MOLECULAR AND CELLULAR BIOLOGY, July 1990, p. 3596–3606
0270-7306/90/073596-11$02.00/0

Vol. 10, No. 7

Structural Analysis of the Mouse *mdr*1a (P-Glycoprotein) Promoter Reveals the Basis for Differential Transcript Heterogeneity in Multidrug-Resistant J774.2 Cells

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In multidrug-resistant mouse J774.2 cells, the differential overproduction of functionally distinct phosphoglycoprotein isoforms reflects the amplification or transcriptional activation or both of two *mdr* gene family members, *mdr*1a and *mdr*1b. The *mdr*1a gene is a complex transcriptional unit whose expression is associated with multiple transcript sizes. Independently selected multidrug-resistant J774.2 cell lines differentially overexpress either 4.6- and 5.0-kilobase (kb) or 4.7- and 5.1-kb *mdr*1a transcripts. However, abundant overproduction of the *mdr*1a gene product was observed only in cell lines which overexpressed the 4.6- and 5.0-kb mRNAs. In order to determine the basis for *mdr*1a transcript heterogeneity and the relationship between transcript size and steady-state *mdr*1a protein levels, genomic and cDNA sequence analyses of the 5′ and 3′ ends of the *mdr*1a gene were carried out. Promoter sequence analysis and primer extension mapping indicated that *mdr*1a transcripts were differentially initiated from two putative promoters to generate either 5.1- and 4.7-kb or 5.0- and 4.6-kb transcripts in four multidrug-resistant J774.2 cell lines. Sequence analysis of 3′ cDNA variants and a 3′ genomic fragment revealed that the 5.1- and 5.0-kb mRNAs had identical 3′-untranslated regions which differed from those of the 4.7- and 4.6-kb mRNAs as a result of the utilization of a more downstream alternative poly(A) addition signal. Transcript initiation from the putative upstream promoter correlated with a 70 to 85% decrease in steady-state *mdr*1a protein levels relative to transcript levels. In addition, the identification of putative AP-1 and AP-2 promoter elements suggests a possible role for protein kinase A and protein kinase C in the regulation of *mdr*1a. The implications of these findings for *mdr* gene expression and regulation are discussed.

Acquired or inherent resistance of human tumors to a wide variety of structurally and mechanistically unrelated antineoplastic agents frequently results in treatment failure (13). Rodent and human cell lines selected in vitro for resistance to a single cytotoxic drug have been developed as model systems for studying the molecular basis of multidrug resistance (11, 52). These cell lines exhibit cross-resistance to a broad spectrum of structurally and functionally diverse lipophilic cytotoxic compounds derived primarily from natural products, such as plant alkaloids and antibiotics of fungal origin. The basis for the multidrug resistance (MDR) phenotype appears to involve decreased net intracellular accumulation of drugs due to an energy-dependent drug efflux (9, 25, 45) which correlates with the increased expression of *mdr* genes. In humans and rodents, *mdr* genes comprise a small gene family of two (*mdr*1 and *mdr*2) and three (*mdr*1a, *mdr*1b, and *mdr*2) members, respectively (6, 23, 35). Increased expression of the *mdr*1-class genes, a single *mdr*1 gene in humans (44, 51), and the *mdr*1a and/or *mdr*1b gene in rodents (23), often accompanied by gene amplification, results in the overproduction of a family of integral membrane phosphoglycoproteins (P-glycoproteins) with M_rs of 130,000 to 180,000 (14, 16, 26). P-glycoproteins are hypothesized to function as energy-dependent drug efflux pumps with broad substrate specificity (11, 52). Transfection studies have demonstrated that the expression of *mdr*1-class genes is sufficient to confer the MDR phenotype to drug-sensitive cells (17, 48). The functional role of the *mdr*2-class genes has not yet been elucidated (19, 53).

We have previously reported that the three murine *mdr* genes are differentially overexpressed in independently selected MDR J774.2 macrophagelike cells. Overexpression of *mdr*1a or *mdr*1b or both, which encode 120- and 125-kilodalton (kDa) P-glycoprotein precursors, respectively, was a consistent feature of all MDR J774.2 cell lines (23). In addition, a single cell line, J7.V3-1, was observed to switch gene expression from *mdr*1b to *mdr*1a during the course of stepwise selection for resistance to vinblastine. This resulted in a switch from the overproduction of the 125-kDa precursor to that of the 120-kDa precursor and correlated with 3.5- to 5-fold-higher levels of resistance to vinblastine, taxol, and doxorubicin in the absence of any detectable increase in the amount of immunoreactive P-glycoprotein. These findings indicated that the *mdr*1a and *mdr*1b gene products were functionally distinct (14, 23, 30).

Direct comparison of 3′-untranslated regions and protein-coding sequences suggested that the murine *mdr*1a and *mdr*1b genes arose recently from duplication of a common precursor gene. Despite the evolutionary relatedness of *mdr*1a and *mdr*1b, each demonstrates unique features. The *mdr*1a gene is a complex transcriptional unit capable of generating mRNA species of 4.6, 4.7, 5.0, and 5.1 kilobases (kb), compared with a single 4.6-kb mRNA species associated with *mdr*1b (23). The 4.6- and 5.0-kb and 4.7- and 5.1-kb transcript pairs are differentially overexpressed in similar amounts in independently selected MDR J774.2 variants, but

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only cells which overexpress the 4.6- and 5.0-kb transcripts exhibit abundant steady-state levels of the *mdr*1a gene product. In order to investigate the basis for *mdr*1a transcript heterogeneity and its relationship to steady-state *mdr*1a protein levels, a detailed analysis of 5' and 3' *mdr*1a genomic and cDNA clones was undertaken. It was concluded that (i) differential *mdr*1a transcript initiation from two putative promoters correlates with differences in steady-state *mdr*1a protein levels and (ii) additional transcript heterogeneity arises from the usage of two different poly(A) addition signals. In addition, the presence of putative AP-1 and AP-2 promoter elements suggests that the *mdr*1a gene may be regulated by transcription factors which mediate signal transduction pathways involving both protein kinase A and protein kinase C.

MATERIALS AND METHODS

Cell culture. The independently isolated MDR variants of the J774.2 macrophagelike cell line were selected and maintained as described previously (14, 40).

Isolation and subcloning of genomic clones. λV1.1a, a 3.0-kb genomic fragment encoding the 5' end of the *mdr*1a gene, was isolated from a λgt10 library constructed with *Eco*RI-digested DNA from the vinblastine-resistant J7.V1-1 cell line. λV1.1a was identified by cross-hybridization to a 1.4-kb *Eco*RI genomic clone encoding the homologous region of the *mdr*1b gene (D. Cohen, unpublished data). It was subcloned into the *Eco*RI site of pGEM7-Zf(+) (Promega Biotec) and designated pV1.1a. pV11-6 (4.1 kb) is an *Eco*RI genomic subclone (23) containing the last two exons and additional downstream sequences of the *mdr*1a. It was subcloned from a larger genomic fragment isolated from a λEMBL3 library prepared with J7.V1-1 DNA which was partially digested with *Eco*RI to give an average size of 12 to 21 kb. Screening was done by plaque hybridization with the hamster P-glycoprotein cDNA fragment pCHP1 (39).

