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Cloning and regulation of the rat *mdr2* gene

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ABSTRACT

We have cloned the complete cDNA encoding the rat *mdr2* gene by a combination of library screening and the polymerase chain reaction. The sequence of rat *mdr2* cDNA is highly similar to other members of the *mdr* gene family but the initiation of transcription, tissue distribution and regulation of expression of rat *mdr2* diverge from the other isoforms. Primer extension analysis showed rat *mdr2* mRNA to have a major transcription start point at -277 and a minor one at approximately -518. We constructed gene specific probes for rat *mdr2* and *mdr1b* and compared the expression patterns of these two genes. The highest expression of *mdr2* mRNA was in the muscle, heart, liver and spleen. Both *mdr2* and *1b* mRNA levels were elevated in the livers of rats treated with CCl_4 or following partial hepatectomies although the time course of induction of each gene differed. *Mdr1b* increased by 12 to 24 hours while *mdr2* did not increase until 48 hours. Treatment of isolated hepatocytes or RC3 cells with cycloheximide did not effect *mdr2* mRNA. In contrast, *mdr1b* expression was increased. These data suggest that rat *mdr2*, unlike *mdr1b*, is not regulated by a negative trans-acting protein factor.

INTRODUCTION

Expression of the membrane-bound drug efflux pump, P-glycoprotein, is a serious impediment to successful chemotherapy of many human tumors. Future efforts at circumventing this form of drug resistance will rely on a full understanding of the structures of the multidrug resistance (*mdr*) genes which encode the P-glycoproteins. The *mdr* gene family consists of two members in humans and three in rodents. cDNAs for the two human (MDR 1 [1] and MDR 2 [2]) and three mouse (*mdr1a*, *mdr1b* and *mdr2* [3, 4]) and hamster (pgp 1, pgp 2, and pgp 3 [5]) *mdr* genes have been isolated. We have described recently the isolation of the complete cDNA for one member of the rat *mdr* gene family (*mdr1b*) [6].

The ability of P-glycoprotein to function as an ATP-driven drug efflux pump has been extensively investigated. Several studies have shown direct binding of photoaffinity drug analogs to P-glycoprotein as would be expected of a drug transporter [7]. Partially purified P-glycoprotein is an active drug-stimulated ATPase [8-11]. Membrane vesicles prepared from multidrug resistant human cells [12], CHO cells [13] and rat intestinal mucosal cells [14] mediate ATP-dependent drug transport. P-glycoproteins encoded by the *mdr* 1 genes confer drug resistance to previously drug sensitive cells [4, 15-18]. Expression of *mdr1* genes is often increased in cell lines selected for drug resistance [19-22]. These data support the theory that P-glycoprotein mediates drug resistance by pumping cytotoxic drugs from cells.

The normal physiological function of P-glycoprotein remains unknown. P-glycoprotein is expressed in the epithelial cells of tissues typically associated with transport of compounds across cell membranes. For example, the bile canalicular cells of the liver, the kidney proximal tubule cells, the intestinal brush border cells, and the endothelial cells of the blood brain barrier all express P-glycoprotein [23-26]. This has lead to the hypothesis that one physiologic function of P-glycoprotein is to protect organisms from xenobiotics [27-29] or to participate in the cellular secretion of endogenous compounds such as steroids [30-32] or peptides [33, 34]. Recent investigations demonstrate that P-glycoprotein may also function as a membrane-bound chloride channel [35, 36], much like the closely related cystic fibrosis transmembrane conductance regulator [37].

In spite of a high sequence homology to the *mdr1* genes, the possible physiological role of the *mdr2* gene product is less clear. Expression of the *mdr2* gene in cells does not confer multidrug resistance [3, 4]. The tissue distribution of *mdr2* expression differs from that of the other mdr genes [26, 38, 39]. The expression of *mdr2* is generally not increased in cell lines selected for resistance [3]. Nevertheless, the structure of the gene and predicted protein contain features which suggest a role very similar to the other isoforms. Some chimeric genes constructed of fragments of the mouse *mdr1b* and *mdr2* genes encode P-glycoproteins capable of conferring drug resistance suggesting that portions of the *mdr2* gene can produce functional P-glycoprotein domains [40, 41]. In addition, recent studies with isoform specific antibodies localized *mdr2* P-glycoprotein to bile canalicular membranes of hepatocytes [42]. This evidence suggests that although *mdr2* is not involved in drug resistance it may still function in some transport capacity.

Expression of the *mdr1* genes in the rat is induced *in vivo* and *in vitro* by treatment with various xenobiotics and inhibitors of protein synthesis [29, 43]. In addition, partial hepatectomy elevates *mdr* gene expression in rat liver [44]. In the course of our studies we noted that the three *mdr* genes respond differently to xenobiotic exposure (Unpublished data). Therefore, it is of

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particular interest to obtain more information about the expression, regulation and function of *mdr2* in the rat. In this paper we describe the cloning and characterization of the rat *mdr2* cDNA. Using the rat *mdr2* cDNA and the recently cloned rat *mdr1b* cDNA, we derived gene specific probes for the RNase protection assay. We used this technique to illustrate tissue specific expression of these genes and to show a divergence in regulation between these two genes.

MATERIALS AND METHODS

Cloning and characterization of the rat *mdr2* cDNA

A cDNA library was constructed in λgt10 bacteriophage using poly A^+ RNA isolated from the liver of a rat treated with aflatoxin B1. This treatment enhances *mdr* expression in the rat [28]. 5×10^5 plaques were screened using a segment of the rat *mdr1b* cDNA labelled with [α-^{32}P]dCTP by the random primer method [45]. This probe, 2B13−155, recognizes all three mdr genes in the rat [6]. Plaque lift filters were hybridized and washed under low stringency conditions to permit identification of clones containing the *mdr2* gene. The bacteriophage DNA was first digested with *BamHI* or *SalI* and then treated with T7 gene 6 exonuclease [46] to provide a single strand DNA template for sequencing with the Sequenase 2.0 system (U.S. Biochemical, Cleveland, OH). The largest clone identified as *mdr2* was approximately 3.2 kb and was subcloned as four *EcoRI* fragments into pGEM7Zf(+). Rescreening of the cDNA library with probe made from a fragment of the 5′ end of this clone did not yield any clones containing the remaining 5′ sequence.

Two primers were made to obtain the 5′ end of the cDNA by the polymerase chain reaction. The 3′ antisense primer, 5′-GGCAGCTAGTTCTTTGTCACTG-3′, was selected directly from the 5′ end of the partial rat *mdr2* cDNA sequence. The 5′ sense primer, 5′-TGCGGCCAACACGCGCGTGA-3′, was selected as having a high probability of being conserved in the rat *mdr2* gene from a comparison of known *mdr* sequences. The polymerase chain reaction (PCR) was performed with cDNA which had been synthesized from rat liver RNA by reverse transcription with an *mdr2* gene specific primer corresponding to bases 830 to 851 of the final cDNA. Direct sequencing of the PCR product confirmed that it was the 5′ end of the rat *mdr2* cDNA. The PCR product was cloned directly into the pT7Blue plasmid (Novagen, Madison, WI) to allow further characterization.

