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Regulation of the expression of the oncogene *EVI1* through the use of alternative mRNA 5′-ends

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Abstract

The *EVI1* gene plays important roles in development and leukemogenesis. Recently, human *EVI1* has been shown to give rise to at least six different mRNA variants with alternative 5′-ends, only some of which are conserved in mice. In order to gain a basic understanding of the regulation and potential biological importance of these alternative transcripts, we confirmed their expression by Northern blot, and, using real time quantitative RT-PCR, compared their abundance and stability under different conditions. The general expression patterns of the *EVI1* 5′-end variants in a panel of 20 human tissues were similar, but particularly high or low levels of some of them were noted in certain tissues. Pronounced differences in the expression of the 5′-end variants were noted in response to all-*trans* retinoic acid: in a human teratocarcinoma cell line, only the *EVI1* transcript variants containing alternative exons 1a and 1b were upregulated in response to this agent. This induction required transcriptional activity of RNA polymerase, but was also associated with a substantial increase in the stability of these mRNA variants.
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Keywords: EVI1; Oncogene; 5′-end variability; Gene expression; mRNA stability

1. Introduction

The *EVI1* gene codes for a zinc finger protein presumed to act as a transcription factor, and plays important roles in leukemogenesis and as a regulator of mammalian development. It is overexpressed in patients with myeloid leukemia either due to prognostically unfavourable rearrangements of its gene locus in chromosome band 3q26, or to still unknown reasons (Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2003; Vinatzer et al., 2003). Its role as an oncogene is confirmed by analyses in murine model systems (Louz et al., 2000; Cuenco and Ren, 2004). *EVI1* also has important functions in normal mammalian development. Mice with a homozygous deletion of the sixth exon of this gene exhibited pleiotropic developmental defects, and died around day 10.5 of embryonic development due to cardiac failure after sudden, massive hemorrhaging (Hoyt et al., 1997). In further support of a role of *EVI1* in development, its expression is upregulated in response to the differentiation inducing agent all-*trans* retinoic acid (ATRA) (Xi et al., 1997; Kazama et al., 1999), and ectopic expression of *EVI1* in the murine teratocarcinoma cell line F9 reproduces the effect of ATRA on the neuronal differentiation of these cells (Kazama et al., 1999).

The human *EVI1* gene gives rise to at least six mRNA variants with different 5′-termini (Fig. 1). The exon 1a-(acc. no. AF487424 (Vinatzer et al., 2003)), 1b-(acc. no.

Abbreviations: ATRA, all-*trans* retinoic acid; ActD, actinomycinD; azaC, 5-azacytidine; bp, base pairs; DMSO, dimethyl sulfoxide; EVI1, ecotropic viral integration site 1; FBS, fetal bovine serum; kb, kilobasepairs; MDS1, myelodysplastic syndrome 1; PBS, phosphate buffered saline; RTQ-RT-PCR, real time quantitative reverse transcriptase polymerase chain reaction; SD, standard deviation; SSC, sodium chloride sodium citrate; SDS, sodium dodecyl sulfate; UTR, untranslated region.

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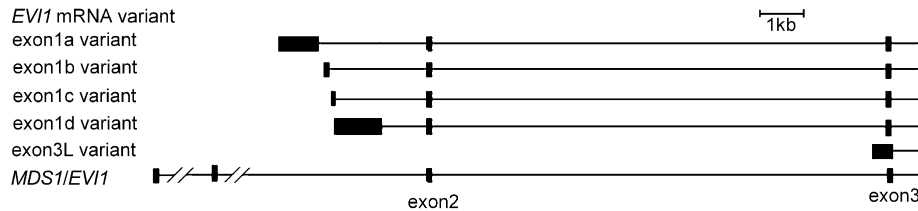

Fig. 1. Schematic representation of the different 5′-ends of the human *EVI1* mRNA. Exons are represented by boxes, and introns by lines. The names of the mRNA variants are shown to the left. The accession numbers and originally reported names for the transcript variants are as follows: exon 1a, acc. no. AF487424, clone 39 (Vinatzer et al., 2003); exon 1b, acc. no. AF487422, clone 6 (Vinatzer et al., 2003); exon 1c, acc. no. X54989, 'exon 1' (Morishita et al., 1990); exon 1d, acc. no. AF164154, clone 9; *MDS1/EVI1*, acc. no.s S69002 and U43292, *MDS1/EVI1* (Fears et al., 1996); exon 3L, acc. no. AF487423, clone 19 (Vinatzer et al., 2003).

AF487422 (Vinatzer et al., 2003)), 1c-(acc. no. X54989 (Morishita et al., 1990)), and 1d-(acc. no. AF164154) containing mRNA variants use different transcription initiation sites, which leads to the incorporation of different first exons that are all joined to exon 2 using the same splice acceptor site. Four different splice variants of the exon 1d-containing *EVI1* 5′-UTR have been deposited in the database (acc. no.s AF164154–AF164157), but so far only one of them was confirmed independently (Vinatzer et al., 2003). In the *MDS1/EVI1* mRNA (acc. no.s S69002, U43292 (Fears et al., 1996)), the first two exons of *MDS1*, a gene located upstream of *EVI1* and also expressed on its own, are joined to exon 2 of *EVI1*. In the exon 3L-variant (acc. no. AF487423 (Vinatzer et al., 2003)), both exons 1 and 2 of *EVI1* are replaced by the genomic sequence immediately upstream of exon 3. An ATG codon in the context of a Kozak consensus is present in exon 3 of *EVI1*, and is likely to be used for translation initiation in most of the *EVI1* transcript variants. The *MDS1/EVI1* mRNA, however, is translated from an ATG codon located in the *MDS1* portion of this transcript, leading to the production of a protein in which 188 amino acids with homology to the so-called PR domain are joined to the amino-terminus of the otherwise unchanged EVI1 protein (Fears et al., 1996). The EVI1 and MDS1/EVI1 proteins exhibit partially antagonistic and partially similar biological properties (Soderholm et al., 1997; Sitailo et al., 1999; Vinatzer et al., 2001; Cuenco and Ren, 2004). Of interest, only the exon 1a-containing transcript variant and the *MDS1/EVI1* mRNA are conserved between humans and mice.

