# A small upstream open reading frame causes inhibition of human major vault protein expression from a ubiquitous mRNA splice variant

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**Abstract Overexpression of the major vault protein (MVP) has been linked to a multidrug resistance (MDR) phenotype. We describe a ubiquitously expressed MVP mRNA splice variant (long (L)-MVP) differing from the regular isoform (short (S)-MVP) within the 5′-leader. Only L-MVP mRNA contains a small upstream open reading frame which was proven to inhibit in vitro and in vivo MVP expression in *cis*. L-MVP represented an almost constant portion of total MVP mRNA in diverse normal tissues, but was more variable in malignant cell types. MDR sublines with altered MVP expression displayed changed S-MVP/L-MVP ratios as compared to their drug-sensitive counterparts. Our results suggest alternative splicing as one mechanism for regulation of MVP expression.** 

*Key words:* Major vault protein; Lung resistance protein; Alternative splicing; 5′-Untranslated region; Upstream open reading frame; Translational repression

## 1. Introduction

Vaults are ubiquitously expressed ribonucleoprotein particles containing multiple copies of three different proteins as well as an untranslated RNA [1]. The evolutionary conservation and widespread expression of vaults suggest a basic, yet unknown, cellular function. The three-dimensional hollow structure points to a role in transport processes [2] and indeed vaults were suggested to regulate nucleo-cytoplasmic transport [3]. The major vault protein (MVP) is identical to the lung resistance-related protein (LRP) [4]. Vaults are overexpressed in a variety of multidrug-resistant (MDR) cell lines [5] and MVP expression predicts chemotherapy failure in several malignancies [6]. Little is known about factors involved in the regulation of MVP expression. In normal tissues, high amounts are present in potentially drug-exposed epithelia and macrophages [7]. MVP expression can be up-regulated by a short-term exposure to chemotherapeutic or differentiation-inducing agents [8,9]. Discordance between MVP mRNA and protein expression in several cell types points to a substantial influence of posttranscriptional regulatory mechanism [8–10].

Here we describe and characterise a ubiquitously expressed but translationally repressed MVP mRNA splice variant which has very recently also been identified in an MVP-promoter study [11]. The expression pattern in normal and tumour tissues as well as MDR cell models suggested that alternative splicing might be involved in posttranscriptional regulation of MVP expression.

## 2. Materials and methods

### 2.1. Cell lines

The brain tumour cell line KM-YH was established from patient material; MR-1 glioblastoma cells were a gift of Dr. T. Kurata, Tokyo, Japan. Data on 16 NSCLC cell lines, BEAS-2B and F2000 have been described elsewhere [12]. Generous donors and characterisation of MDR cell models have been described [13]: GLC4 and GLC4/ADR [14]; SW1573, SW1573/2R120 and SW1573/2R160 [15]; 8226/S, 8226/DOX6 and 8226/DOX40 [16].

### 2.2. cDNA, monoclonal antibodies (mAbs) and chemicals

Multiple tissue cDNA panels (MTC Panels I and II, Human Tumor MTC Panel) were obtained from Clontech (Palo Alto, CA, USA). Two MVP mAbs LRP56 (IgG2b, Sanbio, Uden, The Netherlands) and LRP (IgG1, Pharmingen/Transduction Laboratories, San Diego, CA, USA) were used for immunofluorescence staining and Western blot analysis, respectively. The isotype control antibody and all other chemicals used were obtained from Sigma (St. Louis, MO, USA).

### 2.3. EST clones, sequence analysis

EST cDNA clones IMAGE:2222005, 363900, 2276385 and DKFZp434L1720 were obtained from the German Human Genome Project Resource Center (RZBD, Berlin, Germany) and the UK HGMP Resource Centre (Cambridge, UK). For clones IMAGE:2222005 and 2276385 cDNA 5′-sequences were determined by automated sequencing (VBC Genomics, Vienna, Austria) and deposited in GenBank/EMBL/DDBJ (accession numbers AJ291369, AJ291368). For amplification of the intron sequence the Expand Long Template PCR System (Roche Molecular Biochemicals, Mannheim, Germany) was used, and for primer-mediated mutagenesis recombinant pfu DNA polymerase (Stratagene, La Jolla, CA, USA) was used. Polymerase chain reaction (PCR) products have been introduced for sequencing into pCR2.1-TOPO vector (Invitrogen, Groningen, The Netherlands).

