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Cloning and Initial Analysis of the Human Multidrug Resistance-Related MVP/LRP Gene Promoter

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The lung resistance-related protein (LRP) was identified as the human major vault protein (MVP), and is overexpressed in various multidrug-resistant cancer cell lines and clinical samples. We characterized DNA sequences upstream to the transcription initiation site of the *MVP* gene in the human non-small cell lung cancer cell line SW-1573. A 1.9-kb and a shortened 0.7-kb fragment of the 5′-upstream genomic region show strong promoter activity in chloramphenicol acetyltransferase (CAT) reporter assays. The promoter is TATA-less and contains an inverted CCAAT-box and a Sp1 site located near to a p53 binding motif. An alternative 3′-splice site of intron 1 results in a splicing variant within the 5′-untranslated region of MVP mRNA. © 2000 Academic Press

Key Words: **multidrug resistance; human major vault protein/lung resistance-related protein; gene promoter.**

Vaults are the largest known ribonucleoprotein complexes (13 MDa) and are highly conserved throughout evolution. These cellular particles consist of a small untranslated RNA and of three proteins: the lung resistance protein LRP (1), identified as the major vault protein MVP (2; to follow the more recent nomenclature, we will hereafter refer to LRP as MVP), and the minor vault proteins p193 and p240.

Distribution of MVP in normal human tissues showed that of other drug-resistance-related proteins, with high expression in tissues chronically exposed to xenobiotics. In the context of drug resistance vaults are suggested to be involved in the nucleocytoplasmic drug transport with respect to the defense against xenobiotics (for review: 3). Expression of MVP in clinical specimen had been observed in a variety of human tumors. It revealed for certain malignancies that MVP expression might be predictive of response to chemotherapy and prognosis, as repeatedly reported for, e.g., acute myeloid leukemia, multiple myeloma, and advanced ovarian carcinoma (3).

Previous studies have demonstrated that vaults are up-regulated in different multidrug resistant (MDR) cancer cell lines (e.g., 4, 5). Increased levels of MVP have been reported in numerous cell lines after selection with a wide panel of cytostatic drugs, such as doxorubicin, mitoxantrone, methotrexate, etoposide, vincristine, cytarabine and cisplatin (6–12). Up-regulation of both MVP and the minor vault protein p193 have been published very recently (13). Moreover, differentiating agents like sodium butyrate (14), the carcinogen benzopyrene (15), and phorbolester (11) were shown to activate MVP transcription. By contrast, tumor necrosis factor-α either applied externally or after gene transduction led to down-regulation of MVP transcription (16). Reversal to a sensitive phenotype by expression of two MVP-specific ribozymes indicated that MVP may be directly involved in multidrug resistance (14).

To our knowledge thus far, no reports have analyzed the molecular basis for this phenomenon of modulating *MVP* gene transcription by external stimuli. Investigating the regulation of the *MVP* gene is essential to clarify its role in resistance of various tumors to chemotherapeutic drugs. In this study we approached this issue by focussing on the transcription initiation site of the *MVP* gene in the non-small cell lung cancer cell line SW-1573 which shows moderate expression of MVP (while its doxorubicin-resistant derivative SW-1573/2R120 up-regulates MVP transcription, 1). We present the organization of the genomic region upstream of the identified transcription initiation site and show its strong promoter activity.

The nucleotide sequences reported in this paper have been submitted to the EMBL/GenBank database under Accession Nos. AJ238509–AJ238519.

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MATERIALS AND METHODS

Cell culture. The human non-small cell lung cancer cell line SW-1573 was kindly provided by Dr. Henricus J. Broxterman (Free University Hospital, Amsterdam, The Netherlands), and grown in RPMI 1640 medium at 37°C, 5% CO_2 as described (17). Colon carcinoma cell lines HCT15 and HCT116 were cultured in RPMI 1640 medium at 37°C, 5% CO_2 as described previously (16).

Isolation and amplification of human genomic DNA. For isolation of SW-1573 genomic DNA, 1×10^7 cells were trypsinized, suspended in 1 ml RPMI 1640 medium, and washed twice by centrifugation (900 rpm, 4°C, 5 min) with ice-cold PBS. Cells were resuspended in 1 ml freshly prepared extraction buffer [100 mM Tris–Cl (pH 8.0), 50 mM EDTA, 500 mM NaCl, 10 mM β-mercaptoethanol] and were lysed by two cycles of freezing in a dry-ice/ethanol bath and thawing at room temperature. Then the procedure described by Dellaporta *et al.* was proceeded (18). Genomic DNA was dissolved in 10 mM Tris–Cl (pH 8.0) containing 0.1 mg/ml RNase A (Roche Diagnostics, Mannheim, Germany) overnight at 4°C.

To amplify genomic sequences of the *MVP* gene, the GeneAmp PCR Reagent Kit including AmpliTaq Gold DNA polymerase (Perkin–Elmer, Weiterstadt, Germany) was used according to the manufacturer's recommendations. For amplification of intron 1 of the *MVP* gene, Combipol DNA polymerase (InViTek GmbH, Berlin, Germany) was used following the manufacturer's instructions. The MVP primers used for conventional polymerase chain reaction (PCR) are: MVP_2, 5′-AGGCAGCCCTTTATTTCAGAGAAT; MVP_4, 5′-AGGTTCATCCTGTGTCGTCTC; MVP_9, 5′-GACAAGGTGCTGTCGGACATGAAGAGA; MVP_10, 5′-TTCCCAAGTTTCTCAATGTCTGGGC; MVP_11, 5′-AGCAGAAGCAAGGCCCTTCAAAGCAAG; MVP_15, 5′-TCAGACTCAGAAGATTCAGTCCAGG; MVP_17, 5′-CCTGATGATGGTGGCCTGAATGATC.

