driven by the Rous sarcoma virus long terminal repeat promoter. Colonies growing in 600 $\mu g\ ml^{-1}$ hygromycin B 2 weeks after transfection were expanded and analyzed for expression of the inserted gene. Transfectants of the mixed population at exponential growth phase were harvested by EDTA treatment, washed three times, and resuspended in PBS. Cells (5×10^4) were injected into the tail veins of C57BL/6 mice. Three weeks later, mice were sacrificed, and visible metastases in lungs were examined with a dissecting microscope. The expression of the inserted gene was analyzed using the Northern blot analysis.

RESULTS

Differential Screening. We screened replica filters of B16-F10 cDNA libraries with the cDNA probes synthesized from B16-F10 and B16-BL6 poly(A)$^+$ RNAs. We adopted a nonradioisotope digoxigenin-labeling system for differential screening. Plaque spots were clearer than those obtained using the radiolabeling method. We isolated 133 clones in the first screening from 2×10^5 clones of the B16-F10 cDNA library. After the third screening, 31 cDNA clones that were highly expressed in B16-F10, compared with B16-BL6, were isolated. These 31 clones were classified into 22 independent clones by plaque hybridizations with each other.

Partial DNA Sequencing. We determined the partial DNA sequences of isolated cDNAs and compared them with other known cDNA sequences using the Genbank/European Molecular Biology Laboratory data base (Table 1). Several isolated cDNAs had the same 3′ end sequences as the previously reported genes and were predicted to code the same genes. However, the homology was not always complete, even compared with mouse cDNAs, and some changes existed in noncoding regions.

We isolated two independent clones each of laminin receptors (TI-4), J1 protein (TI-201), tyrosinase-related protein 2 (TI-62), glucose-regulated protein 78 (TI-115), and heat shock protein 70 (TI-41). Three independent clones each of ferritin light chain and elongation factor 1α (TI-32) were also isolated. Although total sequences of the clones were not determined, only one clone, named TI-227, showed no significant homology with formally reported genes.

Northern Blotting. We performed Northern blot analysis using isolated cDNAs and compared the level of expression in B16-F10,