### 2.4. MVP plasmid construction

The expression vector constructs S-MVP-1 and S-MVP-2 were obtained using the EST clones IMAGE:2276385 and 2222005, respectively. cDNA inserts were cloned between the *Sal*I and *Not*I sites of pCMV-SPORT6. For construction of long (L)-MVP an *Eco*RI and *Not*I cDNA fragment from DKFZp434L1720 was introduced into pCMV-SPORT6. To generate the S-MVP-3 construct a *Xba*I fragment from L-MVP was replaced by the corresponding fragment from S-MVP-2. To introduce mutations into the upstream open reading frame (uORF) a primer-mediated mutation procedure was chosen.

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*Abbreviations:* L-MVP, long MVP mRNA isoform; LRP, lung resistance protein; MDR, multidrug resistance; MVP, major vault protein; uORF, upstream open reading frame; S-MVP, short MVP mRNA isoform; UTR, untranslated region

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First, using the primers M13-reverse 5′-GGAAACAGCTATGACCATG-3′ and MVP-10-as 5′-CTAGAAGTGCAGGTAGCTG-3′ a 150 bp upstream fragment was amplified. Then either a primer containing a point mutation in the uORF start codon (MVP-M1-s 5′-CCTGCACTTCTAGTTGCCTCCCCAGG-3′) or a frameshift (−1) following the start codon (MVP-M2-s 5′-CCTGCACTTCTAGATGTCCCCAGGTT-3′) were combined with MVP-22-as: 5′-CACAGGGTTGGCCACTGTGCA-3′ to amplify mutated 280 bp downstream fragments. Fragments were fused together (overlapping regions are underlined) by amplification with primers M13-reverse and MVP-22-as. The *Eco*RI and *Bst*XI fragments were exchanged with the respective fragment in L-MVP resulting in L-MVP-mut1 and L-MVP-mut2 constructs. The sequences of all constructs were proven by automated sequencing.

### 2.5. In vitro transcription/translation

In vitro transcription/translation experiments with the respective MVP constructs were performed using the TNT coupled reticulocyte lysate system (Promega, Madison, WI, USA) according to the manufacturer's protocol. Incorporated radioactivity was quantified using the Molecular Dynamics phosphoimager system (Sunnyvale, CA, USA). Results are given as means of at least three independent experiments.

### 2.6. Transfection experiments, Western blotting and immunofluorescence staining

Cells were transfected with MVP constructs by electroporation using standard protocols. For transient experiments, cells were harvested after a 48 h incubation and total cell extracts prepared as described [12]. For stable transfection, MVP constructs were co-transfected with a puromycin resistance construct in a ratio 10:1. Resistant clones were isolated, propagated and total cell extracts prepared. SDS–PAGE and Western blot analysis were performed as described [12]. MVP expression in NSCLC cells was quantified relatively (arbitrary units) to extracts of strongly MVP-expressing embryonic fibroblasts F2000 [9]. All results are means of at least three experiments.

### 2.7. Reverse transcription (RT)-PCR

MVP mRNA isoform expression in tissues and cell lines was determined by RT-PCR analysis. Either total cellular RNA was reverse transcribed [12] or cDNA panels (Clontech) were used. PCR was performed [12] using several oligonucleotide primers: MVP-3-s 5′-TTCTGGATTTGGTGGACG-3′ and MVP-3-as 5′-ACTTCTCTCCCTTGACCA-3′ amplified a 284 bp fragment within the MVP ORF; MVP-49-s 5′-GTTCCCCATCTGAGGCGT-3′ and MVP-50-as 5′-CTGCCGGATGTAGGTCTT-3′ amplified a 235 bp fragment of L-MVP and a 193 bp fragment of S-MVP; MVP-44-s 5′-GGTTCATCCTGTGTCGTCTC-3′ corresponds to a sequence within the uORF and amplified, in combination with MVP-50-as, a 181 bp product specific for L-MVP. PCR amplification was performed in the exponential proportion of the reaction. Quantification of MVP isoforms was done at 25–30 cycles, depending on the MVP mRNA expression level. Results given are the means of three independent experiments. Standard deviations were below 13%.