Variability at the 5′-ends of mRNAs may affect the structure of the resulting proteins, as is the case for MDS1/EVI1. If it involves only the 5′-untranslated region (UTR) of an mRNA, it may affect quantitative aspects of protein production at the levels of transcription initiation (Minegishi et al., 1998; Orban and Olah, 2003), transcript stability (Chen et al., 1998; Tebo et al., 2000), and translation efficiency (van der Velden and Thomas, 1999; Wilkie et al., 2003). Except for a few reports on the expression and the biological properties of the MDS1/EVI1 mRNA and protein, virtually no information is available regarding the expression patterns and biological significance of the different *EVI1* mRNA variants. The hitherto only study in which the expression levels of different *EVI1* 5′-end variants – namely, of the exon 1d type mRNA and of *MDS1/EVI1* – were compared, revealed that expression of the former, but not of the latter, was associated with a bad prognosis in myeloid leukemia (Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2003). Previous studies, measuring the sum of all *EVI1* transcript variants, which we suggested to be designated c*EVI1* (Vinatzer et al., 2003), had failed to reveal this correlation. In the present study, we began to investigate the biological importance of the variability at the 5′-end of the *EVI1* mRNA by comparing the expression levels and stability of these mRNA variants in different human tissues and in response to the physiological *EVI1* inducer, ATRA.

2. Materials and methods

2.1. Cell lines and culture conditions

The cell lines used for this study are: MPD, HEL, and HNT-34, all of human myeloid origin, the human renal epithelial cell line 293T, the human teratocarcinoma cell line PA-1, and the murine teratocarcinoma cell line F9. All cell lines were cultured in RPMI-1640 Medium with L-Glutamine (Invitrogen, Groningen, The Netherlands) supplemented with 10% fetal bovine serum (FBS; Invitrogen) in a humidified incubator with 5% CO_2 at 37 °C. For PA-1 and HNT-34 cells, FBS was heat inactivated for 30 min at 60 °C prior to use. F9 cells were cultured on a gelatinized surface.

Stock solutions of all-*trans* retinoic acid (ATRA) and actinomycin D (ActD) were prepared in DMSO, those of 5-azacytidine (azaC) in PBS. The final concentrations of these reagents were 100nM for ATRA, 10 μg/ml for ActD, and 5 μM for azaC. Controls for ATRA-treated cultures were incubated with an equal volume of DMSO. ATRA, ActD, and azaC were purchased from Sigma (St. Louis, MO, USA).

2.2. RNA extraction and Northern blots

Total RNA was extracted from cell lines using Trizol reagent (Invitrogen) according to the manufacturer's

instructions. An RNA panel representing 20 different human tissues was purchased from Clontech (Palo Alto, CA, USA). For Northern experiments, 10 μg of total RNA from the indicated cell lines were used. Formaldehyde gel electrophoresis, transfer to nylon membranes, labelling of probes, and hybridization were carried out using standard procedures. After several washes with 1x SSC and 0.1% SDS at 42 °C, membranes were exposed to a Phosphoimager screen. Before rehybridization, blots were stripped by submerging them twice into boiling water. The probes used were as follows: for exon 1a, a 1 kb BglII fragment containing exclusively exon 1a sequences was isolated from plasmid DKFZp686J18113 (acc. no. of insert: BX640908). For exon 1b (acc. no. AF487422), a 107 bp fragment containing the entire exon 1b sequence and 10 bp from exon 2 was amplified by PCR from an appropriate plasmid. For exon 1d (acc. no. AF164154), a 1.1 kb HaeIII fragment containing exclusively exon 1d sequences was isolated from plasmid c9/pGEM-T Easy. For exon 3L, a 550 bp fragment containing sequences exclusive to the originally isolated exon 3L clone (acc. no. AF487423) and an EST clone overlapping with its 5′-end (acc. no. CB241806) was amplified from cDNA of a human leukemia cell line by nested PCR. A probe consisting of two restriction fragments containing the first 1.3 kb and the last 1.0 kb of the EVI1 coding region was hybridized to the membranes after the 5′-end specific probes for the purpose of size comparison. The GAPDH probe (acc. no. BC025925) was an RT-PCR product obtained using the primers indicated in Table 1.

2.3. cDNA synthesis, conventional RT-PCR, and real time quantitative (RTQ) RT-PCR

For cDNA synthesis, 0.5–1 μg of each RNA sample were digested with DNAse I (Roche Diagnostics, Mannheim, Germany) and reverse transcribed using the M-MLV enzyme (Invitrogen) and random hexamer primers (Invitrogen). The absence of genomic DNA from the cDNA samples was verified by PCR with primers located in introns of the *NF1* gene. Conventional PCR was carried out using Taq polymerase (Invitrogen) and the oligonucleotide primers listed in Table 1 at standard conditions with the indicated annealing temperatures.