Genome walking. For isolation of 5′-flanking sequences of the *MVP* gene, a nested PCR genome walking method adapted from Shyamala and Ames (19) was used. Five μg genomic DNA of the human cell line SW-1573 was digested with the restriction endonucleases *Kpn*I or *Eco*RV (Roche Diagnostics). DNA was purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and eluted in 60 μl 10 mM Tris–Cl (pH 8.0). Ten μl were ligated into a *Kpn*I or *Eco*RV digested Bluescript II KS vector (Stratagene, Amsterdam, The Netherlands). For genome walking beyond the putative transcription start point of the *MVP* gene, a gene specific primer MVP_# and a nested primer MVPnested_# were designed according to the published cDNA sequence (GenBank Accession No. X79882). The MVP primers used for genome walking are: MVP_1, 5′-TCCAGCACATGGATATAGTGGTATG; MVPnested_1, 5′-GGATGCGGATGATGAACTCTTCA; MVP_3, 5′-AAGTGCAGGTAGCTGCAACAAAC; MVPnested_3, 5′-CAACAAACGCCTCAGATGGGGAA; MVP_3/c, 5′-GTTTGTTGCAGCTACCTGCACTT; MVPnested_3/c, 5′-TTCCCCATCTGAGGCGTTTGTTG.

In the primary PCR, the two genomic libraries were amplified in a total volume of 50 μl containing 10 pmol of each MVP_# and the vector primer M13 reverse 5′-CAGGAAACAGCTATGACCATG, 200 μM dNTP, 2 mM $MgCl_2$, 1.25 units AmpliTaq Gold DNA polymerase (Perkin–Elmer), 1× reaction buffer, and 2 μl of the 1:20 diluted ligation mix. The PCR products were purified and the secondary PCR was performed with the gene specific nested primer MVPnested_# and the nested vector primer T3 5′-AATTAACCCTCACTAAAGGG. The PCR conditions used for both reactions were 95°C for 10 min, 30 cycles with 95°C for 20 s, 60°C for 30 s, 72°C for 5 min, and 72°C for 10 min.

Two fragments of the 5′-flanking genomic region of the human *MVP* gene of approximately 1.9 and 0.7 kb in size were successfully amplified.

Nucleotide sequence analysis. DNA fragments of interest were size-separated in 1% agarose gels, excised and purified with the QIAEX II Gel Extraction Kit (Qiagen). Fragments were cloned into pCR2.1 vector of the TA Cloning Kit (Invitrogen) and DNA of individual colonies was sized. Both strands of inserts were sequenced by Invitek GmbH (Berlin, Germany) on a ABI 373 DNA Sequencer using the Dye Terminator Cycle Sequencing Kit (Perkin–Elmer). Primer design, database searching and *in silico* analysis of sequences was performed with the Heidelberg Unix Sequence Analysis Resources (HUSAR) version 5.0 at the German Cancer Research Center (Heidelberg, Germany). Sequence analysis of putative promoter sequences was done by searching the TRANSFAC database using the MatInspector software (http://genomatix.gsf.de).

Construction of MVP promoter–harboring reporter plasmids. The two MVP promoter fragments were cloned into pCR2.1 as described above resulting in the clones pCR-MVP1.9 and pCR-MVP0.7. Both MVP promoter fragments were excised by using *Spe*I/*Xba*I (Roche Diagnostics) for construction of the CAT-reporter plasmids. The respective *Spe*I/*Xba*I MVP promoter fragments were inserted into the *Xba*I site of the pCAT-Basic plasmid (Promega, Mannheim, Germany) to drive CAT-gene expression. Due to the compatibility of the *Spe*I and the *Xba*I restriction sites this cloning strategy creates both, sense and antisense MVP promoter constructs (pCAT-MVPprom1.9s and pCAT-MVPprom1.9as, as well as pCAT-MVPprom0.7s and pCAT-MVPprom0.7as). Orientation of the pCAT-MVPprom1.9 constructs were determined by *Hin*dIII digestion, since this promoter fragment harbors a unique *Hin*dIII restriction site. The orientation of the pCAT-MVPprom0.7 constructs were determined by *Ava*I digests, since this fragment carries a unique *Ava*I restriction site. For both MVP promoter fragments, sense and antisense CAT-reporter constructs were isolated for CAT assays. Reporter constructs were sequenced on both strands prior to use.

Transfection and CAT-enzyme-linked immunosorbent assay (ELISA). For determination of potential promoter activity the respective CAT-reporter plasmids pCAT-MVPprom0.7s, pCAT-MVPprom0.7as, pCAT-MVPprom1.9s, pCAT-MVPprom1.9as and control plasmids (promoter-less pCAT-Basic, SV40-promoter-harboring pCAT-Control) were transfected into the human colon carcinoma cell lines HCT15 and HCT116 using lipofectin (Gibco BRL, Karlsruhe, Germany) according to the manufacturer's instructions. For the transfections 10 μg plasmid DNA and 15 μg lipofectin were used.

