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Vascular Permeability Factor Expression Influences Tumor Angiogenesis in Human Melanoma Lines Xenografted to Nude Mice

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We studied the expression of the angiogenic factor vascular permeability factor (VPF, also called vascular endothelial growth factor), in human melanoma cells in vitro and in vivo. Melanoma lines that develop tumors with a low metastatic potential in nude mice were found to have low expression levels of VPF in vitro, and the VPF expression levels in melanoma lines that yield highly metastatic xenografts were high. However, in vivo the correlation between VPF mRNA levels and the frequency of metastasis was lost; in all xenografts equally high levels of VPF mRNA were found, independent of the parental cell line. Hence, in vivo VPF gene expression was upregulated in the low expressing lines. The external factor responsible for this induction may be hypoxia, given that we found that low oxygen tension caused a (reversible) increase in the VPF mRNA levels in otherwise low expressing melanoma lines in vitro. A melanoma line with an inducible VPF expression was engineered into a line with a constitutive VPF expression. In the xenografts from this line a change in the vascular architecture was seen, indicating that the pattern or the level of VPF expression is important for tumor angiogenesis in melanoma xenografts. (Am J Pathol 1995, 146:197-209)

Vascular permeability factor (VPF), also known as vascular endothelial growth factor, is a glycosylated, secreted protein factor that increases blood vessel permeability, stimulates endothelial cell division *in vitro*, and induces angiogenesis *in vivo*.^{1,2} Two dif-

ferent receptors for VPF have been characterized: fms-like tyrosine kinase (flt) and fetal liver kinase 1 (flk-1/kinase insert domain-containing receptor). These appear to be expressed almost specifically by endothelial cells and hematopoietic cells.³⁻⁶ VPF is an important angiogenic factor, along with other factors such as acidic and basic fibroblast growth factor (aFGF, bFGF) and transforming growth factor- β (TGF- β).^{7,8} Apart from its expression by some normal, well-vascularized tissues and embryonic tissues, and during wound repair,⁹⁻¹² VPF has been found in many tumors and tumor cells.¹³ In glioblastoma VPF expression was found to be highest near necrotic areas, and clusters of newly formed capillaries were found around the sites of VPF production,^{14,15} suggesting that VPF can be recruited to augment angiogenesis if the tumor vasculature and therefore the oxygen supply is insufficient. The role of VPF in tumor angiogenesis was confirmed by blocking its activity in tumors by a monoclonal antibody, and by application of a dominant-negative VPF receptor mutant.^{16,17} These treatments led to a decrease in angiogenesis and to slower tumor growth.

Several protein variants of VPF exist because of alternative splicing of the VPF mRNA. The molecular variants differ in their efficiency of secretion. The smaller forms (VPF₁₂₁ and VPF₁₆₅) are efficiently secreted by the producing cells, and can easily reach their target cells. The larger forms (VPF₁₈₉ and VPF₂₀₀) are retained at the extracellular matrix, but biologically active parts of these proteins can be released by plasmin.¹⁸⁻²⁰ Theoretically, different cell types may benefit from particular VPF variants. Tissue-specific expression of certain VPF messenger variants has indeed been demonstrated.^{19,21}

As a consequence of its proposed role in tumor angiogenesis, VPF may also facilitate metastasis, as

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this process is dependent on the vascular bed.²² To study the relation between VPF expression and metastasis, a panel of human melanoma cell lines was used. Xenografts of these melanoma lines in nude mice give rise to tumors with distinctly different biological behavior. Some of these lines develop into rapidly metastasizing tumors, whereas other lines metastasize at a low frequency or very slowly (see ref. 23; J.R. Westphal, J.A.W.M. van der Laak, C.J.M. Schalkwijk, D.J. Ruiter, R.M.W. de Waal, manuscript in preparation). The metastatic phenotype of these lines was found to correlate with the expression of a series of genes or antigens, such as urokinase-plasminogen activator and its type 1 inhibitor, various integrins, the epidermal growth factor receptor, thymosin β 10, and calcyclin.²⁴⁻²⁸ We report here that low and highly metastatic melanoma lines have distinctly different expression patterns of VPF. Highly metastatic melanoma lines have constitutively high levels of VPF expression, whereas the less metastatic lines have a low level of VPF expression in culture, which is elevated in mouse xenografts. *In vitro* data suggest that hypoxia may be an important trigger in this upregulation of VPF gene expression. Transfection experiments show that an alteration of VPF expression in melanoma xenografts profoundly affects vascular architecture. Hence, at least in this melanoma model, the VPF expression pattern is an important determinant of angiogenesis, and possibly of angiogenesis-dependent biological behavior.

Materials and Methods

Cell Culture

Human melanoma cell lines were cultured as previously described.²³ Transfected melanoma lines were cultured in medium supplemented with 200 μ g/ml hygromycin B (Boehringer Mannheim, Germany). Human brain capillary pericytes were isolated and cultured as described elsewhere.²⁹ U937 cells and Balb-3T3 cells were from American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Flow Laboratories, Irvine, UK) with 10% fetal calf serum (FCS, Gibco BRL, Paisley, UK), 2 mmol/L L-glutamine and 40 μ g/ml gentamycin (Schering Corporation, Amstelveen, The Netherlands).

Melanoma Xenografts

Human melanoma cells were trypsinized and 2×10^6 cells were injected s.c. into BALB/c *nu/nu* mice as previously described.²³ Xenografts were dissected at

different time points after inoculation, measured, rapidly frozen in liquid nitrogen, and stored at -70 C.

Isolation of RNA

RNA was isolated from cultured cells either using LiCl/urea,³⁰ or using guanidinium chloride.³¹ RNA from tumors and normal murine tissues was isolated after disruption in guanidinium isothiocyanate by CsCl centrifugation.³² RNA concentration was determined spectrophotometrically at 260 nm.³²

Northern Blotting and Probes Used

Samples of total RNA were denatured in 50% formamide in formaldehyde/phosphate buffer (65 mg/ml formaldehyde, 25 mmol/L $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.0) for 15 minutes at 55 C, run on a 1% agarose gel in formaldehyde/phosphate buffer, and blotted to nitrocellulose membranes.³² As a probe for VPF mRNA, a fragment was used which comprised the protein coding region of VPF₁₆₅. To isolate this fragment, a reverse transcriptase (RT) polymerase chain reaction (PCR) was performed on RNA from phorbol 12-myristate 13-acetate-treated U937 cells,¹ using reverse primer (5'-TTCCTCCTGCCCGGCTCACCG-3') and forward primer (5'-CCCGGTCGGGCCTCCGAAACCA-3'). Blunt-ended PCR products were cloned into *Sma*I-digested pUC19 vector DNA and characterized by sequencing. PCR products from the alternatively spliced messengers for VPF₁₂₁, VPF₁₆₅, and VPF₁₈₉ were identified. A 0.6-kb VPF₁₆₅ insert was isolated as an *Xba*I/*Kpn*I-fragment and labeled. Control hybridizations were performed with a human ubiquitin probe,³³ a human β -actin probe,³⁴ or with a *Drosophila* ribosomal RNA probe. Fragments were labeled with [α -³²P]dATP after random hexamer priming.³⁵ Northern hybridizations were performed in 50% formamide, $6 \times$ SSC, 0.1% SDS, 0.1 mg/ml denatured herring sperm DNA and $5 \times$ Denhardt's³² at 45 C, followed by washing in $0.2 \times$ SSC, 0.1% SDS at 65 C. Intensities of hybridization bands were scanned with an Ultrosan XL Enhanced Laser Densitometer from LKB (Bromma, Sweden).

Analysis of Alternatively Spliced VPF mRNA

2 to 8 μ g of total RNA isolated from cell lines or melanoma xenografts was reverse transcribed using 0.5 μ g reverse primer (5'-TTCCTCCTGCCCGGCTCACCG-3'), 0.4 mmol/L deoxyribonucleotides (dNTPs; Pharmacia LKB, Woerden, The Netherlands), five

units avian myeloblastosis virus (AMV)-RT (Stratagene, San Diego, CA), five units RNasin (Boehringer Mannheim), and reaction buffer provided by Stratagene, in final volumes of 10 μ l, for 1 hour at 42 C. After ethanol precipitation and redissolving in 10 μ l distilled water, half of the cDNA was used in PCRs containing 0.3 μ g reverse primer (see above), 0.3 μ g forward primer (5'-GCACCCATGGCAGAAGGAGGA-3'), 0.2 mmol/L dNTPs, and 0.25 units Supertaq polymerase and reaction buffer provided by HT Biotechnology (Cambridge, UK) in final volumes of 25 μ l. 20 cycles of 1 minute at 96 C, 1.5 minutes at 52 C, and 4 minutes at 72 C were performed. 5 μ l of every reaction mix was transferred to a new tube containing the same reagents, for one more cycle (6 minutes at 72 C). 10- μ l samples of the products were separated on a 1.5% agarose gel, Southern blotted to nitrocellulose and probed with the VPF₁₆₅ fragment in formamide hybridization mix at 42 C.