Sybr Green Technology (Sybr Green PCR Master Mix, ABI, Foster City, CA, USA) was employed for all RTQ-RT-PCR experiments. Reactions were carried out in an ABI Prism 7700 Sequence Detection System (ABI) in a total volume of 20 μl using the conditions recommended by the manufacturer. Oligonucleotide primers were designed using the Primer Express software program (ABI). Their sequences are indicated in Table 2. All primer pairs were validated by amplifying serial dilutions of cDNA from MPD or HNT-34 cells. When plotting the known cDNA concentration versus the measured expression, slopes close to −3.3, indicating an amplification efficiency near 100%, were obtained for all assays. For each data point, duplicate or triplicate samples were analyzed in parallel. Expression of the *EVI1* mRNA variants relative to the housekeeping gene *GAPDH* was calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). The one-sided error bars shown in the

Table 1
List of primers used for conventional PCR and RT-PCR

Primer name[a]	Sequence	GenBank acc. no.	Nucleotide position	Annealing temperature
h*GAPDH* fwd	AAGGTGAAGGTCGGAGTCAACG	BC025925	64–85	62 °C
h*GAPDH* rev	CAGCCTTCTCCATGGTGGTGAA	BC025925	382–361	62 °C
hc*EVI* fwd	AGCAACGTCGAATCAAGACCTGCTTCAGAT	X54989	2110–2139	62 °C
hc*EVI* rev	ACTGACTGTAAGAGCTCACTGGCCTCAGGT	X54989	2381–2361	62 °C
h*EVI*ex1a fwd	TATTGCTGAGTTGAGGCCATAG	AF487424	372–393	58 °C
h*EVI1*ex1b fwd	GGGCTGCTTATCTACGTTGC	AF487422	1–18	58 °C
h*EVI*ex3L fwd	AGATTTGTGAACTGGCTTGAGA	AF487423	127–148	58 °C
h*EVI*ex1a,1b,3L rev	TTGAAAATGCTGAGTGAGGAGT	X54989	424–403	58 °C
h*EVI1*ex1d-rtq fwd	CTTCTTGACTAAAGCCCTTGGA	AF164154	1234–1255	60 °C
h*EVI1*ex1d-rtq rev	GTACTTGAGCCAGCTTCCAACA	AF164154	1357–1335	60 °C
h*MDS1/EVI1* fwd	GGGGCAGGACTAGGAATATGGAC	S69002	2362–2384	58 °C
h*MDS1/EVI1* rev	TTGCCGTTCTTCGTGGATATCCG	X54989	327–305	58 °C
h*NF1*intron23 fwd	TGTAAAACGACGGCCAGTTTGAACTCTTTGTTTTCA	AC004526	77,853–77,871	55 °C
h*NF1*intron24 rev	CAGGAAACAGCTATGACCGGAATTTAAGATAGCTAG	AC004526	78,119–78,102	55 °C
mEvi1 fwd	CTCGAGTACCTGGCTGCTGCTGAT	BC076620	526–549	65 °C
m*Mds1/Evi1* fwd	CACATTCAAGAGCCATGCTCTCCTG	MAJ10015	151–175	67 °C
m*Evi1*, m*Mds1/Evi1* rev	GGTCCTCTTCACTCTTCATGAACAGC	BC076620	823–798	65/67 °C
mβmicrog. fwd	GCATGACAGTATGGCCGAGCC	X01838	361–381	63 °C
mβmicrog. rev	GCTCAGCTATCTAGGATATTTCC	X01838	594–572	63 °C

Shown are the names, sequences, source sequence accession numbers, relative positions, and annealing temperatures of the primers used for conventional PCR and RT-PCR. The exon 1d-specific primers are able to amplify all reported splice variants of the exon 1d-containing 5′-UTR.

[a] Names of primers for human genes are preceded by the letter h; names of primers for murine genes by the letter m. fwd, forward primer; rev, reverse primer.

Table 2
List of primers used for RTQ-RT-PCR

Primer name[a]	Sequence	GenBank acc. no.	Nucleotide position
h*GAPDH*-rtq fwd	TGCACCACCAACTGCTTAGC	BC025925	511–530
h*GAPDH*-rtq rev	GGCATGGACTGTGGTCATGA	BC025925	597–578
h*EVI1*ex1a-rtq fwd	TATTGCTGAGTTGAGGCCATAG	AF487424	372–393
h*EVI1*ex1b-rtq fwd	TGCGGTCTGGACACGTCTC	AF487422	56–74
h*EVI1*ex1a/b-rtq rev	CTTCCAACATCTGGTTGACTGG	X54989	140–119
h*EVI1*ex1d-rtq fwd	CTTCTTGACTAAAGCCCTTGGA	AF164154	1234–1255
h*EVI1*ex1d-rtq rev	GTACTTGAGCCAGCTTCCAACA	AF164154	1357–1335
h*EVI1*ex3L-rtq fwd	GGTATCTTAGTGTATATCTTGCCCTTTGT	AF487423	263–291
h*EVI1*ex3L-rtq rev	GCGCAATGTCTGCAACTACTCT	AF487423	380–359
h*MDS1/EVI1*-rtq fwd	CCAGTTATGGATGGGAGATCTTAGAC	S69002	2456–2481
h*MDS1/EVI1*-rtq rev	CCAGCGAATCTAATGTACTTGAGC	X54989	167–144
h*Smad2*-rtq fwd	TTGTGCAGAGCCCCAATTG	BC014840	1315–1333
h*Smad2*-rtq rev	GACTGAGCCAGAAGAGCAGCAA	BC014840	1438–1417