Cloning of 5' *mdr*1a cDNAs by PCR. Total RNA was isolated from subconfluent MDR J774.2 cells. cDNA was prepared by reverse transcription of 1 μg of total RNA by using 100 ng of random primers and 5 to 10 U of avian myeloblastosis virus reverse transcriptase under conditions recommended by the manufacturer (Amersham Corp.). cDNA samples corresponding to 50 to 100 ng of RNA were used for enzymatic amplification by the polymerase chain reaction (PCR) by using 2.5 U of recombinant *Taq* DNA polymerase and commercially available kit reagents (Amplitaq; Perkin Elmer Cetus). Each reaction contained 20 pmol of reverse and forward PCR primers. Each primer included 5' adapter sequences to facilitate subsequent cloning. The sequence of the primer-adapters was as follows (adapter sequences are underlined): SBH-66 (forward), 5'-(*Cla*I)TA ATCGATGGTCCCATCTTCCAAGGCTCT-3' and SBH-20 (reverse), 5'-(*Xba*I, *Sal*I, *Sph*I)GATCTAGAGTCGACGC ATGCTAAGGAGAAAAGCTGCAC-3'. A total of 40 cycles of PCR were carried out in 50-μl volumes by using a thermal cycler (Perkin Elmer Cetus). Each cycle included 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C. Samples were analyzed on 2% agarose gels (Nusieve GTG; FMC Bioproducts), stained with ethidium bromide, transferred to a Zetaprobe nylon membrane (Bio-Rad Laboratories) with 0.4 N NaOH, and neutralized with 2× standard saline citrate (SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) prior to hybridization. Membranes were hybridized to a 0.9-kb *Sph*I-*Eco*RI fragment isolated from pV1.1a. PCR fragments were purified by phenol-chloroform-isoamyl alcohol extraction followed by ethanol precipitation and cloned into the *Cla*I and *Sph*I sites of pGEM7-Zf(+) (Promega) for subsequent sequence analysis.

Isolation of *mdr*1a 3' cDNA variants. Isolation of the cDNA pV1.35, encoding the short variant of the *mdr*1a 3'-untranslated region, was described previously (23). pV1.10, the long *mdr*1a 3' cDNA variant, was screened from a J7.V1-1 cDNA library constructed directly in pUC18 by hybridization with pC1.5, an *mdr*1b full-length cDNA (23).

Sequence analysis. Overlapping deletion subclones of pUC18 and pGEM7-Zf(+) inserts were constructed in both directions by successive exonuclease III and S1 nuclease digestions (21). Supercoil sequencing was done by the method of Hsu (22) by using the USBioclean kit (U.S. Biochemical Corp.). Nucleotide sequence analysis was done from both strands or from multiple independent overlapping clones on one strand with the Sequenase Version 2.0 or Taquence sequencing kit (U.S. Biochemical Corp.). The nucleotide sequences were analyzed and assembled as previously described (23). Restriction maps were generated on the Plasmid Description Language program of Craig Werner, as modified by L. S. Kirschner.

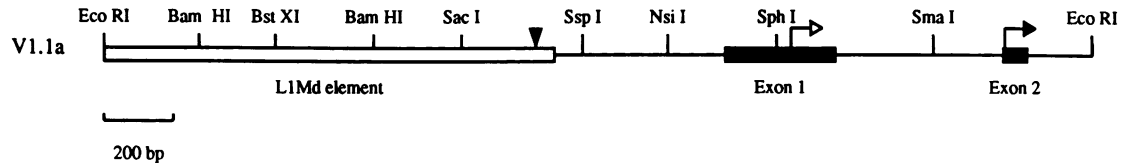
Primer extension mapping. The 5' ends of the *mdr*1a transcripts were mapped by using the protocol of Kingston (27). Total RNA (50 μg) was hybridized to 5×10^5 cpm of a 30-base-pair (bp) extension primer which was end labeled with [γ-^{32}P]ATP by polynucleotide kinase. The sequence of the 30-mer was 5'-CACCAAGATGTAGACTTTGGAAGAA GCGGC-3'. Annealing was carried out for 16 h at 30°C. Extension of annealed primers was done with 40 U of avian myeloblastosis virus reverse transcriptase (Promega) at 42°C for 90 min.

Nucleic acid blot analysis. The conditions for Northern (RNA) blot analysis were as described previously (23). Samples for RNA slot blot analysis were diluted in 10× SSC and applied to the wells of a slot blot manifold (Bethesda Research Laboratories) under a low vacuum. Hybridization to β-actin served to normalize sample loading. Autoradiographic signals were quantitated with a laser densitometer (LKB Instrument, Inc.). Only exposures which produced signals within the linear range of the film (Kodak XAR-5) were used to calculate the relative levels of *mdr*1a transcripts in each cell line.

Quantitative immunoblot analysis. P-glycoprotein levels were quantitated essentially as described previously (30). In brief, immunoblot analysis was done by the method of Sarkar et al. (43), employing sequential incubations of a rabbit polyclonal antipeptide antibody specific for the *mdr*1a gene product (23) and 5×10^5 cpm of [^{125}I]protein A (Amersham Corp.) per ml. Quantitation was done by excising the band corresponding to the 130-kDa mature *mdr*1a P-glycoprotein from the nitrocellulose blot and gamma counting.

RESULTS

Isolation and sequence analysis of the *mdr*1a 5' genomic region. A 1.4-kb genomic fragment containing the promoter region of the mouse *mdr*1b gene was found to cross-hybridize by Southern blot analysis to an additional 3.0-kb amplified fragment in MDR J774.2 cell lines (Cohen, unpublished data). The high degree of sequence homology previously noted between the *mdr*1a and *mdr*1b genes (23) suggested that the 3.0-kb cross-hybridizing fragment might contain the homologous promoter region of *mdr*1a. This fragment was subsequently isolated from a λgt10 library

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ttcaaatgtgtgtgacagatcaatcaatgattctgtgaattc   +882
                                    EcoRI
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FIG. 1. Nucleotide sequence and restriction map of the *mdr*1a 5′ genomic fragment λV1.1a. Exons are indicated by boldface capital letters in the sequence and by solid bars in the restriction map. The empty and filled arrowheads indicate transcription and translation initiation sites, respectively. The inverted triangle marks the position of the 5′-TAATAAAAAA-3′ consensus sequence (29) at the 3′ end of the L1Md repetitive element (open bar). The sequence is numbered relative to the putative major transcription initiation site at position +1. The L1Md element and the putative ATG translation initiation codon are underlined in the sequence. The nucleotide sequence of the *mdr*1a 5′ genomic clone has been submitted to the GenBank and EMBL Data Bank with accession number M33580.

constructed with *Eco*RI-digested DNA from the J7.V1-1 cell line. Sequence analysis of the isolated clone, designated λV1.1a, and comparison with the *mdr*1a 5′ cDNA sequence (see Fig. 4) confirmed its identity as a 2,873-bp 5′ genomic fragment containing the first noncoding exon (exon 1, 319 bp) and the first coding exon (exon 2, 71 bp) of the *mdr*1a gene (Fig. 1). Intervening sequences of 684 bp and a minimum of 195 bp were located downstream of exon 1 and exon 2, respectively (Fig. 1).