Determination of the Species Origin of VPF mRNA

1 to 12 μ g total RNA isolated from human cell lines, murine tissues, or melanoma xenografts was reverse transcribed using 0.5 μ g reverse primer (5'-TTGGT-GAGGTTTGATCCGCAT-3'), 0.4 mmol/L dNTPs, 5 units AMV-RT, 10 units RNasin, and reaction buffer provided by the supplier of AMV-RT (Stratagene) in final volumes of 10 μ l for 1 hour at 37 C. After ethanol precipitation and redissolving in 10 μ l distilled water, half of the cDNA was used in PCRs containing 0.3 μ g reverse primer (see above), 0.3 μ g forward primer (5'-CGAAACCATGAACTTCTGCT-3'), 0.2 mmol/L dNTPs, 0.25 units Supertaq polymerase, and reaction buffer provided by HT Biotechnology, in final volumes of 25 μ l. 20 cycles of 1 minute at 96 C, 1.5 minutes at 48 C, and 2.5 minutes at 72 C were performed. 1- μ l samples of the products were transferred to new tubes with all the PCR reagents, and subjected to one more cycle (10 minutes at 72 C). PCR products were then precipitated and redissolved. One-third was digested with five units *StyI*, which only cleaves the human product,^{2,36} separated on a 1.5% agarose gel, Southern blotted, and hybridized with the VPF₁₆₅ probe.

Analysis of Secreted VPF Protein

Melanoma cells were cultured for 24 hours in serum-free medium when indicated with 100 μ g/ml heparin. Conditioned media were centrifuged and used for VPF analysis. To correct for differences in cellular den-

sity, cells were scraped and lysed, and the cellular protein content was determined by a standard protein assay (Bio-rad, Veenendaal, The Netherlands). Based on this protein determination, samples of conditioned media derived from equal amounts of cell material were taken, and proteins were acetone precipitated, electrophoresed on 17.5% polyacrylamide gels under reducing conditions, and electroblotted to nitrocellulose membranes. Antisera against VPF were raised in rabbits using purified VPF produced in a bacterial expression system. Antiserum was diluted 1:100 or 1:250 for detection of VPF on Western blots, and the immune reactions were visualized using the chemiluminescent substrate AMPPD (Tropix, Westburg, Leusden, The Netherlands) following the manufacturer's protocol. The crude antisera had some non-specific reactivity toward some bands of higher molecular weight than VPF on Western blots. Where indicated this was diminished by affinity purification of anti-VPF antibodies using purified *Escherichia coli*-produced VPF absorbed by nitrocellulose membranes.³⁷

VPF Induction Experiments

The response to serum, fibroblast-conditioned medium, or growth factors/cytokines was tested after preculturing subconfluent melanoma cell cultures in DMEM without serum for 24 hours. Medium was then replaced for 4 or 24 hours by DMEM with 10% FCS, or by DMEM (with and without FCS) conditioned by Balb-3T3 cells for 24 hours. Alternatively, serum-free medium was replaced for 4 hours by DMEM containing 0.1% bovine serum albumin and one of the following growth factors or cytokines: 5 ng/ml EGF (Collaborative Research, Bedford, MA); 5 ng/ml TGF- α (Bachem, Bubendorf, Switzerland); 2 ng/ml TGF- β 1 (R & D Systems, Minneapolis, MN); 10 ng/ml platelet-derived growth factor AA (PDGF-AA, a gift from Dr. C.H. Heldin, Uppsala, Sweden); 100 ng/ml bFGF (a gift from Scios Inc., Mountain View, CA); ~100 ng/ml VPF₁₆₅ (in culture supernatant of COS cells transfected with plasmid containing VPF₁₆₅ cDNA under control of an SV40 early promoter); 50 ng/ml TNF- α ; 100 units/ml interleukin-4 (IL-4); 400 units/ml interferon gamma (IFN- γ) (TNF- α , IL-4, and IFN- γ were from Boehringer Ingelheim, Germany) 100 units/ml IL-1 β ; 50 units/ml IL-2 (IL-1 β and IL-2 were from Genzyme, Sanbio, Uden, The Netherlands). Hypoxia experiments were performed using subconfluent melanoma cell cultures in 10 mmol/L Hepes-buffered DMEM with 10% FCS. Rubber-capped flasks were flushed for 1 hour at room temperature with 30 volumes of nitrogen made oxygen-free using a BASF R

3-11 catalyst (BASF, Ludwigshafen, Germany). Oxygen-free CO₂ was added up to 5%, and the flasks were then placed back at 37 C for the indicated times. At the end of each incubation, gas samples were tested for O₂ content by gas chromatography, and the pH of the media were determined. In some cases, the P_{O₂} of the media were also determined (CIBA-Corning 288 Blood Gas System, Houten, The Netherlands). Control experiments were performed (in air/5% CO₂) to investigate the influence of the pH of the medium. To achieve a low final pH, cells were grown in HEPES-buffered medium for several days, and to achieve a high pH medium was replaced for 6 hours by HEPES-buffered fresh medium preincubated in air for 1 hour.

Production of Stably Transfected Melanoma Lines

The protein coding region of VPF₁₂₁ was cloned directionally as a *Xba*I/*Kpn*I fragment into the expression vector EBOpLPP,³⁸ by which the sequence was brought under control of SV40 transcriptional elements. This plasmid also codes for the hygromycin phosphotransferase (HPH) gene. The recombinant plasmid and the empty vector were digested with *Apa*I, thus eliminating some nonessential sequences. The residual 8-kb fragment was transfected into Mel57 cells by calcium phosphate precipitation followed by a 3-minute shock with 25% DMSO.³¹ After 48 hours the cells were trypsinized, seeded sparsely in culture flasks, and selected in medium with 200 µg/ml hygromycin B (Boehringer Mannheim). Single resistant colonies were removed by scraping and grown until analysis of VPF expression, and storage of stocks in liquid nitrogen was possible.

Histological Analysis of Tumors from Transfected Melanoma Lines

Transfected melanoma lines were inoculated in nude mice as described above. Every line was injected in at least two mice on both flanks. Tumor volumes were measured weekly, and growth curves were prepared. Tumors of sizes between 150 and 800 mm³ were excised 30 to 60 days after inoculation, and cut into three fragments. One part was formalin-fixed and used to study overall tumor morphology. The other parts were snap frozen in liquid nitrogen; one was used for RNA isolations, the other to study the vascular patterns and the organization of stroma and extracellular matrix. Cryosections were stained with monoclonal 9F1, which reacts specifically with mouse endothelium (J.R. Westphal, J.A.W.M. van der Laak,

C.J.M. Schalkwijk, D.J. Ruiter, R.M.W. de Waal, manuscript in preparation), and which is suitable for the examination of tumor vasculature. Also, tumor sections were stained with a polyclonal rabbit anti-mouse laminin antiserum (provided by Dr. J. van den Born, Department of Nephrology, Nijmegen, The Netherlands).

Results

Analysis of VPF mRNA in Cultured Melanoma Cells

The four melanoma cell lines IF6, Mel57, BLM, and MV3 differ in metastatic potential upon s.c. injection in nude mice.²³ To determine whether this behavior correlates with VPF expression, RNA was isolated from these cell lines and examined by Northern analysis. VPF mRNA was readily detectable in RNA from the cell lines BLM and MV3 (Figure 1) as a major band of 3.7 kb. RNA from the cell lines IF6 and Mel57 showed only very weak hybridization signals with the VPF probe. The highest VPF mRNA levels were thus found in the cell lines BLM and MV3, which were