## 3. Results

### 3.1. 5′-Region of the MVP gene

Using the published human MVP cDNA sequence (X79882) [4] several homologous clones were identified in an EST database differing within the 5′-untranslated region (UTR) of the MVP gene. Two of four EST sequences contained an additional 41 bp stretch located 35 bp upstream of the initiation start codon. Consequently, we amplified the corresponding region from cDNA and genomic DNA derived from A549 cells. RT-PCR resulted in the amplification of two DNA fragments differing by 41 bp in length. Amplification from genomic DNA led to one 10 kb fragment. The respective PCR products were cloned and sequenced (accession numbers AJ291366, AJ291367, AJ291364, AJ291365). Fig. 1 summarises the situation at the 5′-region of the MVP

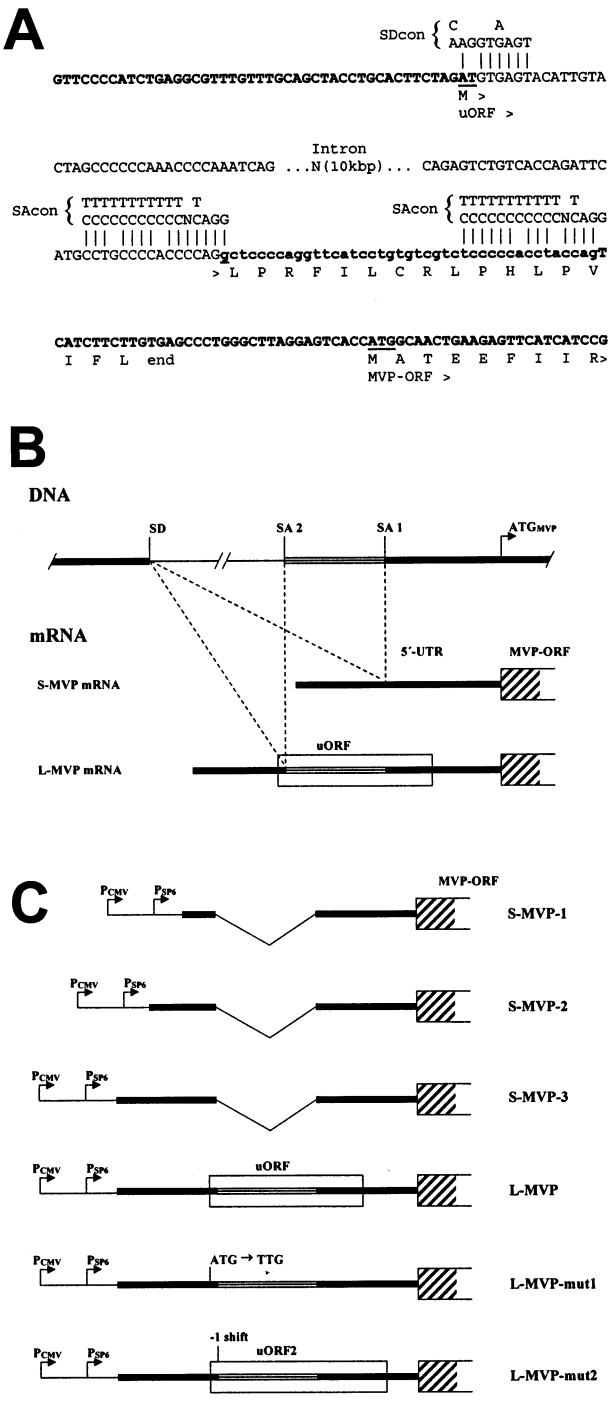


Fig. 1. 5′-Region of the MVP gene: genomic sequence, mRNA isoforms and expression constructs. A: Genomic sequence; SD and two different SA sites are indicated by the respective consensus sequences (SDcon and SAcon, respectively). Bold face depicts exons. The additional 41 bp in L-MVP are shown in lowercase letters. The translation of the uORF and nine N-terminal residues of the MVP ORF are given. Start codons are underlined. Usage of the downstream SA site does not lead to an ATG start codon and consequently a uORF as in case of the upstream SA site. B: Generation of S-MVP and L-MVP by alternative splicing (broken lines). The