***mdr*1a transcripts are initiated from distinct upstream and downstream promoters.** The technique of primer extension was employed to map the initiation site(s) of *mdr*1a transcripts in a series of three vinblastine-selected MDR J774.2 cell lines, which were previously shown to differentially overexpress distinct *mdr*1a mRNA size species (23). Extension was carried out from a 30-mer complementary to the *mdr*1a cDNA sequence from −42 to −72 with respect to the putative AUG translation initiation codon (Fig. 1). A doublet corresponding to the putative major transcription initiation site of a downstream promoter region was detected in mRNA from J7.V3-1 cells but not in mRNA from J7.V1-1 or J7.V2-1 cells (Fig. 2, left panel). These bands were also absent in the parental J774.2 cell line, the colchicine-selected J7.C1-100 cell line which does not express *mdr*1a (23), and tRNA when it was used as a control for nonspecific hybridization. The position of the more proximal band in the major doublet was arbitrarily designated +1 (positions discussed below are with respect to this assignment unless otherwise stated). A single major primer extension product corresponding to a putative upstream promoter was detected in mRNA from the J7.V1-1 and J7.V2-1 cell lines but not in mRNA from the J7.V3-1 cell line (Fig. 2, right panel). A minor primer extension product was seen in J7.V1-1 cells. The signals were absent in control lanes. The primer extension product corresponding to the putative major upstream initiation site was 227 bp longer at the 5′ end than that corresponding to the major downstream initiation site at position +1. The pattern of promoter utilization in the cell lines described above was confirmed by assaying for the presence of promoter-specific transcript sequences, by using the highly sensitive PCR (data not shown). This technique also demonstrated that only the downstream promoter is active in the brain, heart, and lung of the normal mouse (data not shown), where high levels of *mdr*1a expression have been reported (8).

Comparison of mouse *mdr*1a and human *mdr*1 promoter regions. Sequence comparison of the mouse *mdr*1a promoter region with the previously reported human *mdr*1 promoter region (50) revealed a high degree of structural homology (Fig. 3). They shared ~70% nucleotide sequence identity between positions −240 and +21. The intron-exon boundaries delineating the first noncoding exon (exon 1), which contained regulatory elements for a putative downstream promoter, were identical for the two genes. The likely intron −1-exon 1 boundary in the mouse *mdr*1a gene was inferred from homology to the human *mdr*1 gene. This homology extended well into intron −1. The intron −1-exon 1 boundary of the human *mdr*1 gene was determined by comparison with a cDNA variant corresponding to transcripts initiated from an upstream promoter (49, 50). The mouse *mdr*1a and human *mdr*1 promoter sequences between +1 and the exon 1-intron 1 boundary exhibited no significant sequence homology but were essentially conserved in length. The lack of sequence conservation in this region was consistent with the absence of any recognizable regulatory elements. However, the nearly precise conservation of exon length suggests that this region may be important for transcript function. Three GC box-like sequences, which are recognized by transcrip-

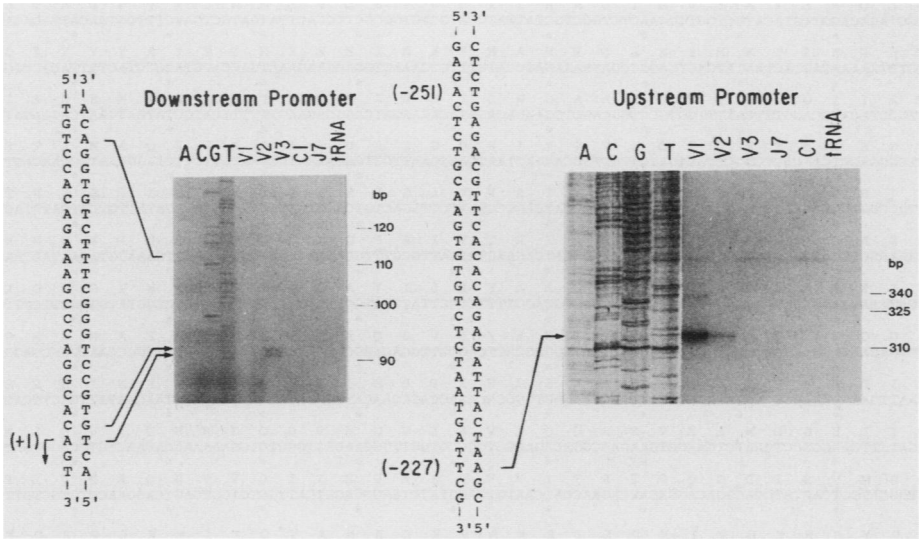

FIG. 2. Primer extension mapping of *mdr*1a transcription initiation sites. Primer extension products were analyzed alongside sequencing ladders extended with the same 30-bp primer (see Materials and Methods). Arrows indicate the primer extension products corresponding to transcription initiation sites in the promoter sequence of λV1.1a. The number in parentheses indicates the position relative to the major downstream transcription initiation site at position +1. The number of nucleotides from the 5′ end of the extension primer is indicated to the right of each panel. Samples in the left and right panels were electrophoresed for 1.5 and 3.5 h, respectively, on a 6% polyacrylamide-8.3 M urea sequencing gel at ~1,500 V.

tion factor SP-1 (10), and a single AP-1 (1) consensus sequence were conserved in position between the two genes, as was the major downstream transcription initiation site. The mouse *mdr*1a AP-1 sequence was canonical (1), while the human *mdr*1 AP-1 sequence was identical to that reported for the interleukin-1-responsive element in the interleukin-2 promoter (34).

Despite many structural elements shared between the mouse *mdr*1a and human *mdr*1 downstream promoters, the former contained several unique putative regulatory sequences absent in the latter (Fig. 3). Whereas the human *mdr*1 promoter did not contain a TATA box-like element, an AT-rich sequence, resembling the TATA box for the rat interleukin-6 gene (36), was present between −29 and −36 in the mouse *mdr*1a promoter. The nearly canonical CAAT box, 5′-GGTCAAACT-3′, identified between positions −80 and −88 in mouse *mdr*1a, differed from the consensus eucaryotic CAAT box sequence, 5′-GGYCAATCT-3′ (2), by a single nucleotide. This element differed from the human *mdr*1 CAAT box in both position and sequence (Fig. 3). A nearly canonical AP-2 sequence (33), 5′-GCCCAGGC-3′, was also present in the mouse *mdr*1a promoter but not in the human *mdr*1 promoter.