In vivo treatment

Male Fischer 344 rats were maintained on a 12-h light/dark cycle and fed standard laboratory chow ad libitum. Rats weighed 150−250g at the time of experimentation. Rats were fed a diet of 0.05% phenobarbital for one week prior to treatment with a single dose of 0.33 ml/kg CCl_4. Phenobarbital treatment has previously been shown to enhance the effect of CCl_4 [47]. Partial hepatectomies were performed according to the method of Higgins and Anderson [48]. RNA for RNase protection assays was isolated from the tissues by guanidinium thiocyanate extraction and cesium chloride-gradient separation [49].

In vitro experiments

Hepatocytes were isolated by *in situ* perfusion of the livers of rats and cultured as previously described [29]. The RC3 cell line used in this study originated as a single cell clone of the rat H4-II-E hepatoma cell line (ATCC CRL 1548). Cycloheximide treatment of the cells was carried out at nonlethal doses as previously described [43]. RNA was isolated from the cells by detergent lysis and phenol extraction as previously described [29].

Primer extension analysis

Primer extension analysis was used to map the distance of the transcription start point (*tsp*) from the start of translation [50]. An 18-mer oligonucleotide corresponding to nucleotides 10−27 of the noncoding strand relative to the ATG start codon was end labelled with γ-[^{32}P]ATP and used as a primer for the reverse transcription of 50 μg rat liver RNA. This primer contains significant mismatch to the sequence of the rat *mdr1b* and the mouse *mdr1a* genes to reduce the chance of cross hybridizing to these mRNAs. The extension products were electrophoresed through an 8% denaturing polyacrylamide gel and then visualized by autoradiography. A sequence ladder was run on this gel to permit sizing of the extension product.

RNase protection assay

The *mdr2* probe was synthesized from a DNA fragment corresponding to positions 2457 to 2813 of the cloned cDNA. The *mdr1b* probe was a 313 nt segment corresponding to nucleotides 212−524 of the *mdr1b* cDNA [6] and the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) probe was a 249 nt segment corresponding to nucleotides 2−250 of the G-APDH cDNA [51]. The *mdr2* probe had 34 and 28% mismatch to the corresponding regions of the rat *mdr1b* and the mouse *mdr1a* cDNA sequences, respectively and the *mdr1b* probe had 43 and 31% mismatch to the rat *mdr2* and the mouse *mdr1a* cDNA sequences. In each comparison, numerous sites of multiple base mismatch of 3 or more bases occurred. Each antisense probe was transcribed with T7 polymerase in the presence of [α-^{32}P]CTP as described in the Ambion Maxiscript kit (Ambion, Austin, TX). The probes were purified on a 5% polyacrylamide gel. Total RNA was hybridized with 90,000 cpm (*mdr1b* or *mdr2*) and 30,000 cpm (GAPDH) of the probes and digested with RNase A and T1 (0.5 and 20 U, respectively) at 30°C for 1 hour as described in the Ambion RPAII kit (Ambion, Austin, TX). These digest conditions and the numerous probe mismatches assure that this assay is gene specific. RNA was hybridized to all three probes simultaneously to allow direct comparison. The protected fragments were separated on a 5% denaturing polyacrylamide gel and visualized by autoradiography. Pilot experiments demonstrated that each probe yielded only one fragment and that the probes were present in excess to assure quantitative results. Normalization of *mdr* to GAPDH expression was performed by densitometric analysis with the ImageQuant package (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Cloning and characterization of the rat *mdr2* gene

Twenty one positive plaques were identified, nineteen of which were successfully purified and their DNA was isolated. *EcoRI* digestion of the DNA from these bacteriophage identified three phage carrying an insert which contained several *EcoRI* sites.

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gcggccaaca cgcgcgtgaa gttcaggctg agATGGATCT TGAGGCAGCA AGAAACGGAA CAGCGCGGCG CCTGGACGGC GACTTTGAAC TAGGCAGCAT CAGCAACCAG AGCAGAGAAA 88
                                     M  D  L  E  A  A  R  N  G  T  A  R  R  L  D  G  D  F  E  L  G  S  I  S  N  Q  S  R  E  29

AAAAGAAGAA AGTGAATTTA ATTGGCCCGT TGACACTGTT CCGATACTCT GATTGGCAGG ATAAATTGTT TATGCTCCTG GGCACCGCCA TGGCCATAGC TCACGGATCA GGTCTTCCCC 208
 K  K  K  K  V  N  L  I  G  P  L  T  L  F  R  Y  S  D  W  Q  D  K  L  F  M  L  L  G  T  A  M  A  I  A  H  G  S  G  L  P  69

TTATGATGAT AGTCTTTGGA GAAATGACAG ATAAGTTTGT AGATAATGCT GGGAACTTTT CCTTGCCAGT GAATTTTTCA TTGTCAATGC TAAATCCAGG AAGAATTCTG GAAGAAGAAA 328
 L  M  M  I  V  F  G  E  M  T  D  K  F  V  D  N  A  G  N  F  S  L  P  V  N  F  S  L  S  M  L  N  P  G  R  I  L  E  E  E  109

TGACTAGATA TGCATACTAC TATTCGGGAC TAGGTGGTGG AGTTCTTTTG GCTGCCTATA TCCAAGTCTC CTTCTGGACT TTGGCAGCTG GCCGACAAAT AAGGAAAATC AGGCAAAAAT 448
 M  T  R  Y  A  Y  Y  Y  S  G  L  G  G  G  V  L  L  A  A  Y  I  Q  V  S  F  W  T  L  A  A  G  R  Q  I  R  K  I  R  Q  K  149

TTTTTCACGC CATCCTTCGA CAAGAAATGG GCTGGTTTGA TATCAAGGGC ACCACCGAAC TCAACACGCG GCTGACAGAT GACATCTCCA AAATCAGTGA AGGAATTGGT GACAAGGTTG 568
 F  F  H  A  I  L  R  Q  E  M  G  W  F  D  I  K  G  T  T  E  L  N  T  R  L  T  D  D  I  S  K  I  S  E  G  I  G  D  K  V  189

GAATGTTCTT TCAAGCAATA GCCACGTTTT TTGCAGGATT CATAGTGGGG TTCATCAGAG GCTGGAAACT CACCCTCGTG ATCATGGCCA TCACCGCCAT CTTGGGGCTC TCTACAGCCG 688
 G  M  F  F  Q  A  I  A  T  F  F  A  G  F  I  V  G  F  I  R  G  W  K  L  T  L  V  I  M  A  I  T  A  I  L  G  L  S  T  A  229

