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Tumoral Environment Triggers Transcript Anomalies in Established Tumors: Induction of Altered Gene Expression and of Aberrant, Truncated and B2 Repeat-Containing Gene Transcripts[1]

Pieter Rottiers, Marjory Desmedt, Hans Dooms, Roland Contreras and Johan Grooten

Department of Molecular Biology, Molecular Immunology Unit, Flanders Interuniversity Institute for Biotechnology and University of Gent, Gent, Belgium

Abstract

In addition to eugenetic changes, cancerous cells exhibit extensive modifications in the expression levels of a variety of genes. The phenotypic switch observed after inoculation of T lymphoma cells into syngenic mice illustrates the active participation of tumoral environment in the induction of an aberrant gene expression pattern. To further substantiate this contribution, we performed polymerase chain reaction (PCR)-based subtraction suppression hybridization (SSH) to identify genes that are differentially expressed in tumor-derived EL4/13.3 cells compared to the same cells isolated from cultures. Besides a number of unknown genes, the subtracted library contained several known genes that have been reported to be expressed at increased levels in tumors and/or to contribute to carcinogenesis. Apart from clones representing translated transcripts, the subtracted library also contained a high number of clones representing B2 repeat elements, *viz.* short interspersed repetitive elements that are transcribed by RNA polymerase III. Northern blotting confirmed the induction of B2 transcripts in tumor tissue and also revealed induction of chimeric, B2 repeat-containing mRNA. The appearance of chimeric transcripts was accompanied by aberrant, shorter-than-full-length transcripts, specifically from upregulated genes. Accordingly, in addition to altered gene expression, tumoral environmental triggers constitute a potent mechanism to create an epigenetic diversity in cancers by inducing extensive transcript anomalies.

Keywords: mouse cancer, differential gene expression, microenvironment, SINE, subtraction suppression hybridization.

Introduction

Malignant outgrowth of cells is the result of a complex, multistep process that involves genetic alterations in cellular oncogenes and/or tumor-suppressor genes as well as changes in expression levels of a large number of genes [1,2]. The resulting altered growth characteristics and diminished sensitivity to programmed cell death combine with cycles of phenotypic diversification and subsequent host selection to generate a dominant malignant cell population [3]. Misexpressed genes contribute to these processes of transformation and tumor progression by modifying expression levels of cell adhesion receptors, growth factors and growth factor receptors, and/or by interfering with more complex cellular functions. Thus, increased cell motility [4,5], reduced genomic stability [6], resistance to host immune surveillance [7] and to stress generated by nutrient depletion and hypoxia [8] have been correlated with altered gene expression and tumor progression.

The high number and diversity of genes with altered expression pattern identified so far in various cancers indicates a profound perturbation of transcriptional regulation in tumor cells. Changes in DNA methylation [9], gene translocation [10], and gain or loss of function mutations in transcription factors and signaling proteins [11] seem to account for the majority of differentially expressed genes in tumor cells. Also, changes in cellular physiology induced by hypoxia [12], hypoglycemia [13] and acidosis [14] contribute to changes in gene expression. Furthermore, relaxation of the RNA splicing fidelity and retrotransposal integration of mobile genetic sequences may generate various transcript isoforms. Alternative splicing leading to multiple protein isoforms has been observed with CD44 [15] and with distinct tumor suppressors such as WT1 and APC [16,17]. Insertion of noncoding, rodent-specific B2 elements into genes has been reported to cause premature termination and aberrant splicing of the affected genes [18,19]. B2 elements are short interspersed repeats of 180 bp, present in the genome at a frequency of nearly 10^5 [20] and are thought to be involved in retrotransposition [21].

Although altered gene expression as a result of eugenetic and epigenetic changes in tumor cells has been extensively documented, it remains unclear to what

Abbreviations: G3PDH, glyceraldehyde-3-phosphate dehydrogenase; pol II, RNA polymerase II; RT-PCR, reverse transcription polymerase chain reaction; SINE, short interspersed nucleotide element; SSH, subtraction suppression hybridization; TAA, tumor-associated antigen.

Address all correspondence to: Dr. Johan Grooten, Department of Molecular Biology, University of Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium. E-mail: johan.grooten@dmb.rug.ac.be

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extent altered gene expression occurs constitutively or on the contrary is driven by signal transduction, triggered by the tumoral microenvironment. Among the over 500 differentially expressed genes, identified by comparing gene expression profiles in neoplastic gastrointestinal and normal tissues [22], part may require environmental signals for expression. To address this question, we previously analyzed phenotypic changes in tumor tissues. By comparing murine T lymphoma cells grown as solid tumors with the same cells grown in tissue culture, we observed a dramatic phenotypic switch of the tumor-derived cells, involving not only expression of normal T cell differentiation antigens, but also of tumor-associated and ectopic antigens [23]. This approach therefore allows identification of gene expression induced during tumor outgrowth or dictated by the tumoral environment and excludes genes that are constitutively expressed as part of neoplastic transformation. In the present study, this induction of gene expression was analyzed by applying polymerase chain reaction (PCR)-based subtraction suppression hybridization (SSH). An extensive pattern of gene induction was observed, indicating that a sizeable fraction of misexpressed genes in tumor cells is upregulated in response to tumoral environmental triggers. Most strikingly, this induction was accompanied by a prominent induction of B2 repeat sequence-containing transcripts and of aberrant, shorter-than-full-length transcripts of specific genes. Combined, these results indicate that tumoral environmental triggers favor phenotypic and functional divergence within tumor cells not only via altered gene expression but also by generating abnormal transcripts, *viz.* truncated and chimeric messengers from specific, upregulated genes.

Materials and Methods

Animals

Female C57BL/6 (H-2^b) mice were purchased from The Broekman Institute (Someren, The Netherlands). Nu/Nu mice were purchased from Iffa-credo (Saint Germain-sur-l'Arbresle, France). All mice were 9 to 14 weeks old at the time of the experiments.

Cells and Culture Conditions

All cell lines were mycoplasma-free and were routinely propagated in culture for periods of up to 2 months. Cultures of the murine tumor cell lines, B16BL6 (melanoma), EL4/13.3 (T-lymphoma) [23], L929 (fibrosarcoma), F9 (teratocarcinoma), TL-Rnu1 (T-lymphoma) and LLC (lung carcinoma), were grown in RPMI 1640 medium (Life Technologies, Paisley, UK), supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM) (Life Technologies) and 2-ME (5×10^{-5} M; BDH, Poole, UK). The nontransformed, influenza hemagglutinin (HA)-specific and H-2^b-restricted $CD4^+$ T lymphocyte clone, T-HA, was established in our laboratory from lymph nodes of HA-immunized C57BL/6 mice [24]. The cells were maintained long-term in culture by bi-weekly restimulation with specific antigen and IL-2 as described previously [24].

Tumor Tissue Samples

Cells were harvested from the cultures and washed thoroughly with cold LPS-free PBS; 10^5 cells in a volume of 0.2 ml LPS-free PBS were injected subcutaneously into the right flank in 6-weeks-old syngeneic C57BL/6 mice or Nu/Nu mice. Well-established tumors with a diameter of approximately 5 to 8 mm were excised under sterile conditions. Single cell suspensions in PBS were obtained by gently squeezing tumor pieces with a pincet and by filtering through a sterile 70 μm nylon cell strainer. Necrotic cells and other debris were removed by centrifugation on a Histopaque-1077 density gradient (Sigma-Aldrich, Irvine, UK) for 25 minutes at 2000 rpm. Routinely, trypan-blue exclusion revealed a cell viability of over 95%. Light scatter analysis combined with indirect immunofluorescence measurement of the tumor-associated antigen (TAA), HTgp-175 (23), revealed less than 5% contaminating TAA^- host cells in the resulting cell suspension. Cells were cultured in standard medium, subcloned by limiting dilution cloning, and/or frozen in liquid nitrogen for RNA preparation.