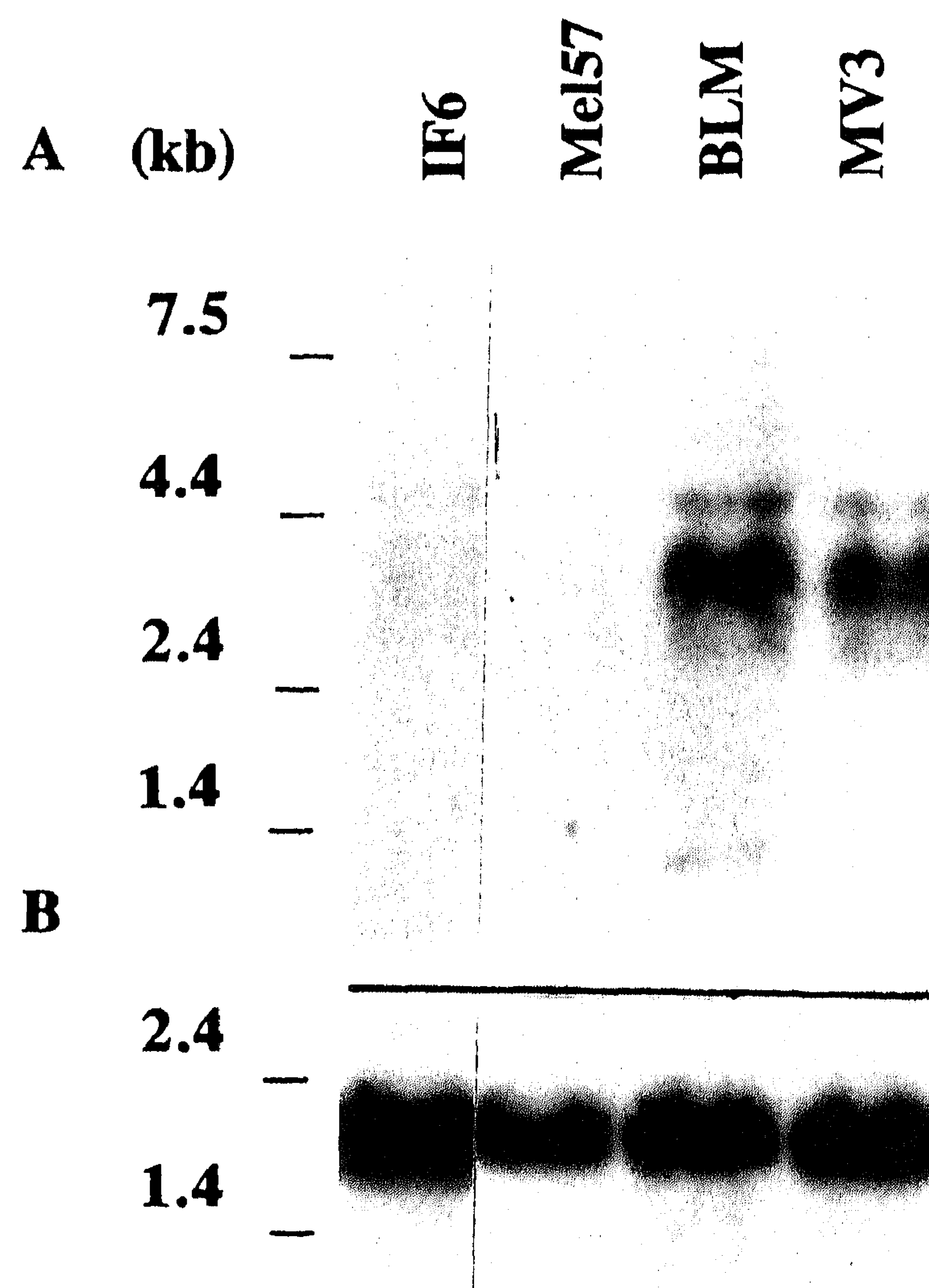


Figure 1. Northern blot analysis of VPF mRNA in melanoma cell lines. 10-µg samples of LiCl/urea isolated RNA were loaded in each lane. (A) VPF hybridization. (B) Ubiquitin hybridization. Only relevant parts of the autoradiograms are shown. Positions of RNA size markers are shown on the left.

shown in earlier studies to give rise to highly metastatic tumors in nude mice. The other lines, which had a low level of VPF mRNA expression, produced tumors with a much lower metastatic frequency.²³

The observed difference in expression of VPF mRNA is not likely to be caused by a genomic rearrangement or an amplification of the VPF gene. Southern analysis of the genomic DNA isolated from these melanoma lines and from normal human buffy coat showed no obvious differences in intensities of the bands hybridizing with a VPF probe, nor were shifts of bands or extra bands observed in any of the melanoma lines (data not shown).

Alternatively spliced messengers of VPF cannot be distinguished by Northern blotting. To determine whether the melanoma lines differ in the ratio of these mRNA variants, an RT-PCR was performed, which amplified the alternatively spliced region of the messenger. RNA from all the melanoma cell lines yielded the same pattern in that the product from the RNA coding for VPF₁₂₁ was the most prominent (52 to 70%), followed by the VPF₁₆₅ product (26 to 42%). The VPF₁₈₉ mRNA was in all cases found to be a minor species (4 to 8%), whereas no VPF₂₀₆ mRNA was found in any of the melanoma cell lines (Figure 2, lanes 2 to 5). No correlation between the biological behavior of the melanoma lines and the predominance of the VPF variants produced could therefore be demonstrated. The observed ratio of splice variants does not seem to be specific for melanoma cells or even for tumor cells, as the same ratio of splice variants was found in RNA from the human lymphoma cell line U937 and in RNA from normal human pericytes (Figure 2, lanes 6 and 7).

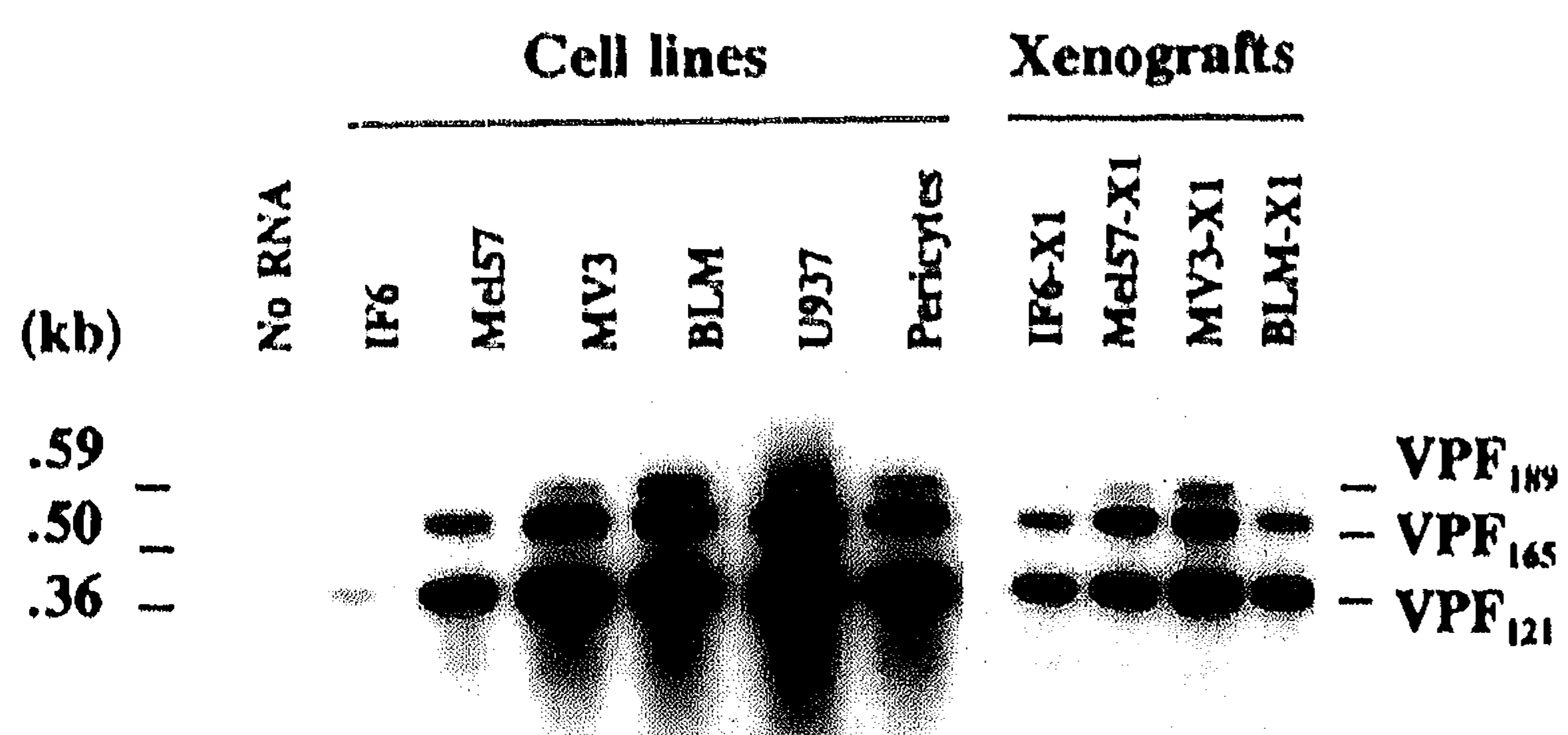


Figure 2. Analysis of VPF mRNA splice variants. RT-PCR was performed on RNAs isolated from various sources, amplifying the alternatively spliced region as indicated in Materials and Methods. (Left) Human cell lines: IF6, Mel57, MV3, BLM (melanoma cells), U937 (histiocytic lymphoma cells), pericytes (from human brain capillaries). (Right) Melanoma xenografts; see also Figure 1 legend. The left and the right panels are from different gels. Southern blots were hybridized with a VPF probe. Positions of DNA size markers are shown on the left. Expected PCR products: VPF₁₂₁ (381 bp), VPF₁₆₅ (513 bp), VPF₁₈₉ (585 bp), VPF₂₀₆ (636 bp). Note that no PCR products are expected from mouse VPF mRNA, as the primers do not match the murine sequence completely.

Levels of Secreted VPF Protein Correlate with the VPF mRNA Levels

The amount of VPF protein secreted from the melanoma cell lines was determined by culturing the cells in serum-free medium containing heparin, to release also the longer VPF variants from the extracellular matrix.²⁰ The proteins in the conditioned medium were Western blotted and stained with a polyclonal antibody against VPF. In the conditioned media from cell lines BLM and MV3 detectable amounts of VPF were present. Several bands with apparent molecular weights between 17 and 25 kd were identified (Figure 3), which agree with the expected sizes of non-glycosylated and glycosylated VPF₁₂₁ and VPF₁₆₅.^{19,20} VPF₁₈₉ and VPF₂₀₆ (expected around 30 kd) could not be found, supporting the finding that their mRNAs are poorly expressed in melanoma cells (Figure 2; the band in the MV3 lane at 38 kd is probably caused by a dimer of VPF₁₂₁ or by a nonspecific reaction rather than by a monomeric form of one of the larger VPF variants). In conditioned media from cell lines IF6 and Mel57 no VPF could be detected, which agrees with the fact that these cells contain low amounts of VPF mRNA (Figure 1). These results in-