Shown are the names, sequences, source sequence accession numbers, and relative positions of the primers used for RTQ-RT-PCR. The annealing temperature for all RTQ-RT-PCR primers was 60 °C. The exon 1d-specific primers are able to amplify all reported splice variants of the exon 1d-containing 5′-UTR.
[a] The letter h in front of primer names designates primers specific for human genes. fwd, forward primer; rev, reverse primer.

figures correspond to the difference between $2^{-\Delta\Delta Ct}$ and $2^{-(\Delta\Delta Ct-SD)}$.

2.4. Determination of mRNA half lives

PA-1 cells pretreated with DMSO, ATRA, or azaC for 18 h, HNT-34 cells, or MPD cells were incubated with 10 μg/ml ActD for 0, 1, 2, 3, and 4 h. The amounts of the *EVI1* or *Smad2* mRNAs were measured by RTQ-RT-PCR and calculated relative to those of the *GAPDH* mRNA, which is essentially stable over the time periods used for analysis (half life >20 h (Ranganna and Yatsu, 1997)). The natural logarithms of the normalized expression values were plotted against time. The slope k of the resulting curve was used to calculate mRNA half life ($\tau_{1/2}$) according to the formula $\tau_{1/2}=-0.693/k$. Correlation coefficients (R^2) for the mRNA decay curves were on average 0.90 (range, 0.78 to 0.99).

2.5. Plasmids, transient transfections, and luciferase assays

pTK luc was constructed by introducing the BamHI/BglII fragment of pBL-CAT2, which contains the tk promoter, into the BglII site of pGL3basic (Promega). To search for sequence elements conferring regulation by ATRA to the *EVI1* promoter, the following genomic DNA fragments were cloned into pTK luc or pGL3promoter (Promega): fragment B2, −2.7 to −0.8 kb relative to exon 1a; fragment B1, −0.9 to +0.1 kb relative to exon 1a; fragment A, −1.1 to +0.6 kb relative to exon 1b (including the entire exons 1a and 1b); and fragment EPP1/1, −0.4 to +3.5 kb relative to exon 1b (including exons 1b–d, the entire intron 1, and exon 2). As a positive control for ATRA responsiveness, pRARE-TK-luc/pGL2 (kindly provided by Dr H. Harant, Novartis, Vienna, Austria) was used. The respective empty vectors were used as negative controls. The renilla luciferase plasmid pRL-SV40 (Promega) served as a control for transfection efficiency. For transient transfections of 293T cells, DAC-30 (Eurogentech, Seraing, Belgium) was used according to the manufacturer's instructions. Expression vector pSG5 (Stratagene, La Jolla, CA, USA) with the *RARα* cDNA inserted (pSG5/*RARα*, kindly provided by Dr H. Harant, Novartis, Vienna, Austria) was cotransfected with the luciferase reporter plasmids and pRL-SV40. One day after transfection, 100 nM ATRA was added to half of the wells, and an equivalent amount of DMSO to the other half. On the next day, cells were lysed, firefly and renilla luciferase activities were measured (Dual luciferase reporter assay system, Promega), and the former expressed relative to the latter. Duplicate samples were assayed for each data point.

3. Results

3.1. Northern analysis confirms the expression of the 5′-end variants of the EVI1 mRNA

Most of the alternative 5′-ends of the *EVI1* mRNA have been identified using PCR-based cloning strategies. To proof that they are bona fide transcripts, and not artifacts of PCR procedures, we carried out Northern analyses using total RNA extracted from the human cell lines HEL and HNT-34. Probes specific for exons 1a, 1b, 1d, and 3L did indeed detect transcripts in these cell lines (Fig. 2). The small size and low expression (Morishita et al., 1990; Vinatzer et al., 2003) of exon 1c make it impossible to generate a useful probe. Expression of *MDS1/EVI1* has been demonstrated by Northern analysis previously (Fears et al., 1996). The low signal intensity obtained for the exon 1b-containing *EVI1* transcript is probably due to the small size of this exon, and consequently, of the probe specific for it. Exon 1d-containing transcripts, on the other hand, appear to be expressed at low levels, as also suggested by the PCR experiments described below. The transcripts hybridizing to the exon-specific probes are identical in size to those

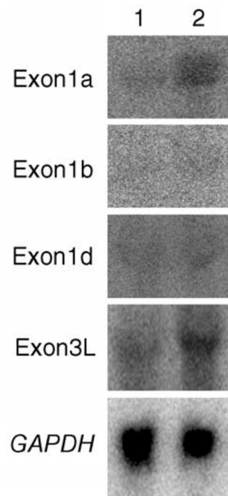

Fig. 2. Northern analysis confirming the expression of *EVI1* transcript variants containing exons 1a, 1b, 1d, or 3L in human cell lines. Lane 1, HEL, and lane 2, HNT-34 cells. Both cell lines are of hematopoietic origin. HNT-34 cells contain a t(3;3)(q21;q26) rearrangement of the *EVI1* locus (Hamaguchi et al., 1997). 10 μg of total RNA was loaded per lane. The probes used are specific for the respective alternative first exons. The transcripts they detect are identical in size to transcripts hybridizing to a probe encompassing 2.3 kb of the *EVI1* coding region. A probe specific for the housekeeping gene *GAPDH* was used to control for the amount of RNA in each lane.

hybridizing to a probe encompassing a region common to all *EVI1* 5′-end variants.