isoforms differ with respect to the additional 41 bp in L-MVP mRNA (thin line). Hatched box and the open box depict the MVP ORF and the uORF, thick and thin lines, exons and introns, respectively. C: MVP constructs: promoters for in vitro ($P_{SP6}$) and in vivo ($P_{CMV}$) transcription are indicated. The S-MVP constructs 1, 2 and 3 vary only in the length of their 5′-UTR upstream of the alternative splice site.

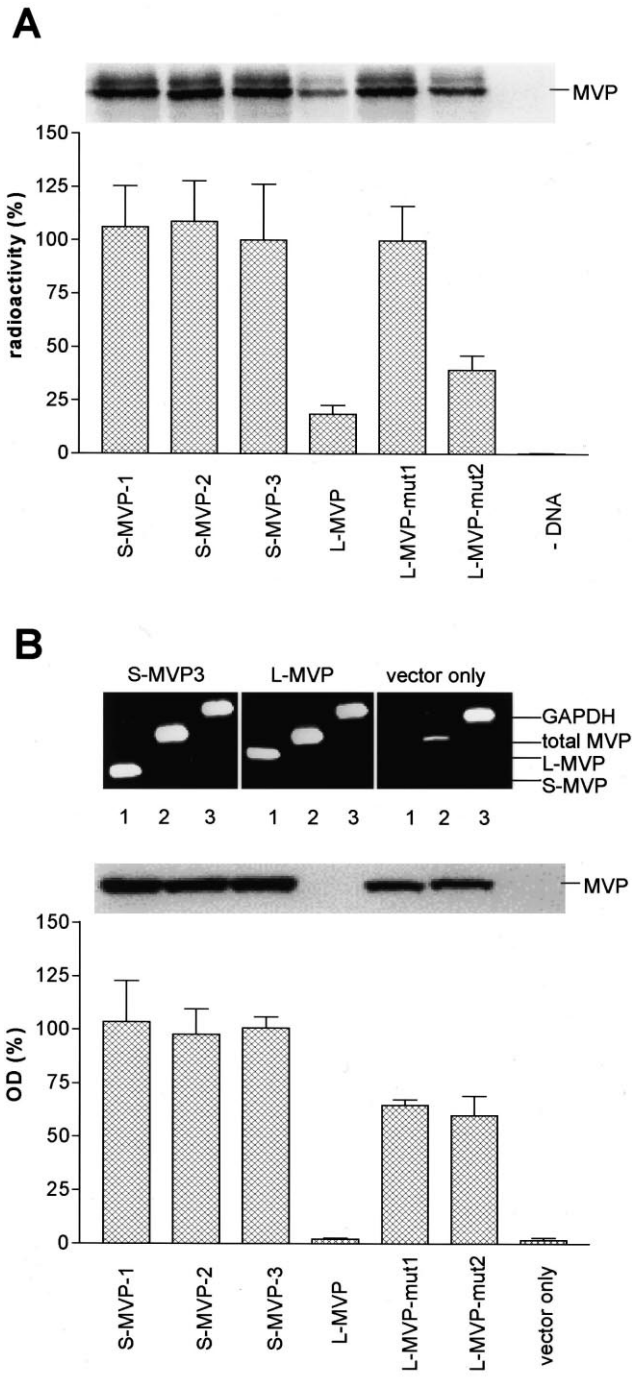


Fig. 2. MVP expression from MVP constructs. A: In vitro MVP expression from the indicated constructs. B: Transient expression of MVP in KM-YH cells from the indicated constructs. MVP mRNA isoform (upper panel) and MVP expression (lower panel) were analysed by RT-PCR and Western blot, respectively. For RT-PCR (upper panel) the following primers were used: MVP-49-s, MVP-50-as, lane 1; MVP-3-s, MVP-3-as, lane 2; GAPDH primers, lane 3. Representative results for S-MVP-3 and L-MVP are shown. In case of Western blot analysis (lower panel), bars represent optical density (OD) values from scanned autoradiograms (means, S.D. of three independent experiments). One representative experiment is shown as inset. In A and B values for S-MVP-3 were arbitrarily set as 100%. C: MVP expressions in stable transfected KM-YH cell clones (vectors as indicated) were analysed by immunofluorescence (upper panel) and Western blot (lower panel). MR-1 glioblastoma cells served as MVP positive control.