Cells were harvested 96 h after transfection and suspended in 500 μl ice-cold TE-buffer containing the protease inhibitors aprotinin (10 mg/ml) and phenylmethylsulfonyl fluoride (0.1 mg/ml) and lysed by 5 cycles of freezing-thawing and cell debris was removed by centrifugation at 14,000 rpm, 4°C for 10 min. Two hundred μl of the respective cell lysates were subjected to the CAT-ELISA in duplicates as recommended by the manufacturer (Roche Diagnostics). Absorbance was measured at 492 nm in a microplate reader and CAT values were calculated from the CAT-standard curve using the EasySoftG200/Easy-Fit software (SLT-Labinstruments, Crailsheim, Germany). The amount of CAT-protein was normalized to the protein content of the respective lysate, which was determined by using the Coomassie Plus Protein Assay reagent (Pierce, Rockford, IL).

Poly(A)$^+$ RNA isolation and construction of a solid-phase cDNA library. Poly(A)$^+$ RNA from the human cell line SW-1573 was isolated using the Oligotex Direct mRNA Isolation Mini Kit (Qiagen) according to the manufacturer's instructions. To construct a solid phase cDNA library covalently bound to oligo$(dT)_{30}$ latex beads (20), the instructions were followed including the mRNA oligo$(dT)_{30}$ latex complex formation step. The complex was precipitated by centrifugation for 10 min at room temperature and the pellet was washed once in 1× Expand reverse transcriptase reaction buffer (Roche Diagnostics). The precipitate was dissolved in 120 μl of the same buffer containing 1 mM dNTPs (Perkin–Elmer), 10 mM DTT, 25 μg bovine serum albumin (Amersham Pharmacia Biotech, Freiburg, Germany), 100 units RNase inhibitor (Amersham Pharmacia Biotech), 500 units Expand reverse transcriptase (Roche Diagnostics), and was incubated at 42°C for 90 min. The reverse transcription mix

was denatured at 95°C for 3 min and cooled immediately on ice. The cDNA oligo(dT)$_{30}$ latex complex was washed twice with ice-cold H_2O and dissolved in 100 μl of the terminal deoxynucleotidyl transferase reaction mixture (Roche Diagnostics) containing 1× reaction buffer, 1 mM dATP, 1.5 mM $CoCl_2$ and 100 units terminal deoxynucleotidyl transferase. The reaction was carried out at 37°C for 10 min and terminated by addition of 10 μl 500 mM EDTA. The resulting oligo(dA)-tailed cDNA oligo(dT)$_{30}$ latex complex was washed twice with H_2O.

To prepare the template for 5′ rapid amplification of cDNA ends (RACE), we added an adapter sequence containing a restriction site for *Sal*I for cloning to both ends of the cDNA. The latex complex was dissolved in 100 μl of a second strand synthesis mixture containing 20 pmol UNI-(T_{17}) adapter primer, 5′-GGCCACGCGTCGAC-TAGTAC(T_{17}) (Life Technologies, Eggenstein, Germany), 200 μM dNTPs, 1× reaction buffer II and 10.5 units enzyme mix of the Expand High Fidelity PCR Kit (Roche Diagnostics). Double stranded cDNA synthesis was carried out in a T-Gradient thermal cycler (Biometra, Göttingen, Germany) with 2 cycles of the following program: 95°C for 2 min, 36°C for 30 s, heating to 68°C with 0.25°C/s, 68°C for 15 min. The mixture was heated to 95°C for 5 min, cooled immediately on ice and the oligo(dA)-tailed cDNA oligo(dT)$_{30}$ latex complex was centrifuged as above, washed twice, and stored in 10 mM Tris–Cl (pH 8.0) at 4°C. The supernatants of the washing steps with the double-stranded, UNI-(T_{17})-flanked cDNA were collected, purified, and used for 5′-RACE.

5′-RACE analysis and reverse transcription PCR (RT-PCR). For rapid amplification of the 5′-ends of MVP cDNA, the same experimental approach as for genome walking, a combination of ligation and nested PCR, was used. First, UNI-(T_{17})-flanked cDNA was totally amplified with the UNI adapter primer harboring an unique *Sal*I restriction site (5′-GGCCACGCGTCGACTAGTAC) as previously described (21). PCR products were purified and concentrated by ammonium acetate/ethanol precipitation. Eight μg PCR product were digested with *Sal*I, purified and precipitated, and 1.6 μg were ligated to 0.4 μg of compatibly *Sal*I digested Bluescript II KS vector (Stratagene) by heating the ligation mix 60°C for 2 min, cooling to 14°C with 0.01°C/s, and incubating at 14°C over night in a T-Gradient thermal cycler (Biometra). The T4 DNA ligase (Invitrogen, Groningen, The Netherlands) was added at 14°C. Both the primary PCR with 10 pmol of each primer MVP_1 and the vector primer M13 reverse, and the secondary PCR with 10 pmol of each primer MVPnested_1 and the vector primer T3, were exactly carried out as described for genome walking. Due to the ligation of fragments in both directions to the vector, we alternatively used the vector primers M13 −20 5′-TGTAAAACGACGGCCAGTGA and T7 5′-AATTAACCCTCACTAAAGGG, respectively. RT-PCR with poly(A)$^+$ RNA was performed with the Gene Amp RT-PCR Kit (Perkin–Elmer) according to the manufacturer's specifications. Fragments were cloned and sequences were analyzed as described above.