3.2. Expression of the EVI1 5′-end variants in different human tissues

Because the 5′-end specific probes did not yield robust signals in Northern blots, we used real time quantitative (RTQ) RT-PCR to assess the expression of the *EVI1* transcript variants in a panel of human tissues. The very low abundance of the exon 1c-type transcript, which had previously prompted us to search for alternative 5′-ends (Vinatzer et al., 2003), made it impossible to reliably measure its expression even by RTQ-RT-PCR. This mRNA variant was therefore disregarded for the analyses described in this report. The tissue distribution of the other *EVI1* 5′-end variants is shown in Fig. 3.

In order to get a rough estimate of the relative expression levels of the 5′-end variants to each other, they were amplified in parallel assays from prostate cDNA. The results of these experiments suggested that the amounts of the exon

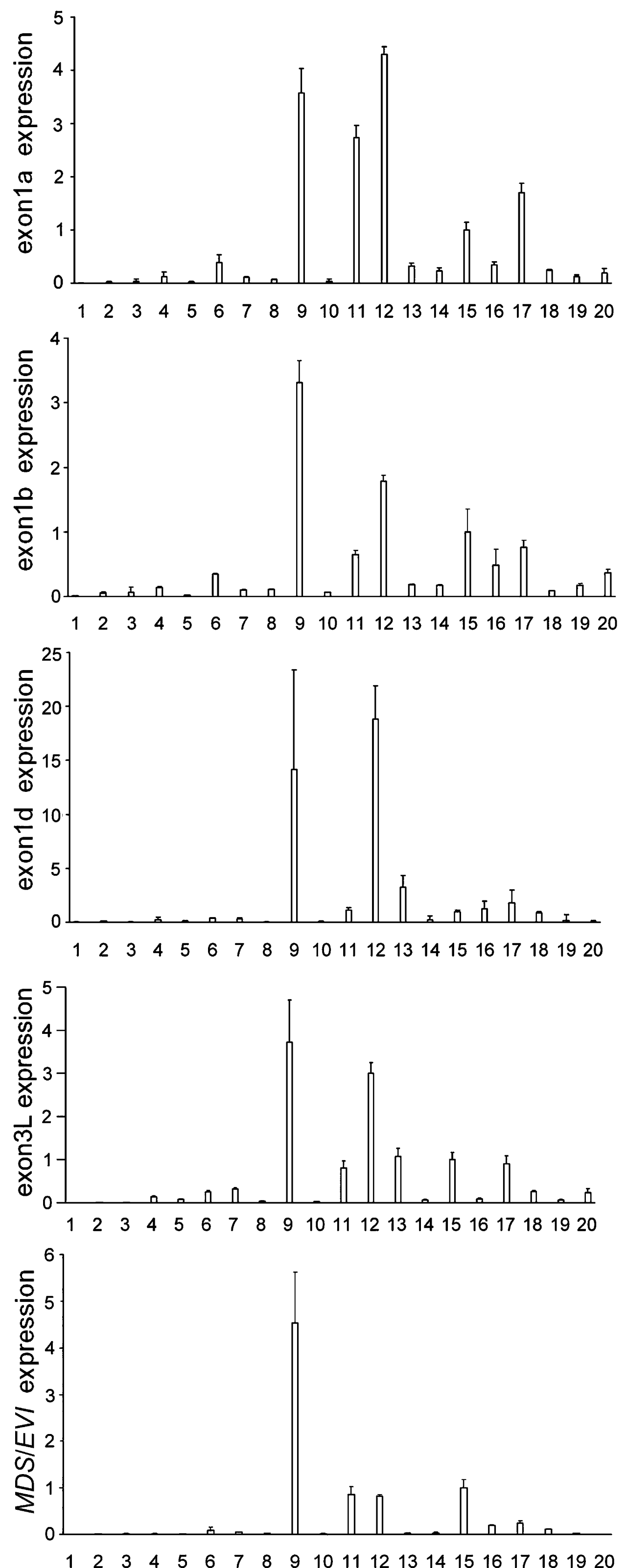

Fig. 3. Expression of the *EVI1* 5′-end variants in a panel of human tissues. A commercially available panel of RNAs from 20 different human tissues was analyzed for the expression of the exon 1a-, 1b-, 1d-, and 3L-type transcripts and of *MDS1/EVI1* by RTQ-RT-PCR. Expression values and experimental error were calculated according to the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001), using *GAPDH* as a control for the amount of cDNA in each sample, and prostate as a reference tissue. Note that the scales of the *y*-axes cannot be compared among each other, because they reflect expression relative to prostate, not absolute expression levels. 1, skeletal muscle; 2, bone marrow; 3, fetal liver; 4, fetal brain; 5, adrenal gland; 6, colon; 7, small intestine; 8, thymus; 9, lung; 10, salivary gland; 11, kidney; 12, stomach; 13, testis; 14, placenta; 15, prostate; 16, spleen; 17, uterus; 18, heart; 19, whole brain; 20, cerebellum.