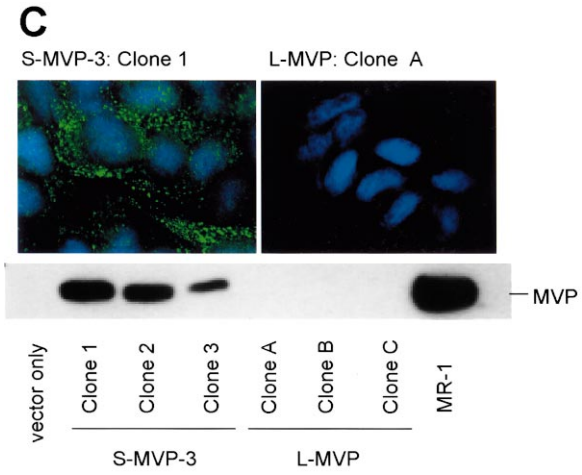


Fig. 2. (*continued*).

gene on the genomic and transcript level. Consensus splice donor (SD) and splice acceptor (SA) sequences (Fig. 1A) suggested the presence of a 10 kb intron located in the 5′-UTR of the MVP gene which was proven by the sequence of the shorter cDNA fragment. The longer cDNA fragment contained an additional 41 bp stretch derived from the 3′-end of the intron sequence. This situation together with the presence of second consensus SA sequence (Fig. 1A) indicated that the two mRNA isoforms originate from alternative splicing (Fig. 1B). The L-MVP mRNA contained an initiation start codon located in the 5′-UTR thus opening a uORF encoding 18 amino acids, whereas the short one (S-MVP mRNA) lacked any upstream initiation start codons (Fig. 1A,B).

To analyse the influence of the 5′-leader sequence on MVP expression, constructs that differ in their 5′-UTR (Fig. 1C) have been created. To determine an influence of the far-end 5′-sequence on MVP expression, which varies between X79882 and EST sequences, three different constructs for S-MVP slightly differing in the length of the 5′-UTR were used (S-MVP-1 to -3). The MVP expression constructs S-MVP-3 and L-MVP differ only with respect to the 41 bp stretch caused by alternative splicing. To test the influence of the uORF, either the initiation start codon was mutated (L-MVP-mut1) or a 1 bp deletion was introduced (L-MVP-mut2).

### 3.2. Expression of MVP from mRNA isoforms

Fig. 2 summarises the expression of MVP from the investigated MVP constructs (Fig. 1C) in an in vitro transcription/translation system (Fig. 2A) and when transfected into MVP-negative KM-YH cells in either a transient (Fig. 2B) or a stable (Fig. 2C) setting. In both the in vitro and in vivo assays the three S-MVP constructs (S-MVP-1 to -3) led to a comparable synthesis of a 110 kDa protein which represented MVP as proven by immunoblot detection (Fig. 2A,B). When using the L-MVP construct in vitro only around 20% of MVP as compared to the S-MVP constructs was expressed (Fig. 2A). A point mutation within the uORF start codon (L-MVP-mut1) completely abolished suppression of MVP expression. Also a changed nature of the uORF (L-MVP-mut2) significantly up-regulated MVP expression, however, only to around 40% as compared to S-MVP constructs. In a transient setting

in vivo (Fig. 2B, lower panel), MVP expression from the L-MVP construct was completely suppressed and comparable to the vector control. Both L-MVP-mut1 and L-MVP-mut2 partially (60–80% of S-MVP) released suppression of MVP expression. The lack of MVP expression from L-MVP did not rest on a markedly altered stability of L-MVP mRNA as demonstrated by RT-PCR (Fig. 1B, upper panel). In the case of stable transfection, the use of S-MVP but not L-MVP constructs led to MVP-expressing subclones, as could be demonstrated by immunoblot and immunofluorescence analysis (Fig. 2C). Transient transfection experiments using the NSCLC cell line VL-4 led to comparable results with regard to MVP (not shown).