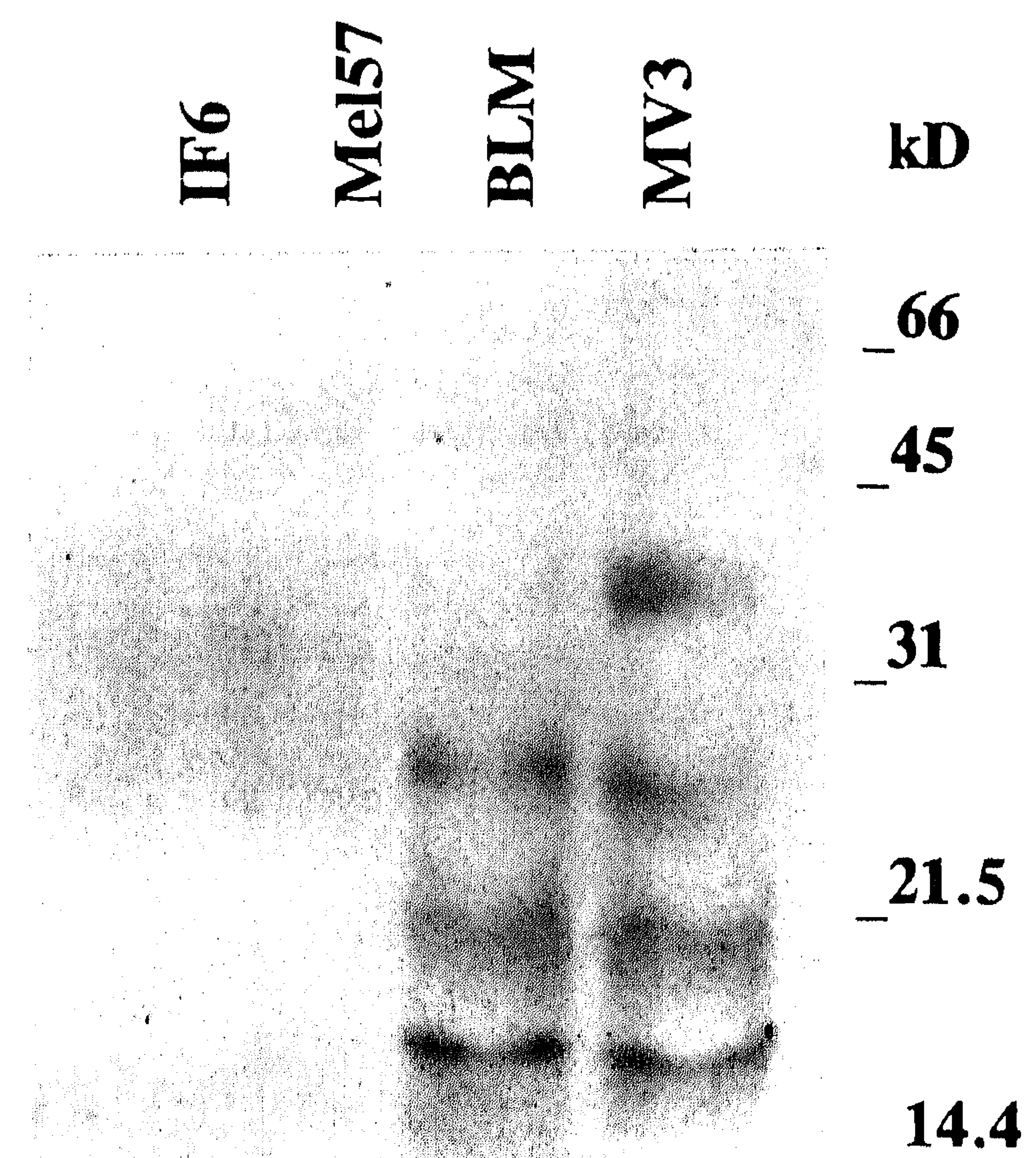


Figure 3. Western blot analysis of VPF in conditioned media from melanoma cells. Confluent cultures of melanoma cells were grown in serum-free medium with 100 µg/ml heparin for 24 hours. Precipitated proteins from 0.225 to 0.7 ml conditioned media (but derived from the same amount of cellular mass) were subjected to SDS-PAGE under reducing conditions, Western blotted, and stained with affinity-purified antiserum against VPF. Molecular weight markers are shown on the right.

dicate that the levels of VPF mRNA in melanoma cells are reflected in the levels of VPF protein expression.

Analysis of Melanoma Xenograft RNA

To determine whether VPF mRNA levels change when melanoma cell lines form tumors in nude mice, RNA isolated from melanoma xenografts was examined. The four melanoma lines that were also used in the above experiments (IF6, Mel57, BLM, and MV3) were injected in mice, and the resulting tumors were collected at different time points after injection. All xenografts contained comparable levels of VPF mRNA (Figure 4), including those derived from the lines IF6 and Mel57, which had hardly detectable levels of VPF mRNA in culture. Scanning of autoradiograms from blots containing both cell line RNAs and xenograft RNAs (not shown) revealed that the average VPF mRNA level in xenografts was 75% of the average level in the cultured cell lines BLM and MV3 (range 30 to 170%). The small differences between the VPF mRNA levels in xenografts were independent of the parental cell line. The levels of VPF mRNA did tend to decrease with increasing tumor size for those tumors that were derived from the IF6, Mel57, and MV3 lines, but in tumors derived from cell line BLM the opposite effect was seen, namely an increase in the VPF mRNA level with increasing tumor size (Figure 4). No change

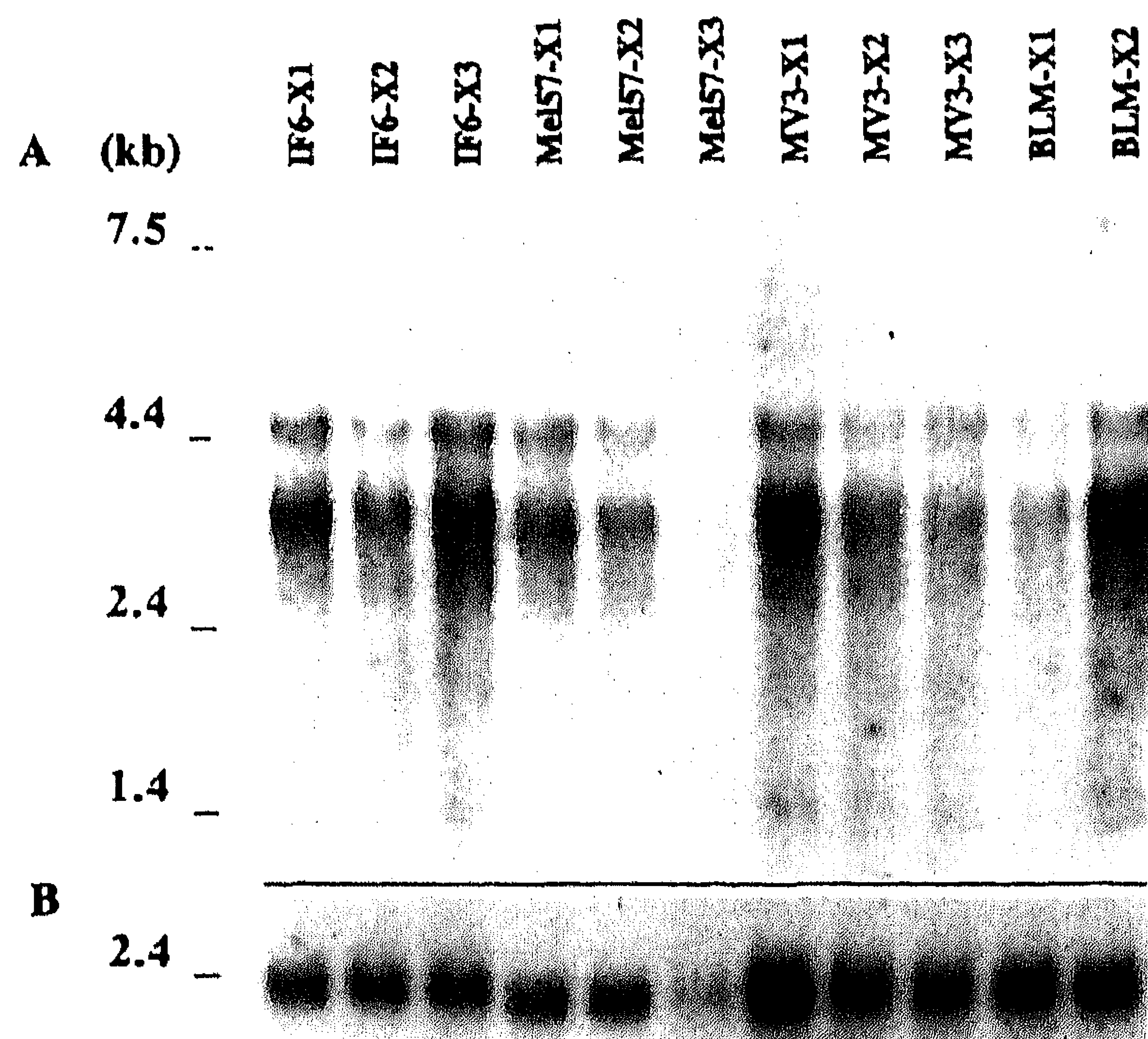


Figure 4. Northern blot analysis of VPF mRNA in melanoma xenografts. 10 to 16- μ g samples of total RNA were loaded in each lane. Parental cell line and number of the xenograft are given with the age and the estimated tumor volume. IF6-X1: 31 days, 180 mm³; IF6-X2: 31 days, 720 mm³; IF6-X3: 45 days, 320 mm³; Mel57-X1: 31 days, 80 mm³; Mel57-X2: 31 days, 1200 mm³; Mel57-X3: 45 days, 1340 mm³; MV3-X1: 31 days, 490 mm³; MV3-X2: 31 days, 3400 mm³; MV3-X3: 45 days, 18,750 mm³; BLM-X1: 21 days, 6000 mm³; BLM-X2: 24 days, 10000 mm³. (A) VPF hybridization. (B) Ubiquitin hybridization. Only relevant parts of the autoradiograms are shown. Positions of RNA size markers are indicated on the left.

in the splicing pattern was found in xenografts: the ratios of alternative splicing products were equal to those in the cultured cell lines (Figure 2, lanes 8 to 11).