Initiation from a putative upstream promoter was reported previously for the human *mdr*1 promoter (49, 50). The human *mdr*1 upstream promoter was shown to be at least 18 kb from the downstream promoter, as determined by field inversion gel electrophoretic analysis (6). The putative mouse *mdr*1a upstream and downstream promoters also appeared to be separated by intron sequences (Fig. 1 and 3). However, the distance between the two promoters could not be determined, since λV1.1a did not contain the upstream promoter and cDNAs containing the additional 227-bp 5′ sequence corresponding to transcripts initiated from the upstream promoter have not yet been isolated.

Initial use of the entire λV1.1a insert as a Southern blot hybridization probe indicated that it contained mouse repetitive sequences (data not shown). A search of GenBank revealed 98.6% identity between the first 1,304 bp of λV1.1a (Fig. 1) and the 3′-most end of the L1Md-A2 element (29), a

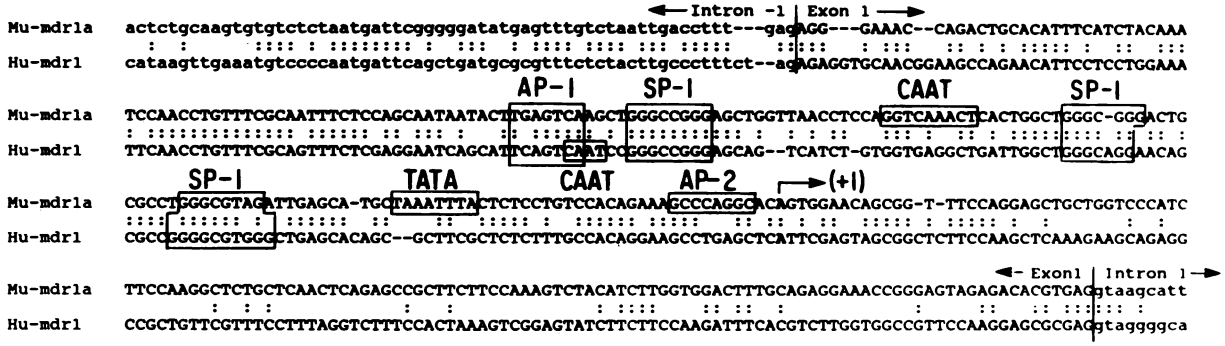

FIG. 3. Sequence comparison of the mouse *mdr*1a and human *mdr*1 promoter regions. The sequence of the 5′ noncoding exon of mouse (Mu) *mdr*1a was compared with that of human (Hu) *mdr*1 (50). Gaps (indicated by dashes) were introduced to maximize alignment. Colons indicate identical nucleotides. Putative regulatory elements are in boxes. The conserved major transcription initiation site is indicated at position +1.

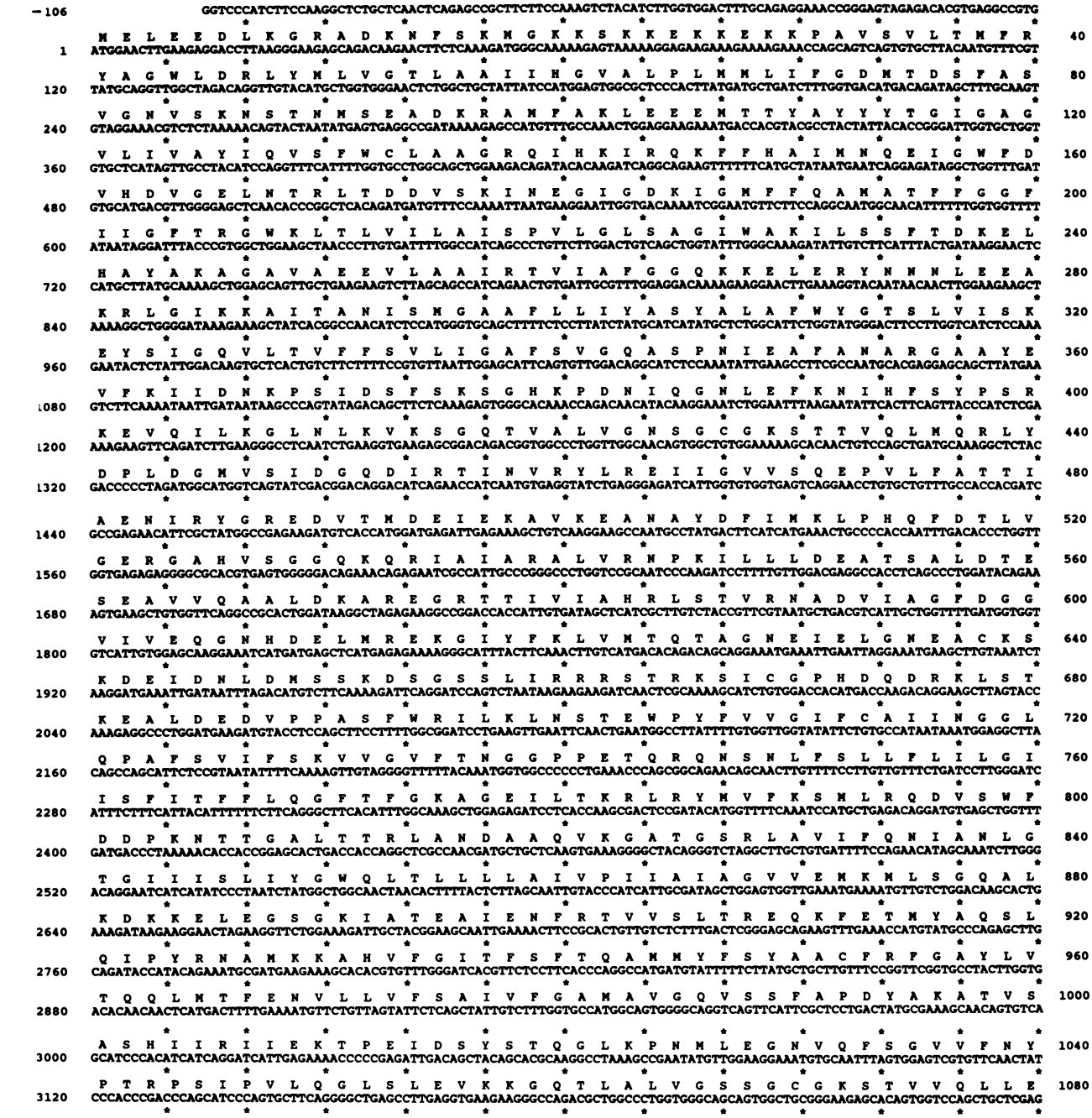

FIG. 4. Complete cDNA nucleotide sequence and deduced amino acid sequence of mouse *mdr*1a. Nucleotides are numbered in the 5′-to-3′ orientation relative to the ATG initiator codon at position +1. The nucleotide at position −106 corresponds to the 5′ end of the forward primer used for PCR cloning of the 5′-most cDNA fragment. The sequence ends at the distal polyadenylation site at +4788. The positions of the translation termination codon (TGA) and the two putative poly(A) addition signals (TATAAA and AATAAA) are underlined. The polyadenylation sites determined by sequence analysis of 3′ cDNA variants are indicated as follows: (A)n. The nucleotide sequence of the *mdr*1a cDNA has been submitted to the GenBank and EMBL Data Bank with accession number M33581.