1a-, 1b-, and 3L-containing transcripts and of MDS1/EVI1 were in a similar range, while the exon 1d-type transcript appeared to be expressed at considerably lower levels.

3.3. Stability of the EVI1 mRNA 5′-end variants

We next investigated whether the use of alternative first exons would affect the stability of the *EVI1* mRNA by comparing the half lives of the 5′-end variants in the human myeloid cell lines HNT-34 and MPD. While HNT-34 cells express *EVI1* due to the presence of a t(3;3)(q21;q26) (Hamaguchi et al., 1997), no cytogenetically detectable 3q26 rearrangements are present in MPD cells (Paul et al., 2000), and the mechanism for the expression of *EVI1* in these cells (Vinatzer et al., 2003) is unknown. HNT-34 and MPD cells were treated with the RNA polymerase II inhibitor actinomycin D (ActD), and the amounts of the different *EVI1* mRNA variants were determined by RTQ-RT-PCR at various time points after ActD addition. mRNA half lives were calculated from the slopes of the decay curves. As shown in Table 3, the half lives of the exon 1a-, 1b-, 1d-, and 3L-type *EVI1* transcripts were similar in HNT-34 cells, ranging from 1.4 to 1.7 h. *MDS1/EVI1* is expressed at extremely low levels in this cell line so that its half life could not be determined. The same was true for the exon 1d-type transcript in MPD cells. The other *EVI1* mRNA variants, i.e., the exon 1a-, 1b-, and 3L-type transcripts and *MDS1/EVI1*, decayed with half lives ranging from 2.2 to 2.5 h in MPD cells, again showing little variation among each other. However, all transcripts appeared to be more stable in MPD than in HNT-34 cells. The stability of the mRNA for the TGF-β regulated transcription factor *Smad2*, on the other hand, was identical in the two cell lines (Table 3).

Table 3
Half lives of the *EVI1* mRNA 5′-end variants in the cell lines HNT-34 and MPD

	HNT-34	MPD
EVI1 exon 1a	1.57±0.34	2.22±0.47
EVI1 exon 1b	1.51±0.16	2.43±0.65
EVI1 exon 1d	1.74±0.38	N.e.
EVI1 exon 3L	1.40±0.18	2.16±0.04
MDS1/EVI1	N.e.	2.48±0.08
Smad2	2.14±0.30	2.12±0.02

HNT-34 or MPD cells were treated with the RNA polymerase inhibitor ActD for 0, 1, 2, 3, and 4 h. The abundance of each *EVI1* mRNA variant was measured by RTQ-RT-PCR. The natural logarithm of its expression relative to the long-lived *GAPDH* mRNA was plotted against time. Correlation coefficients (R^2) of the resulting mRNA decay curves were at least 0.78 (average, 0.90). Their slopes k were used to calculate transcript half lives according to the formula $\tau_{1/2} = -0.693/k$. Half lives are given in hours and represent the averages ± standard deviations of two or three independent experiments. N.e., not expressed at sufficiently high levels for accurate half life determination. The half life of the *Smad2* mRNA was measured as a control.

3.4. Differential responsiveness of the EVI1 5′-end variants to induction by all-trans retinoic acid (ATRA)

The differentiation inducing agent ATRA has been shown to increase c*EVI1* mRNA levels (Xi et al., 1997; Kazama et al., 1999), but the 5′-end mRNA variants of this gene were not distinguished in these reports. Therefore, the human teratocarcinoma cell line PA-1 was treated with ATRA for different periods of time, and RNA was extracted and subjected to RT-PCR. Expression of the exon 1a- and exon 1b-containing transcripts was elevated in response to ATRA, with maximal induction occurring after 12 to 24 h (Fig. 4A). This result was confirmed by RTQ-RT-PCR (Fig. 4B). The exon 1d- and exon 3L-type mRNAs and *MDS1/EVI1*, on the other hand, were not induced beyond the detection limit at any time point investigated (Fig. 4A). In contrast, in the murine teratocarcinoma cell line F9, *Mds/Evi1* exhibited a robust induction in response to ATRA, but with delayed kinetics as compared to the exon 1a-containing *Evi1* transcript (Fig. 4C; note that these two *Evi1* 5′-end variants are the only ones known in the mouse). Some aggregation of PA-1 cells was observed after 72 h of ATRA treatment, but no gross morphological alterations were observed after 24 and 48 h with ATRA, i.e., the times of maximal *EVI1* induction, in either PA-1 or F9 cells.

We next asked whether the induction of the exon 1a-containing mRNA variant occurred at the level of transcription initiation or at a later step of gene regulation. PA-1 cells were incubated with DMSO or ATRA in the presence or absence of ActD. After 9 h, RNA was extracted and subjected to RTQ-RT-PCR. ActD strongly reduced the induction of the exon 1a- and exon 1b-type transcripts by ATRA (Fig. 4D, and data not shown), indicating that de novo transcription was required for this effect. We therefore searched for a retinoic acid response element in the promoter regions and first intron of the *EVI1* gene. Two overlapping DNA fragments representing 2.6 kb of the exon 1a promoter, a fragment containing the sequences upstream of exon 1b, and a 4 kb fragment containing intron 1 were cloned into luciferase reporter vectors, and transfected into 293T cells along with an expression plasmid for the retinoic acid receptor alpha (RAR-α). While a control plasmid containing a known retinoic acid response element did show ATRA-inducible luciferase activity in this assay, no induction was observed with any of the *EVI1* promoter fragments (data not shown).