The VPF messengers found in melanoma xenografts could derive from melanoma cells, but also from host cells within the tumor (stromal cells) or from small amounts of surrounding tissue (eg, skin). VPF mRNA was indeed found in some murine tissues such as skeletal muscle and heart, but was not detected in normal mouse skin (Figure 5). These normal mouse tissues are not readily comparable, however, with host tissue within melanoma xenografts. To confirm the melanoma origin of the xenograft VPF mRNA unequivocally, an RT-PCR assay was carried out which discriminates between human and murine VPF sequences. RNA was reverse transcribed and amplified using a set of primers based on sequences common to both the human and murine VPF messenger. PCR products were then digested with *StyI*, which cleaves the human, but not the murine sequence. As predicted, a 0.35-kb band was found when RNA from murine tissues was used, whereas two products of ~0.25 and 0.1 kb (the latter only slightly visible) arose from human RNA (Figure 6). Although xenograft

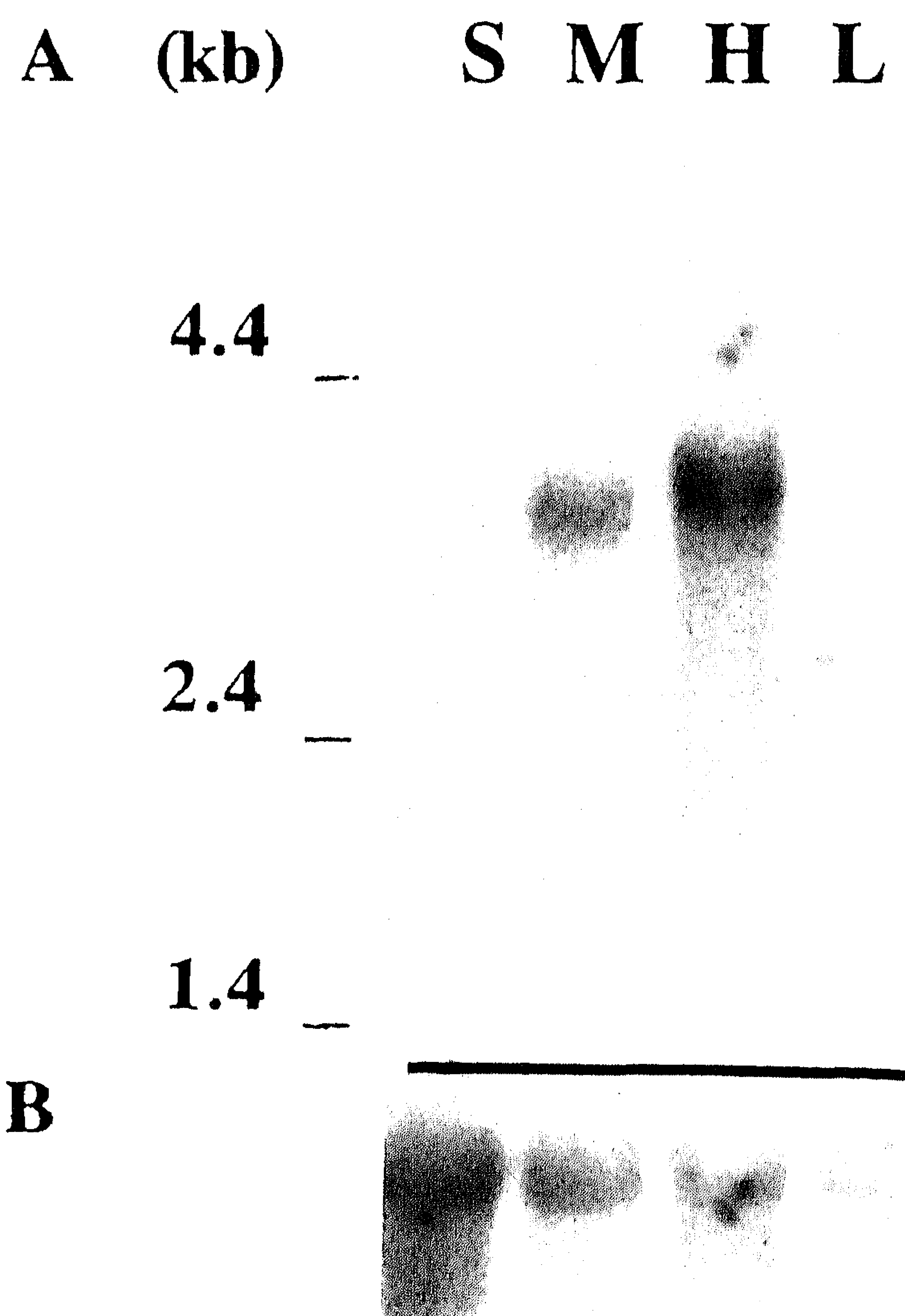


Figure 5. Presence of mRNA for VPF in mouse organs. 10 to 16- μ g samples of total RNA were loaded in each lane. S, skin; M, skeletal muscle; H, heart; L, liver. (A) VPF hybridization. (B) the 28S signal upon hybridization with a ribosomal probe. Positions of RNA size markers are shown on the left.

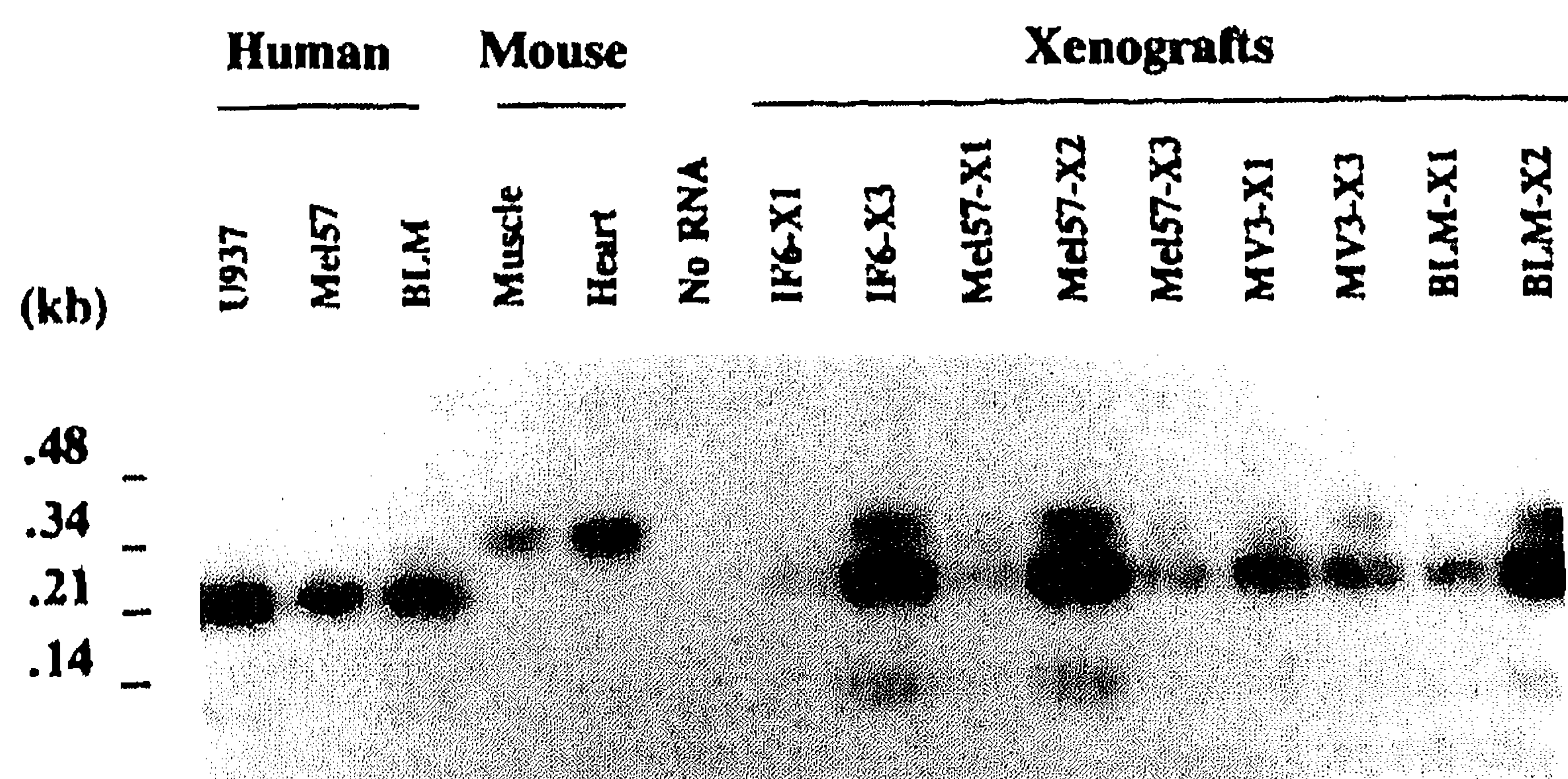


Figure 6. Determination of the species origin of VPF mRNA. RT-PCR followed by StyI-digestion was performed on RNA from various sources to determine the origin of the VPF messengers, as explained in Materials and Methods. Lanes 1 to 3, human cell line RNAs; lanes 4 to 5, mouse RNAs (skeletal muscle and heart); lane 6, no RNA; lanes 7 to 15, melanoma xenograft RNAs (see also legend to Figure 4). A Southern blot was hybridized with a VPF probe and washed in $2 \times$ SSC at 50°C. Subsequent washing in $0.2 \times$ SSC at 50°C did not change the pattern. Positions of DNA size markers are indicated on the left. Expected fragments from human VPF mRNA, 256 and 90 bp; from murine VPF mRNA, 343 bp.