RNA Preparation

Total RNA was prepared from preconfluent cell cultures or frozen tumor-derived cells using RNAzol B (Cinna/Biotecx Laboratories, Houston, TX) following the manufacturer's instructions. Poly(A)$^+$ RNA was prepared with a FastTrack kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions.

Construction of a Subtracted EL4/13.3 Tumor cDNA Library

Tester (tumor-derived EL4/13.3 cells) and driver (cultured EL4/13.3 cells) cDNA synthesis and subtraction were done essentially with a PCR-select kit (Clontech Laboratories, Palo Alto, CA) using Advantage KlenTaq polymerase (Clontech). Briefly, cDNA was synthesized from two populations of poly(A)$^+$ RNA (2 μg of each); tester and driver cDNA were digested with *Rsa*I; tester cDNA was ligated with adaptors; a first hybridization was performed to equalize and enrich for differentially expressed sequences, and a second to generate templates for PCR amplification. For each subtraction, two PCR amplifications were conducted in 25 μl. The primary PCR reaction was done for 30 cycles (10 seconds denaturation at 94°C, 30 seconds annealing at 68°C, and 1.5 minutes extension at 72°C) and a final extension for 5 minutes at 72°C in a Perkin-Elmer GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT). The amplified products were diluted 10-fold in deionized water. One microliter of the product was then used as a template in secondary (nested) PCR for 12 cycles using the same parameters as for the primary PCR. The pattern of the amplified pool was analyzed on a 2% agarose gel.

Subtraction efficiency was analyzed by PCR performed on 10-fold diluted subtracted and unsubtracted secondary PCR products with the G3PDH 3′ and 5′

primers from the PCR-select kit and CD32 primers (upstream: 5′-TGGACAGCCGTGCTAAATCT-3′; downstream: 5′-GGTTGGCTTTGGGGATAGA-3′. The PCR reaction mixture contained 0.2 mM dNTP, 200 nM primers, 0.5 units Advantage KlenTaq polymerase and standard PCR buffer. Samples were amplified during 18, 23, 28 and 33 cycles (30 seconds denaturation at 94°C, 30 seconds annealing at 60°C and 2 minutes extension at 68°C) in a Perkin-Elmer GeneAmp PCR System 2400.

A library from the subtracted PCR fragments was constructed by insertion of subtracted cDNA, pooled from eight nested PCR reactions and purified on MicroSpin S-300 HR columns (Pharmacia Biotech, Uppsala, Sweden), into a T/A vector (pGEM-T Easy vector; Promega Biotec, Madison, WI) and by electroporation into DH5α.

Differential Screening and Analysis of the Subtracted Library

Three hundred randomly selected cDNA clones, isolated from the EL4/13.3 tumor-subtracted library, were amplified separately for 25 cycles under conditions of nested PCR. Fifteen microliters of the amplified cDNA was mixed with 0.3 N NaOH/15% Ficol/0.5% bromophenol blue (10 μl) and dotted in an array onto Hybond N^+ membranes (Amersham Pharmacia Biotech, Rainham, UK) for duplicate screening. These DNA dot blots were hybridized with ^{32}P-labeled probes made from the subtracted cDNA pool ("+" probe) and from a second, reverse subtraction ("−" probe). The latter probe was prepared by PCR-Select subtraction using the original tester cDNA as a driver and the driver cDNA as a tester. Hybridizations were carried out according to standard protocols. For cDNA sequence analysis, clones of interest were picked, plasmid DNA prepared using Wizard Miniprep columns (Promega), and DNA sequencing performed in both directions with an automatic ABI373 DNA sequencer using universal primers (SP6 and T7). Nucleic acid sequences were compared to the EMBL and dbest databases using the Blast program.

Northern Blotting

Total (20 μg) or poly(A)$^+$ (1.5 μg) RNA from cultured cells or tumor tissues were denaturated with glyoxal at 50°C for 60 minutes, and size-fractionated on 1% agarose gel containing 20 mM Na phosphate buffer. Equalization of total RNA was confirmed by ethidium bromide staining of agarose gels. Gels were submerged in 10× SSC and capillary transferred to Hybond N^+ membranes. Filters were hybridized with PCR-amplified inserts of subtracted clones according to standard procedures. Amplified DNA fragments were purified from dNTPs using Wizard PCR Preps columns (Promega) and ^{32}P-labeled using a random-primed DNA labeling kit (Boehringer, Mannheim, FRG) according to the manufacturer's instructions. Hybridization with the subtracted probes was followed by hybridization with a probe specific for G3PDH to monitor the amount of RNA in each lane. The integrity of total RNA was confirmed by agarose gel electrophoresis. Relative intensities of 18*S*/28*S* rRNA between various RNA samples on the agarose gels were similar to ratios of G3PDH hybridization intensities, confirming the validity of G3PDH as a probe for normalization. Radioactive bands were quantified by a PhosphorImager 425 (Molecular Dynamics, Sunnyvale, CA).

Reverse Transcription (RT) PCR

Twenty micrograms total RNA was treated with 2 U RQ1 RNase-free DNase I (Promega). After phenol/chloroform extraction, 1 μg of the RNA was reverse-transcribed after addition of an oligo-dT primer (Boehringer) with superscript II reverse transcriptase (Life Technologies, Paisley, UK) in the presence of RNase block (Stratagene Cloning Systems, La Jolla, CA). The primers used for PCR amplification were 5′-AAGAAGAAGTACGGCGTCTCA-3′ (forward primer F1: Cathepsin S), 5′-CTTCTGGTGTGTCTGAAGAGAG-3′ (reverse primer R1: B2 repeat) and, as well as 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′ (β-actin). The PCR reaction mixture contained 1.5 mM $MgCl_2$, 0.4 mM dNTP, 200 nM primers and 0.5 U of Goldstar taq polymerase (Eurogentec, Seraing, Belgium). Samples were amplified during 35 cycles (45 seconds denaturation at 94°C, 30 seconds annealing at 60°C and 2 minutes extension at 72°C) in a Peltier thermal cycler 200 (MJ Research, Watertown, MA). PCR products were analyzed on 2% agarose gel and visualized by ethidium bromide staining. The PCR fragments were cloned and their DNA sequence determined as described above.

Results

PCR-Based Subtraction Suppression Hybridization Reveals Extensive Differential Gene Expression between Cultured and Tumor-Derived Thymoma Cells

We previously reported that growth as solid tumor induced in murine T-lymphoma EL4/13.3 cells not only expression of the T cell differentiation antigens CD3, CD4, CD32 and MTH, but also ectopic expression of PG-M and expression of the tumor-associated antigen HTgp-175 [23]. In this study, the contribution of tumoral environmental triggers to the aberrant gene expression pattern of tumor cells was analyzed in more detail by performing PCR-based SSH [25]. cDNA, isolated from cultured EL4/13.3 cells, was used to drive subtraction in the tester cDNA isolated from tumor-derived EL4/13.3 cells. The resulting subtracted PCR fragments produced a distinct pattern of amplified cDNA fragments that was absent when driver was not included (Figure 1*A*). This indicates that the enriched PCR fragments emanated from sequences present in tumor-derived cDNA, but absent or rare in cDNA from the cultured counterpart.