The negative results of the promoter analysis prompted us to ask whether the stabilities of the exon 1a- and exon 1b-containing *EVI1* transcripts were altered in response to ATRA. PA-1 cells were treated with DMSO or ATRA, and the stability of the exon 1a-containing transcript variant was measured as described above. Its half lives in the presence of DMSO and ATRA were 1.3 and 4.1 h, respectively. We were unable to repeat this experiment due to the usually very low basal expression of *EVI1* in PA-1 cells. To

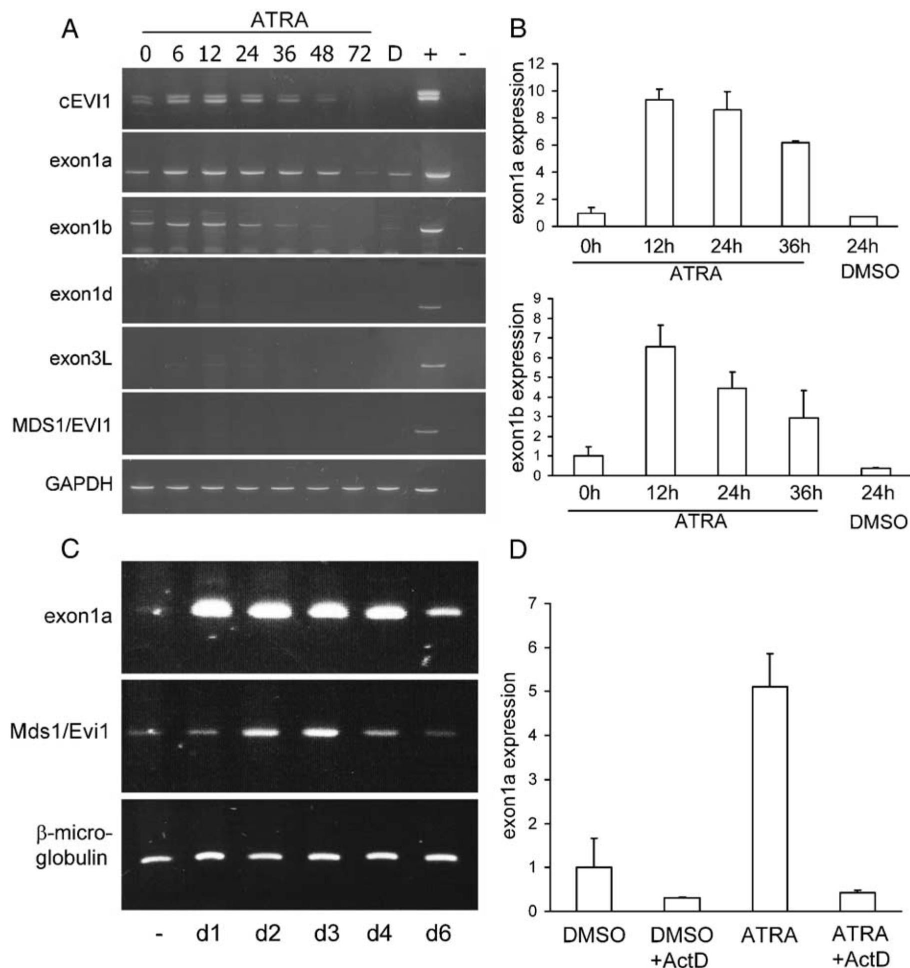

Fig. 4. Regulation of the *EVI1* mRNA variants by all-*trans* retinoic acid (ATRA). (A) Time course analysis of human PA-1 cells treated with 100nM ATRA. Conventional RT-PCR was used to measure the expression of c*EVI1*, of the exon 1a-, 1b-, 1d-, and 3L-type transcripts, of *MDS1/EVI1*, and of *GAPDH* as a control for the amount of cDNA in each sample. Time of treatment with ATRA is indicated in hours. D, 24 h treatment with DMSO; +, positive control; −, negative control (H_2O). Basal *EVI1* levels in the absence of ATRA varied between experiments, but were typically lower than in the experiment shown. (B) RTQ-RT-PCR on the same samples as in Fig. 4A confirms the induction of the exon 1a- and exon 1b-type transcripts by ATRA. Expression values and experimental error were calculated according to the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001), using *GAPDH* as a control for the amount of cDNA in each sample, and the 0 h time point as a reference sample. (C) Time course analysis of murine F9 cells treated with 100 nM ATRA. The exon 1a-containing transcript, *Mds1/Evi1*, and β-microglobulin as a control for the amount of cDNA in each sample were amplified by conventional RT-PCR. Time of treatment with ATRA is indicated in days. (D) The RNA polymerase inhibitor ActD prevents induction of the exon 1a-containing transcript variant by ATRA. PA-1 cells were treated with DMSO or ATRA (100nM) in the presence or absence of ActD (10 μg/ml) for 9 h. The amount of the exon 1a-containing transcript was measured by RTQ-RT-PCR, and normalized to the amount of *GAPDH* and expressed relative to the DMSO-treated sample using the $\Delta\Delta C_t$ method.

circumvent this problem, we tested whether 5-azacytidine (azaC), an agent that induces the transcription of many genes by inhibiting DNA methyltransferase, but to our knowledge has not been reported to affect the stability of any mRNA, would raise *EVI1* transcript levels in PA-1 cells. This was found to be the case (data not shown). We therefore compared the stabilities of the exon 1a- and exon 1b-containing transcripts in the presence of azaC or ATRA (Fig. 5). The half life of the exon 1a-containing transcript, calculated from two independent experiments, was 1.82 ± 0.22 h in the presence of azaC, and 3.95 ± 0.08 h in the presence of ATRA. Similarly, the half life of the exon 1b-containing transcript was 1.57 ± 0.13 h in the presence of azaC, and 4.41 ± 0.37 h in the presence of ATRA (R^2 of all mRNA decay curves >0.85; average, 0.91). These results confirm the notion that ATRA increases the stability of the exon 1a- and exon 1b-containing *EVI1* mRNAs.