RNAs were amplified to different extents, leading to varying amounts of total PCR product, a consistent pattern was seen. In all cases, the human-specific band of 0.25 kb was the most prominent product. The mouse-derived 0.35-kb band was also present in all xenograft RNAs, but its intensity was only 14 to 25% of that of the human-derived band. The majority of the xenograft VPF mRNA is thus synthesized by the human melanoma cells. For IF6 and Mel57 cells this implies that the expression of VPF is distinctly higher *in vivo* than *in vitro*, and that *in vivo* their VPF expression is no longer different from that in the cell lines BLM and MV3.

Influencing VPF Gene Expression

Apparently, VPF mRNA levels are induced in IF6 and Mel57 cells during tumorigenesis in nude mice. To investigate possible mechanisms that could contribute to this induction, Mel57 cells were exposed *in vitro* to factors that may also have been present *in vivo*. After serum deprivation, the cells were incubated for 4 or 24 hours in serum-containing medium, in medium conditioned by mouse fibroblasts (Balb-3T3 cells) with or without serum, or in serum-free media to which a growth factor or cytokine (listed in the Materials and Methods section) was added. No increase in the VPF mRNA levels was found (not shown). IF6 cells also did not respond to addition of serum or Balb-3T3-conditioned medium. Hence, growth factors or cytokines present in serum or fibroblast-conditioned medium, or added separately, apparently do not augment VPF messenger levels under these conditions. When Mel57 cells were cultured in low oxygen tension for 6 or 24 hours, however, a dramatic increase in the VPF messenger content was found (Fig-

ure 7). After reoxygenation of the flasks these levels started to decrease; within 3 hours a reduction of 50% was observed (Figure 7, lanes 5 and 6). No effect on the VPF mRNA levels was seen when the pH of the medium was varied between 7.2 and 8.1 (Figure 7, lanes 1 and 2), which excludes the possibility that pH differences due to the hypoxic treatment caused the observed induction. In IF6 cells cultured under low oxygen tension for 24 hours, a dramatic induction of the VPF mRNA level was found as well (not shown). The absolute induction of VPF messenger levels reached in Mel57 and IF6 cells after 24 hours of hypoxia cannot be measured, as hybridizing bands are hardly visible in the uninduced state. The maximally induced VPF mRNA levels in Mel57 and IF6 cells were 1.3- and twofold the already high basal level in MV3 cells, respectively. The high level of VPF mRNA in MV3 cells could further be increased by a factor of three when cultured in low oxygen levels (not shown). These results show that hypoxia increases VPF mRNA levels in melanoma cells such that cell lines with distinctly different levels under normal conditions reach comparable levels under conditions of low oxygen tension. Relative levels of VPF splice variants were analyzed and found unaltered upon hypoxic shock in Mel57, IF6, and MV3 cells (not shown).

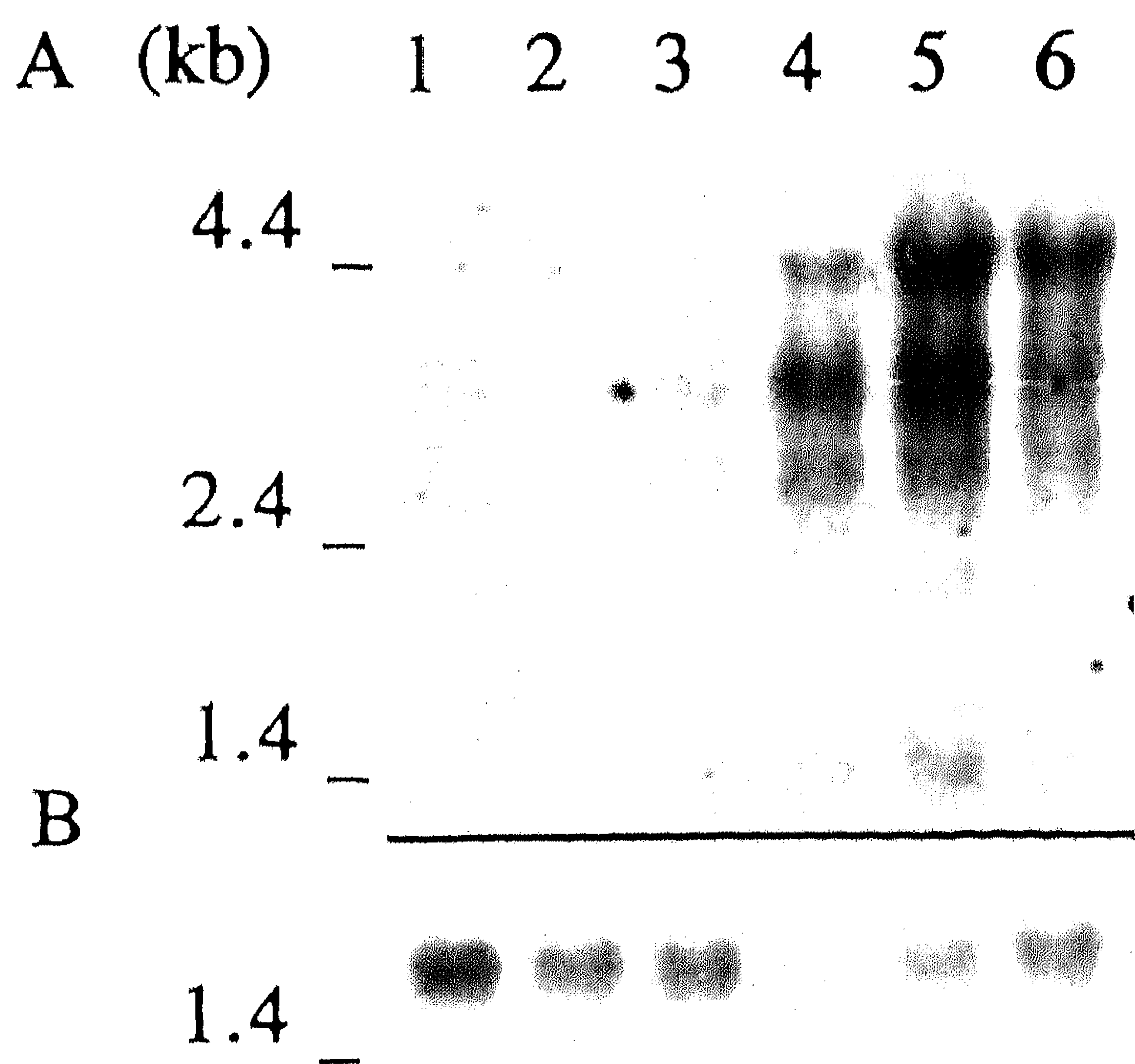


Figure 7. Induction of VPF mRNA in Mel57 cells by hypoxia. Cells were cultured under nitrogen, as described in Materials and Methods. At the end of the incubations, oxygen levels in the gas phase were 0.5 to 1.0% and P_{O_2} values in the media were 2.8 to 3.0 kPa. Control cells were incubated in air/5% CO_2 in media of different pH. Final pH values of the media are indicated. 8 to 22 μ g samples of guanidium chloride isolated RNA were loaded in each lane. Lane 1: air/ CO_2 , pH 8.1; lane 2: air/ CO_2 , pH 7.2; lane 3: N_2 -flushed and 5 hours in air/ CO_2 , pH 7.5; lane 4: 6 hours in N_2 / CO_2 , pH 7.8; lane 5: 24 hours in N_2 / CO_2 , pH 7.5; lane 6: 20 hours in N_2 / CO_2 and 3 hours in air/ CO_2 , pH 7.4. (A) VPF hybridization. (B) β actin hybridization. Ubiquitin was not used as a control, because the expression of this gene may be altered in stress situations. Positions of RNA size markers are shown on the left.

Production of a VPF-Overproducing Transfectant Melanoma Line

To determine whether the pattern of VPF expression, constitutive *versus* inducible, actually makes a difference in the process of angiogenesis and perhaps metastasis during the development of the melanoma xenografts, we transformed a VPF inducible line into a VPF constitutive line by stably transfecting a construct containing the protein coding region of VPF₁₂₁, the VPF variant most abundantly expressed in melanoma cells (Figure 2), into cell line Mel57. The resulting transfectants should have a constitutive VPF expression (as in lines MV3 or BLM) instead of an inducible VPF expression. As a control, Mel57 cells were also transfected with vector DNA without a VPF sequence. A number of stable transfectant lines were examined for expression of recombinant VPF RNA, which is easily recognizable as it is much shorter than endogenous VPF mRNA (see Figure 8). The transfectant line I-3 had the highest level of recombinant VPF RNA and was used for further study. Medium conditioned by these cells contained clearly detectable levels of nonglycosylated and glycosylated VPF₁₂₁ visible as bands of 17 and 20 kd on a Western blot stained with polyclonal anti-VPF. The vector-transfected line E2, which did not show any signs of (recombinant) VPF RNA or protein expression (Figure 8), was used as control in further experiments.