TTTGGGCAAA GATACTCTCA ACATTCAGTG ACAAAGAACT AGCTGCCTAT GCAAAAGCAG GTGCCGTGGC GGAAGAGGCT CTGGGAGCCA TCAGGACCGT GATAGCTTTC GGGGGCCAGA 808
 V  W  A  K  I  L  S  T  F  S  D  K  E  L  A  A  Y  A  K  A  G  A  V  A  E  E  A  L  G  A  I  R  T  V  I  A  F  G  G  Q  269

ACAAAGAGCT AGAAAGGTAT CAGAAGCATT TAGAAAATGC CAAAAAGATT GGAATTAAAA AGGCTATCTC GGCCAACATC TCCATGGGCA TTGCCTTTTT GTTAATATAT GCATCCTATG 928
 N  K  E  L  E  R  Y  Q  K  H  L  E  N  A  K  K  I  G  I  K  K  A  I  S  A  N  I  S  M  G  I  A  F  L  L  I  Y  A  S  Y  309

CACTGGCCTT CTGGTATGGA TCCACTCTGG TTATATCAAA AGAATATACA ATTGGAAATG CCATGACAGT GTTCTTCTCA ATCCTCATTG GGGCCTTCAG TGTGGGGCAG GCTGCCCCCT 1048
 A  L  A  F  W  Y  G  S  T  L  V  I  S  K  E  Y  T  I  G  N  A  M  T  V  F  F  S  I  L  I  G  A  F  S  V  G  Q  A  A  P  349

GTATTGATGC TTTCCCCAAT GCTAGAGGAG CAGCCTATGT GATCTTTGAC ATTATTGATA ATAATCCTAA AATTGACAGT TTTTCAGAGA GAGGACACAA GCCAGACAGC ATCAAAGGAA 1168
 C  I  D  A  F  P  N  A  R  G  A  A  Y  V  I  F  D  I  I  D  N  N  P  K  I  D  S  F  S  E  R  G  H  K  P  D  S  I  K  G  389

ATTTGGAGTT CAGTGACGTT CACTTTTCCT ACCCATCTCG GGCTAATATC AAGATCTTGA AGGGCCTCAA CCTGAAGGTG AAGAGCGGGC AGACGGTAGC CCTGGTTGGC AACAGTGGCT 1288
 N  L  E  F  S  D  V  H  F  S  Y  P  S  R  A  N  I  K  I  L  K  G  L  N  L  K  V  K  S  G  Q  T  V  A  L  V  G  N  S  G  429

GTGGGAAAAG CACAACTGTC CAGCTGCTGC AGAGGCTCTA CGACCCCACA GAGGGTACGA TTAGCATCGA TGGGCAGGAC ATCCGGAACT TTAACGTCAG GTGTCTAAGG GAATTCATCG 1408
 C  G  K  S  T  T  V  Q  L  L  Q  R  L  Y  D  P  T  E  G  T  I  S  I  D  G  Q  D  I  R  N  F  N  V  R  C  L  R  E  F  I  469

GCGTGGTGAG TCAAGAGCCG GTACTGTTCT CTACCACGAT TGCTGAAAAT ATCCGCTATG GCCGTGGGAA TGTAACAATG GATGAGATTA AAAAAGCTGT CAAAGAGGCT AATGCCTATG 1528
 G  V  V  S  Q  E  P  V  L  F  S  T  T  I  A  E  N  I  R  Y  G  R  G  N  V  T  M  D  E  I  K  K  A  V  K  E  A  N  A  Y  509

ACTTCATCAT GAAACTGCCA CAGAAATTTG ACACCCTGGT TGGTGACAGA GGGGCGCAGC TGAGCGGGGG ACAGAAACAG AGGATCGCCA TTGCTCGTGC CTTGGTCCGC AACCCCAAGA 1648
 D  F  I  M  K  L  P  Q  K  F  D  T  L  V  G  D  R  G  A  Q  L  S  G  G  Q  K  Q  R  I  A  I  A  R  A  L  V  R  N  P  K  549

TCCTCCTGCT GGACGAGGCC ACGTCAGCCT TGGACACAGA AAGCGAAGCT GAGGTGCAGG CCGCTCTGGA TAAGGCCAGA GAAGGCCGGA CCACCATCGT GATAGCTCAC CGACTGTCAA 1768
 I  L  L  L  D  E  A  T  S  A  L  D  T  E  S  E  A  E  V  Q  A  A  L  D  K  A  R  E  G  R  T  T  I  V  I  A  H  R  L  S  589

CTGTCCGGAA TGCAGATGTC ATCGCTGGGT TTGAGGATGG CGTCATCGTG GAGCAAGGAA GCCACAGTGA GCTGATAAAG AAGGAAGGGA TCTACTTCAG ACTTGTTAAC ATGCAGACAT 1888
 T  V  R  N  A  D  V  I  A  G  F  E  D  G  V  I  V  E  Q  G  S  H  S  E  L  I  K  K  E  G  I  Y  F  R  L  V  N  M  Q  T  629

CAGGAAGCCA GATCCTGTCA GAAGAATTTG AAGTTGAGCT AAGTGATGAA AAGGCTGCTG GAGGTGTGGC CCCAAATGGC TGGAAAGCAC GCATATTTAG GAATTCTACG AAGAAAAGTC 2008
 S  G  S  Q  I  L  S  E  E  F  E  V  E  L  S  D  E  K  A  A  G  G  V  A  P  N  G  W  K  A  R  I  F  R  N  S  T  K  K  S  669

TGAAAAGTTC ACGGGCGCAT CAAAATAGGC TGGATGTGGA AACCAATGAA CTTGATGCAA ACGTGCCACC AGTGTCTTTT CTGAAGGTCT TAAGACTGAA TAAAACAGAG TGGCCCTACT 2128
 L  K  S  S  R  A  H  Q  N  R  L  D  V  E  T  N  E  L  D  A  N  V  P  P  V  S  F  L  K  V  L  R  L  N  K  T  E  W  P  Y  709

TTGTGGTGGG GACACTCTGT GCCATTGCCA ACGGGGCCCT CCAGCCGGCA TTCTCCATCA TCCTGTCAGA GATGATAGCT ATCTTTGGCC CTGGGGATGA CACAGTAAAG CAACAGAAGT 2248
 F  V  V  G  T  L  C  A  I  A  N  G  A  L  Q  P  A  F  S  I  I  L  S  E  M  I  A  I  F  G  P  G  D  D  T  V  K  Q  Q  K  749

GTAACATGTT CTCGCTGGTC TTCTTGGGCC TAGGAGTCCA CTCCTTCTTT ACTTTCTTCC TTCAGGGTTT CACATTCGGG AAAGCTGGCG AGATCCTCAC CACAAGGCTC CGGTCCATGG 2368
 C  N  M  F  S  L  V  F  L  G  L  G  V  H  S  F  F  T  F  F  L  Q  G  F  T  F  G  K  A  G  E  I  L  T  T  R  L  R  S  M  789