4. Discussion

Variability at the 5′-end of certain mRNAs plays important roles in the regulation of protein function and/or abundance. *EVI1*, a gene with pivotal roles in mammalian development and in leukemogenesis, has recently been shown to give rise to at least six mRNAs differing from each other at their 5′-termini. To date, virtually no information regarding the regulation of the expression of most of these transcript variants was available. In this report, we confirmed their expression by Northern blot, measured their

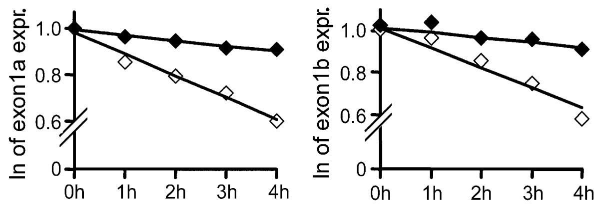

Fig. 5. Stabilization of exon 1a- and exon 1b-containing EVI1 transcripts in response to retinoic acid. PA-1 cells were incubated with ATRA (closed symbols) or azaC (open symbols; see Section 3.4 for explanation) for 18 h, and then treated with the RNA polymerase inhibitor ActD for the times indicated on the x-axes. The amounts of the exon 1a- or exon 1b-containing transcripts were measured by RTQ-RT-PCR. The natural logarithms of their expression relative to GAPDH and the 0 h time point were plotted against time. The half lives of the mRNAs were calculated from the slopes of the decay curves. The experiments shown were repeated with similar results. The deduced average half lives and standard deviations are given in Section 3.4.

basal stability, and investigated their abundance both in different human tissues and in response to all-*trans* retinoic acid.

Of interest, we found that the *EVI1* 5′-end variants respond differently to retinoic acid: while in the murine teratocarcinoma cell line F9 both *Mds/Evi1* and the exon 1a-containing transcript (which are the only *Evi1* 5′-variants known in murine cells) were induced by ATRA, pronounced induction of the exon 1a- and 1b-containing, but not the exon 1d- or 3L-containing transcript variants or of *MDS1/EVI1*, was observed in the human cell line PA-1. This may reflect differences between cell lines, or between species. However, both in PA-1 and in F9 cells, *EVI1* induction by ATRA was strongly reduced by coincubation with the transcriptional inhibitor, ActD (Fig. 4D, and data not shown), indicating that it is at least in part due to an increased rate of transcription initiation. Despite of this, using luciferase reporter gene assays we were not able to identify a retinoic acid responsive element in the genomic regions upstream of exon 1a and 1b or in the first intron of the *EVI1* gene. Rather, comparison of the half lives of the exon 1a- and exon 1b-containing transcripts in the presence and absence of ATRA revealed that this agent substantially increased their stabilities. These apparently contradictory observations can be reconciled by the assumption that *EVI1* induction by ATRA takes place at the levels of both transcription initiation and mRNA stabilization, similar to what has been observed in other cases of gene induction by physiological regulators (Faour et al., 2003; Mifflin et al., 2004). In the experiments shown in Fig. 4D, we cotreated cells with ATRA and ActD for the relatively short period of 9 h in order to prevent cell poisoning. Over this time, transcript stabilization in the absence of de novo transcription apparently is insufficient to cause a substantial alteration of *EVI1* mRNA levels. On the other hand, the effect of ATRA on the rate of transcription initiation may be relatively small, and could have gone undetected in our reporter gene assays because transcriptional induction of such reporter constructs is sometimes strongly diminished as compared to a native promoter (Muller et al., 2000; Raouf et al., 2000).

The observation that selective induction of certain *EVI1* 5′-end variants by ATRA is in part mediated by mRNA stabilization is of particular interest because retinoic acid regulates the vast majority of its target genes at the level of transcription initiation through its nuclear receptors, the transcription factors RAR and RXR (Bastien and Rochette-Egly, 2004), and only a comparatively small number of genes has been shown to be regulated by this agent at the level of mRNA stability (Wang and Christakos, 1995; Liaudet-Coopman and Wellstein, 1996; Grosjean et al., 2001). The fact that only some, but not all, *EVI1* 5′-end variants are regulated in this manner may indicate that the responsible sequence elements are located in the regions specific to these transcript variants, i.e., their 5′-ends. Indeed, preliminary experiments from our laboratory indicated that the exon 1a-containing 5′-UTR is able to confer ATRA responsiveness to a heterologous luciferase gene. If confirmed, these results would add *EVI1* to a so far relatively short list of genes whose mRNA half lives are regulated by their 5′-UTRs (Chen et al., 1998; Tebo et al., 2000), and offer an interesting model system to study the largely unexplored regulation of mRNA stability by ATRA.

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