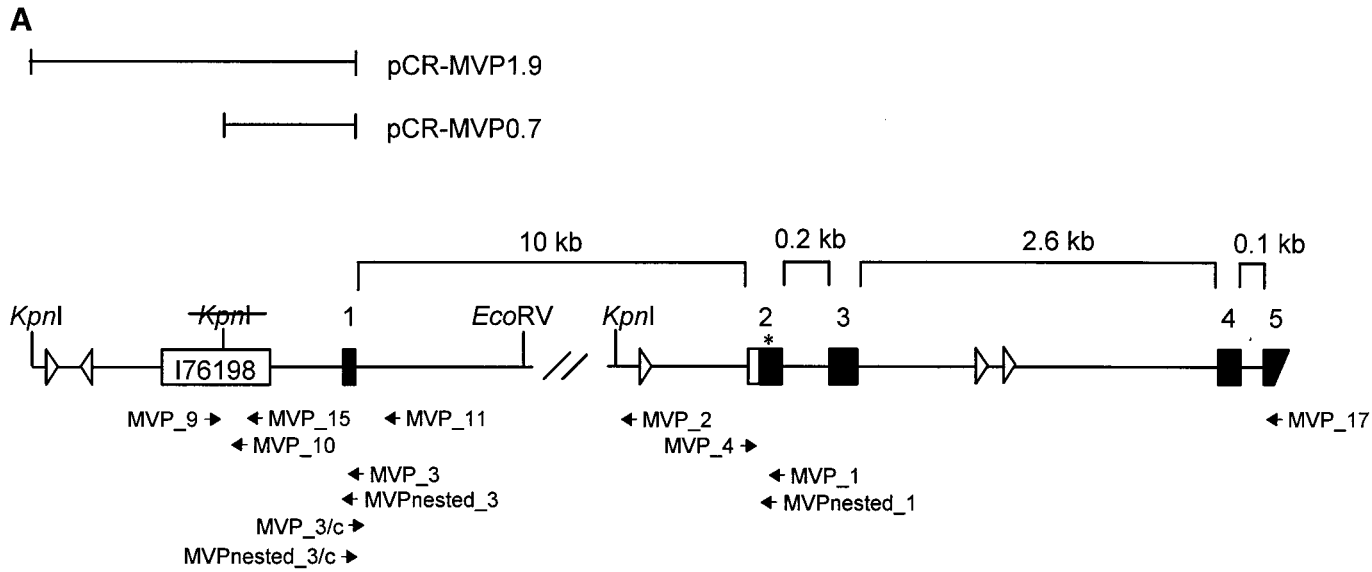
RESULTS AND DISCUSSION

Genomic organization of the 5′-regulatory region of the MVP gene. To isolate putative regulatory sequences of the *MVP* gene, genomic DNA fragments of the non-small cell lung cancer cell line SW-1573 were ligated into the Bluescript II KS vector. We performed nested PCR experiments by combining two MVP-specific primers with vector primers. This genome walking strategy enabled us to amplify the 5′-upstream genomic region of the *MVP* translational start site. The exon–intron boundaries of intron 1 were mapped by genome walking, and the complete intron 1 as well as the introns 2–4 were amplified and sequenced (Fig. 1A). Altogether, we isolated 14.6 kb of the *MVP* genomic 5′-region. The sequence data have been submitted to the GenBank data library under Accession Nos. AJ238509–19 (During preparation of the manuscript, a genomic sequence with the Accession No. AC009133 was published in GenBank. This sequence contains the complete *MVP* gene consisting of 15 exons and confirms our results.).

Genome walking toward the putative MVP promoter region produced two fragments from the *Kpn*I genomic DNA library with 702 bp and 1882 bp *MVP*-specific sequence, respectively. The resulting clone pCR-MVP0.7 (harboring 702 bp from −679 to +23 of the sequence in Fig. 1B) is a shortened variant of clone pCR-MVP1.9 (harboring 1882 bp from −1859 to +23), missing 1180 bp from the 5′-end. However, in the sequence of pCR-MVP1.9 the expected *Kpn*I site could not be found. Furthermore, there was no *Kpn*I site in any matching sequence identified by database search. Thus, we concluded that star activity of *Kpn*I as indicated by the manufacturer may have produced the fragment of clone pCR-MVP0.7.

Nucleotide sequence of the MVP 5′-genomic regulatory region. Sequence analysis of the 1.9 kb putative MVP promoter revealed several consensus elements for binding of several known transcription factors, such as Y-box and E-box, and binding sites for Sp1, p53, and signal transducer and activator of transcription (STAT) proteins (Fig. 1B).