CCTTCAAAGC AATGCTAAGA CAGGACATGA GCTGGTTTGA CGATCATAAA AACAGTACTG GTGCCCTCTC TACAAGACTC GCCACAGACG CTGCGCAGGT CCAAGGAGCC ACAGGAACCA 2488
 A  F  K  A  M  L  R  Q  D  M  S  W  F  D  D  H  K  N  S  T  G  A  L  S  T  R  L  A  T  D  A  A  Q  V  Q  G  A  T  G  T  829

GGTTGGCTTT AATTGCACAG AACACAGCCA ACCTTGGAAC GGGTATTATT ATATCATTTA TTTACGGTTG GCAACTGACA CTTCTGCTCT TATCAGTTGT TCCATTCATT GCTGTAGCGG 2608
 R  L  A  L  I  A  Q  N  T  A  N  L  G  T  G  I  I  I  S  F  I  Y  G  W  Q  L  T  L  L  L  L  S  V  V  P  F  I  A  V  A  869

GAATTGTTGA AATGAAAATG TTGGCTGGCA ACGCCAAGAG AGATAAAAAG GAGATGGAAG CTGCTGGAAA GATTGCAACA GAGGCAATAG AAAATATTCG GACTGTTGTA TCCTTGACCC 2728
 G  I  V  E  M  K  M  L  A  G  N  A  K  R  D  K  K  E  M  E  A  A  G  K  I  A  T  E  A  I  E  N  I  R  T  V  V  S  L  T  909

AAGAGAGAAA ATTTGAGTCA ATGTATGTTG AAAAATTACA CGGACCTTAC AGGAATTCAG TGCGGAAGGC TCACATCTAC GGCATCACTT TTAGCATCTC ACAAGCATTC ATGTACTTTT 2848
 Q  E  R  K  F  E  S  M  Y  V  E  K  L  H  G  P  Y  R  N  S  V  R  K  A  H  I  Y  G  I  T  F  S  I  S  Q  A  F  M  Y  F  949

CTTATGCTGG CTGCTTTCGA TTTGGTTCTT ACCTCATTGT GAATGGACAC ATGCGCTTCA AGGATGTCAT CCTGGTGTTC TCAGCAATCG TGCTTGGTGC AGTGGCTCTA GGACATGCCA 2968
 S  Y  A  G  C  F  R  F  G  S  Y  L  I  V  N  G  H  M  R  F  K  D  V  I  L  V  F  S  A  I  V  L  G  A  V  A  L  G  H  A  989

GCTCATTTGC TCCAGACTAT GCAAAAGCCA AGCTGTCTGC AGCATACTTA TTCAGTCTGT TTGAAAGACA ACCTCTGATT GACAGCTACA GCAGAGAAGG AATGTGGCCG GATAAGTTTG 3088
 S  S  F  A  P  D  Y  A  K  A  K  L  S  A  A  Y  L  F  S  L  F  E  R  Q  P  L  I  D  S  Y  S  R  E  G  M  W  P  D  K  F  1029

AAGGAAGCGT GACATTCAAT GAAGTTGTGT TCAATTATCC CACCCGGGCC AATGTGCCAG TGCTTCAGGG GCTGAGCCTC GAGGTGAAGA AGGGGCAGAC CCTGGCCCTG GTGGGCAGTA 3208
 E  G  S  V  T  F  N  E  V  V  F  N  Y  P  T  R  A  N  V  P  V  L  Q  G  L  S  L  E  V  K  K  G  Q  T  L  A  L  V  G  S  1069

GTGGCTGCGG GAAGAGCACC GTGGTCCAGC TGCTCGAGCG CTTCTACGAC CCCATGGCCG GAACAGTGCT CCTCGATGGT CAGGAAGCAA AGAAACTCAA TGTCCAGTGG CTCCGAGCTC 3328
 S  G  C  G  K  S  T  V  V  Q  L  L  E  R  F  Y  D  P  M  A  G  T  V  L  L  D  G  Q  E  A  K  K  L  N  V  Q  W  L  R  A  1109

AACTTGGCAT TGTGTCCCAG GAGCCCATCC TGTTTGACTG CAGCATCGCC AAGAACATCG CCTACGGAGA CAACAGCCGT GTCGTGTCTC AGGATGAGAT TGTGAGGGCG GCCAAGGAGG 3448
 Q  L  G  I  V  S  Q  E  P  I  L  F  D  C  S  I  A  K  N  I  A  Y  G  D  N  S  R  V  V  S  Q  D  E  I  V  R  A  A  K  E  1149

CCAACATCCA CCCCTTCATT GAGACACTGC CCCAAAAGTA TGAAACAAGA GTAGGAGACA AGGGGACACA GCTCTCTGGA GGCCAGAAAC AGAGGATTGC TATCGCCCGA GCCCTCATCA 3568
 A  N  I  H  P  F  I  E  T  L  P  Q  K  Y  E  T  R  V  G  D  K  G  T  Q  L  S  G  G  Q  K  Q  R  I  A  I  A  R  A  L  I  1189

GACAGCCTCG GGTCCTACTG CTGGATGAAG CCACGTCGGC TTTGGACACT GAGAGTGAAA AGGTCGTCCA GGAAGCGCTG GACAAAGCCA GGGAAGGCCG CACCTGCATT GTGATCGCGC 3688
 R  Q  P  R  V  L  L  L  D  E  A  T  S  A  L  D  T  E  S  E  K  V  V  Q  E  A  L  D  K  A  R  E  G  R  T  C  I  V  I  A  1229

ACCGCCTGTC CACCATCCAG AACGCAGACT TGATCGTGGT GATCGACAAC GGCAAGGTCA AGGAGCACGG CACCCACCAG CAGCTGCTGG CCCAGAAAGG CATCTATTTC TCCATGGTCA 3808
 H  R  L  S  T  I  Q  N  A  D  L  I  V  V  I  D  N  G  K  V  K  E  H  G  T  H  Q  Q  L  L  A  Q  K  G  I  Y  F  S  M  V  1269

ACATTCAAGC TGGCACACAG AACTTATGAa cttgttacag tatattttta aaataaattc caatcgtttt tt                                                3912
 N  I  Q  A  G  T  Q  N  L  -                                                                                                     1278
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Figure 1. Nucleotide sequence of the rat *mdr2* cDNA and corresponding amino acid sequence. Nucleotides are numbered in a 5′-to-3′ orientation starting at the ATG start codon. The amino acid symbols are aligned with the third nucleotide of each codon. The amino acids corresponding to the nucleotide-binding site motif A and ATP-binding active transporter consensus sequence are underlined as are the start codon, the first in-frame stop codon and the potential polyadenylation signal.