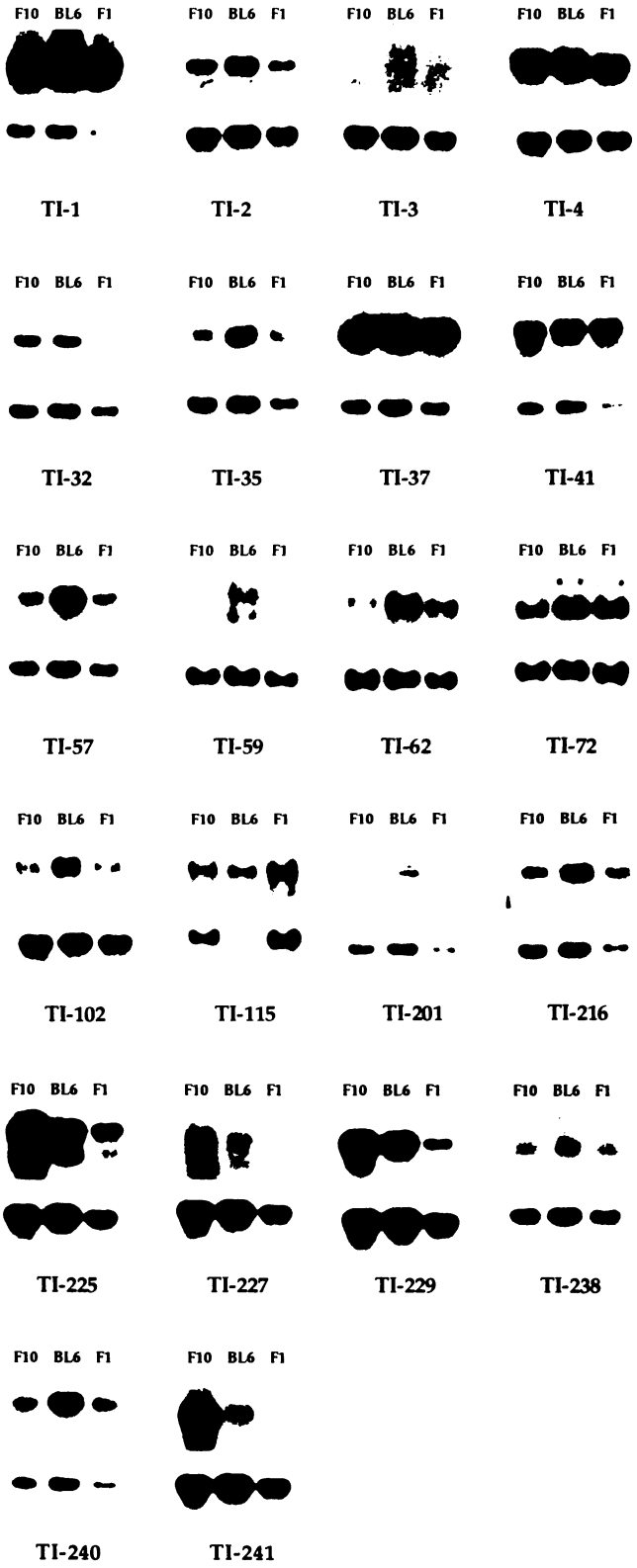

Fig. 1. Northern blot analysis of various isolated genes expressed in B16-F1, B16-BL6, and B16-F10 cells. Samples of poly(A)$^+$ RNA (0.5 μg) were electrophoresed in 1% formaldehyde-agarose gels and transferred to nylon membranes. The membranes were hybridized with the isolated cDNA probe (*top*) and mouse actin probe (*bottom*).

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                                        Met Met Leu Gln His Pro Gly Gln Val Ser Ala Ser
                GAA GAC TGG AGC AAA ATG ATG CTT CAA CAT CCA GGC CAG GTC TCT GCC TCA
                          9          18          27          36          45

Glu Val Ser Ala Thr Ala Ile Val Pro Cys Leu Ser Pro Pro Gly Ser Leu Val Phe
GAA GTC AGT GCG ACC GCC ATT GTC CCC TGC CTC TCA CCT CCT GGG TCA CTG GTA TTT
        60          69          78          87          96          105

Glu Asp Phe Ala Asn Pro Thr Pro Phe Val Lys Glu Glu Leu Arg Phe Ala Ile Gln
GAG GAT TTT GCT AAC CCG ACA CCC TTT GTC AAG GAA GAG CTG AGA TTC GCC ATC CAG
        117         126         135         144         153         162

Asn Lys His Leu Cys His Arg Met Ser Ser Ala Leu Glu Ser Val Thr Val Asn Asn
AAT AAA CAC CTC TGC CAT CGG ATG TCC TCT GCG CTT GAG TCA GTT ACC GTC AAC AAC
        174         183         192         201         210         219

Arg Pro Leu Glu Met Ser Val Thr Lys Ser Glu Ala Ala Pro Glu Glu Asp Glu Arg
AGA CCC CTG GAG ATG TCA GTC ACC AAG TCT GAG GCG GCC CCT GAA GAA GAT GAG AGG
        231         240         249         258         267         276

Lys Arg Arg Arg Arg Glu Arg Asn Lys Ile Ala Ala Ala Lys Cys Arg Asn Lys Lys
AAA AGG AGG CGG CGA GAA AGA AAT AAA ATT GCT GCT GCC AAG TGT CGA AAC AAG AAA
        288         297         306         315         324         333

Lys Glu Lys Thr Glu Cys Leu Gln Lys Glu Ser Glu Lys Leu Glu Ser Val Asn Ala
AAG GAG AAG ACA GAG TGC CTG CAG AAA GAG TCA GAG AAA CTG GAG AGT GTG AAT GCT
        345         354         363         372         381         390

Glu Leu Lys Ala Gln Ile Glu Glu Leu Lys Asn Glu Lys Gln His Leu Ile Tyr Met
GAG CTG AAG GCC CAG ATT GAG GAG CTG AAG AAT GAG AAA CAG CAT TTG ATA TAC ATG
        402         411         420         429         438         447

Leu Asn Leu His Arg Pro Thr Cys Ile Val Arg Ala Gln Asn Gly Arg Thr Pro Glu
CTC AAC CTG CAC CGG CCC ACC TGC ATC GTC CGG GCT CAG AAT GGA CGG ACA CCG GAA
        459         468         477         486         495         504

Asp Glu Arg Asn Leu Phe Ile Gln Gln Ile Lys Glu Gly Thr Leu Gln Ser TER
GAC GAG AGG AAC CTC TTT ATC CAA CAG ATA AAA GAA GGA ACA TTG CAG AGC TAA GCA
        516         525         534         543         552         561

GAG GTG GCA CGG
        573
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Fig. 2. DNA sequence of the TI-241 cDNA clone. The predicted amino acid sequence of open reading frame is shown above the nucleotide sequence.