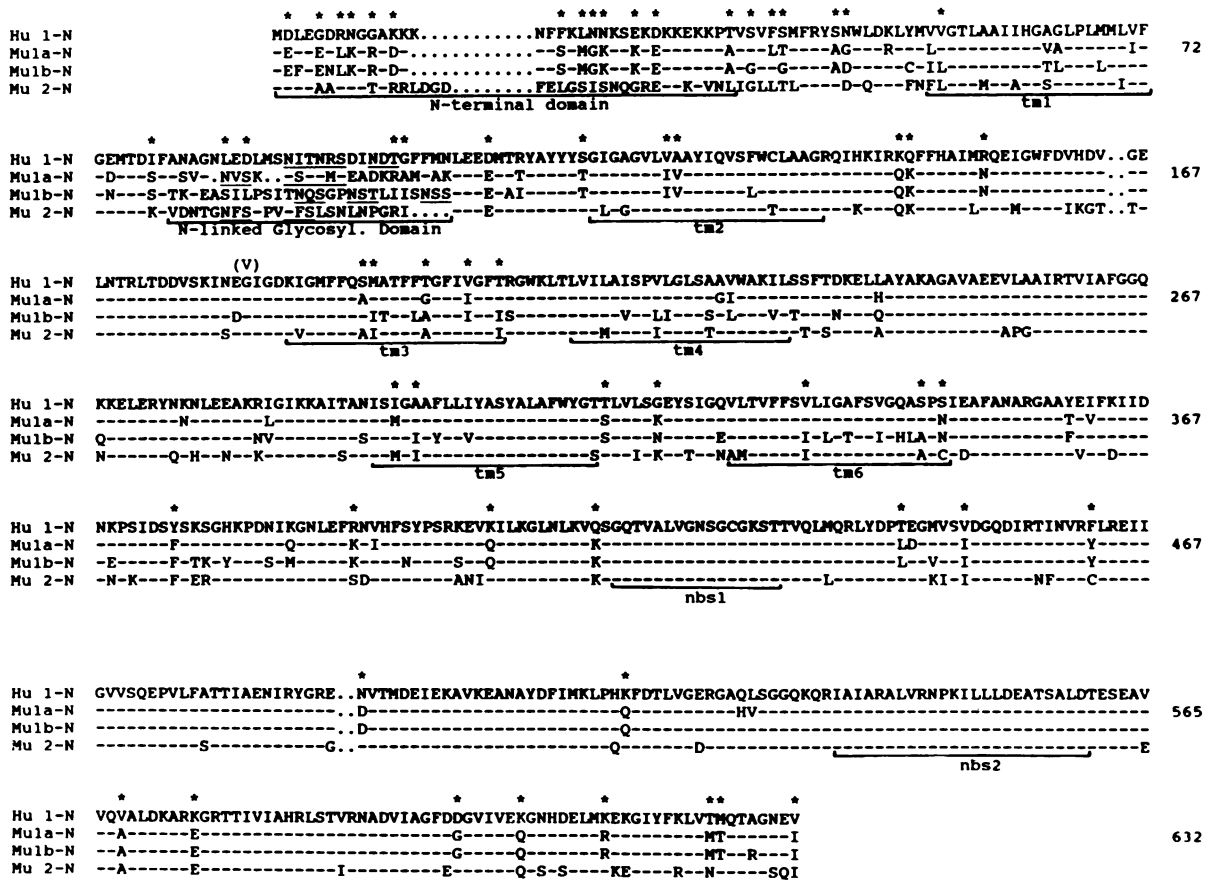

FIG. 5. Comparison of deduced amino acid sequences of the three mouse *mdr* proteins and the human *mdr*1 protein. The predicted amino acid sequences of the amino-terminal halves of the mouse *mdr*1a, *mdr*1b (18), and *mdr*2 (19) proteins are aligned with the corresponding sequences of the human *mdr*1 protein (5). The beginning of the alignment is indented to facilitate comparison with the previously reported carboxyl-terminal alignment (23). Gaps, indicated by dots, are introduced to optimize the alignment. The entire sequence for the human *mdr*1 is shown. For the mouse *mdr* proteins, only the residues which differ from the human *mdr*1 sequence are shown; identical amino acids are marked with a dash. Asterisks mark positions where at least two distinct mouse *mdr* proteins differ from the human *mdr*1 (23). Predicted transmembrane domains (tm) and nucleotide binding sites (nbs) are as determined for the human *mdr*1 sequence (5). Putative N-linked glycosylation sites corresponding to the consensus sequence Asn-X-Ser or Asn-X-Thr are underlined in the N-linked glycosylation domain. Other abbreviations: Mu, mouse; Hu, human; N, amino terminus.

member of the mouse long interspersed L1Md repetitive family. The significance of this repetitive sequence with respect to *mdr*1a gene regulation is unknown.

Cloning of an *mdr*1a 5′ cDNA by PCR. A 3,691-bp portion of the 3′-most *mdr*1a cDNA sequence, containing 87% of the protein-coding region, was recently reported from the analysis of overlapping clones (23). Isolation of cDNA clones from a library by conventional screening methods had previously failed to yield additional 5′ clones. Alternatively, enzymatic amplification of cDNA by PCR was used to carry out the primer-directed amplification and cloning of *mdr*1a 5′ cDNA sequences. *mdr*1a gene-specific forward and reverse primer sequences (amplimers) were chosen from regions of divergence between reported mouse *mdr* cDNA sequences (positions +29 to +50 in the forward direction and +1039 to +1021 in the reverse direction). 5′ Adapter sequences for infrequently cleaving restriction enzymes were added to the amplimers to facilitate cloning of the PCR product (see Materials and Methods). The ~1-kb target region of the amplimer pair overlaps the 5′-most previously isolated *mdr*1a cDNA clone (23) and includes 106 bp of 5′-untranslated sequences. A single major PCR product of the expected size was detected by both ethidium bromide staining and blot hybridization in cDNA prepared from J7.V1-1 and J7.V3-1 RNA but was not detected in cDNA from parental J774.2 cells in which *mdr*1a has been shown to be transcriptionally silent (S. I. Hsu, unpublished data). Several independent clones of the 1-kb PCR product from J7.V1-1 cells were analyzed by sequence analysis. No PCR-associated misincorporations were detected.