### 3.3. Expression of the two MVP mRNA isoforms in benign and malignant tissues and cells

mRNA preparations from several normal and malignant tissues and cell lines were investigated by an RT-PCR setting allowing the simultaneous detection of L-MVP and S-MVP isoforms (Fig. 3A). In each RNA pool analysed, both MVP mRNA isoforms were detectable; however, S-MVP was always the dominant isoform. Fig. 3B summarises the ratio between the two MVP mRNA isoforms in a panel of normal tissues, demonstrating that the suppressed L-MVP isoform represents almost constantly about 35–45% of total MVP mRNA. Only the brain cDNA contained merely 28% L-MVP mRNA. In malignant tissues the MVP mRNA isoform ratio was more variable as compared to normal tissues (Fig. 3C,D). When analysing a panel of NSCLC cell lines ($N = 16$) L-MVP/S-MVP ratios between 0.27 and 1 were detected (Fig. 3D). When the NSCLC cell lines were grouped regarding their isoform ratio $<$ and $>0.5$, the low isoform ratio indicated a high MVP expression ($30.5 \pm 27.6$; $N = 6$) as compared to the high ratio ($7.4 \pm 8.7$; $N = 10$) ($P < 0.05$, unpaired $t$-test), independent of the general MVP mRNA expression level (not shown).

### 3.4. Expression of the two MVP mRNA isoforms in MDR cell models

Three MDR cell models containing drug-sensitive parental cell lines and drug-selected sublines were investigated (Fig. 4). In the case of the GLC4 cells a concordant increase of both MVP mRNA isoforms was detectable in the markedly MVP-overexpressing subline GLC4/ADR, resulting in a moderate increased isoform ratio. In contrast, in the SW1548 cell model the MVP-overexpressing subline 2R120 displayed a signifi-