B16-BL6 and B16-F1 cells (Fig. 1). TI-227 (novel gene), TI-225 (mutant polyubiquitin), TI-241 (LRF-1 homologue), and TI-229 (pyruvate kinase) were all expressed higher in B16-F10 cells than in B16-BL6 cells. Sequence analysis revealed that TI-225 was 99.6% identical to mutant polyubiquitin (Ubiquitin C), whose partial sequence was reported. We determined the entire coding region (accession no. D50527). Its 3′ variation region was thought to be responsible for the different expression pattern between TI-72 (ubiquitin) and TI-225.

Triosephosphate isomerase, 10-formyltetrahydrofolate dehydrogenase, tyrosinase-related protein 2, cytochrome *c* oxidase, ATP synthetase α subunit, RNA helicase, and ribosomal protein (L37, J1, acidic phosphoprotein) were expressed more in B16-BL6 cells than in B16-F10 cells. The other isolated cDNA probes did not show significant differences in the expression levels between B16-F10 and B16-BL6 cells compared with mRNA levels of control actin. Laminin receptor, EF-1α, and heat shock protein have been reported to be expressed in association with metastasis (11–14). However, we did

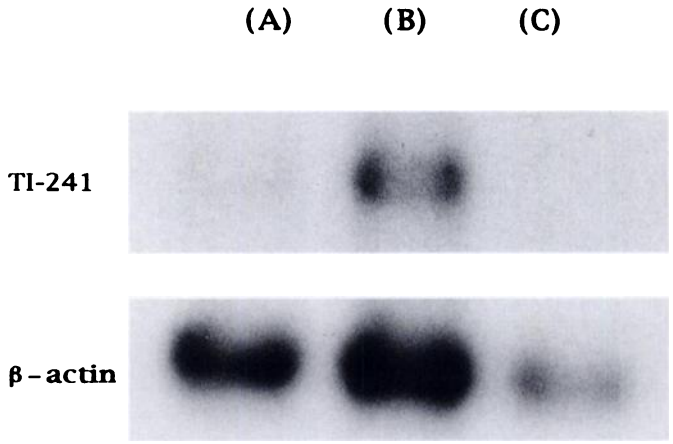

Fig. 3. Northern blot analysis of TI-241 expressed in B16-F1 (*A*), B16-F1 of the TI-241 transfectant (*B*), and B16-F1 of the pDR-2 transfectant (*C*). Samples of poly(A)$^+$ RNA (2.0 μg) were electrophoresed in 1% formaldehyde-agarose gels and transferred to nylon membranes. The membranes were hybridized with the TI-241 cDNA probe (*top*) and mouse actin probe (*bottom*).

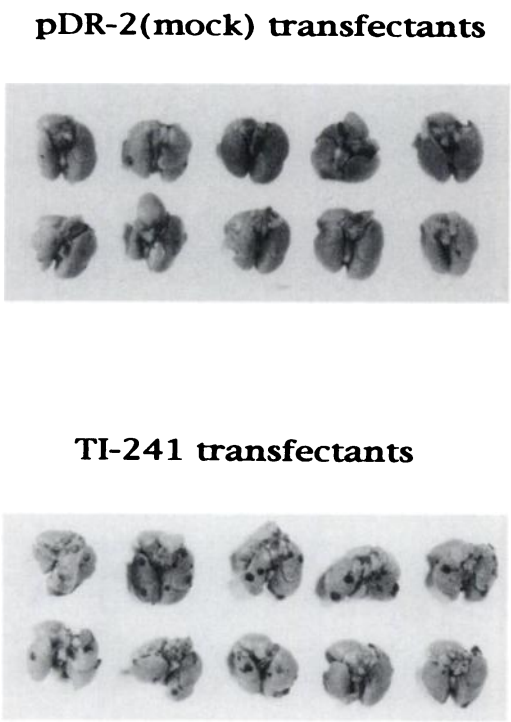

Fig. 4. Experimental metastatic potential of pDR-2 and TI-241 transfectants. Lungs were removed on day 21 after tumor inoculation and fixed in Bouin's solution.

not detect any significant difference in the expression levels of these genes between B16-F10 and B16-BL6 cells.