VPF-transfected line I-3, control transfected line E2, and the parental cell line Mel57 were injected into

nude mice. The tumors that developed after inoculation of line I-3 still expressed the 1-kb recombinant VPF RNA, but the endogenous 3.7-kb VPF messenger was detectable in these tumors as well (Figure 8). The upregulation of VPF mRNA was only a minor contribution to the total amount of VPF RNA produced in these tumors; the band intensities of VPF mRNA were only about 50% of the intensities of the recombinant VPF RNA bands. Tumors from control transfected line E2, as expected, had elevated levels of VPF mRNA but did not show recombinant VPF RNA. Tumors from line E2 did express the vector-encoded hygromycin phosphotransferase RNA (not shown).

Vascular Architecture in Transfectant Xenografts

Tumors from the parental and the transfected cell lines did not differ significantly in growth rate, nor did they differ in their extent of necrosis (as judged from HE-stained cross sections). Staining of cross sections with the monoclonal antibody 9F1, which reacts specifically with mouse endothelium, showed that the vascular pattern in tumors from VPF-transfected line I-3 was clearly different from the pattern in tumors from the control lines. Tumors from control lines Mel57 and E2 had similar vascular patterns. Typically, in tumors from control lines blood vessels always appeared separately. Large vessels with lumina were visible as well as smaller microvessels and capillary sprouts in which no lumen could be observed upon light microscopy. Between different regions within one tumor, differences in vessel density did occur: some tumor parts were well vascularized and viable, whereas other parts were poorly vascularized and highly necrotic. In regions with a low density of vessels, as in the section shown in Figure 9A, vessels were surrounded by a layer of viable tumor cells, while at a greater distance tumor tissue was necrotic. Tumors from VPF-transfected line I-3 had quite a different arrangement of blood vessels (Figure 9B). Vessels formed a dense network around nodules of tumor cells. The endothelial staining in cross sections appeared to be largely continuous, suggesting that there is a three-dimensional plexus of blood vessels surrounding the tumor nodules. Within the tumor nodules very few or no blood vessels were present, often leading to viable layers of tumor cells surrounding necrotic centers.

Staining for the extracellular matrix component laminin showed that in tumors from both the control

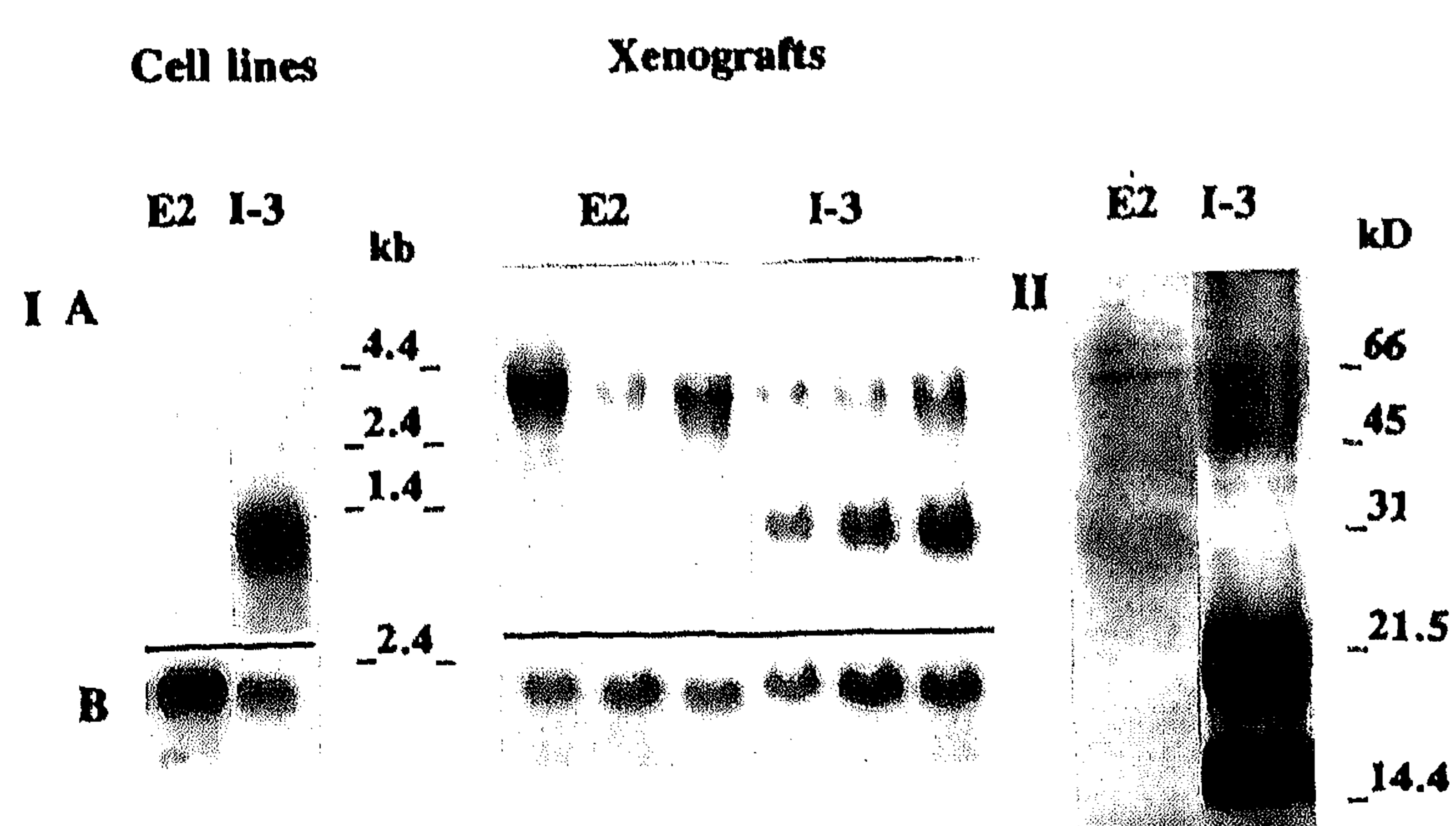


Figure 8. Expression of recombinant VPF RNA and protein by transfected melanoma lines. (I) RNA isolated from control- (E2) and VPF (I-3)-transfected cell lines (left, 10 to 15 μ g RNA loaded in each lane) and from their respective xenografts in nude mice (center, 22 to 30 μ g of RNA loaded in each lane). (IA) VPF hybridization (with endogenous VPF messenger bands at 3.7 kb, and recombinant VPF RNA bands at 1 kb). (IB) Ubiquitin hybridization. Only relevant parts of the Northern blots are shown. (II) Proteins from 0.2 ml serum-free conditioned media of transfected lines E2 and I-3 were acetone precipitated, electrophoresed on SDS-PAGE, electroblotted, and stained with crude antiserum against VPF. Specific staining for VPF₁₂₁ is observed at 17 to 20 kd. Also some nonspecific staining of higher molecular weight bands is visible in both lanes. Positions of molecular weight markers are indicated on the right.