Since the rat *mdr1b* cDNA contains no *EcoRI* sites, these three clones appeared promising. Partial sequence data were obtained for 16 of the bacteriophage inserts. The sequences of thirteen clones were identical to the rat *mdr1b* cDNA while the sequences of the three clones containing *EcoRI* sites were similar to the mouse *mdr2* gene. The largest rat *mdr2* insert was approximately

Table I. Comparison of rat, hamster, mouse and human *mdr*/MDR gene sequences

Genes[a]	Human *MDR1*	*MDR2*	Mouse *mdr1a*	*mdr1b*	*mdr2*	Hamster *mdr1a*	*mdr1b*	*mdr2*	Rat *mdr1b*
				Percent of nt homology[b]					
Rat *mdr2*	72.6	86.6	72.3	70.8	94.0	72.3	70.7	90.4	72.1
Rat *mdr1b*	79.1	71.5	82.5	90.4	69.6	81.8	85.3	72.5	100
Hamster *mdr2* (pgp3)	74.0	87.2	72.7	71.4	91.1	74.2	72.8	100	
Hamster *mdr1b* (pgp2)	79.8	71.6	82.4	86.4	69.1	83.6	100		
Hamster *mdr1a* (pgp1)	84.0	73.0	90.1	81.2	71.4	100			
Mouse *mdr2*	71.1	86.1	71.1	69.4	100				
Mouse *mdr1b* (mdr1)	78.7	70.6	84.2	100					
Mouse *mdr1a* (mdr3)	82.2	71.6	100						
Human *MDR2* (MDR3)	74.9	100							

[a] Alternative gene designations are in parentheses.
[b] Comparison of the rat, mouse, hamster and human *mdr/MDR* gene sequences. The percentage identity was calculated using the NALIGN program of the PCGene package (Intelligenetics, Palo Alto, CA). The complete sequences of these genes, coding and non-coding, was used for this comparison. The accession numbers of the sequences used for this comparison are as follows: hamster *mdr1a*, M60040; hamster *mdr1b*, M60041; hamster *mdr2*, M60042, [5]; human *MDR1*, M14758, [54]; human *MDR2*, M23234, [2]; mouse *mdr1a*, M33581, [4]; mouse *mdr1b*, m14757, [55]; mouse *mdr2*, J03398, [3] and rat *mdr1b*, M62425 [6].

3.2kb and contained three *EcoRI* sites. The cDNA sequence was identified by comparison to the *mdr2* genes of human and mouse. This sequence begins approximately 800 bases 3′ to the start of translation. The remaining 5′ region of the cDNA was obtained by the polymerase chain reaction using gene specific primers.

The sequence of the rat *mdr2* cDNA is presented in figure 1. The sequence is 3912 nt long with a longest open reading frame of 3837 nt. The first ATG in this open reading frame is at nucleotide 33. The assignment of this as the first amino acid in the encoded protein is, in part, based on a comparison to the mouse, human and hamster *mdr2* genes. The ATG initiation codon is preceded at position −3 by a purine and followed at position +4 by G. Both of these features are characteristic of a genuine eucaryotic initiation site [52, 53]. The first termination codon in this reading frame occurs at nucleotide 3867. The 43 nucleotide 3′-noncoding region contains a potential polyadenylation signal sequence, AATAAA at nucleotide 3892.

Comparison of this rat *mdr* gene with other known mdr sequences reveals a high degree of sequence identity with other *mdr2* genes. A comparison of this gene to the other mammalian *mdr* genes is shown in Table I. The entire available sequences of these genes was used for this comparison [2−6, 54, 55]. This table shows also the alternate names by which certain *mdr* genes are known. The rat *mdr2* cDNA has the greatest similarity to the mouse *mdr2* cDNA with an overall identity of 94%. This sequence has been submitted to the GenBank database under accession No.L15079.

The rat *mdr2* open reading frame potentially encodes a protein of 1278 amino acids. Many of the characteristics of the previously described P-glycoproteins are conserved in this protein. Examination of the putative rat *mdr2* protein sequence suggests that it would contain 12 transmembrane regions. Each half of the protein contains sequences consistent with a nucleotide binding site motif and an ATP-binding transporter consensus sequence [56]. Nine potential glycosylation sites (NXS or NXT) occur in the entire sequence; however, if the transmembrane regions are positioned in the arrangement that has been suggested for other P-glycoproteins, then only two sites would be located extracellularly [3]. These sites would be within the first extracellular loop toward the amino terminus of the protein. Potential glycosylation sites have been described in other P-glycoproteins at the same relative location.

Primer extension analysis

Primer extension of control rat liver RNA yielded a major 304 nt product and a minor product at around 545 nt (Fig. 2). Identical size extension products were observed in RNA isolated from a rat liver 48 hours after partial hepatectomy (Data not shown). These data show that the major *tsp* is 277 nt 5′ to the start codon. This is a longer segment of 5′-nontranslated mRNA than that identified for the rat and mouse *mdr1b* genes and for the human MDR1 gene. The rat *mdr1b* has a single *tsp* at −156 [43], *tsps* for the mouse *mdr1b* have been reported at −148 [57] and −151 [58] and two major *tsps* have been demonstrated for the human MDR1 at −136 and −140 [1]. The *tsps* for *mdr2* genes of other species have not been published.

Mdr1b and *mdr2* tissue distribution in the rat

We used the RNase protection assay to evaluate tissue specific expression since this assay is more sensitive, specific and quicker than Northern analysis. Liver, muscle, heart, and spleen had the highest level of *mdr2* mRNA expression (Fig. 3). Expression of *mdr2* mRNA was also detected at lower levels in the lung and brain. Rat adrenal tissue did not express *mdr2* or *mdr1b* (data not shown), which agrees with previous observations of rat adrenals [59]. While signal strength was very low in Northern blots using a rat *mdr2* specific probe, the same tissue distribution was observed (data not shown). In addition, the size of the *mdr2* transcript was similar to that found in mouse tissue (4.5 kb) [38]. *Mdr1b* expression in the rat differs from *mdr2* expression in that the tissues with the highest *mdr1b* levels are the lung and liver with lower levels detected in the kidney, small intestine and spleen

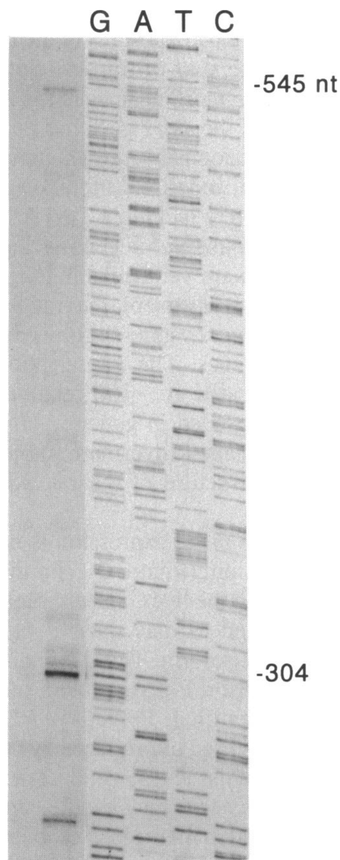

Figure 2. Primer extension analysis to determine the transcription start point of rat *mdr2*. Total RNA (50 μg) was hybridized to a γ-[^{32}P]ATP end-labelled oligo probe and transcribed with avian myeloblastosis virus reverse transcriptase. The reaction products were separated by electrophoresis in a denaturing 8% polyacrylamide gel and visualized by autoradiography. A dideoxy sequencing ladder (**GATC**) was used to measure the size of the extension products.