DNA Transfection. *TI-241* was identified as a gene which was expressed at a higher level in B16-F10 than in B16-BL6. B16-F10 demonstrates extremely higher lung colonization potential than do B16-F1 and B16-BL6. Therefore, B16-F10 could possess certain molecules important for lung colonization of the cells circulating in the blood. Since B16-F1 is the origin of B16-F10 selected for lung colonization *via* i.v., we assume that B16-F1 is a better negative control to evaluate the roll of TI-241 in lung colonization *via* i.v.

The sequence of the TI-241 entire coding region is shown in Fig. 2 (accession no. D50524). We inserted the coding region into the pDR-2 vector and transfected it into B16-F1 cells. Since B16-F1 is a subline of the B16 melanoma cell line and not a clone, B16-F1 cells are heterogeneous, and there is always the risk of picking up originally highly metastatic clones in the cloning procedure. Thus, we decided to evaluate the metastatic potential of TI-241 transfectant cells in the mixed population. Their mRNA level was much higher than that of the parental F1 cells and mock transfectant cells (Fig. 3). We injected transfectant cells into tail veins of C57BL/6 mice. Three weeks later, mice were sacrificed, and visible metastases in lungs were examined (Fig. 4). The transfectant cells showed clearly elevated experimental metastatic potential compared with mock transfectant controls (Table 2). We measured the levels of TI-241 mRNA in the lung metastasis nodules formed by the transfectants and control cells, and observed higher expression of TI-241 in the colonies formed by the transfectants than controls (Fig. 5).

DISCUSSION

Recently, many genes have been isolated using differential screening methods. The most important factor for successful studies using differential screening techniques is thought to be the choice of cell lines. If cell lines distant in nature are used, many unrelated genes could be isolated. On the other hand, screening itself may be difficult when we use cell sublines too close in nature. To study critical gene expression associated with metastatic phenotypes, the differential screening of cDNAs prepared from metastatic and nonmetastatic cells have been used by several groups. Metastasis-related genes, such as *nm23*, *PGM21*, *MTS-1*, *WDNM*, and *EF-1α*, were all isolated using this method (4–7, 13).

We performed differential screening using cDNAs from two highly metastatic sublines of B16 melanoma (B16-F10 and B16-BL6). B16-F10 cells form many lung colonies when transplanted i.v. but not s.c. In contrast, B16-BL6 cells produce lung nodules when transplanted s.c. but not i.v. The aim of comparing two highly metastatic cell lines was to identify genes related to different molecular mechanisms between experimental metastasis introduced i.v. and spontaneous metastasis from the subcutis. Thus, we reduced the risk of picking up abundant unrelated genes, most of which were metabolic and growth-related genes. These genes have been identified from highly metastatic clones that were often more proliferative and metabolically active (5).

By comparing 200,000 cDNA clones, we isolated 22 independent clones differentially expressed in B16-F10 and B16-BL6 cells. Some of the isolated genes have been reported by other groups in association with tumorigenicity and metastatic potential (11–19). But, Northern blot analysis resulted in the isolation of only four genes encoding TI-225, TI-227, TI-241, and TI-229 that were highly expressed in B16-F10 cells. TI-227 represents the only novel cDNA insert identified in this screening. Northern blot analysis identified a mRNA of 3500 nucleotides. Sequence analysis of TI-227 revealed the insert to be 3751 bp, with no obvious open reading frame or signal for translation identification (accession no. D50523). We have not been able to extend the sequence of mRNA further than the 5′ end by primer extension. The human homologue of TI-227 (TI-227H, accession no. D50525), which was isolated from the human fibroblast cDNA library, showed 88.7% homology with TI-227 at its 3′ end. This result suggests that TI-227 is conserved during evolution. Even if this gene may not be translated, the possibility that it plays some role in expression control still remains.