The inverted CCAAT box (Y-box) is located at nt −279 to −275 (where +1 refers to the 5′-end of the MVP cDNA with the GenBank Accession No. X79882; Fig. 1B), which is designated to be a recognition site for Y-box binding transcription factors such as YB-1 and NF-Y. In the context of multidrug resistance, the involvement of YB-1 in mediating effects of external stimuli such as cytostatic drugs (actinomycin D, cisplatin, mitomycin C, etoposide) and UV light have already been reported (22–24). Translocation of YB-1 from the cytoplasm into the nucleus have been observed after treatment of cell cultures with DNA-damaging agents and irradiation (25, 26). Correlation of an nucleic YB-1 expression and intrinsic expression of the multidrug resistance gene 1 (MDR1) have been shown within mammary carcinomas (27) and osteosarcomas (28). Since MVP expression regulation, like those for MDR1, have also been described after exposure to an entire panel of cytostatic drugs (6–12), the potential binding of YB-1 to the inverted CCAAT box within the MVP promoter might be an causal event for triggering the effects of such external stimuli. NF-Y is reported to activate transcription of MDR1 by recruiting the histone acetyl transferase P/CAF to the MDR1 promoter (29). The identification of an inverted CCAAT box within the MVP promoter and the observation that MVP is transcriptionally induced by the histone deacetylase inhibitor sodium butyrate (14) suggests



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      KpnI
-1859 GGTACCTGCAGTGGCATGATCTTGGCTCACTGCAATCTCCACCTCCTGGGTTGAAGCGATTCTCCTACCTCAGTCTCCTGAGTAGCTGGGATTAC
-1764 AAGGACACGTCACCACGCCTAGCTAATTTTTGTATTTTCTAGTAGAGAGGGGGTTTCGCCATGTTGGCGAGGCTGGTCTTGAACTCCTGACCTAA
-1669 AGCTTCCCAAAGTGCCTGCCTCAGCTTCCCAAAGTGCTGGAATTACAGGCGTGAGCTGCTGCACTCAGCCATAAATCGTGTCACTTTTCCACTTA
-1574 AAATTTTCCAAGGGATTCCATCTTGCCAGGGAAGAGCATGTCAAAGGAGAATCCAAGCGCTTTTCCCGCCACCTCCAGTTCTCTGCACTCTTTTT
-1479 TTTGTTTTTGTTTTTGGCGAGTAGGGGAGACGGAGTGTAGCTGTGTCACCGAGGCTGGAGTGCAGTAGTGCGATCTTGGCTCATTGCAACCTCCA
-1384 CCTCCCGGGTTCAAGCGATTCTCCTGCCTCAGCCTTCCAAGTAGCTGGGATTACAGGCGCCCACCACCACGCCCAGCTAATTTTTTGTGTTGGCC
-1289 AGGCTGGACTCAAACTCCTGACCTCGAGAGTTGCCCACCTCAGCCTCCCAAAGTGCTGGCATTACAAGCGTGAACCACCATGCCCGGCCTGCACT
-1194 CTTCTTTGAACAGAACTCTGTTCTTGTCCTGGGGCCTATACCCTTGCCATTCCCCTGCCCAGTATGTTCCTCCCTGTTCTTCACATTACCTGTGC
-1099 CTTCCTGTCAATCAAGATCTTTGCCTCTCACCCTCTCTGAGGTCCAACCCTGAACCTCAGGTCCTCCAAGACAGAGGCCTGGGGACCTGCATGTT
-1004 TAACCAGCTCCCCAAGTGATGAGTGAGGTCCAGGCAGGTATGGTGTACACCACCTCCATCCCTTCCATGTATCTTACCTTCCTCTTCTCCAGGAA
 -909 GCTCAGCCCGGAGCCAGAAACGGGAGGCCCGCCTGGACAAGGTGCTGTCGGACATGAAGAGACACAAGAAGCTGGAGGAGCAGATCCTTCGTACC
 -814 GGGAGGGACCTCTTCAGCCTGGACTCGGAGGACCCCAGCCCCGCCAGCCCCCCACTCCGATCCTCCGGGGAGTAGTCTCTTCCCTCGGCAGCGGAA
                                                                                    delC
 -719 ATACTGATTCCCACTGCTCCTGCCTCTAGGGTGCAGTGTCCGTACCTGCTGGAGCCTGGGCCCTCCTTCCCCAGCCCAGACATTGAGAAACTTGG
                                                                                                    A>C
      lentiviral TATA upstream element
 -624 GAAGAAGAGAGAAACCTCAAGCTCCCAAACAGCACGTTGCGGGAAAGAGGAAGAGAGAGTGTGAGTGTGTGTGTGTTTTTTCTATTGAACACC
                    T>A  G>A
                                lentiviral poly(A) downstream element direct repeats
 -529 TGTAGAGTGTGTGTGTGTTTTCTATTGAACACCTATAGAGAGAGTGTGTGTGTTTTCTATTGAACATCTATATAGAGAGAGTGTGTGAGTGTG
                T>A       delA       T>A                poly(A)
 -434 TGTTTTCTATTGAACACCTATTCAGAGACCTGGACTGAATTTTCTGAGTCTGAAATAAAAGATGCAGAGCTATCATCTCTTAAAAGGAGGGGCTG
                                                                                CCAAT box
 -339 TAGCTGTAGCTCAACAGTTAGGCCCCACTTGAAGGGAGAGGCAGAATTGTACTCACCCAGATTGGAAAATGAAAGCCAGATGGGTAGAGGTGCCC
           MyoD
 -244 TCAGTTAGCACCTGTCCCATCTCGGGCCCTCCAACTCCTCCCAGTCCCACTCCAGTGCAGCCAGCTGGCTCCAAGGTAGAAACCCATGAGCACTC
                                                GATA                E box                Sp1
 -149 AGGGAGCAGTGTGCCTTCAGCTGCAGCAGAAGCAGCCCGGAGGATAAAATGAGAACCAGCTGCACACGGGCCCTTTAACTCCCAAGCCCCACCCC
           p53                STAT                          +1
  -54 TGGGCTTGGCCTGCCTTGCCCTGCCGGGAAGTGATCCCCAAGGCAGGGTGAGAGTTCCCCATCTGAGGCGTTTGTTGCAGCTACCTGCACTTCTA
  +33 GATgtgagtacattgtactagcccccca
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FIG. 1. Genomic organization of the 5′-regulatory region of the *MVP* gene. (A) Exon–intron boundaries of the genomic 5′-region. The exons are numbered and marked with black rectangles. The size of the introns (upper panel) and the position of primers used in this study (lower panel) are indicated. Sequences isolated by genome walking with MVP-specific primers are: 1.9 kb upstream of exon 1 with MVPnested_3, resulting in the clones pCR-MVP1.9 and pCR-MVP0.7, respectively; 1.6 kb downstream of exon 1 with MVP_3/c; 1.3 kb upstream of exon 2 with MVPnested_1. The 10-kb intron 1 was amplified with the primers MVP_3/c and MVP_2; the region between exons 2 and 5 was amplified with the primers MVP_4 and MVP_17. The translation start point is marked as an asterisk and *Alu*-repeats are indicated with triangles; *Kpn*I, *Eco*RV, restriction sites derived from genomic libraries. (B) Nucleotide sequence of the *MVP* 5′-genomic