The efficacy of subtraction was further verified on the basis of the abundance in secondary PCR products of G3PDH and CD32 PCR fragments before and after subtraction. G3PDH is a housekeeping gene that is similarly expressed in both cell populations, whereas CD32 is a target transcript, absent in cultured cells but present in tumor-derived cells [23]. Figure 1*B* shows a 400-fold reduction of

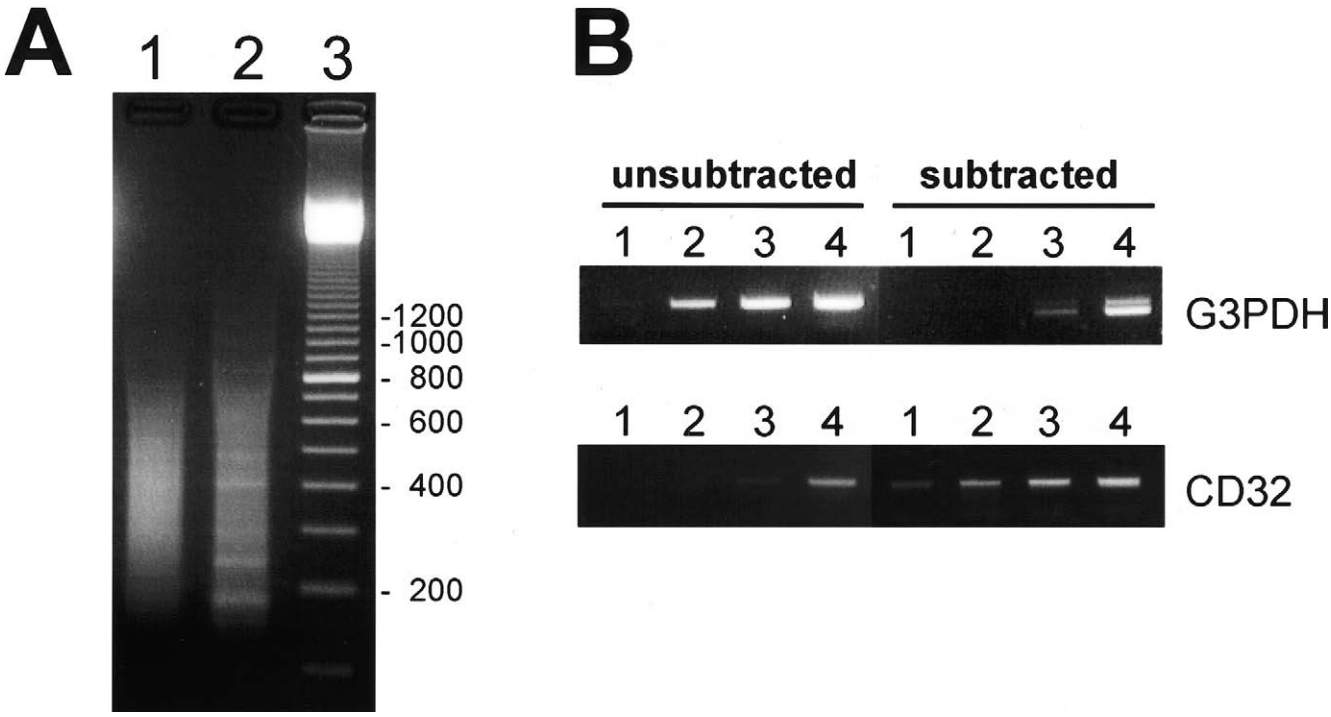

Figure 1. *Construction of a representative cDNA library from tumor-derived EL4/13.3 cells, subtracted against cultured EL4/13.3 cells. Tester cDNA was cut with RsaI, adaptor-ligated and, after hybridization in the presence (subtracted) or absence (unsubtracted) of RsaI-digested driver cDNA, amplified by adaptor-specific (primary PCR) and nested (secondary PCR) primers. (A) Secondary PCR products of unsubtracted (lane 1) and subtracted (lane 2) tester cDNA. A 100-bp ladder was used as size marker (lane 3). (B) PCR analysis of subtraction efficiency. PCR was performed on unsubtracted and subtracted secondary PCR products with G3PDH and CD32 5′ and 3′ primers and examined after 18 (lane 1), 23 (lane 2), 28 (lane 3) and 33 (lane 4) cycles. Five cycles correspond roughly*

G3PDH cDNA in the subtracted sample, while the amount of CD32 cDNA increased more than 400-fold, confirming that subtraction was successful. These estimates are based on the observation that five PCR cycles correspond to a 20-fold difference in cDNA content (Clontech). Next, the pool of subtracted, tumor-enriched cDNA fragments was cloned and the inserts of 300 randomly selected clones were tested by dot-blot hybridization with probes made from the subtracted cDNA pool ("+" subtracted probe) and from a second, reverse subtraction ("−" subtracted probe; see *Materials and Methods* section). Differential hybridization was observed for 62 cDNA clones (20%) (not shown). From these, 45 hybridized strongly with the "+" subtracted probe and were retained for nucleotide sequence analysis of the insert. In general, sequences of 300 to 400 nucleotides long were obtained, sufficient to identify the corresponding genes.

Among the 17 distinct cDNA clones identified (Figure 2), three corresponded to unknown genes and were each represented by a single clone. Several of the remaining genes have been reported to be expressed at high levels in tumors or have been described in relation to tumor development and progression. This is the case for osteopontin [26], cathepsin S [27], PG-M [28], CD32 [29], stathmin [30] and B2 repeat sequence [31]. Also, the presence of CD32 in the subtracted library is in agreement with our previous report that this receptor is strongly upregulated in tumor-derived EL4/13.3 cells. A third set comprises cDNA clones encoding the 78-kDa glucose-regulated protein (GRP78), the 70-kDa heat-shock protein (HSP70) and cytochrome *b*. These proteins exert stress- or redox-related functions and promote tumor progression or survival by protecting the tumor cells against conditions of stress [32–34]. Finally, increased expression of histone H1(0), IgE-binding factor, IL7Rα, mitochondrial 60*S* ribosomal protein L3, and NADH-ubiquinone oxidoreductase has not yet been functionally implicated in cancer.

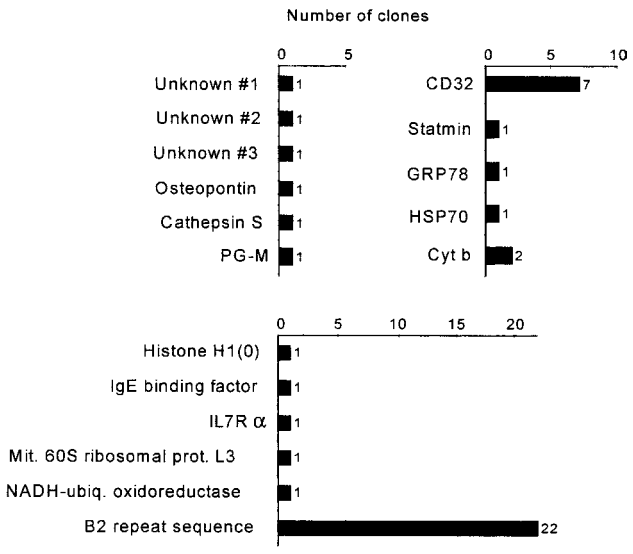

Figure 2. *Identity of 45 cDNA clones isolated from the subtracted library.*

B2 Repeat Sequences are Strongly Upregulated During Tumor Growth

A striking feature of this limited analysis of the subtracted cDNA library is the high representation of clones encoding so-called B2 repeat sequences, a class of short interspersed nucleotide elements (SINE) with retrotransposon activity. Although this high representation may reflect a bias of the PCR-based subtraction method towards amplification of small PCR fragments and/or B2 repeats, an upregulation of these elements in EL4/13.3 tumor tissue is similarly plausible. To discriminate between both possibilities, we performed Northern blot analysis using total RNA from tumor-derived and cultured EL4/13.3 cells, and from normal T lymphocytes as a control. As shown in Figure 3*A*, small B2 RNA was only detected in material from EL4/13.3 cells and not from the nontransformed control, which confirms previous reports documenting an upregulation of small B2 RNA as a result of transformation [35]. However, B2 repeat sequences were approximately 4-fold more abundant in tumor-derived cells compared to their cultured counterparts. This increment was due to increased levels of small B2 repeat RNA of 200 to 600 nucleotides long but also to induction of large B2-containing RNA of 0.6 to 5.0 kb long. This large B2-containing RNA was absent in cultured EL4/13.3 cells, identifying growth as a solid tumor as cause of its induction.