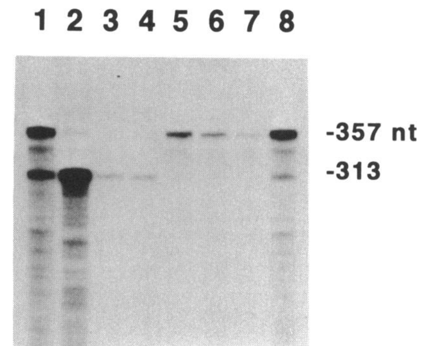

Figure 3. Analysis of tissue specific expression of *mdr2* and *mdr1b* in rat tissues by RNase protection. Lane 1, liver; 2, lung; 3, kidney; 4, small intestine; 5, skeletal muscle; 6, cardiac muscle; 7, brain; and 8, spleen. 25 μg total RNA from the indicated tissues was hybridized with 90,000 cpm of each probe. Samples were subjected to RNase digestion and separated on a denaturing 5% polyacrylamide gel. Autoradiography was performed at −70°C with intensifying screens. The 357 nt protected band corresponds to the *mdr2* probe and the 313 nt band to the *mdr1b* probe.

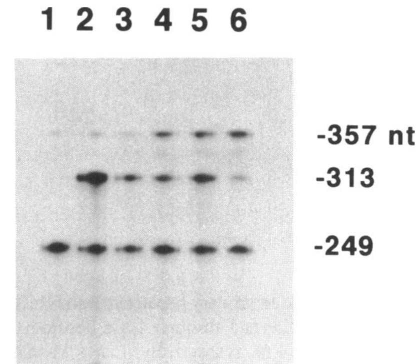

Figure 4. The induction of *mdr2* and *mdr1b* in rat liver by CCl_4 treatment. Lane 1 is control rat liver. Lanes 2 through 6 are 12 hr, 1 d, 2 d, 3 d, and 4 d after CCl_4 treatment, respectively. The RNase protection assay was done as outlined in Materials and Methods using 10 μg total RNA. The GAPDH probe produces the 249 nucleotide band.

(Fig. 3). This distribution is similar to that reported for the mouse [38] and hamster [26]. *Mdr* expression in the liver appears higher in this figure than in later figures because more RNA was used and long exposures were made in order to see the very low expression in some extrahepatic tissues. The GAPDH probe was not used in this experiment since GAPDH expression varies widely between tissues.

Induction of *mdr* expression in liver by CCl_4

The expression of *mdr2* and *mdr1b* was increased at different times following administration of CCl_4 (Fig. 4). *Mdr2* expression was elevated at 2 days and remained elevated at 3 and 4 days. Densitometric analysis showed a maximum increase of *mdr2* expression of 19 fold over control at 4 days. Expression at each time point was compared after normalization for GAPDH expression. *Mdr1b* expression was elevated by 12 hours after CCl_4 treatment and had begun to decrease by 4 days after treatment. Densitometric analysis of *Mdr1b* expression showed a maximum increase at 12 hours from a very low control level. A higher control signal could be obtained with longer film exposure time; however, the other signals then exceed the linear range of the film making accurate densitometry impossible.

Induction of *mdr* in regenerating liver

Figure 5 shows the changes in *mdr2* and *mdr1b* expression in the regenerating liver. *Mdr2* mRNA levels decreased at 12 hours following partial hepatectomy and increased at 48 and 102 hours. Densitometric analysis of *mdr2* expression showed a 2 fold increase at 102 hours over control after normalization to GAPDH. However, since GAPDH expression also increased during regeneration, this analysis may underestimate the increase in *mdr2* expression. *Mdr1b* expression increased from a very low level to a maximum at 24 hr after partial hepatectomy and declined at 48 and 102 hr.

Cycloheximide mediated *mdr* induction

The ability of cycloheximide to induce *mdr* gene expression in primary cultures of rat hepatocytes was analyzed with the RNase protection assay. Cells treated with cycloheximide had a 3- to 5-fold increased expression of *mdr1b* at all time points tested

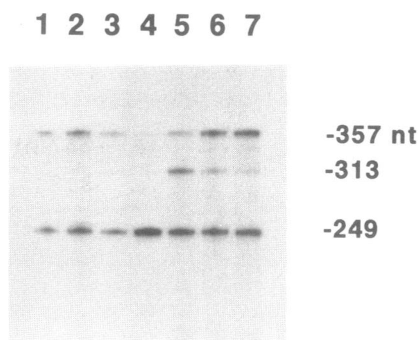

Figure 5. *Mdr* expression following partial hepatectomy. Lane 1 is control rat liver. Lanes 2 through 7 are 3, 6, 12, 24, 48 and 102 hr after partial hepatectomy, respectively. RNase protection analysis was done as outlined in the Materials and Methods using 10 μg total RNA.

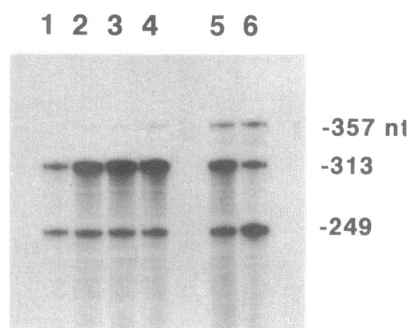

Figure 6. *Mdr1b* and 2 expression in primary hepatocytes and RC3 cells following inhibition of protein synthesis. Lanes 1 through 4 are hepatocytes treated with cycloheximide for 0, 6, 12 and 24 hr, respectively. Lanes 5 and 6 are RC3 cells treated with cycloheximide for 24 and 0 hr, respectively. Cells were treated with 10 μM cycloheximide in 0.1% DMSO for the indicated times prior to harvesting of the RNA. Gene expression was determined by RNase protection as outlined in Materials and Methods using 10 μg total RNA.

(Figure 6). Cycloheximide did not increase *mdr2* expression in these same cells (Figure 6). Interestingly, the expression of *mdr2* relative to *mdr1b* is consistently lower in control hepatocytes than in control liver. An increase in *mdr* expression in isolated primary rat hepatocytes has previously been described [60] . In the rat hepatoma cell line, RC3, cycloheximide also increased the expression of *mdr1b* approximately 6-fold without increasing *mdr2* expression.

DISCUSSION

We have shown that *mdr2* in the rat is similar to the other *mdr* genes in structure yet differs in its initiation of transcription, tissue distribution and regulation of expression. The predicted protein sequence of the rat *mdr2* cDNA contains 12 transmembrane regions, two nucleotide binding regions, and two extracellular glycosylation sites. These characteristics are similar to other previously described P-glycoproteins.