The 4,894 bp of the complete *mdr*1a cDNA sequence assembled from overlapping clones is shown in Fig. 4. The sequence contains a long open reading frame between positions −105 and +3944, in which position 1 has been assigned to the first ATG codon in the cDNA sequence. The size of the open reading frame is 1,276 amino acids, with a calculated minimum molecular mass of approximately 140,000 daltons, which is in good agreement with the 120,000-dalton estimated size of the P-glycoprotein precursor encoded by *mdr*1a (16). The discrepency in size is most likely due to the gel conditions used for precursor analysis (15).

Members of the mouse *mdr* class I shared amino acid sequence homology at the N terminus (94% for *mdr*1a versus *mdr*1b), suggesting that this lysine-rich region (Fig. 5) may be important for P-glycoprotein structure and function in the mouse. However, comparisons of *mdr*1a and *mdr*1b with

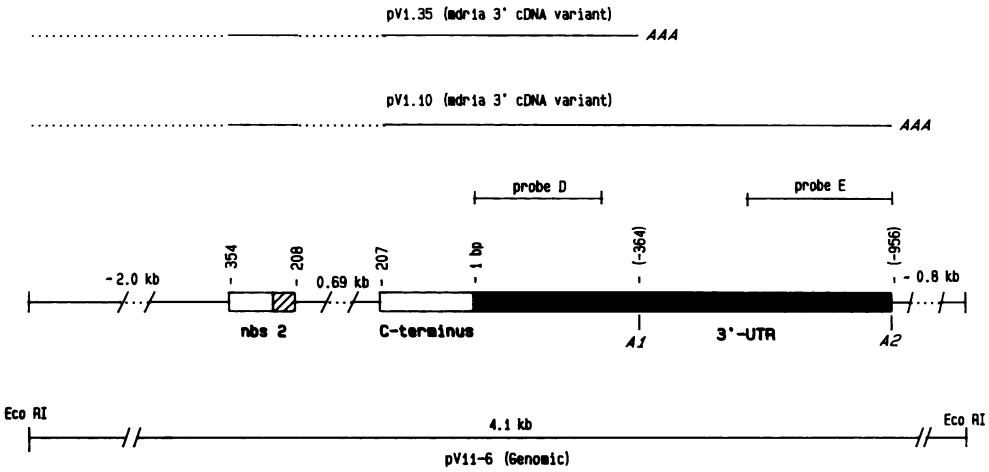

FIG. 6. Mouse *mdr*1a 3′ genomic and transcript organization. The intron-exon organization of the *mdr*1a 3′ genomic fragment pV11-6 (see Materials and Methods) was determined by comparison with the cDNA variants pV1.10 and pV1.35. Positions of the intron-exon boundaries are numbered in the 5′-to-3′ orientation relative to the first nucleotide of the TGA termination codon at position +1. The consensus sequence for the second fold of the carboxyl-terminal nucleotide-binding site (nbs) is indicated by a hatched box, while the solid bar represents 3′ untranslated (3′-UTR) sequences. A1 and A2 mark the proximal and distal polyadenylation sites, respectively.

*mdr*2 revealed a divergence (18, 19) in the N-terminal domain (<20% identity) and the first putative extracellular loop (<15% identity) which encodes potential N-linked glycosylation sites (Fig. 5).

***mdr*1a 3′ transcript heterogeneity is generated by alternative usage of different poly(A) addition signals.** Isolation of the cDNA pV1.35 encoding the 3′-untranslated region of *mdr*1a was previously described (23). A longer 3′ cDNA variant, pV1.10, which differed from pV1.35 by the addition of 591 bp at its 3′ end, was subsequently isolated (Fig. 6) and sequenced. Both pV1.35 and pV1.10 contained consensus poly(A) addition signals 12 and 21 bp, respectively, upstream of a poly(A) tail. Comparison of the sequences of these two 3′ cDNA variants with the sequence of a 3′ *mdr*1a genomic fragment revealed that pV1.35 and pV1.10 were generated by the alternative usage of two poly(A) addition signals, designated A1 and A2, rather than by alternative splicing (Fig. 6).

Hybridization probes specific for transcripts containing the 3′ untranslated sequences associated with polyadenylation at A1 and A2 were used in Northern blot analysis to determine the polyadenylation pattern used to generate the four *mdr*1a mRNA size species (Fig. 7). Probes D and E were 282-bp *Mae*III and 349-bp *Pst*I-*Eco*RI fragments isolated from pV1.35 and pV1.10, respectively. Probe D, which contained sequences common to pV1.35 and pV1.10, hybridized to 4.7- and 5.1-kb *mdr*1a transcripts in J7.V1-1 and J7.V2-1 cells and to 4.6- and 5.0-kb *mdr*1a transcripts in J7.V3-1 and J7.T1-50 cells. The 4.6- and 4.7-kb mRNA size species were previously referred to as a common 4.6-kb size species (23). The broadness of the lower bands in J7.V1-1 and J7.V2-1 cells was likely due to cross-hybridization of probe D to the predominant 4.6-kb *mdr*1b transcripts in these cell lines, since the homology between *mdr*1a and *mdr*1b in the proximal 3′ untranslated region is significant (23). Probe E hybridized to the 5.1-kb transcript in J7.V1-1 and J7.V2-1 cells and the 5.0-kb transcript in J7.V3-1 and J7.T1-1 cells but not to the 4.6- and 4.7-kb transcripts in these cells. Thus, A1 and A2 are used alternatively for polyadenylation in each cell line to generate *mdr*1a transcripts which differ by 591 bp at the 3′ end (Fig. 8). A similar pattern of polyadenylation has been reported for the human aromatase P-450 gene (47).

Relative steady-state *mdr*1a protein levels correlate with differential initiation from two promoters. A site-directed polyclonal antibody (anti-*mdr*1a) specific for the *mdr*1a gene product (23) detected abundant overproduction of the 130-kDa *mdr*1a gene product only in J7.V3-1 (Fig. 9, right panel). Overexpressed *mdr*1a transcripts in J7.V3-1 were initiated from the putative downstream promoter (Fig. 2, left panel). Overproduction of a 132-kDa *mdr*1a protein was much less abundant in membrane fractions from J7.V1-1 and J7.V2-1 cells (Fig. 9, right panel), despite levels of *mdr*1a mRNA overexpression comparable to those observed in J7.V3-1. J7.V1-1 and J7.V2-1 cells overexpressed *mdr*1a transcripts initiated from the putative upstream promoter (Fig. 2, right panel). The small size difference between the *mdr*1a gene

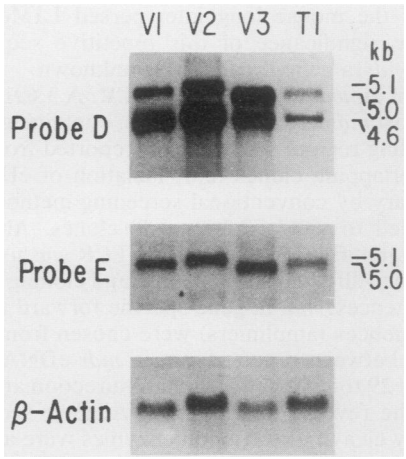

FIG. 7. Northern blot analysis of *mdr*1a transcript 3′-end formation. Total cellular RNA (10 μg) isolated from the cell lines indicated was resolved by electrophoresis, transferred to nylon membranes, and sequentially hybridized with ^{32}P-labeled cDNA probes as indicated in each panel. Transcript sizes were estimated relative to the migration of 18 and 28S rRNA.