To verify whether the increment of B2-containing RNA in tumor-derived EL4/13.3 cells is a general feature of established tumors, we performed an identical Northern blot analysis on RNA samples from various tumors and their

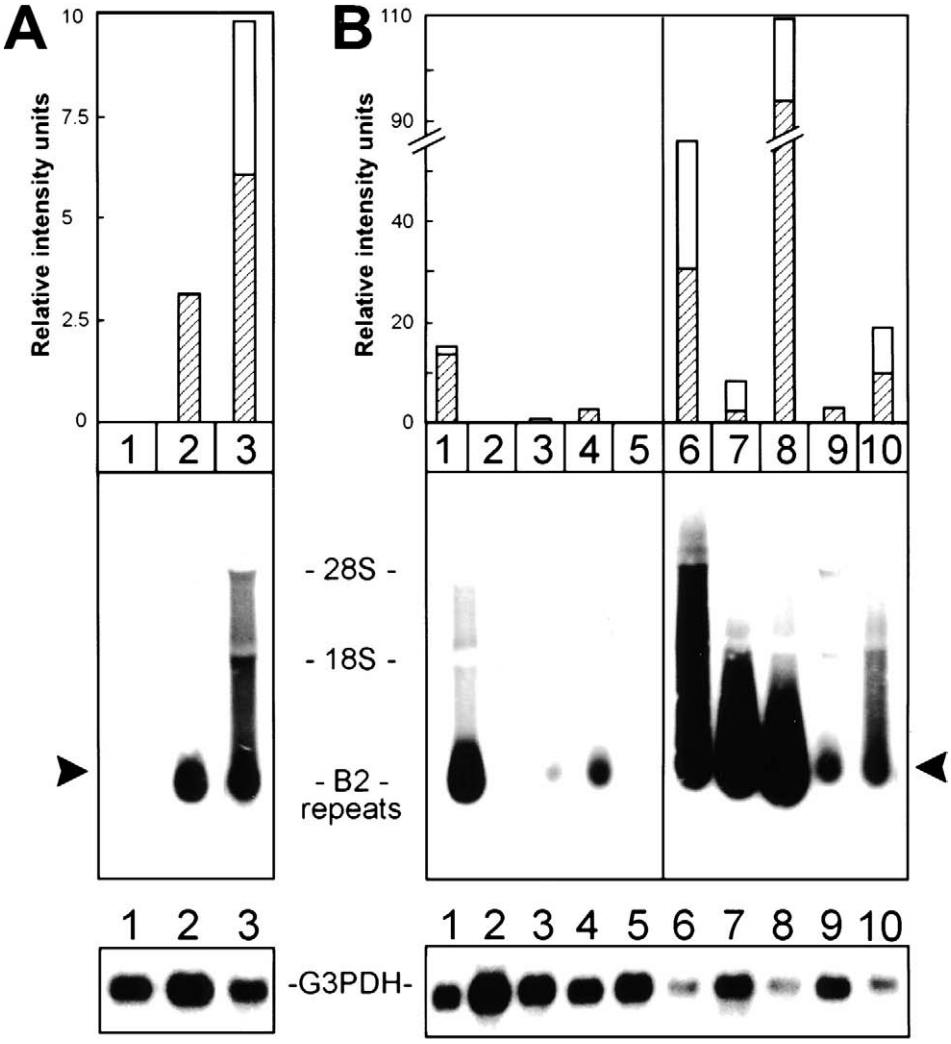

Figure 3. *Northern blot analysis of B2 repeat elements. Total RNA samples were size-fractionated on a 1% agarose gel and transferred to Hybond N$^+$ membranes. Bound RNA was hybridized to radiolabeled B2 cDNA fragments obtained from the subtracted library. (A) Total RNA samples from long-term cultured, antigen-dependent CD4$^+$ T lymphocytes (lane 1), cultured EL4/13.3 cells (lane 2) and tumor-derived EL4/13.3 cells (lane 3). (B) Total RNA from various cultured cell lines (lanes 1 to 5) and their corresponding tumor tissues (lanes 6 to 10): L929 (lanes 1 and 6), B16BL6 (lanes 2 and 7), F9 (lanes 3 and 8), TL-Rnu1 (lanes 4 and 9) and LLC (lanes 5 and 10). Autoradiographs were obtained after exposure for 1 day. As a quantitative control of RNA loading, the same blots were probed with a G3PDH cDNA fragment. Arrows indicate the position of B2 repeat element RNA (200 to 600 bp). The location of rRNA (28S: 4850 bp and 18S: 1740 bp) is indicated as 28S and 18S. The bars on top of each autoradiograph indicate hybridization intensity for B2 repeat element RNA (hatched bars) and larger B2-containing RNA (open bars), apparent as a rising smear. Intensities were quantified by phosphor-imaging and normalized to the G3PDH level of cultured B16BL6 cells. Normalization to 28S rRNA in an ethidium bromide-stained gel gave similar results (not shown).*

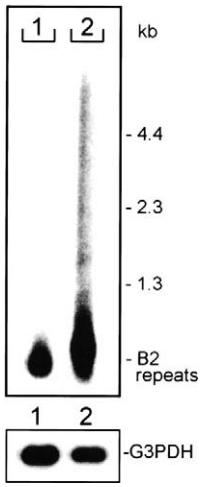

Figure 4. *Northern blot analysis of B2-containing transcripts in poly(A)$^+$ RNA from cultured (lane 1) and tumor-derived (lane 2) EL4/13.3 cells. Poly(A)$^+$ RNA was size-fractionated on a 1% agarose gel, transferred to Hybond N$^+$ membranes, hybridized with radiolabeled B2 cDNA fragment and visualized by autoradiography. As a quantitative control of mRNA loading, the same blot was probed with a G3PDH cDNA fragment. The locations of RNA size markers are shown on the right.*

cultured counterparts. As shown in Figure 3*B*, a similar pattern of increased levels of small but especially large B2 RNA was observed in fibrosarcoma (L929), melanoma (B16BL6), teratocarcinoma (F9), and lung carcinoma (LLC) tissues, except for the T-lymphoma TL-Rnu1. Consequently, upregulation of small B2 repeat RNA and induction of large B2-containing RNA are widespread in murine tumors.

B2-Containing Transcripts Represent Aberrant mRNA Transcripts

In order to verify whether poly(A)$^+$ RNA messengers are included in the B2-containing RNA population, poly(A)$^+$ RNA was isolated from tumor-derived and cultured EL4/13.3 cells, and assayed by Northern blotting for the presence of B2 elements (Figure 4). Again, large B2-containing sequences ranging from 0.6 to 5.0 kb were detected exclusively in tumor-derived EL4/13.3 cells. Accordingly, at least a fraction of the large B2-containing RNA revealed in tumor tissue represents not merely RNA but also large pol II transcripts of translated genes.