that similar mechanisms could regulate *MVP* gene expression.

An E-box element was identified at site nt −85 to −77 providing binding possibilities for members of the myc/max transcription factor family (30, 31). Correlation of myc expression with poor prognosis have been published for several cancers, and involvement of myc in drug resistance toward e.g., cisplatin and etoposide is also known (32, 33).

A binding site for the ubiquitous transcription factor Sp1 was identified at nt −64 to −55. This consensus sequence occurs predominantly in TATA-less promoters; the here described MVP promoter sequence and the MDR1 promoter are both TATA-less with Sp1 binding sites (34, 35). This Sp1 binding site is closely linked to a putative palindromic p53 tumor suppressor protein binding site at nt −53 to −34. The involvement of p53 in drug resistance and on expression modulation of MDR1 and the multidrug resistance-associated protein 1 (MRP1) have been investigated in numerous studies (35), and effects of wild-type and mutant p53 on MDR1 promoter have been analyzed in several reports (e.g., 36). Moreover, it was reported that p53 is able to repress transcription initiation of Sp1 by interaction between both proteins, as it was shown for MRP1 (37). In multiple myeloma patients with a p53 deletion, LRP expression was more frequently observed than in those without such a deletion (38).

A database search with the BLASTN program identified a 794-bp cDNA (GenBank Accession No. I76198), of which the 3′-end with nt 794–230 is homologous to the *MVP* gene nt −353 to −916 (Figs. 1A and 1B). Sequence features or putative functions of this cDNA were actually not further characterized, although a previous publication refers to the sequence (39). The sequence of a I76198 specific RT-PCR product from SW-1573 poly(A)$^+$ RNA confirmed that this transcript also exists in the analyzed cell line. A genome walking approach into the 5′-region of I76198 with specific primers produced a 1.2-kb fragment with identical sequence to the *MVP* gene 5′-region, indicating that the genomic locus of I76198 is closely linked to the MVP promoter. Our sequence is identical to all 38 expressed sequence tags (ESTs) identified in the GenBank database. In contrast, we found seven nucleotide substitutions with respect to I76198 (Fig. 1B), but no open reading frame could be detected in both sequences. Searching the TRANSFAC database of promoter elements reveals that this region of the *MVP* gene contains a lentiviral TATA upstream element at nt −603 and five 35-bp direct repeats between nt −568 and nt −390, which were defined as lentiviral poly(A) downstream elements in the database. Furthermore, we identified a putative eukaryotic polyadenylation signal at nt −376 of the *MVP* gene 5′-region, and all 38 identified ESTs have a poly(A) stretch that would arise from the usage of this signal. These findings could indicate that I76198 may be of viral origin, e.g., a long terminal repeat of a lentivirus, which is transcribed and polyadenylated in human cells. Although it is expected that transcription of both loci occurs independently, there could be possible consequences for the regulation of the *MVP* gene.

MVP gene promoter activity in CAT reporter gene assay. To test whether the isolated 5′-end sequences of the *MVP* gene possess promoter activity, the CAT-reporter assay was performed using CAT-ELISA. The CAT-reporter constructs harbor the 1.9-kb 5′-end of the *MVP* gene in sense or antisense orientation (pCAT-MVPprom1.9s and pCAT-MVPprom1.9as) to drive the CAT-gene expression. The 0.7-kb fragment, which is a shortened variant of the 1.9 kb fragment missing 1180 bp from the 5′-end was also utilized in either sense or antisense orientation (pCAT-MVPprom0.7s and pCAT-MVPprom0.7as) to control CAT-expression.