By primer extension analysis we have shown that the rat *mdr2* gene has a major start site at −277 and a minor one at around −518 (Figure 2). In addition, several other very weak extension products were observed in long exposures of the primer extension gels. This suggests that the start of transcription may not be as rigorously controlled in the *mdr2* gene as in the *1b* gene. In fact, even the major start site of the *mdr2* gene has faint bands around it, suggesting that transcription is not always initiated at precisely the same nucleotide. Such indefinite initiation of transcription is characteristic of promoters lacking a TATA box [61]. It has been reported that the mouse *mdr2* gene lacks a TATA box, although no sequence data are yet available [62]. A comparison of the primer extension analysis of the rat *mdr2* gene with information about the rat *mdr1b* gene suggests differences in the promoters of these two genes. The transcription start point of the rat *mdr1b* gene is located at −156 relative to the start of translation [43] and is preceded in genomic DNA by a TATA box 31 nucleotides upstream (Silverman, submitted). We are currently analyzing the structure of the rat *mdr2* promoter in greater detail.

The tissue specificity of *mdr2* expression is notably different from the *mdr1b* expression (Figure 3). In the rat, *mdr2* is expressed predominantly in the liver, spleen, skeletal and cardiac muscle. In the skeletal and cardiac muscle *mdr* expression is almost exclusively of the 2 isoform. This distribution may provide clues to the function of *mdr2* if P-glycoprotein substrates specific to these tissues can be identified. Perhaps *mdr2* plays a role in the transport of metabolites used or produced by these high energy consuming tissues. Muscle tissue also conducts extensive protein assembly and degradation. *Mdr2* may handle components of this process since P-glycoprotein has recently been shown to be capable of transporting small peptides [33]. Further experiments examining whether the *mdr2* P-glycoprotein can transport any of these potential substrates are required to more fully understand its physiological role.

We noted several differences between the regulation of *mdr2* and *mdr1b*. Both *mdr1b* and *mdr2* were induced in rat liver after treatment with CCL_4 although the time course of induction differed. *Mdr1b* expression is elevated 12 hours after treatment and had begun to decrease again by 4 days after treatment. *Mdr2* expression is not increased until 2 days after CCl_4 administration and remains elevated 4 days after treatment. Similar induction of *mdr* expression was also measured by *in situ* hybridization (Nakatsukasa, in preparation).

Previous reports from this laboratory, using a non-*mdr* class specific probe, demonstrated that *mdr* expression increased following partial hepatectomy [44]. In the current study we used the highly specific RNase protection assay to show that both *mdr2* and *mdr1b* are increased in regenerating liver. *Mdr2* increases at the later time points of 48 and 102 hours. This response of *mdr2* to partial hepatectomy is particularly interesting since this gene responded similarly to CCl_4 treatment. The *mdr2* gene may be responding to the liver regeneration induced in each case. *Mdr1b* is maximally increased at 12 hr after partial hepatectomy and decreases at later time points. *Mdr1b* must respond to some component of the CCl_4 effect in addition to the regeneration induced since the increase in *mdr1b* expression occurs earlier in the case of CCl_4 than in partial hepatectomy.

Our studies with hepatocytes and cell lines show that expression of *mdr2* is not induced by protein synthesis inhibition; however, *mdr1b* can be readily induced by treatment with protein synthesis inhibitors (Figure 6). This result agrees with our previous findings in which cycloheximide increased *mdr* expression in culture

hepatocytes and RC3 cells as measured by a non class specific *mdr* probe [43]. Nuclear run-on analyses demonstrated that this increased expression is due to increased transcription. We proposed that the inhibition of protein synthesis released the *mdr1b* gene from the effect of a negative trans-acting protein factor. Further evidence of a negative trans-acting factor has recently been provided by analysis of the promoter region of the rat *mdr1b* gene (Silverman, submitted). Lack of induction by protein synthesis inhibition suggests that the *mdr2* gene in the rat is not under the control of a negative trans-acting factor.

In conclusion, we have shown that the rat *mdr2* cDNA is highly homologous to previously identified members of this gene family. The tissue specific expression, transcription start site, and regulation of *mdr2* by various stimuli are clearly divergent from the rat *mdr1b* gene. Further analysis of *mdr2* P-glycoprotein function and of *mdr2* promoter structure will provide insight into the normal physiological role and regulation of this protein.