Confirmation that large B2-containing RNA actually comprises pol II mRNA transcripts was obtained by isolation of a B2-containing cathepsin S messenger from tumor-derived cDNA (Figure 5*A*). This fragment was isolated by RT-PCR with a forward primer F1 which anneals with a region of exon 3 of the cathepsin S gene and a reverse primer R1 which is complementary to the 3′ end of the consensus B2 sequence (Figure 5*B*). The same fragment could not be amplified in samples from cultured EL4/13.3 cells nor from normal T lymphocytes. Sequencing of the PCR fragment revealed normal exon 3 sequences with the B2 repetitive element located in an unidentified sequence downstream of exon 3 (Figure 5*C*). This B2 element is more than 90% homologous to the consensus B2 sequence

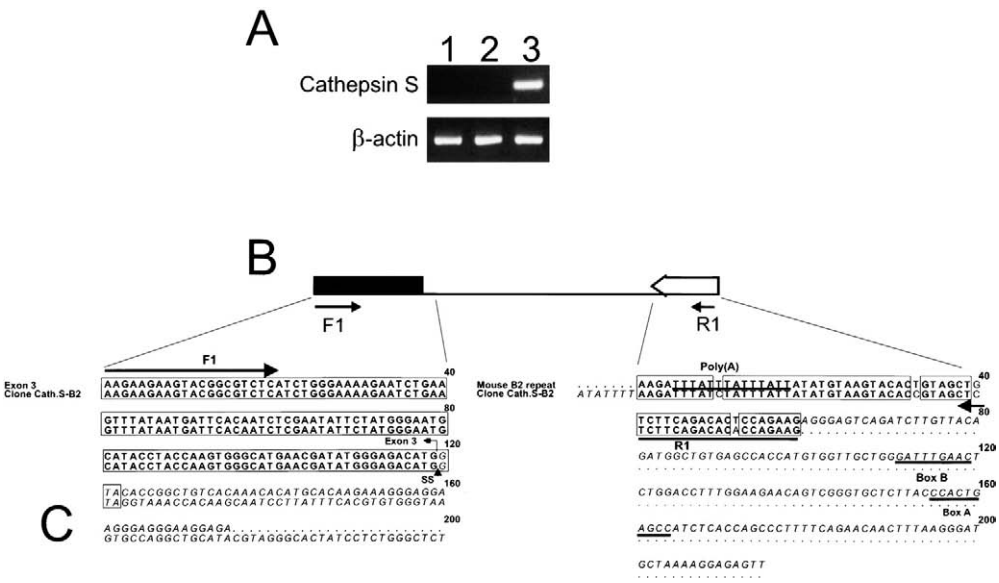

Figure 5. *Identification of a B2-containing cathepsin S transcript. (A) RT-PCR detection of a B2-containing cathepsin S transcript in CD4$^+$ T lymphocytes (lane 1), cultured EL4/13.3 cells (lane 2) and tumor-derived EL4/13.3 cells (lane 3). Amplification with specific β-actin primers is shown as a control for RNA-reversed transcription. (B) Scheme of the amplified B2-containing cathepsin S transcript. The open arrowhead box shows the location and orientation of the B2 element. The black box represents exon 3 of cathepsin S. PCR primers F1 and R1 are indicated. (C) Sequence analysis of the exon 3-putative intron 3 region of the cathepsin S gene and the B2 element. Sequences are aligned with exon 3 and flank of the Mus musculus cathepsin S gene (accession no. AF051727), and with published mouse B2 element (36). Boxes indicate identities. Arrows above and below the DNA sequence show the location of the forward and reverse primers used in PCR. The A and B boxes of the split polymerase III promoter and the poly(A) addition signals are underlined. The canonical splicing donor site is noted as ss. The nucleotide sequence between exons 3 and 4 of the cathepsin S gene is not available.*

[36] and is oriented in the opposite direction of the cathepsin S gene. As the template for RT-PCR was treated with DNase in order to remove contaminating genomic DNA, the presence of a putative intron sequence indicates that aberrant splicing generated the B2-containing cathepsin S mRNA. Hence, relaxation of RNA splicing fidelity may contribute to formation of B2-containing transcripts in tumor tissues.

Induction of Aberrant RNA Transcripts in Progressively Growing Tumors

The appearance in tumor tissues of B2-containing mRNA and of aberrant spliced forms of these transcripts indicates that growth as a tumor induces in the implanted cells aberrant gene transcription and/or RNA processing. Such dysregulation of the transcriptional machinery of the cell might also have consequences for transcription of specific genes. To verify this possibility, we probed Northern blots of the various cultured cell lines and their corresponding tumor tissues with a selection of cDNA fragments isolated from the subtracted library, representing upregulated genes, as well as with probes specific for housekeeping genes, representing genes with unaltered expression levels. As shown in Figure 6, full-length transcripts for osteopontin, CD32, cathepsin S and cytochrome *B* are indeed induced or strongly upregulated in most tumors, accompanied by a pronounced appearance of smearing bands of less than full-length size. GRP78 and HSP70 do not show a differential expression of full-length RNA but again exhibit a remarkable increase in truncated gene transcripts in the various tumors, but not in the cultured counterparts. Similarly to the B2-containing transcripts, the TL-Rnu1 lymphoma was also here the only exception, manifesting neither an increase in full-length transcripts nor in truncated transcripts for all six genes assayed. Noticeably, not all differentially expressed genes are equally upregulated in all tumor types (*cf.* Figure 6*A*, the strong upregulation of cytochrome *b* and the weak induction of cathepsin S in B16BL6 tumors *versus* the opposite in L929 tumors) nor is the pattern of smearing bands similar for all tumor tissues (*cf.* Figure 6*A*, GRP78 where a distinct band is visible in both LLC and EL4/13 but not or less in B16BL6 and F9). Finally, the appearance of smearing bands in tumor-derived material is not valid for all expressed genes as demonstrated by the absence of smearing bands with the housekeeping genes, β-actin and G3PDH.

Occurrence of smearing bands may reflect artificial RNA degradation in the tumor-derived material. However, the particularities of the smears observed with the different tumors and genes strongly argue against artificial RNA degradation as cause of these smears. The restriction of these smears to specific genes, their lack of correlation with gene expression levels (apparent in F9 tumors where GRP78 exhibits extreme smearing and where cytochrome *b* only leads to moderate smearing despite its strong expression), the occurrence of a distinct pattern within these smears for some tumors and genes, and the absence of smears in TL-Rnu1 tumors despite high, constitutive expression are difficult to reconcile with artificial RNA degradation. Also, the presence of intact 28*S* and 18*S* ribosomal RNA in all samples argues against this possibility (Figure 6*B*). Therefore, the appearance of aberrant, truncated gene transcripts in the various tumor tissue samples must reflect (post)transcriptional anomalies, generated by an active mechanism.

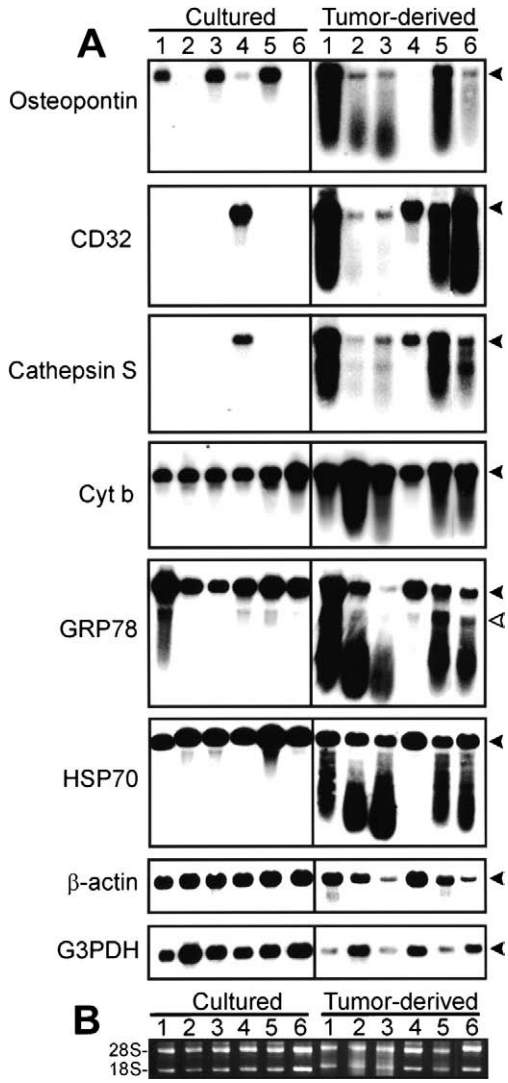

Figure 6. *Northern blot analysis of a selection of cDNA clones from the subtracted library on various cultured cell lines and their corresponding tumor tissues. (A) Total RNA from L929 (lane 1), B16BL6 (lane 2), F9 (lane 3), TL-Rnu1 (lane 4), LLC (lane 5) and EL4/13.3 (lane 6) were hybridized with the selected cDNA fragments. Autoradiographs were obtained after exposure for 1 day. Closed arrowheads indicate full-length transcript; the open arrowhead indicates a distinct pattern within the shorter than full-length smears. Two identical membranes were utilized to examine the expression of all clones. After each hybridization reaction, membranes were stripped and exposed for autoradiography for 48 hours to check for complete stripping before a new probe was added. (B) 28S and 18S rRNA in an ethidium bromide-stained gel, representative of the two gels.*

Aberrant, B2-Containing and Truncated Gene Transcripts are Generated by a Reversible Mechanism

Aberrant gene transcripts may result from irreversible changes in the genome of the cell or from reversible aberrations in DNA transcription and/or RNA processing.