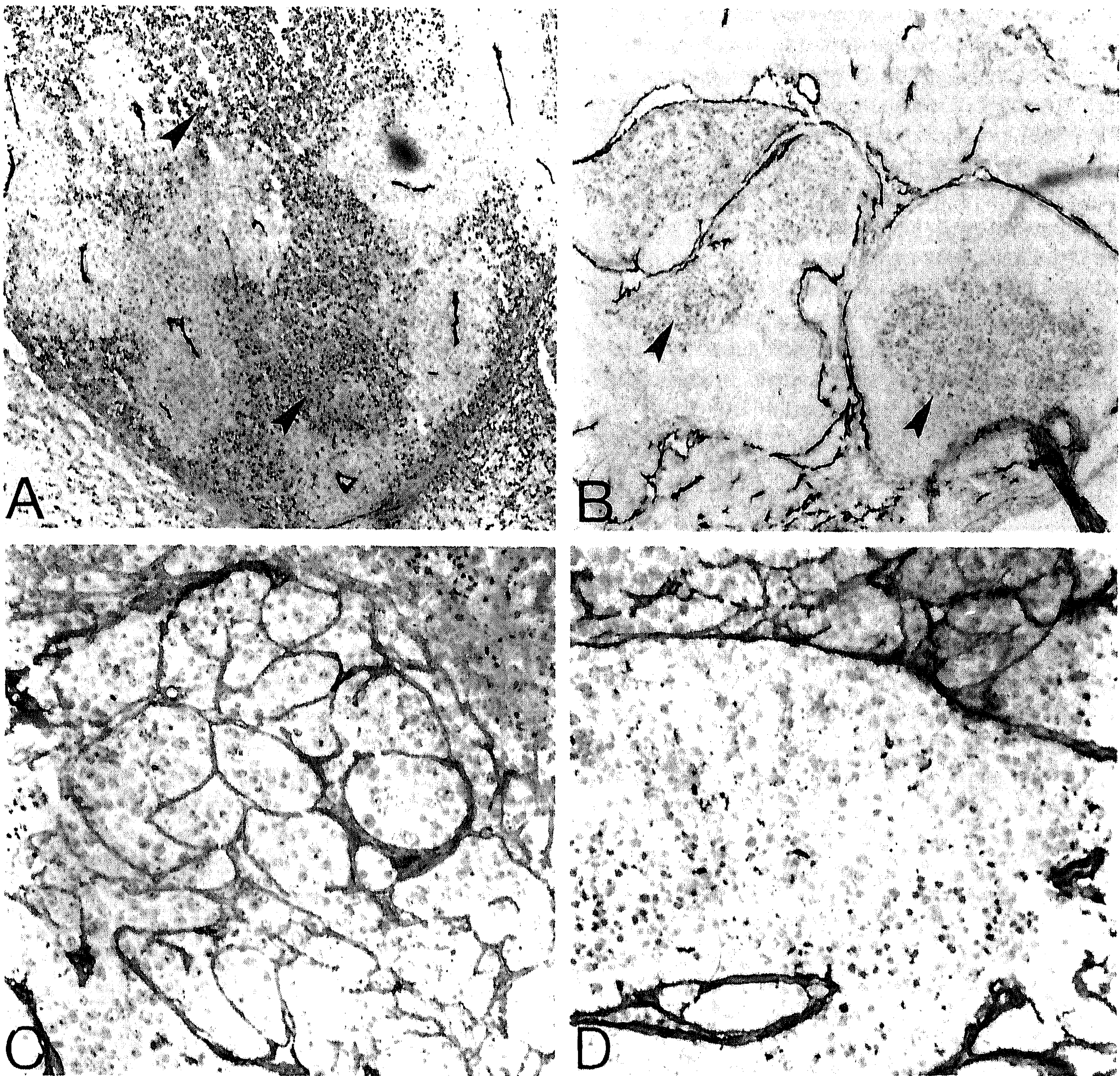


Figure 9. Visualization of tumor vessels and laminin in tumors from transfected melanoma lines. Cross sections were stained with monoclonal 9F1 for endothelium blood vessels (A, B), magnification 60 \times , or with rabbit anti-mouse laminin (C, D), magnification 300 \times . (A, C) Cross sections of line E2 derived tumors. (B, D) Cross sections of tumors from VPF-transfected line I-3. Arrowheads point to areas of necrosis.

lines and the VPF-overproducing line tumor nodules were surrounded and separated from each other by stromal septa (Figure 9, C and D). However, on the average the tumor nodules in the line I-3 tumors were larger than those in control tumors, and the stromal component consisted for a large part of endothelial cells (Figure 9B), unlike the situation in control tumors, in which only single blood vessels were seen (Figure 9A). The change in the pattern and level of VPF expression has therefore dramatically changed the vascular architecture in the tumors from this transfectant melanoma line.

Discussion

The initial goal of this investigation was to determine whether the level of VPF expression in several human melanoma lines correlated with the metastatic potential of these lines in nude mice. We did observe this correlation in cultured melanoma cells: lines IF6 and Mel57, which produce rarely or slowly metastasizing tumors, had much lower levels of VPF mRNA and secreted less VPF protein than lines BLM and MV3, which develop into highly metastatic tumors in mice (see ref. 23; J.R. Westphal, J.A.W.M. van der Laak,

C.J.M. Schalkwijk, D.J. Ruiter, R.M.W. de Waal, manuscript in preparation). However, these differences in VPF expression were no longer observed *in vivo*; tumors from all four melanoma lines had high levels of VPF mRNA, produced mainly by the melanoma cells. Therefore the highly metastatic melanoma lines have a constitutive VPF expression, which apparently is not dependent on the extracellular environment, while the less metastatic lines can have the same level of VPF expression, but only if it is induced by external factors.

The upregulation of VPF expression in IF6 and Mel57 cells *in vivo* might have been caused by protein factors released by, eg, stromal cells. In several cell lines, induction of VPF expression by TGF- β and PDGF-BB has been demonstrated.³⁹⁻⁴¹ We did not find any induction of VPF mRNA by various growth factors or cytokines, serum, or conditioned media of fibroblasts in the melanoma cells IF6 and Mel57. We cannot, however, exclude the possibility that other growth factors or combinations of such factors can cause an increased VPF expression in these cells.

One of the mechanisms that may lead to enhanced VPF expression *in vivo* is hypoxia, given that a dramatic upregulation of VPF messenger levels was evident after incubation of Mel57 and IF6 cells under low oxygen pressure. VPF expression was already known to be induced by hypoxia in glioma and hepatoma cells^{14,42}; also, the expression of a number of other genes is induced by hypoxia. The intracellular mechanisms responsible for this kind of induction are only beginning to be understood.^{43,44} There is evidence that an intracellular heme protein is involved in hypoxia-induced expression of both VPF and erythropoietin, and this effect of hypoxia on glioma and hepatoma cells is mimicked by cobalt chloride.⁴² VPF mRNA levels in Mel57 cells were also upregulated by cobalt chloride (not shown), suggesting a regulatory mechanism that is common to many cell types. In MV3 cells, which have a higher expression of VPF mRNA than Mel57 and IF6 cells when cultured under normoxic conditions, VPF mRNA induction by hypoxia was relatively small. These cells may have undergone an irreversible switch to a high VPF producing phenotype, which would make them less sensitive to VPF-inducing mechanisms.

Hypoxia is thought to be one of the major causes of tumor necrosis. *In situ* hybridization studies have shown that VPF mRNA levels were highest around necrotic sites in glioblastoma lesions, suggesting an involvement of hypoxia in the regulation of VPF expression.^{14,15} However, in other tumor types most tumor cells produced VPF mRNA in abundance with only slightly increased levels being found adjacent to necrotic areas.⁴⁵ In our study we found two sets of

melanoma lines, one in which VPF mRNA levels were upregulated by hypoxia, and a second in which VPF mRNA levels were only slightly affected by oxygen tension. Further evidence for the assumption that hypoxia is the major trigger for the VPF mRNA upregulation observed by us *in vivo* can only be provided by *in situ* hybridization or by immunohistochemistry. These experiments are in progress in our laboratory.

A relation between tumor angiogenesis and metastasis has been postulated on theoretical grounds and has also been demonstrated in practice in several tumor types including melanoma.^{8,46-49} Given that VPF is a potent angiogenic factor, it might also play an important role in the development of the metastatic phenotype of melanoma xenografts. Although the xenografts of all melanoma lines examined showed comparable VPF expression, this expression in the less metastatic lines IF6 and Mel57 has to be upregulated first *in vivo*. A lower expression level of VPF in early IF6 and Mel57 xenografts might result in a delayed development of the vascular bed compared with BLM and MV3 xenografts, which might eventually hamper the opportunities for dissemination. To separate the effect of VPF expression on the vasculature from the effects of other factors (differentially) produced by the various melanoma lines, it is necessary to manipulate VPF expression in one of the melanoma lines.

By transfection into cell line Mel57 of a VPF₁₂₁ sequence, we were able to produce an Mel57-derived cell line that had a constitutively high expression of VPF. In this way it was possible to study the biological behavior of tumors from melanoma lines with an inducible or a constitutive VPF expression in an otherwise unchanged genetic background. The arrangement of the tumor vasculature indeed turned out to be quite different in tumors resulting from the constitutive VPF-expressing transfectant line. In another study,⁵⁰ two types of vascular branching in tumors were distinguished. "Tumor-penetrating branches of variable diameter" were found in some tumors, and "lateral surface branches that formed an arborizing and anastomosing plexus of interconnecting vessels" were found in other tumors. The vasculature in tumors from the control melanoma lines resembled the first type, whereas in tumors from the VPF-transfected line the vasculature appeared to be more like the second type. An altered VPF expression obviously is sufficient for a switch from one vascular type to the other.

Studies from others, in which Chinese hamster ovary cells or HeLa cells were transfected with VPF, showed that overexpression of VPF can lead to a growth advantage in nude mice, combined with higher angiogenic activity.^{51,52} The tumors from the

VPF-transfected melanoma line used in our study did not show such behavior; they did not have a faster growth rate, and angiogenesis was qualitatively rather than quantitatively different. Obviously, angiogenesis is too complex a process to have its outcome predicted by the level of VPF expression only. Similarly, the vasculature in xenografts from transfectant line I-3 might have been expected to be very much like that in xenografts from other constitutively VPF-producing melanoma lines such as BLM and MV3, but this was not at all the case (J. R. Westphal, J.A.W.M. van der Laak, C.J.M. Schalkwijk, D.J. Ruiter, R.M.W. de Waal, manuscript in preparation). VPF expression in line I-3 was probably much higher than in lines BLM and MV3, and transfectant lines with a lower recombinant VPF expression than line I-3 should therefore be studied. Even then, VPF is probably not the only factor relevant to angiogenesis in these tumors; a number of other factors directly or indirectly involved in angiogenesis may be differentially expressed between BLM and MV3 on one hand and Mel57 and its transfectants on the other hand. Differences in expression of various integrins and of proteins involved in the plasminogen activator pathway have indeed been shown between some of these melanoma lines.^{25,24} The vascular phenotype in any tumor will be the result of a large number of factors influencing angiogenesis, but our study shows that VPF is at least one of the important factors governing angiogenesis in these melanoma xenografts.

Further study has to be undertaken to completely understand the way the aberrant vascular phenotype in tumors from line I-3 evolves. Furthermore, it is an interesting question whether the altered VPF expression pattern leads to a different degree of vascular permeability for, eg, fibrinogen or for labeled tracers.¹⁰ Experiments addressing this issue are currently in progress.

We also hope to answer the initial question whether VPF expression is important for the metastatic potential of melanoma xenografts in nude mice. However, as the constitutive expression of VPF by the Mel57-derived cell line I-3 led to a vascular phenotype unlike that of other constitutive VPF-producing lines such as BLM and MV3, the metastatic frequency of line I-3-derived tumors may not simply be expected to increase to the frequencies found for tumors from lines BLM and MV3. Although it is still of interest to study the metastatic potential of tumors from line I-3, xenografts from other transfectant melanoma lines with expression levels of recombinant VPF lower than in line I-3 perhaps will provide a better basis for comparing the metastatic potential with lines BLM and MV3. Therefore other transfectant lines will need to be

included in future studies on the correlation between VPF levels, vascular pattern, and metastasis.

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