REFERENCES

1. Ueda,K., Clark,D.P., Chen,C., Roninson,I.B., Gottesman,M.M. and Pastan,I. (1987) J. Biol. Chem. **262**, 505–508.
2. Van der Bliek,A.M., Kooiman,P.M., Schneider,C. and Borst,P. (1988) Gene. **71**, 401–411.
3. Gros,P., Raymond,M., Bell,J. and Housman,D. (1988) Mol. Cell. Biol. **8**, 2770–2778.
4. Devault,A. and Gros,P. (1990) Mol. Cell. Biol. **10**, 1652–1663.
5. Endicott,J.A., Sarangi,F. and Ling,V. (1991) DNA Seq. **2**, 89–101.
6. Silverman,J.A., Raunio,H., Gant,T.W. and Thorgeirsson,S.S. (1991) Gene. **106**, 229–236.
7. Beck,W.T. and Qian,Xiao-D. (1992) Biochem. Pharmacol. **43**, 89–93.
8. Sarkadi,B., Price,E.M., Boucher,R.C., Germann,U.A. and Scarborough,G.A. (1992) J. Biol. Chem. **267**, 4854–4858.
9. Doige,C.A., Yu,X. and Sharom,F.J. (1992) Biochim. Biophys. Acta **1109**, 149–160.
10. Shimabuku,A.M., Nishimoto,T., Ueda,K. and Komano,T. (1992) J. Biol. Chem. **267**, 4308–4311.
11. Ambudkar,S.V., Lelong,I.H., Zhang,J., Cardarelli,C.O., Gottesman,M.M. and Pastan,I. (1992) Proc. Natl. Acad. Sci. USA **89**, 8472–8476.
12. Horio,M., Gottesman,M.M. and Pastan,I. (1988) Proc. Natl. Acad. Sci. USA **85**, 3580–3584.
13. Doige,C.A. and Sharom,F.J. (1992) Biochim. Biophys. Acta **1109**, 161–171.
14. Hsing,S., Gatmaitan,Z. and Arias,I.M. (1992) Gastroenterology **102**, 879–885.
15. Ueda,K., Cardarelli,C., Gottesman,M.M. and Pastan,I. (1987) Proc. Natl. Acad. Sci. USA **84**, 3004–3008.
16. Shen,D., Fojo,A., Roninson,I.B., Chin,J.E., Soffir,R., Pastan,I. and Gottesman,M.M. (1986) Mol. Cell. Biol. **6**, 4039–4044.
17. Gros,P., Ben-Neriah,Y., Croop,J.M. and Housman,D.E. (1986) Nature. **323**, 728–731.
18. Guild,B.C., Mulligan,R.C., Gros,P. and Housman,D.E. (1988) Proc. Natl. Acad. Sci. USA **85**, 1595–1599.
19. Roninson,I.B., Chin,J.E., Choi,K., Gros,P., Housman,D.E., Fojo,A., Shen,D., Gottesman,M.M. and Pastan,I. (1986) Proc. Natl. Acad. Sci. USA **83**, 4538–4542.
20. Shen,D., Fojo,A., Chin,J.E., Roninson,I.B., Richert,N., Pastan,I. and Gottesman,M.M. (1986) Science. **232**, 643–645.
21. Barrand,M.A. and Twentyman,P.R. (1992) Br. J. Cancer **65**, 239–245.
22. Raymond,M., Rose,E., Housman,D.E. and Gros,P. (1990) Mol. Cell. Biol. **10**, 1642–1651.
23. Thiebaut,F., Tsuruo,T., Hamada,H., Gottesman,M.M., Pastan,I. and Willingham,M.C. (1987) Proc. Natl. Acad. Sci. USA **84**, 7735–7738.
24. Thiebaut,F., Tsuruo,T., Hamada,H., Gottesman,M.M., Pastan,I. and Willingham,M.C. (1989) J. Histochem. Cytochem. **37**, 159–164.
25. Georges,E., Bradley,G., Gariepy,J. and Ling,V. (1990) Proc. Natl. Acad. Sci. USA **87**, 152–156.
26. Bradley,G., Georges,E. and Ling,V. (1990) J. Cell. Physiol. **145**, 398–408.
27. Thorgeirsson,S.S., Silverman,J.A., Gant,T.W. and Marino,P.A. (1991) Pharmac. Ther. **49**, 283–292.
28. Burt,R.K. and Thorgeirsson,S.S. (1988) J.N.C.I. **80**, 1381–1386.
29. Gant,T.W., Silverman,J.A., Bisgaard,H.C., Burt,R.K., Marino,P.A. and Thorgeirsson,S.S. (1991) Mol. Carcin. **4**, 499–509.
30. Wolf,D.C. and Horwitz,S.B. (1992) Int. J. Cancer **52**, 141–146.
31. Arceci,R.J., Croop,J.M., Horwitz,S.B. and Housman,D. (1988) Proc. Natl. Acad. Sci. USA **85**, 4350–4354.
32. Ueda,K., Okamura,N., Hirai,M., Tanigawara,Y., Saeki,T., Kioka,N., Komano,T. and Hori,R. (1992) J. Biol. Chem. **267**, 24248–24252.
33. Sharma,R.C., Inoue,S., Roitelman,J., Schimke,R.T. and Simoni,R.D. (1992) J. Biol. Chem. **267**, 5731–5734.
34. Kuchler,K. and Thorner,J. (1992) Proc. Natl. Acad. Sci. USA **89**, 2302–2306.
35. Valverde,M.A., Díaz,M., Sepúlveda,F.V., Gill,D.R., Hyde,S.C. and Higgins,C.F. (1992) Nature. **355**, 830–833.
36. Gill,D.R., Hyde,S.C., Higgins,C.F., Valverde,M.A., Mintenig,G.M. and Sepúlveda,F.V. (1992) Cell. **71**, 23–32.
37. Anderson,M.P., Gregory,R.J., Thompson,S., Souza,D.W., Paul,S., Mulligan,R.C., Smith,A.E. and Welsh,M.J. (1991) Science. **253**, 202–205.
38. Croop,J.M., Raymond,M., Haber,D., Devault,A., Arceci,R.J., Gros,P. and Housman,D.E. (1989) Mol. Cell. Biol. **9**, 1346–1350.
39. Chin,J.E., Soffir,R., Noonan,K.E., Choi,K. and Roninson,I.B. (1989) Mol. Cell. Biol. **9**, 3808–3820.
40. Buschman,E. and Gros,P. (1991) Mol. Cell. Biol. **11**, 595–603.
41. Currier,S.J., Kane,S.E., Willingham,M.C., Cardarelli,C.O., Pastan,I. and Gottesman,M.M. (1992) J. Biol. Chem. **267**, 25153–25159.
42. Buschman,E., Arceci,R.J., Croop,J.M., Che,M., Arias,I.M., Housman,D.E. and Gros,P. (1992) J. Biol. Chem. **267**, 18093–18099.
43. Gant,T.W., Silverman,J.A. and Thorgeirsson,S.S. (1992) Nucl. Acids Res. **20**, 2841–2846.
44. Thorgeirsson,S.S., Huber,B.E., Sorrell,S., Fojo,A., Pastan,I. and Gottesman,M.M. (1987) Science. **236**, 1120–1122.
45. Feinberg,A.P. and Vogelstein,B. (1984) Anal. Biochem. **137**, 266–267.
46. Ruan,C.C. and Fuller,C.W. (1991) Comments **18**(1), 1–8.
47. Bucher,N.L.R. and Malt,R.A. (1971) Regeneration of liver and kidney, 1st edn, Little, Brown, Boston.
48. Higgins,G.M. and Anderson,R.M. (1931) Arch. Pathol. **12**, 186–202.
49. Chirgwin,J.M., Przybyla,A.E., MacDonald,R.J. and Rutter,W.J. (1979) Biochemistry. **18**, 5294–5299.
50. Archer,T.K., Tam,S.-P., Deugau,K.,V and Deeley,R.G. (1985) J. Biol. Chem. **260**, 1676–1681.
51. Fort,P., Marty,L., Piechaczykl,M., Sabrouty,S.E., Dani,C., Jeanteur,P. and Blanchard,J.M. (1985) Nucl. Acids Res. **13**, 1431–1443.
52. Kozak,M. (1987) Nucl. Acids Res. **15**, 8125–8148.
53. Kozak,M. (1986) Cell. **44**, 283–292.
54. Chen,C., Chin,J.E., Ueda,K., Clark,D.P., Pastan,I., Gottesman,M.M. and Roninson,I.B. (1986) Cell. **47**, 381–389.
55. Gros,P., Croop,J. and Housman,D. (1986) Cell. **47**, 371–380.
56. Walker,J.E., Saraste,M., Runswick,M.J. and Gay,N.J. (1982) The EMBO Journal **1**, 945–951.
57. Cohen,D., Piekarz,R., Hsu,S.I.-H., DePinho,R.A., Carrasco,N. and Horwitz,S.B. (1991) J. Biol. Chem. **254**, 2239–2244.
58. Raymond,M. and Gros,P. (1990) Mol. Cell. Biol. **10**, 6036–6040.
59. Fojo,A.T., Ueda,K., Slamon,D.J., Poplack,D.G., Gottesman,M.M. and Pastan,I. (1987) Proc. Natl. Acad. Sci. USA **84**, 265–269.
60. Fardel,O., Loyer,P., Morel,F., Ratanasavanh,D. and Guillouzo,A. (1992) Biochem. Pharmacol. **44**, 2259–2262.
61. Mathis,D.J. and Chambon,P. (1981) Nature. **290**, 310–315.
62. Kirschner,L.S., Greenberger,L.M., I.-Hong Hsu,S., Yang,Chia-P.H., Cohen,D., Piekarz,R.L., Castillo,G., Han,E.Kyu-H., Yu,L. and Horwitz,S.B. (1992) Biochem. Pharmacol. **43**, 77–87.