Accordingly, we verified whether the appearance of aberrant, B2-containing and truncated gene transcripts in tumor-derived cells was retained after *ex vivo* propagation. Northern blot analysis for the presence of B2-containing transcripts and shorter-than-full-length CD32 gene transcripts revealed a complete to nearly complete disappearance of both (post)transcriptional anomalies in tumor-derived EL4/13.3 cells cultured *ex vivo* for 20 days as a pool and as single cell-derived clones (Figure 7). This downregulation of aberrant transcripts, both in the individual clones and in the pool, indicates that at least in a majority of the tumor-derived cells, aberrant gene transcripts are induced by a reversible mechanism. Strikingly, this downregulation was not accompanied by downregulation of the full-length CD32 transcripts, a phenomenon that was also observed for the other differentially expressed genes (result not shown). This retention of expression in the explanted tumor cells clearly confirms the tumor origin of the differential gene expression, and hence excludes contaminating host cells as source.

To further verify the reversible nature of the process, randomly chosen clones were again inoculated and the resulting second-passage tumors were analyzed by Northern blotting (Figure 7). The recurrence of a B2 and CD32 hybridization pattern characteristic of first-passage tumors further confirms the reversible and inducible nature of the mechanism responsible for aberrant gene transcription.

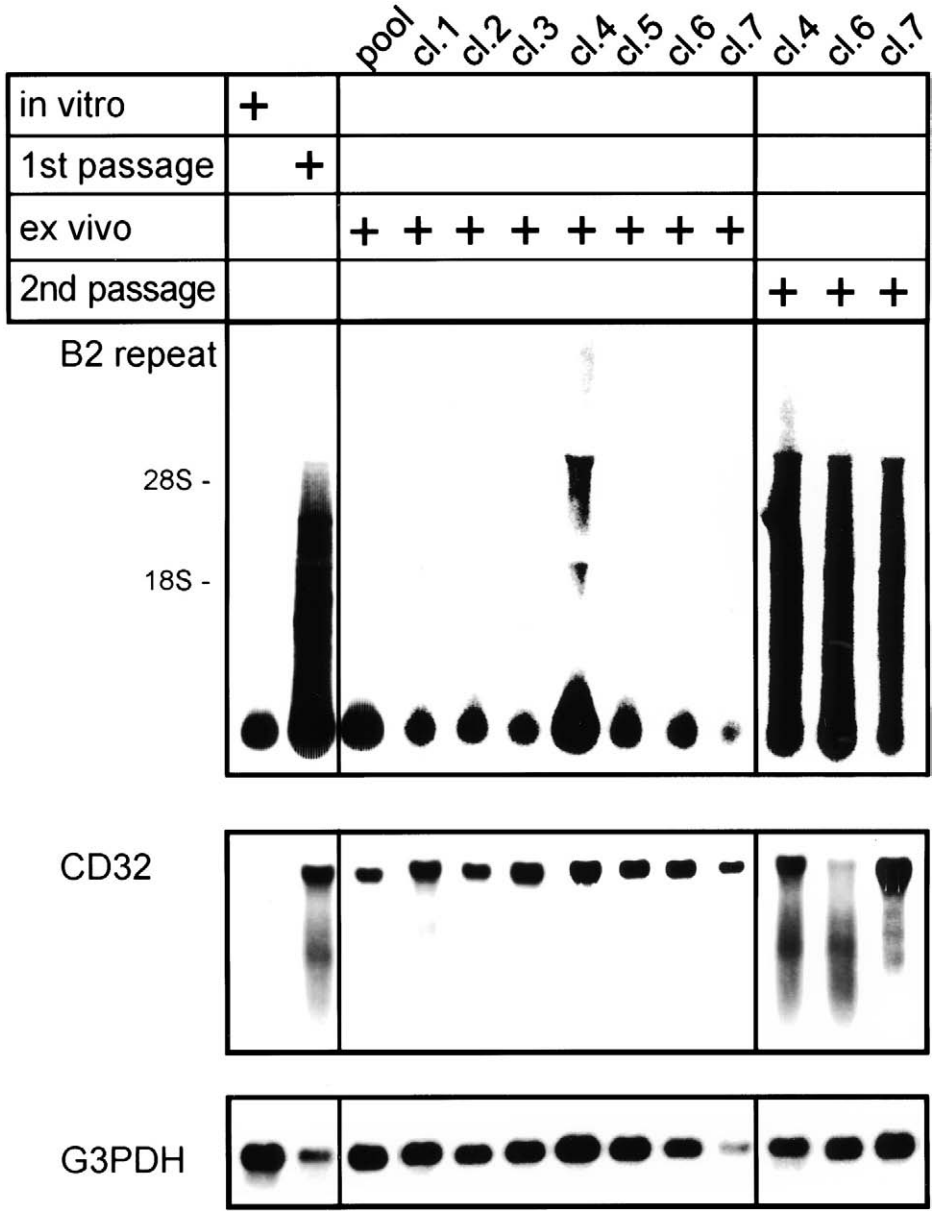

Figure 7. *Northern blot analysis of B2-containing transcripts and gene transcripts from CD32 and G3PDH on EL4/13.3 cells obtained from culture (in vitro), tumor tissue (first-passage), explanted, polyclonal (pool) and monoclonal (cl.1 to cl.7) cultures (ex vivo), and second-passage tumors. The EL4/13.3 subclones cl.1 to cl.7 were derived by limiting dilution cloning of cell suspensions prepared from solid, first-passage tumors. The total RNA blots were sequentially probed with radiolabeled B2, CD32 and G3PDH cDNA fragments with intermittent stripping. Location of rRNA (28S: 4850 bp and 18S: 1740 bp) is shown for the Northern blot of B2 cDNA.*

Therefore, irreversible changes in the genome of the tumor-derived cells as cause of the observed aberrant transcripts can be excluded. Instead, dysregulation of the gene transcription apparatus, triggered by the tumoral environment, must underlie the appearance of B2-containing and truncated transcripts in tumor tissues.

Discussion

Tumorigenesis represents a complex process in which alterations in the structure and expression of the genome play an important role. In the present study, we investigated genetic changes that occur in neoplastically transformed cells during and/or as a result of their outgrowth as a solid tumor. The cell line used for this study, the murine T-lymphoma EL4/13.3, was selected on the basis of our previous observation that growth as a solid tumor of these cells was accompanied by a dramatic phenotypic switch, involving expression of T cell differentiation antigens but also of tumor-associated and ectopic antigens [23]. Application of the PCR-based subtraction method resulted in identification of several known but also unknown genes that were expressed in tumor-derived EL4/13.3 cells but not, or at a lower level, in EL4/13.3 cells grown in tissue culture. For several of these genes, their overexpression in tumor cells and/or their contribution to cancer progression have been reported before. However, except CD32, (increased) expression of these genes was regarded as proper to tumorigenesis *per se*, instead of resulting from an inducible process triggered by tumor-specific growth conditions. This inducible process is not a cell-line-specific feature since a similar gene induction pattern was also observed in a melanoma, a fibrosarcoma, a teratocarcinoma and a carcinoma.