Transfection of the two sense pCAT-MVPprom constructs revealed promoter activity in the two human colon carcinoma cell lines HCT15 and HCT116. Figure 2 demonstrates strong promoter activity of the pCAT-MVPprom0.7s construct indicating slightly higher activity compared to the SV40-promoter driven CAT-expression of the pCAT-Control transduced cells. However, if this 0.7 kb fragment is in antisense orientation, the promoter activity drops dramatically in pCAT-MVPprom0.7as transduced tumor cells. The antisense orientation reduces the promoter activity to a fifth of the promoter activity exerted by the 0.7-kb sense fragment. This observation has been made in both tumor cell lines indicating that the promoter activity for the 0.7-kb fragment is strictly orientation-dependent.

Similar observations were made with the CAT-reporter constructs harboring the larger 1.9-kb portion of the *MVP* gene 5′-region. In contrast to the 0.7-kb sense fragment, the activity of the 1.9-kb sense fragment did not exceed the promoter activity of the SV40-promoter driven CAT-expression and was only a third of the promoter activity determined in the pCAT-MVPprom0.7s-transduced HCT15 and HCT116 cells.

regulatory region. The sequence shown contains the 5′-flanking region, the first exon and the beginning of intron 1 (lowercase letters). All numbering is relative to the 5′-end of the cDNA with the GenBank Accession No. X79882 (+1). The transcription initiation sites identified by 5′-RACE are indicated with asterisks. Transcription factor binding sites with homologies ≥95% to their consensus sequence in the TRANSFAC database are underlined. Nucleotides enclosed in brackets designate the homology to GenBank Accession No. I76198; point mutations identified with respect to I76198 are indicated; nucleotides in italic indicate *Kpn*I restriction sites.

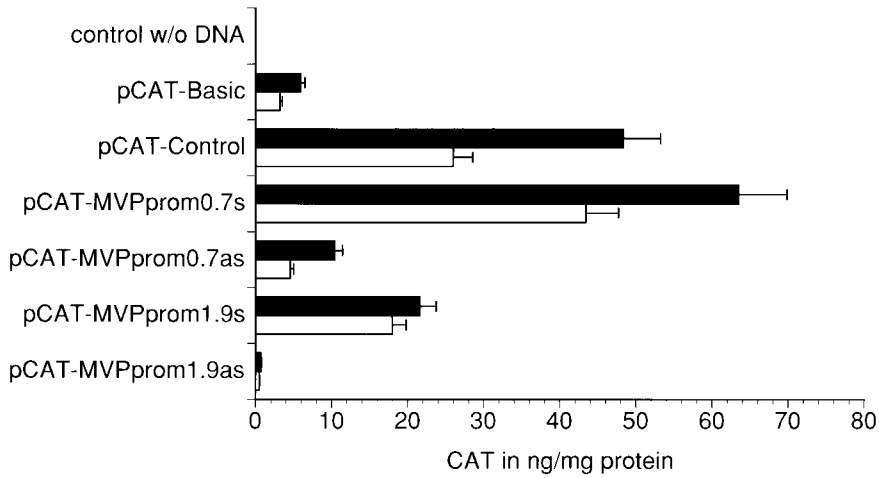

FIG. 2. MVP promoter activity in CAT reporter gene assay. HCT116 (black bars) and HCT15 (white bars) human colon carcinoma cells were transiently transduced with pCAT-MVPprom1.9s, harboring the 5′-flanking regulatory region of the *MVP* gene spanning from +23 to −1859 in sense orientation, or with pCAT-MVPprom0.7s, carrying the promoter fragment from +23 to −679 in sense orientation. The respective antisense constructs pCAT-MVPprom1.9as and pCAT-MVPprom0.7as served as internal controls. Nontransduced cells, cells transduced with the promoter-less pCAT-Basic, and cells transduced with the SV40 promoter-harboring pCAT-Control served as external controls. CAT expression was measured in duplicates by CAT-ELISA as described under Materials and Methods.

The orientation of the 1.9-kb fragment, however, had similar effects on the promoter activity as seen for the smaller 0.7-kb fragment: antisense driven CAT-expression is significantly reduced (3-fold) in both tumor cell lines. The data obtained with the CAT-reporter constructs give strong indication that both the 1.9- and the 0.7-kb fragment of the 5′-end of *MVP* gene harbor sequences are associated with the expression regulation of this resistance-associated gene. The CAT-reporter assays further indicate that this promoter region resides in the 0.7-kb fragment extending from +23 to −679, whereas the larger 1.9-kb fragment may carry regulatory sequences that interfere with the positive regulation of MVP.

Identification of a splicing variant in the 5′- untranslated region (UTR) of MVP mRNA. To map the transcription initiation site of the *MVP* gene, we constructed a solid phase cDNA library of SW-1573 poly(A)$^+$ RNA. A 5′-RACE nested PCR approach was used with double stranded cDNA ligated via *Sal*I restriction site to the Bluescript II KS vector (Figs. 3A and B). Seven independent 5′-RACE clones were sequenced, of which six were mapping to nt +6 and one to nt +3 (Fig. 1B), prompting us to assume the transcription initiation site at nt +6 in the analyzed cell line. Two distinct PCR products of 244 and 280 bp in size amplified with the primers MVPnested_1 and T3 were isolated. Sequence analysis revealed that the small fragment extends the 5′- UTR 74 bp from the translational start point, whereas the large fragment extends the 5′-UTR 119 bp due to a 41-bp insertion between exon 1 and exon 2. Comparison with the flanking genomic sequences of the 10 kb sized intron 1 show that this inserted sequence comes directly from the 5′-flanking region of exon 2, where an alternative splicing site matching the intronic 3′splice site consensus sequence was found (40; Fig. 3C).