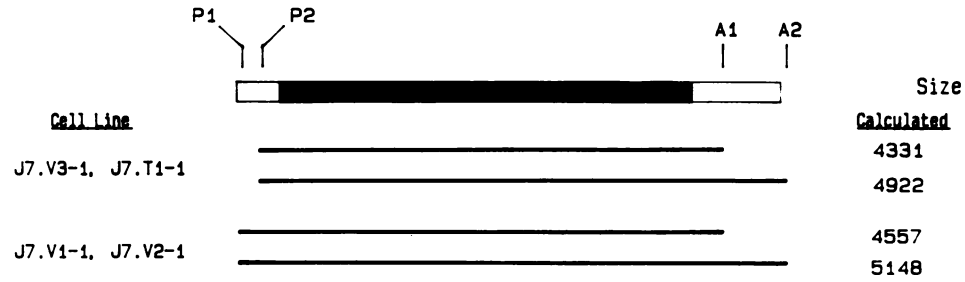

FIG. 8. Basis for *mdr*1a 5′ and 3′ transcript heterogeneity. Generation of *mdr*1a transcript heterogeneity by differential initiation from two promoters (P1 and P2) and alternative usage of two poly(A) addition sites (A1 and A2) is illustrated schematically. The protein-coding region is indicated by a solid bar. Calculated transcript sizes are based on primer extension mapping of the 5′ end and 3′ cDNA sequence analysis.

products in J7.V1-1 and J7.V2-1 as compared with J7.V3-1 may reflect differential N-linked glycosylation, as has been observed for the *mdr*1b gene product (16).

In order to explore the apparent discrepancy in steady-state *mdr*1a protein levels in cells expressing *mdr*1a transcripts initiated from the two putative promoters, *mdr*1a transcript and protein levels were quantitated in the three vinblastine-resistant MDR J774.2 cell lines. To determine the sensitivity of immunoblot quantitation, 2.5 to 12.5 μg of J7.V3-1 membrane protein was sequentially incubated with anti-*mdr*1a and [^{125}I]protein A (see Materials and Methods). There was a sigmoidal relationship between signal intensity and the amount of immunoreactive protein, with the greatest linearity observed between 5 and 10 μg (data not shown). The signal intensities of immunoreactive protein from J7.V1-1 and J7.V2-1 cells were compared with those of a J7.V3-1 protein dilution series within the linear range (30) by gamma counting of bands excised from the nitrocellulose membrane. J7.V1-1 and J7.V2-1 cells produced 15 and 18%, respectively, of the amount of immunoreactive *mdr*1a protein produced in J7.V3-1 cells (Table 1). Quantitation of *mdr*1a transcript levels by slot blot analysis showed that the levels in J7.V1-1 and J7.V2-1 cells were 49 and 114%, respectively, relative to the level in J7.V3-1 cells (Table 1). Thus, the ratio between *mdr*1a P-glycoprotein and transcript levels was 30 and 16% in J7.V1-1 and J7.V2-1 cells, respectively, relative to that in J7.V3-1 cells (Table 1).

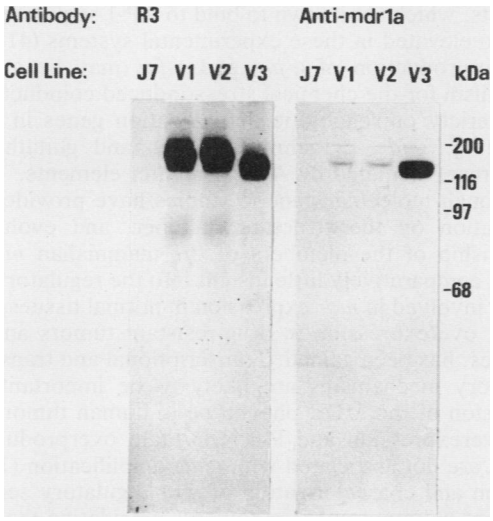

FIG. 9. Immunoblot analysis of mouse *mdr* gene products. Plasma membrane-enriched subcellular fractions (50 μg) were resolved on sodium dodecyl sulfate–7% polyacrylamide gels, electroblotted to nitrocellulose filters, and probed with the antibody indicated in each panel. R3 is a rabbit polyclonal antibody (54) which cross-reacts with *mdr*1a and *mdr*1b gene products. The specificity of the affinity-purified site-directed polyclonal antibody, anti-*mdr*1a, has been described previously (23).

DISCUSSION

The studies described in this report were initiated to determine the basis for mouse *mdr*1a transcript heterogeneity and the relationship between transcript size and steady-state level of P-glycoprotein in a series of independently selected MDR J774.2 cell lines. Primer extension mapping of the 5′ terminus (Fig. 2) and Northern blot analysis of the 3′ terminus (Fig. 7) established that *mdr*1a transcript heterogeneity was generated in individual MDR cell lines by differential initiation from two putative promoters and the alternative usage of different poly(A) addition signals. The structure of each *mdr*1a transcript variant is shown in Fig. 8. The calculated transcript lengths based on primer extension mapping of the 5′ end and cDNA sequence analysis of the 3′ end are in good agreement with values assigned by Northern blot analysis. Small discrepancies are most likely due to the inherent low resolution of Northern blot analysis in the size range of these transcripts.

Transcript initiation from the putative upstream promoter correlates with a 70 to 85% decrease in the *mdr*1a steady-state protein/transcript ratio, compared with initiation from the downstream promoter (Table 1). Several possible mech-

TABLE 1. Comparison of the *mdr*1a steady-state protein/transcript ratio in MDR J774.2 cell lines

Cell line	Relative level of:		Relative protein/transcript ratio[c]
	mRNA[a]	P-gp[b]	
J774.2	0	0	
J7.V1-1	49	15 ± 2.4	30 ± 4.9
J7.V2-1	114	18 ± 5.5	16 ± 4.7
J7.V3-1	100	100	100

[a] Values, obtained by mRNA slot blot analysis with an *mdr*1a gene-specific probe (data not shown), are adjusted for the background signal in parental J774.2 cells in which *mdr*1a is known to be silent, normalized to β-actin, and expressed relative to the value for J7.V3-1 which was set arbitrarily at 100.

[b] P-gp, P-glycoprotein. Values, obtained by quantitative immunoblot analysis with a site-directed polyclonal antibody specific for the *mdr*1a gene product, are expressed relative to the value for J7.V3-1 which was set arbitrarily at 100. Standard deviations reflect the results of two independent experiments.