A common characteristic of the upregulated genes in these various tumors is the concomitant appearance of aberrant gene transcripts. Northern blotting revealed a strong smearing band for all six upregulated genes analyzed, but not for β-actin and G3PDH genes. These aberrant transcripts typically featured a shorter-than-full-length size and a large heterogeneity. Similarly, the upregulated expression of B2 repeat sequences was accompanied by an intense and broad smearing band, corresponding to larger RNA transcripts of 0.6 to 5.0 kb long, as opposed to the small B2 transcripts that typically range in size from 200 to 600 nucleotides. The detection of this smear also in poly(A)$^+$ mRNA preparations and the isolation of a cathepsin S transcript from the mRNA fraction of tumor-derived cells that retained a B2-like element as an exon corroborate our conclusion that these large B2 transcripts comprise chimeric B2-containing messengers.

It is well-known that solid tumors may contain necrotic regions, representing cells with degraded RNA. This necrotic material might be responsible for both types of smearing bands observed in tumor tissues. However, the large B2-containing transcripts in tumor tissues represent transcripts with increased instead of decreased lengths. Therefore, these aberrant transcripts are unlikely to be the result of contaminating necrotic material. Also, the similar percentage of dead cells in cultured and tumor-derived cells confirms that the aberrant transcripts shorter than full-length size do not originate from necrotic cells. As a consequence, the observation of chimeric messengers as well as truncated transcripts of specific upregulated genes points at a specific phenomenon induced by the tumoral environment.

B2 sequences are SINEs that are present in the genome at a frequency of nearly 1×10^5 [20]. Although RNA pol III specifically transcribes these B2 elements, the latter are found in the introns and 3′ nontranslated regions of some RNA pol II transcripts. Transcription of B2 repeats is strongly regulated such that most differentiated cells contain little or no B2 RNA. Embryonic cells but also tumor cells have, on the contrary, high levels of B2 transcripts [37]. We also observed this differential expression pattern: no expression in nontransformed T cells, expression in cultured EL4/13.3 thymoma cells, and a further upregulation in solid tumors. Also conditions of stress, including serum starvation and heat shock, induce B2 expression in somatic cells [38,39]. However, this typical stress is unlikely to be responsible for the B2 increment observed in solid tumors, since neither serum starvation nor heat shock increased the abundance of B2 RNA in cultured EL4/13.3 cells (results not shown).

The small, 200 to 600 nucleotides long B2 transcripts may serve as templates for endogenous reverse transcriptase, resulting in their irreversible integration into a different site of the genome [19,21]. Due to this retrotransposition activity, B2 elements can act as insertional mutagens, generating B2-containing or disrupted transcripts [19,40]. Therefore, the high levels of (small) B2 elements in tumor-derived cells might very well account for the concomitant appearance of truncated and B2-containing transcripts. Our results, however, do not support a direct correlation between high levels of B2 transcripts and occurrence of aberrant transcripts. Despite high levels of B2 transcripts, TL-Rnu1 lymphomas and explanted EL4/13.3 cells lack aberrant mRNAs. Also disappearance of B2-containing transcripts after explantation of EL4/13.3 tumor cells to culture and their recurrence in second-passage tumors is incompatible with the irreversible nature of B2 element retrotransposition. Alternatively, B2-containing transcripts in tumor cells might result from contaminating host cells. Negative selection of these cells under culture conditions *ex vivo* would explain the disappearance of these transcripts. Although it is difficult to fully exclude this possibility, the low amount of contaminating host cells present in the EL4/13.3 tumor samples (below 5%), and the absence of aberrant mRNAs in TLRnu lymphomas argue against this alternative interpretation. Therefore, we favor the interpretation that both chimeric and truncated transcripts are the result of a reversible mechanism. The concurrent appearance/disappearance of both transcriptional anomalies might be explained by induction of an anomalous (post)transcriptional process, generating truncated messengers from specific genes and messengers with introgenic B2 sequences. On the other hand, truncated B2-containing messengers from specific genes are a direct consequence of the presence of B2 elements in pol II genes.

In this scheme, increased B2 transcription leads to mRNAs originating at the B2 promoters instead of at the proper pol II promoter. mRNAs originating at the promoter of retrotransposons have been described in the case of the intracisternal A-particle yellow (A_{iapy}) and the hypervariable yellow (A_{hvy}) mutation, where utilization of the cryptic promoter within the intracisternal A-particle results in ectopic expression of the agouti gene [41,42]. Such mRNAs may give rise to truncated, B2-containing messengers as a result of, e.g., premature termination. In the case of the chimeric cathepsin S transcript, the opposite orientation of the B2 sequence, downstream exon 3 of the cathepsin S gene, may generate a truncated chimeric messenger provided the cathepsin S transcript is initiated from the B2 promoter.

It is well-established that chimeric or truncated messengers affect protein expression levels or lead to production of proteins with altered functionality. The strong rise in response to environmental triggers of truncated and chimeric messengers from specific genes may therefore contribute to tumor growth and progression by affecting the levels and/or functionality of the expressed proteins. Similarly, the induction in solid tumors of an abnormal pattern of gene expression provides a tool to the tumor cells for acquiring new functions. The activation of both processes by as yet undefined, tumor-specific growth conditions and/or signals from the tumoral environment constitutes a potent mechanism for creating epigenetic diversity within the tumors itself, as well as between tumor nodules that metastasized to different organs or sites of the body. The relevance for cancer therapy of this environment-driven generation of aberrant gene transcripts and abnormal gene expression, and its occurrence in tumors of various histological origin certainly warrants further research efforts into the identification of the molecular events that cause these (post)transcriptional anomalies and of the environmental triggers that activate their generation.

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References

[1] Sager R (1997). Expression genetics in cancer: shifting the focus from DNA to RNA. *Proc Natl Acad Sci USA* **94**, 952–955.

[2] Pearson PL, and Van der Luijt RB (1998). The genetic analysis of cancer. *J Intern Med* **243**, 413–417.

[3] Nicolson GL, and Moustafa AS (1998). Metastasis-associated genes and metastatic tumor progression [In Process Citation]. *In Vivo* **12**, 579–588.

[4] Vermeulen SJ, Bruyneel EA, Bracke ME, De Bruyne GK, Vennekens KM, Vleminckx KL, Berx GJ, van Roy FM, and Mareel MM (1995). Transition from the noninvasive to the invasive phenotype and loss of alpha-catenin in human colon cancer cells. *Cancer Res* **55**, 4722–4728.

[5] Akiyama SK, Olden K, and Yamada KM (1995). Fibronectin and integrins in invasion and metastasis. *Cancer Metastasis Rev* **14**, 173–189.

[6] Umar A, and Kunkel TA (1996). DNA replication fidelity, mismatch repair and genome instability in cancer cells. *Eur J Biochem* **238**, 297–307.

[7] Seliger B, Harders C, Lohmann S, Momburg F, Urlinger S, Tampe R, and Huber C (1998). Downregulation of the MHC class I antigen-processing machinery after oncogenic transformation of murine fibroblasts. *Eur J Immunol* **28**, 122–133.

[8] Sutherland RM (1998). Tumor hypoxia and gene expression—implications for malignant progression and therapy. *Acta Oncol* **37**, 567–574.

[9] Laird PW, and Jaenisch R (1996). The role of DNA methylation in cancer genetic and epigenetics. *Annu Rev Genet* **30**, 441–464.

[10] Sanchez-Garcia I (1997). Consequences of chromosomal abnormalities in tumor development. *Annu Rev Genet* **31**, 429–453.

[11] Look AT (1997). Oncogenic transcription factors in the human acute leukemias. *Science* **278**, 1059–1064.