A search of the database of ESTs and a multiple sequence alignment of 127 identified ESTs highlight additional interesting features. Besides two ESTs possessing this 41-bp intronic insertion, the majority of 76 ESTs match the 3′-proximal end of the cDNA X79882. This sequence segment including the translation termination signal belongs to exon 15 of the *MVP* gene and is strictly polymorph. We identified five frequently occurring nucleotide substitutions (2628C → T, 2665G → A, 2673G → C, 2684G → A, 2730C → T) and two deletions (2673delG, 2716delG) with respect to X79882. In addition, there is a possible hot spot between nt 2690 and 2700: only 9 ESTs are identical to X79882, but the majority of 57 ESTs have two G-insertions after nt 2691 and 2698, respectively. This might cause a frameshift generating a novel COOH-terminus of the putative translation product. The genomic sequence (GenBank Accession No. AC009133), which contains the complete *MVP* gene locus, also carries both G-insertions within the coding region of exon 15 (nt 143855–143598). Taken together, these findings may give evidence for existence of a putative heterozygous region of the *MVP* gene in humans. Functional genomic analysis of both alleles of the *MVP* gene locus and their corresponding transcripts is needed to determine the role of nucleotide polymorphisms in non-MDR1 mediated multidrug resistance. Furthermore, since there is

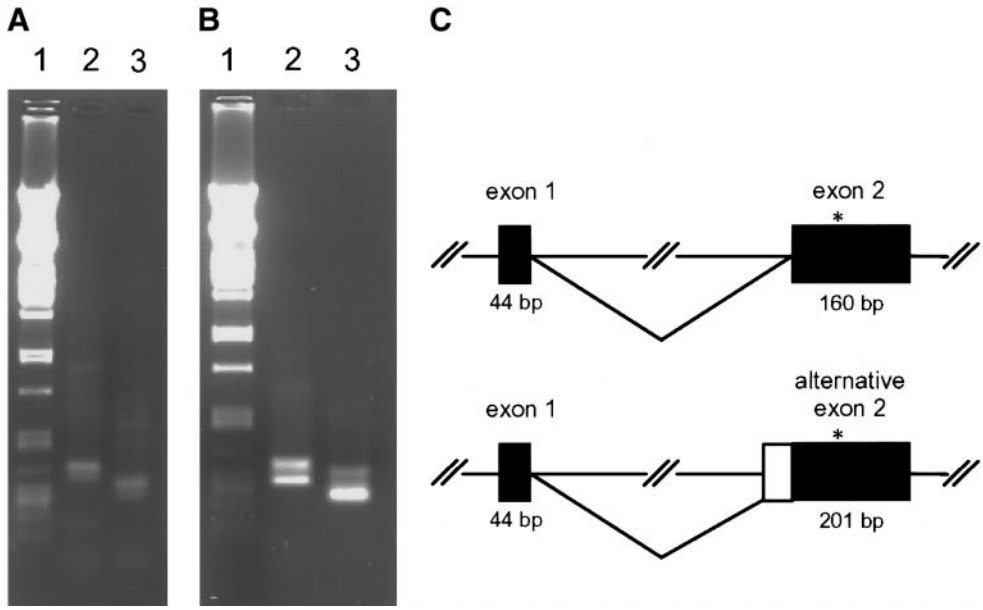

FIG. 3. Identification of a splicing variant in the 5′-UTR of MVP mRNA. (A) Agarose gel of 5′-RACE PCR products of the primary PCR. 1, *Pst*I digest of λ DNA; 2, 5′-RACE using the primers MVP_1 and M13 reverse; 3, 5′-RACE using the primers MVP_1 and M13 −20. The presence of specific PCR products in both reactions indicate that the cDNA fragments were ligated to the Bluescript II KS vector in both directions. (B) Agarose gel of the 5′-RACE PCR products of the secondary nested PCR. 1, *Pst*I digest of λ DNA; 2, nested PCR primed with MVPnested_1 and T3 using purified PCR products as in Lane 2 of A; 3, nested PCR primed with MVPnested_1 and T7 using purified PCR products as in Lane 3 of A. (C) Alternative splicing of intron 1 results in two variant 5′-UTR regions of MVP transcripts. The translation start point is marked with an asterisk.

evidence that MVP and the minor vault protein p193 are concomitantly regulated (13), the p193 gene promoter would be a interesting subject for comparative analysis of gene expression (the human p193 locus of 35 exons is already sequenced as a part of GenBank accession number AC013721).

In conclusion, we have isolated the 5′-flanking region of the human *MVP* gene. A 1.9-kb and a shortened 0.7-kb fragment of this 5′-upstream genomic region show strong promoter activity in CAT reporter assays. The promoter is TATA-less, contains e.g., an inverted CCAAT-box and a Sp1 site located near to a p53 binding motif. A sequence of putative viral origin within the 5′-region of the *MVP* gene is transcribed in the same direction. Furthermore, an alternative 3′-splice site of intron 1 results in a splicing variant within the 5′-untranslated region of MVP mRNA. Based on this knowledge it might be possible to unveil the mechanisms of MVP expression regulation underlying the phenomenon behind multidrug resistance mediated by vaults.

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