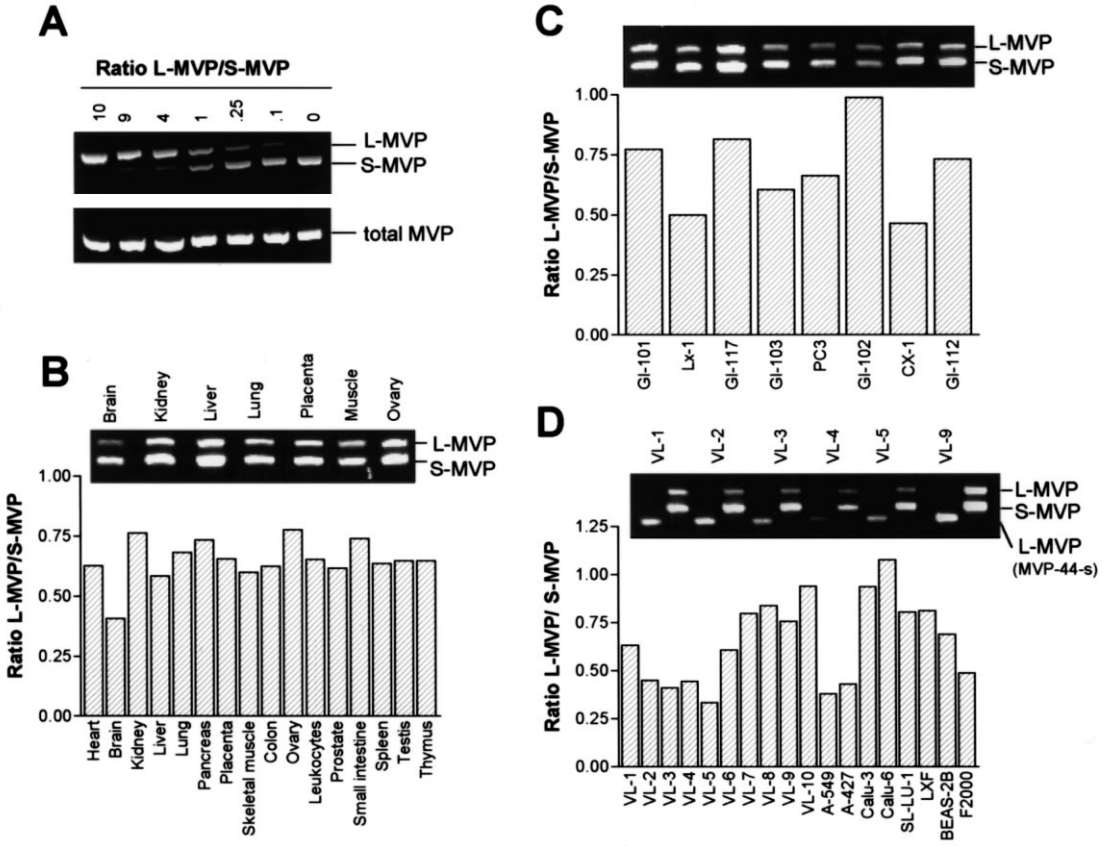


Fig. 3. Expression of MVP mRNA isoforms in normal and malignant cells. A: Simultaneous detection of L-MVP and S-MVP by PCR. Indicated proportions of S-MVP and L-MVP constructs (given as ratio L-MVP/S-MVP) were mixed and PCR performed with the oligonucleotide primers MVP-49-s and MVP-50-as (upper panel). Oligonucleotide primers MVP-3-s and MVP-3-as amplified a fragment of the MVP coding region (lower panel). B–D: Expression of MVP mRNA isoforms in (B) normal tissues, (C) malignant cell lines of mixed histology (GI-101, breast carcinoma; LX-1, GI-117, lung carcinoma; GI-103, pancreatic adenocarcinoma; PC3, prostatic adenocarcinoma; GI-102, ovarian carcinoma; CX-1, GI-112, colon adenocarcinoma) and (D) a panel of 16 NSCLC cell lines, BEAS-2B and F2000 cells was analysed by RT-PCR. Representative experiments are shown as insets. In D the first lane for each cell line in the inset shows amplification products obtained with an upstream primer hybridising in the 41 bp sequence unique to the L-MVP mRNA (MVP-44-s).

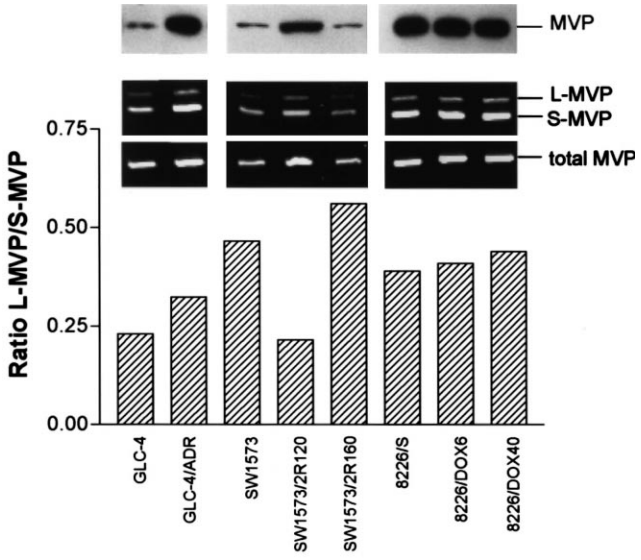


Fig. 4. Expression of MVP mRNA isoforms and MVP in MDR cell models. MVP mRNA isoforms as indicated (middle panel) and derived isoform ratios (bars in lower panel) were determined as described in Fig. 3 and compared to Western blot analysis (upper panel).

cantly reduced isoform ratio as compared to the (low MVP-expressing) parental line. The highly P-glycoprotein-overexpressing, but almost MVP-negative, subline 2R160 was characterised by a slightly enhanced L-MVP ratio as compared to the parental cell line. The multiple myeloma cell line 8226/S and its P-glycoprotein-overexpressing sublines DOX6 and DOX40 all expressed comparable high MVP levels. The MVP mRNA isoform ratio of these cell lines was almost constant.