[12] Maxwell PH, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, Hankinson O, Pugh CW, and Ratcliffe PJ (1997). Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. *Proc Natl Acad Sci USA* **94**, 8104–8109.

[13] Liu X, Gupta AK, Corry PM, and Lee YJ (1997). Hypoglycemia-induced c-Jun phosphorylation is mediated by c-Jun N- terminal kinase 1 and Lyn kinase in drug-resistant human breast carcinoma MCF-7/ADR cells. *J Biol Chem* **272**, 11690–11693.

[14] Toyomizu M, Yamahira S, Shimomura Y, and Akiba Y (1999). Metabolic acidosis inhibits pyruvate oxidation in chick liver by decreasing activity of pyruvate dehydrogenase complex. *Life Sci* **65**, 37–43.

[15] Stickeler E, Runnebaum IB, Mobus VJ, Kieback DG, and Kreienberg R (1997). Expression of CD44 standard and variant isoforms v5, v6 and v7 in human ovarian cancer cell lines. *Anticancer Res* **17**, 1871–1876.

[16] Charlieu SH, Larssont JP, Miyagawa K, Engelkamp D, Rassoulzadegan M, Ross A, Cuzin F, van Heyningen V, and Hastie ND (1995). Subnuclear localization of WT1 in splicing or transcription factor domains is regulated by alternative splicing. *Cell* **81**, 391–401.

[17] Santoro IM, and Groden J (1997). Alternative splicing of the APC gene and its association with terminal differentiation. *Cancer Res* **57**, 488–494.

[18] Pattanakitsakul S, Zheng JH, Natsuume-Sakai S, Takahashi M, and Nonaka M (1992). Aberrant splicing caused by the insertion of the B2 sequence into an intron of the complement C4 gene is the basis for low C4 production in H-2k mice. *J Biol Chem* **267**, 7814–7820.

[19] Michel D, Chatelain G, Mauduit C, Benahmed M, and Brun G (1997). Recent evolutionary acquisition of alternative pre-mRNA splicing and 3′ processing regulations induced by intronic B2 SINE insertion. *Nucleic Acids Res* **25**, 3228–3834.

[20] Rogers JH (1985). The origin and evolution of retroposons. *Int Rev Cytol* **93**, 187–279.

[21] Oberbaumer I (1992). Retroposons do jump: a B2 element recently integrated in an 18*S* rDNA gene. *Nucleic Acids Res* **20**, 671–677.

[22] Zhang L, Zhou W, Velculescu VE, Kern SE, Hruban RH, Hamilton SR, Vogelstein B, and Kinzler KW (1997). Gene expression profiles in normal and cancer cells. *Science* **276**, 1268–1272.

[23] Rottiers P, Verfaillie T, Contreras R, Revets H, Desmedt M, Dooms H, Fiers W, and Grooten J (1998). Differentiation of EL4 lymphoma cells by tumoral environment is associated with inappropriate expression of the large chondroitin sulfate proteoglycan PG-M and the tumor-associated antigen HTgp-175. *Int J Cancer* **78**, 503–510.

[24] Desmedt M, Rottiers P, Dooms H, Fiers W, and Grooten J (1998). Macrophages induce cellular immunity by activating Th1 cell responses and suppressing Th2 cell responses. *J Immunol* **160**, 5300–5308.

[25] Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, and Siebert PD (1996). Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc Natl Acad Sci USA* **93**, 6025–6030.

[26] Feng B, Rollo EE, and Denhardt DT (1995). Osteopontin (OPN) may facilitate metastasis by protecting cells from macrophage NO-mediated cytotoxicity: evidence from cell lines down regulated for OPN expression by a targeted ribozyme. *Clin Exp Metastasis* **13**, 453–462.

[27] Wurl P, Taubert H, Meye A, Dansranjavin T, Weber E, Gunther D, Berger D, Schmidt H, Dralle H, and Rath FW (1997). Immunohistochemical and clinical evaluation of cathepsin expression in soft tissue sarcomas. *Virchows Arch* **430**, 221–225.

[28] Yamagata M, and Kimata K (1994). Repression of a malignant cell-substratum adhesion phenotype by inhibiting the production of the anti-adhesive proteoglycan, PG-M/versican. *J Cell Sci* **107**, 2581–2590.

[29] Zusman T, Gohar O, Eliassi H, Avivi Y, Lisansky E, Sautes C, Even J, Bonnerot C, Fridman WH, Witz IP, and Ran M (1996). The murine Fc-gamma (Fc gamma) receptor type II B1 is a tumorigenicity-enhancing factor in polyoma-virus-transformed 3T3 cells. *Int J Cancer* **65**, 221–229.

[30] Bieche I, Lachkar S, Becette V, Diaz Cifuentes C, Sobel A, Lidereau R, and Currmi PA (1998). Overexpression of the stathmin gene in a subset of human breast cancer. *Br J Cancer* **78**, 701–709.

[31] Blumenstein M, Hossfeld DK, and Duhrsen U (1998). Indirect radiation leukemogenesis in DBA/2 mice: increased expression of B2 repeats in FDC-P1 cells transformed by intracisternal A-particle transposition. *Ann Hematol* **76**, 53–60.

[32] Jamora C, Dennert G, and Lee AS (1996). Inhibition of tumor progression by suppression of stress protein GRP78/BiP induction in fibrosarcoma B/C10ME. *Proc Natl Acad Sci USA* **93**, 7690–7694.

[33] Jäätteï M (1995). Overexpression of hsp70 confers tumorigenicity to mouse fibrosarcoma cells. *Int J Cancer* **60**, 689–693.

[34] Suzuki H, Kumagai T, Goto A, and Sugiura T (1998). Increase in intracellular hydrogen peroxide and upregulation of a nuclear respiratory gene evoked by impairment of mitochondrial electron transfer in human cells. *Biochem Biophys Res Commun* **249**, 542–545.

[35] Ryskov AP, Ivanov PL, Tokarskaya ON, Kramerov DA, Grigoryan MS, and Georgiev GP (1985). Major transcripts containing B1 and B2 repetitive sequences in cytoplasmic poly(A)$^+$ RNA from mouse tissues. *FEBS Lett* **182**, 73–76.

[36] Kramerov DA, Tillib SV, Ryskov AP, and Georgiev GP (1985). Nucleotide sequence of small polyadenylated B2 RNA. *Nucleic Acids Res* **13**, 6423–6437.

[37] Bladon TS, Fregeau CJ, and McBurney MW (1990). Synthesis and processing of small B2 transcripts in mouse embryonal carcinoma cells. *Mol Cell Biol* **10**, 4058–4067.

[38] Edwards DR, Parfett CL, and Denhardt DT (1985). Transcriptional regulation of two serum-induced RNAs in mouse fibroblasts: equivalence of one species to B2 repetitive elements. *Mol Cell Biol* **5**, 3280–3288.

[39] Liu WM, Chu WM, Choudary PV, and Schmid CW (1995). Cell stress and translational inhibitors transiently increase the abundance of mammalian SINE transcripts. *Nucleic Acids Res* **23**, 1758–1765.

[40] Owczarek CM, Layton MJ, Robb LG, Nicola NA, and Begley CG (1996). Molecular basis of the soluble and membrane-bound forms of the murine leukemia inhibitory factor receptor alpha-chain. Expression in normal, gestating, and leukemia inhibitory factor nullizygous mice. *J Biol Chem* **271**, 5495–5504.

[41] Michaud EJ, van Vugt MJ, Bultman SJ, Sweet HO, Davisson MT, and Woychik RP (1994). Differential expression of a new dominant agouti allele (A_{iapy}) is correlated with methylation state and is influenced by parental lineage. *Genes Dev* **8**, 1463–1472.

[42] Argeson AC, Nelson KK, and Siracusa LD (1996). Molecular basis of the pleiotropic phenotype of mice carrying the hypervariable yellow (A_{hvy}) mutation at the agouti locus. *Genetics* **142**, 557–567.