Since metastatic potential is a complex phenotype consisting of multiple steps, we directed our attention to TI-241 (the LRF-1 homologue). LRF-1, which was originally reported to be expressed in rat regenerating liver (20), turned out to be a M_r 21,000 leucine zipper-containing protein and a member of the Jun-Fos family (21, 22). LRF-1 can form homodimers but preferentially forms heterodimeric complexes with c-Jun and JunB in binding to CRE, AP-1, and ATF sites (21, 22). We postulated that TI-241 regulated the expression of various genes as a transcription factor in the complex process of metastasis. A number of studies have demonstrated that one transcription factor could have both positive and negative effects on tumor metastasis, depending on the previous history and transplantation route of the cells. In fact, although B16-F10 transfectants expressing high levels of c-fos and c-jun reportedly reduced experimental meta-

Table 2 *Metastatic potential of B16 melanoma transfectants*

Cells (5×10^4) of each cell line were injected i.v. into five mice. The mice were killed 3 weeks later and examined for metastases. The experiment was repeated twice.

Cell line	Pulmonary metastasis	
	Incidence[a]	No. of colonies[b]
Experiment A		
TI-241 transfectant	5/5	34.2 ± 17.7
Mock transfectant	5/5	8.2 ± 4.0
Experiment B		
TI-241 transfectant	5/5	33.8 ± 15.3
Mock transfectant	5/5	9.6 ± 4.1

[a] Number of mice bearing metastases in the lung/total number of mice inoculated.
[b] Number of lung nodules were expressed as mean ± SD.

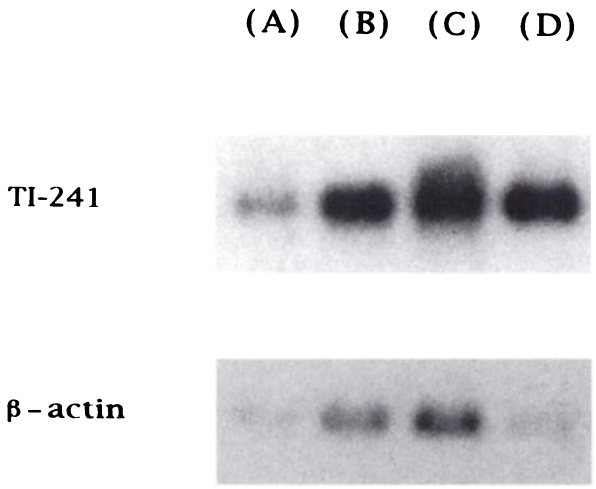

Fig. 5. Northern blot analysis of TI-241 expressed in B16-F1 (*A*) and lung colonies of TI-241 transfectants (*B-D*). Samples of poly(A)$^+$ RNA (2.0 μg) were electrophoresed in 1% formaldehyde-agarose gels and transferred to nylon membranes. The membranes were hybridized with the TI-241 cDNA probe (*top*) and mouse actin probe (*bottom*).

static potential, c-jun expression in other B16 subclones (G3.12, G3.26) was reported to correlate with higher spontaneous metastatic potential (23, 24). The relationship between LRF-1 (or ATF-3, human homologue) and metastatic potential of any cell line has not been reported, to the best of our knowledge. But transfection of the *TI-241* gene in B16-F1 strengthened the experimental metastatic potential in our experiment. At present, it is unclear whether the function of *TI-241* in the metastatic process is specific to B16-F10 or universal. Further analyses of the biological and biochemical changes induced by the *TI-241* gene are now under way.

When the cells are i.v. introduced, the metastatic foci are reported to be greater in F10 than in BL6 (10). Based on that fact, F10 more effectively establishes metastasis, but it may be only because it bypasses the first barrier to invasion. We can suppose that the difference of F10 and BL6 represents the events that occur at the beginning (primary site) and the end (target organ) of the metastatic process, respectively. Since TI-241 is expressed higher in F10 than in BL6, TI-241 can be thought to play a role after the attachment to capillary endothelial cells in the lungs. Although at present the exact function of TI-241 in the metastatic process of B16-F10 is unknown, this gene possibly plays an important role in lung colonization of blood-circulating cells.

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