## 4. Discussion

In eukaryotic cells, translational control in several gene types is mediated via *cis*-acting elements in the 5′- or 3′-UTR of the mRNA. The presence of upstream AUGs and associated uORFs, although generally rare in mRNAs (around 5–10% of the human genes), is a common occurrence in proto-oncogenes, growth factor and growth factor receptor genes as well as other genes involved in cell growth and differentiation [17,18], including for example mdm-2 [19], bcl-2 [20] and TIMP [21]. Repression of translation of the downstream ORF by one up to several uORFs, is a key regulator in tissue-specific gene regulation and developmental control [22]. With regard to MVP, the identification of a uORF in a ubiquitously expressed mRNA isoform might point to an important cellular function of MVP, respectively vaults, as has also been suggested due to the high evolutionary conservation and the widespread expression [4,7].

Several mechanisms have been shown to cause the translational repression of the downstream ORF by a uORF: (1) Ribosomes scan along the 5′-UTR, presumably translate the uORF and are unable to reinitiate at the downstream ORF, as demonstrated for example in case of Her-2/neu [23]. (2) The encoded peptide of the uORF might be translated and inhibits sequence-dependent and in *cis*-downstream translation, probably by stalling the translation machinery, as shown, for example, in the *s*-adenosylmethionine decarboxylase mRNA [24]. (3) Presence of the uORF, its translation or, more likely, translation termination [25] might destabilise the mRNA molecule and enhance its decay rate.

Expression of MVP from L-MVP was intensely repressed in vivo, reaching almost 100% in KM-YH cells. As a general rule single uORFs only weakly (10–40%) inhibit translation. Our results are even more surprising insofar as the uORF in L-MVP has no optimal context for initiation of translation [17]. This suggests a combination of several mechanisms causing the very potent translational suppression in case of L-MVP. A point mutation within the start codon of the uORF as well as insertion of a frameshift within the uORF derepressed translation at almost comparable levels pointing to a predominant sequence-specific activity of the uORF. It has, however, to be taken into account that the altered uORF in construct L-MVP-mut2 is terminated by a different stop codon, thus shortening the spacer between uORF and ORF and perhaps thereby influencing translational repression [26]. Additionally, the release of suppression with both mutated constructs in vivo was not complete (60–80%), suggesting that the translation suppression might not entirely rest on the uORF. For example the presence of secondary structures with a stronger free energy in L-MVP might hamper ribosome scanning [17]. Taken together, several mechanisms are suggested to contribute to the surprisingly high repression of the MVP expression from L-MVP mRNA in vivo. The nature and importance of these mechanisms have to be dissected by further investigations. Results from the in vitro assay differed from the in vivo analysis. The suppression of MVP expression from L-MVP was less pronounced as compared to the in vivo experiments. A point mutation within the start codon of the uORF completely released suppression of MVP expression in vitro. In contrast, insertion of a frameshift within the uORF only weakly up-regulated MVP expression. Thus, the results of the reticulocyte lysate assay point to a loss of the sequence-dependency during preparation of the extracts.

The physiological role of the ubiquitously expressed but translationally suppressed MVP mRNA isoform is enigmatic, in particular as the basic cellular function of MVP is still unclear. In normal tissues, constantly about 30–45% of the total MVP mRNA is present in the form of the repressed L-MVP mRNA. Only the brain tended to contain relatively lower amounts of L-MVP mRNA perhaps corresponding to a general difference of mRNA splicing in neuronal tissues [27]. The enhanced variability of MVP mRNA isoform expression in malignant tissue corroborates data indicating a deregulation of MVP expression during malignant transformation [28]. Regarding drug resistance, MDR sublines characterised by MVP overexpression were demonstrated to differ from the parental line with regard to the MVP mRNA isoform ratio indicating that posttranscriptional events might play an essential role in the acquisition of an MDR phenotype.

Summing up, we demonstrate for the first time that alternative splicing might have an impact on the expression of the MVP gene. Interestingly, the depicted splicing event does not lead to an altered MVP ORF, but rather to an mRNA controlled by a small uORF. Such a complex combination of gene expression regulation by alternative splicing and a translational control via the 5′-leader is almost unique to MVP so far. It suggests the necessity for a precise regulation of MVP expression, as is known for proteins serving essential functions in cell growth, differentiation and tissue homeostasis [17].

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