[c] Values are expressed relative to the value for J7.V3-1 which was set arbitrarily at 100. Values given are ± standard deviations.

anisms may account for this difference. Formation of local secondary structure between the unique 5′ terminus of the upstream transcript and the region surrounding the translation initiation codon may reduce *mdr*1a translational efficiency (20). Translational inhibition due to long-range interactions between 5′- and 3′-untranslated regions has also been reported (28). Isolation and sequence analysis of cDNAs corresponding to *mdr*1a transcripts initiated from the upstream promoter may determine whether secondary structure can account for the lowered protein/transcript ratio associated with this sequence. Transcripts initiated from the upstream promoter may also contain a binding site for *trans*-acting factors which could modulate translational efficiency, perhaps by interacting with ribosomes or initiation factors (38).

Differences in the efficiency of nucleo-cytoplasmic transport of *mdr*1a transcripts may alter steady-state P-glycoprotein levels. A comparison of nuclear versus polysomal *mdr*1a transcript levels may be informative. Although differences in host cell factors unrelated to *mdr*1a transcript structure may also conceivably affect P-glycoprotein levels in MDR J774.2 cell lines, the correlation between *mdr*1a promoter utilization and P-glycoprotein levels in four independently selected cell lines suggests that *mdr*1a transcript structure directly determines, at least in part, the steady-state level of P-glycoprotein. The transfection of appropriate *mdr*1a constructs into a common host may help to determine the extent to which any of the above mechanisms is involved in determining *mdr*1a P-glycoprotein levels.

Sequence analysis indicates that the 5′ intron-exon organization of the mouse and human *mdr*1-class genes is identical (Fig. 3). The structure of the mouse *mdr*1a regulatory region also exhibits significant homology to that of the human *mdr*1 gene (50), consistent with the proposed close evolutionary relationship between these genes (6, 23). The presence of a putative TATA box in the mouse *mdr*1a, which is absent in the human *mdr*1 (Fig. 3), may confer tighter regulation on the precision of transcription initiation, as suggested by the absence of minor primer extension products in mouse *mdr*1a (Fig. 2, left panel). Nonetheless, the major downstream transcription initiation site appears to be identical for these two genes (Fig. 3). Thus, the strong doublet corresponding to the putative mouse *mdr*1a downstream initiation site is unlikely to represent a prematurely terminated reverse transcription product.

Primer extension products corresponding to a putative mouse *mdr*1a upstream promoter were detected in independently derived vinblastine-selected J7.V1-1 and J7.V2-1 cells (Fig. 2, right panel). The major product was extended 227 bp further upstream than that of the downstream promoter at position +1. Similarly, transcripts initiated from the human *mdr*1 upstream promoter in a series of colchicine-selected human KB cell lines were 189 bp longer at the 5′ end than those initiated at the major downstream promoter (49, 50). Interestingly, in human KB cells, both promoters were observed to be simultaneously active, while in J774.2 MDR cell lines, the two promoters were differentially transcribed. Although the presence of the more 5′ extended products in J7.V1-1 and J7.V2-1 indicates the existence of an upstream promoter, the assignment of the positions of the upstream transcription initiation sites must be considered tentative, since the extension products detected may correspond to strong stops of reverse transcription.

An important question to address is whether transcript initiation from the putative upstream promoter, observed in highly resistant J774.2 cell lines, is relevant to *mdr*1a gene regulation in normal mouse cells. This upstream promoter appears to be inactive in the brain, heart, and lung of the normal mouse as well as in drug-sensitive J774.2 cells (Hsu, unpublished data). To fully resolve this issue, a more systematic study of mouse *mdr*1a promoter utilization in diverse normal tissues is required to demonstrate that differential initiation from the two promoters is a physiological mechanism for the regulation of the level of this P-glycoprotein isoform.

The identification of protein kinase A consensus phosphorylation sites in the *mdr*1a and *mdr*1b linker regions (23) and the demonstration that *mdr*1a and *mdr*1b gene products are substrates for in vitro phosphorylation by the purified catalytic subunit of protein kinase A (31) have suggested a role for phosphorylation by protein kinase A in the regulation of P-glycoprotein function. In addition, cyclic AMP may modulate gene expression through the phosphorylation of transcription factors by the catalytic subunit of protein kinase A (32). The presence of putative AP-2 elements (24) in the promoter regions of mouse *mdr*1a (Fig. 3) and *mdr*1b (Cohen, unpublished data) suggests a role for phosphorylation by both protein kinase A and protein kinase C in the regulation of mouse *mdr*1-class genes. The absence of recognizable AP-2 elements in human *mdr*1 may rule out a role for protein kinase A in the regulation of this gene. However, firm conclusions regarding the role of putative AP-2 elements in species-specific *mdr* gene expression must await further studies.

The presence of a conserved AP-1 site in both human *mdr*1 and mouse *mdr*1a gene promoters suggests that this element may play an important role in the regulation of these genes. Circumstantial evidence for this hypothesis comes from observations that *mdr* is induced in a variety of model systems in the liver (3, 4, 12, 46) which show coinduction of both cytochrome P-450 and glutathione *S*-transferase. In the case of placental glutathione *S*-transferase, it has been shown that the induction is mediated by two AP-1-like elements (37, 41, 42). The argument is strengthened by reports that the levels of c-*jun* and c-*fos* proto-oncogene products, which are known to bind to AP-1 sequences (1, 7), are also elevated in these experimental systems (41). Thus, the overproduction of c-*jun* and c-*fos* may be a general mechanism for the chemical stress-induced coinduction of a wide variety of xenobiotic detoxification genes in hepatocytes (e.g., *mdr*, cytochrome P-450, and glutathione *S*-transferase) mediated by AP-1 promoter elements.

Although molecular genetic studies have provided much information on the structure, number, and evolutionary relationship of the members of the mammalian *mdr* gene family, comparatively little insight into the regulatory mechanisms involved in *mdr* expression in normal tissues, as well as *mdr* overexpression in drug-resistant tumors and MDR cell lines, has been gained. Transcriptional and translational regulatory mechanisms are likely to be important in the expression of the MDR phenotype in human tumors, since *mdr* overexpression and P-glycoprotein overproduction in tumors are not associated with gene amplification (13). The isolation and characterization of *mdr* regulatory sequences represent an important step towards elucidating the mechanisms involved in *mdr* gene regulation.

ACKNOWLEDGMENTS

The order of the first two authors is arbitrary.

This work was supported, in part, by Public Health Service grant CA 39821 from the National Institutes of Health and a Bristol-Myers drug resistance grant. S.I.H. and L.S.K., who is a Howard Iser-

mann Scholar, were supported by Public Health Service Medical Scientist Training Program grant 2T32GM 7288 from the National Institute of General Medical Sciences. L.L. was supported by Public Health Service National Research Service Award CA 07764 from the National Institutes of Health.

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